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DEVELOPMENT OF A HARMONISED METHOD FOR THE PROFILING OF AMPHETAMINE

Thèse de doctorat

Présentée à l'Institut de Police Scientifique de l'Université de Lausanne

par

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La Présidente du Jury

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To my parents, William Owen Lock and Eleanor Regina Lock.

"*It is a capital mistake to theorise before one has data. Insensibly one begins to twist facts to suit theories, instead of theories to suit facts* "

A Scandal in Bohemia, The Adventures of Sherlock Holmes.

Sir Arthur Conan Doyle.

FOREWORD

This Ph.D. thesis was carried out in parallel to an European project funded by the 4th framework programme of the European Commission (DG XII, Standards, Measurement and Testing). This project, named SMT CT98-2277 was funded, for the Swiss part, by the Federal Office of Education and Science (Bern, Switzerland). This pioneering study was carried out by four laboratories and three advisors during the period 1999 – 2002 (see list of participants in Annex 0). The ultimate aim of the project, i.e. development of a harmonised method for the profiling of amphetamine, was completely achieved. The resulting method has not only shown to produce interchangeable data but also to be superior in terms of speed and user-friendliness compared to the previous methods. Moreover, statistical data handling methods were compared and the final recommended method optimised in the course of the study proved to be more thorough than any previously published study in the discipline.

Mr. Christos Profilis and Dr. Soren Bowadt and their unit, the DG Research of the EU Commission are gratefully acknowledged for their help, constructive criticism and funding. Also, the Federal Office of Education and Science (Bern, Switzerland) is greatly acknowledged for their financial support to the Swiss partner of the project.

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A. General introduction

A.1 Brief history of amphetamine

Amphetamine is believed to be first synthesised by a German chemist named L. Edeleano in the end of the 19th century [Edeleano, 1887]. After that, it has been largely forgotten for about 40 years.
In 1930, amphetamine was discovered to increase blood pressure and in 1932, amphetamine was first marketed as Benzedrine® by the chemical company Smith, Kline and French and was sold as an inhaler to treat congestion.
In 1935, the stimulant effect of amphetamine is first recognized and physicians successfully use it to treat narcolepsy [Lukas, 1985].
In 1937, the American Medical Association approve the sale of amphetamine in tablet form. It is sold by prescription for use in the treatment of narcolepsy and ADHD (attention deficit hyperactivity disorder).
Amphetamine also found an important military application during World War II and the Korean War (1950-1953). Indeed, it was largely distributed to soldiers in order to improve performance.
It is only in 1970 that amphetamine is listed in the Schedule II of the US Drug Abuse Regulation and Control Act of 1970. In effect, this renders the possession of amphetamine illegal without a prescription.

Then, in the seventies, criminal networks got themselves organised in order to produce and distribute amphetamine and the first clandestine laboratories appeared in the United States and Europe. Frank [Frank, 1983] reported almost 70 illicit production sites in the United States for the period 1978-1981 whereas Sinnema [Sinnema and Verweij, 1981] reported 23 of them in the Netherlands for more or less the same time period.

Nowadays and according to the United Nations World Drug Report [UNODC, 2004], it seems that the number of detected amphetamine clandestine laboratories has increased in recent years after falling in the 1990's. The production is mainly concentrated in Europe and the Netherlands, Poland and Belgium are the most frequently mentioned source countries although some others have also reported the dismantling of amphetamine clandestine laboratories (Russian Federation, Germany, Bulgaria, the Baltic countries, UK and France).
Moreover, in 2002, more than 80 % of all BMK seizures (the main precursor to amphetamine, also known as P2P or 1-phenyl-2-propanone) were made in Europe.

Also, in relation to trafficking, 90 % of all seizures were made in Europe and within Europe, more than 90 % in Western Europe (period 2001-2002). Over the last few years, the world's largest amphetamine seizures have been made in the UK followed by Belgium, the Netherlands, Germany and Sweden. Most amphetamine seized in the EU is produced in the member states. The drugs are subsequently distributed across the EU. According to Europol [Europol, 2001], quantities of up to 30 kg are smuggled to the Nordic member states by car whereas the larger amounts of 100 kg or more are transported in lorries.

As for consumption, it seems that amphetamine is mainly a « northern » problem. Indeed, it is still the number one illicit drug in Sweden and Finland and high levels of abuse have been reported by the UK and Denmark [UNODC, 2004]. No data is available for Norway but it seems that they have a similar situation compared to their neighbour Sweden.
Regarding the new EU member countries, the highest levels are found in Poland and Estonia. High figures are also reported for the Czech Republic but this concerns mainly methamphetamine which is a specific problem in this country.

Finally, with regards to Switzerland, it is quite difficult to obtain reliable statistics as amphetamine is often confounded with other illicit synthetic drugs such as methamphetamine and MDMA. Moreover, there seems to be no distinction between amphetamine in tablet and powder form. Still, according to federal statistics [OFP, 1999], the number of cases involving amphetamine has increased steadily from 4 in 1986 to almost 400 in 1999.
However, there is no indication that amphetamine is a serious issue in Switzerland and there is also no indication of possible home production.

A.2 Concepts of organic profiling [UNODC, 2000]

Amphetamine is not a plant-based drug and is considered to be purely synthetic. It is manufactured in illicit clandestine laboratories and as a consequence of the crude laboratory conditions under which it is produced, the final product is rarely pure. Indeed, depending on the synthetic process it will contain a number of by-products which are generated during the synthesis. Moreover, traces of the starting material and even impurities of the starting material may still be present in the final product.
The presence of by-products and their relative concentrations will depend on many factors such as :

- the synthetic method
- the starting material and its eventual impurities
- the proportions of the chemicals involved in the synthetic method
- general reaction conditions (temperature, pressure, time)
- extraction and purification processes (including cristallisation and washing)

Detailed chemical analysis will enable the detection of all these by-products as well as the measurement of their relative concentrations. By such an approach, a characteristic chemical signature can be assigned to every sample. The information generated can then be used to determine if two or more samples are connected. All relationships between samples may then provide information as to the extent of supply and distribution networks at a local, national or international level.
It may also help in determining common batch* memberships. In the case of amphetamine, it would not determine a geographical origin but a common source such as a batch coming from one illicit laboratory.
Finally, the chemical signature or « profile » may help in determining the method of production and eventually which specific chemicals have been employed. For amphetamine, the production methods are well known and, in the majority of cases, the determination of the synthetic method is quite straightforward. However, this is not the case for other synthetic drugs such as, for example, MDMA.
Profiling is generally used only for intelligence purposes and not as evidence. In the case of amphetamine, it is used in support of law enforcement investigations to :

- confirm or negate a connection between two or more samples.
- provide general intelligence information in relation to the distribution network (at a local, national or international level).
- find links between samples that would not have been suspected by other means.
- monitor the methods used in drug manufacture and identify eventual new trends.

When taking about profiling, one should also bear in mind the concept of batch variation. Indeed, when amphetamine is manufactured, separate and discrete batches of powder may be synthesised at any one time. Because the production conditions may never be reproduced exactly each time, variations will occur in the profile, i.e. different batches from the same clandestine operator or « laboratory » will generate different chemical signatures (so called inter-batch variation). In addition, depending on the size of the batch and because illicit products are usually non-homogeneous, differences may also be seen across a single batch (so-called intra-variation). In general, it is safe to assume that the inter-batch variation will always be bigger than the intra-batch variation. However, it is important to gather as much as possible information on such variations (when possible) in order to carry out proper interpretation of results. Indeed, without any indication of the range of these variations, it is quite difficult to determine by how much samples must differ before they can be assumed to be coming from different batches or sources.

Finally, the strength of evidence of a link between samples is determined by the closeness of their respective profiles but also by the frequency of the particular pattern of the profile. If it can be demonstrated that the pattern of a particular profile is of an unusual type, then the evidential value of the link is increased. Thus, the need of building a reference population of profiles (in a appropriate database for example) in order to improve the interpretation of links between samples.
In profiling, other characteristics can also be important in interpreting links between samples. These are : i) cutting agents, ii) visual and physical characteristics (especially for drugs in tablet form) and iii) packaging. However, these characteristics were not addressed in this project as it focused only on the organic profiling of amphetamine.

* A batch is considered here as an amount of powder coming from one cristallisation process.

A.3 The Project

Development of a harmonised method for the profiling of amphetamine has been a multi-task project which began on February 1st, 1999 and ended on October 30th, 2002, after a 9 months extension period. The ultimate aim of the project was to develop a harmonised, collaboratively tested method for the profiling of illicit amphetamine so that meaningful analytical data, for use in the control of amphetamine, may be distributed on a pan-European basis [Ballany et al., 2001].
Capillary gas chromatography (GC) has been commonly applied to chemical profiling of amphetamine which is mainly due to its user-friendliness and to its high separation power. The existing profiling methods [Jonson et al., 1993, King et al., 1994, Kärkkäinen et al., 1994, Krawczyk et al., 2001] are however developed only for amphetamine synthesized through the so-called Leuckart method. In real life, at least three principle routes employed for the synthesis of amphetamine can be identified, namely (i) the nitrostyrene route, (ii) the reductive amination of benzyl methyl ketone and (iii) the Leuckart synthesis. Regardless that GC has its limitations such as decomposition of thermo-labile compounds, typically found in the amine family, it still can be considered superior to other separation techniques e.g. in terms of high separation power, stable retention times and user-friendliness. It was therefore chosen for this study.

The development of the profiling method was broken down into a seven distinct phases taking into consideration all three types of amphetamine:

1. Synthesis of documented standards for use throughout the project;
2. Study of the stability of the impurities in solution;
3. Optimisation of GC and detector systems;
4. Optimisation of extraction protocols;
5. Determination of variability of results;
6. Investigation of numerical classification schemes for amphetamine;
7. Report preparation and completion.

Moreover, each task was subdivided into subtasks which have been discussed in more detail under each task.
The technical work was performed in four partner laboratories whereas the work programmes were devised together with all partners of the project (four active + three advisers). However, the following description outlines in more detail the work performed by the author of this thesis (further referred to as **partner 4** in the text) :

Task 1 :

21 target compounds were synthesised by the four partners. Partner 4 performed the syntheses of five target compounds (n° 17 to 21, see table 1). GC-MS and FTIR data was collected by partner 4 for these five compounds. Dr Frank Dunand and Dr Amira Abou-Hamdan from the Institute of Mineral and Analytical Chemistry (ICMA, University of Lausanne) are greatly acknowledged for the acquisition of the NMR data. Partners 1 and 2 are likewise acknowledged for the acquisition of the UV data.
Syntheses and analytical data of compounds 1 to 16 were performed and acquired by partners 1, 2 and 3.
The remaining information found in this task was solely the work of partner 4.

Task 2 :

Linearity of GC-FID responses for compounds 17 to 21 was determined by partner 4. The stability of these compounds in various solvents was also determined by partner 4. In addition, a batch of amphetamine sulfate was synthesised (via the Leuckart route) and analysed by partner 4 in order to study the stability of present target compounds. The aim was to determine if the detected target compounds were more stable when present in the amphetamine matrix (and then extracted) as opposed to the synthesised standards directly dissolved in the solvent.
Results for compounds 1 to 16 were obtained from partners 1, 2 and 3.

Task 3 :

For the study of the injection technique (task 3.2), again a Leuckart amphetamine batch was synthesised and analysed by partner 4. The amphetamine extracts were analysed using split and splitless injections with inlet temperatures of 220, 240, 260 and 280°C. The same extracts were sent to partner 2 for analysis and comparison with cool on column injection (reference sample introduction method).

In task 3.3 (choice of column and temperature programme), the amphetamine extracts were analysed by partner 4 with six different temperature programmes (2, 4, 6, 8, 10 and 12°C per minute) and on three different analytical columns (Ultra 1, Ultra 2 and HP 50+). Separation power, resolution and inertness values were then calculated for all conditions. The same work was done by the other partners using their own synthesised amphetamine. In addition, partner 2 briefly studied four supplementary analytical columns.
In task 3.4 (selection of detection technique), a modified Grob sample (test sample consisting of a mixture of alkanes, esters, amines and phenolic compounds) was prepared by partner 4. Repeatability, reproducibility, sensitivity and linearity for all compounds in the test sample were determined for both GC-FID and GC-MS. The same work was carried out in the laboratories of partners 2 and 3. NPD (Nitrogen-Phosphorous Detector) was further evaluated by partner 3.
Finally, the liner study described in task 3.6 was solely the work of partner 4.

Task 4 :

Six amphetamine batches were synthesised by partner 4 and then mixed together to obtain 57 grams of amphetamine sulfate. This amount was necessary to perform all studies throughout the task. Various combinations of buffer types (with different pH), buffer concentrations, buffer volumes, solvents and solvent volumes were tested and studied on the synthesised amphetamine. Results were evaluated according to recovery, sensitivity and repeatability of target compounds. Each partner performed the same experiments on their own synthesised amphetamine. However, partner 4 synthesised an extra batch of nitrostyrene amphetamine in order to get more results about nitrostyrene target compounds.
Matrix effects were also studied by cutting the synthesised amphetamine with various amounts of caffeine. Again, each partner performed the same analyses on their own synthesised amphetamine. This latter remark is also valid for the final subtask (optimisation of solid phase extraction). However, preliminary experiments were performed by partner 2. This lead to the choice of two SPE columns. Each partner evaluated the recovery and repeatability of these two columns and also compared both SPE columns with the optimised liquid-liquid extraction method.

Task 5 :

Task 5 was entirely devised by partner 4. All data was collected, treated and reported by partner 4. In addition, partner 4 synthesised three new batches of Leuckart amphetamine and two new batches of nitrostyrene amphetamine for this task.

Task 6 :

One batch of BMK (precursor) and three new batches of amphetamine were synthesised by partner 4. In addition, six batches of Leuckart amphetamine were synthesised according to the same recipe under controlled conditions. Partner 3 carried out the same syntheses in order to get some insight into the variability between amphetamine synthesised by two operators following the same recipe.
More than 100 illicit samples were also collected thanks to the collaboration of a few police forces (Zurich Canton, Geneva, Neuchatel, Valais, Jura) and special thanks are directed towards Dr Michael Bovens of the scientific section of the Zurich city police for providing the great majority of the samples.
In total (partners 2, 3 and 4), 768 samples were analysed and their results available for data interpretation. These results were shared between the three partners.
Although the various decisions regarding the work programmes and the way forward were the result of group discussions (choice of pre-treatment methods and their evaluation, choice of distance metrics and their evaluation, reduction of target compounds, use of principal component analysis, etc.), task 6 presented in this thesis was rewritten by partner 4 in order to present the results in a different way. Most of the data was also re-evaluated by partner 4 (for example, evaluation of false positives and false negatives).

Chapter 7 :

This task was not part of the project and belongs only to this thesis.

Chapters 8 and 9 :

Were solely the work of the author of this thesis.

1. Task 1 – Synthesis and Identification of Amphetamine and Potentially Route Specific Impurities

1.1 Introduction

The task started by synthesising amphetamine through each of the three synthetic routes (Leuckart, reductive amination and nitrostyrene). The produced amphetamines were then analysed to identify typical synthesis impurities. This data was used parallel to the information found in the literature. The final group of target compounds consisted therefore of compounds synthesised in the four active laboratories and, additionally, of compounds that were identified or tentatively identified in the home-made amphetamine batches.
21 amphetamine impurities were successfully synthesised, and their identity confirmed by various spectroscopic techniques.

1.2 Phenyl-2-propanone syntheses

In this chapter, some details will be given regarding the various syntheses of phenyl-2-propanone. This precursor to amphetamine (and methamphetamine), also known as P2P, phenylacetone, BMK or benzylmethylketone is a controlled substance according to the United Nations Convention against illicit traffic in narcotic drugs and psychotropic substances (1988). For sake of simplicity, this substance will be named BMK throughout this text.
However, this commercial substance, although controlled, can easily be synthesised from uncontrolled precursors. The aim of this chapter is to emphasise the availablity of synthetic methods and ease of synthesis of this vital precursor which is widely used in the manufacture of amphetamine and also methamphetamine.

Numerous published methods are available to synthesise BMK. However, the diagram in the next page illustrates the main routes found in the literature (see figure 1). In summary, BMK can be synthesised from :

- Phenylacetic acid
- Alpha-acetylbenzylcyanide (also known as alpha-acetylphenylacetonitrile or 2-phenylacetoacetonitrile)
- Benzene
- Allylbenzene
- Benzyl cyanide (phenylacetonitrile)
- Benzyl chloride
- Benzaldehyde
- 2-Phenylpropanal (2-phenylpropionaldehyde, hydratropaldehyde)
- Ephedrine, pseudoephedrine, norpseudoephedrine (phenylpropanolamine) or norephedrine
- Phenyl-2-nitropropene (also known as trans-beta-methyl-beta-nitrostyrene)
- Bromobenzene
- Alpha-methylstyrene (2-phenylpropene, isopropenylbenzene)

Diagram of various methods for synthesis of BMK

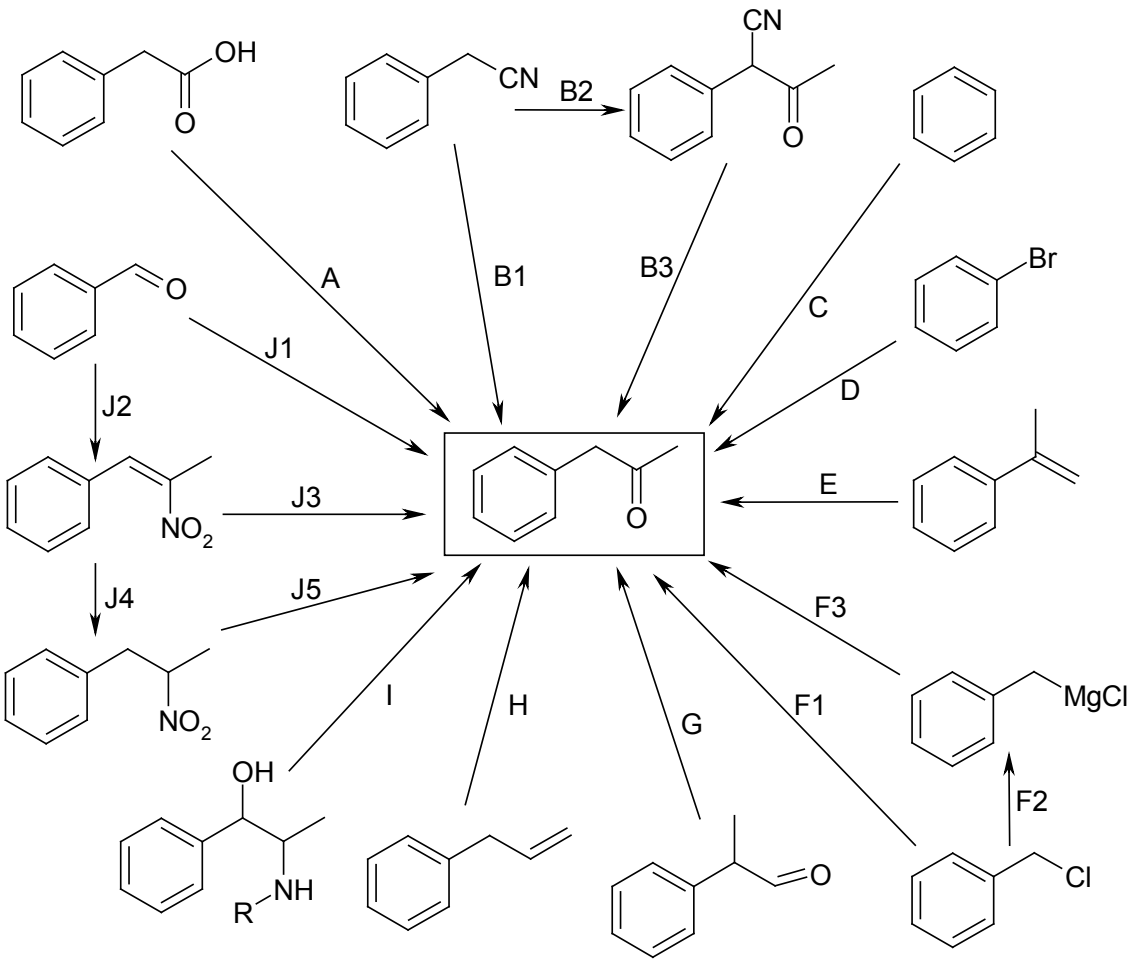


Figure 1 : various precursors and routes in the synthesis of BMK

A :	From phenylacetic acid :	Sodium acetate and acetic anhydride :		[Magidson and Garkusha, 1941] [Forbes and Kirkbride, 1992] [Hurd and Thomas, 1936]
		Acetic anhydride and pyridine :		[King and McMillan, 1951]
		Lead acetate :		[Tsutsumi, 1953] [Allen et al., 1992]
B1 :	From benzyl cyanide :	trimethylaluminium :		[Lau et al., 1997]
B2 :	Benzyl cyanide to alpha-acetylbenzylcyanide :			
		Sodium ethoxide in ethyl acetate		[Julian et al., 1943b]
B3 :	From alpha-acetylbenzylcyanide :		Sulfuric acid Phosphoric acid	[Julian and Oliver., 1943a] [Bobranski and Drabik, 1942]
C :	From benzene :	2-nitropropene and titanium chloride 2-nitropropene and triflic acid chloroacetone and $AlCl_3$: acetone and Mn (III) acetate :		[Lee and Oh, 1988] [Okabe et al., 1989] [Mason and Terry, 1940] [Kurz et al. 1984]

D :	From bromobenzene :	potassium acetylacetonate / CuI in DMF : Heck arylation :	[Sugai et al., 1982] [Kosugi et al., 1984]
E :	From alpha-methylstyrene :	Thallium nitrate :	[McKillop et al., 1970]
F1 :	From benzyl chloride :	Electrolysis with acetic anhydride : Acetonitrile	[Chaussard and Moingeon, 1986] [Bombard et al., 1980] [Jones et al., 1972]
F2 :	Benzyl chloride to benzylmagnesium chloride :		[Newman and Booth, 1945]
F3 :	From benzylmagnesium chloride :	Acetic anhydride :	[Newman and Booth, 1945]
G :	From 2-phenylpropanal :	$HgCl_2$ or sulfuric acid . Bromine / H_2SO_4 :	[Danilow and Danilowa, 1927] [Inoi and Okamoto, 1969]
H :	From allylbenzene :	H_2O_2 / formic acid Pd acetate / benzoquinone	[Fujisawa et al. 1958] [Dal Cason et al., 1984] [Miller and Wayner, 1990]
I .	From ephedrine or pseudoephedrine (R = CH_3). Norephedrine or norpseudoephedrine (R = H)		
		Sulfuric acid and zinc chloride :	[Brauch, 1982] [Blanke and Brauch, 1983]
J1 .	From benzaldehyde :	Phosphonium ylide Via glycidic ester	[Coulson, 1964] [Elks and Hey, 1943]
J2 :	Benzaldehyde to phenyl-2-nitropropene :	nitroethane :	[Shulgin, 1991] [Gairaud and Lappin, 1953]
J3 :	From phenyl-2-nitropropene :	Sodium borohydride : Iron and HCl : Chromium (II) chloride : Tin chloride and Mg : Nickel chloride and Al : $LiAlH_4$:	[Ballini and Bosica, 1994] [Shulgin, 1991] [Hass et al., 1950] [Varma et al. 1985] [Das et al., 1996] [Bezbarua et al. 1999] [Gilsdorf and Nord, 1952]
J4 :	Phenyl-2-nitropropene to phenyl-2-nitropropane :	$LiAlH_4$:	[Gilsdorf and Nord, 1952]
J5 :	From phenyl-2-nitropropane :	NaOH / H_2SO_4 :	[Gilsdorf and Nord, 1952]

Note :

In Switzerland, according to the O Prec-Swissmedic (Ordonnance de l'institut suisse des produits thérapeutiques sur les précurseurs et autres produits chimiques utilisés pour la fabrication de stupéfiants et de substances psychotropes, Nov 8, 1996 ; updated in November 2001), only BMK, phenylacetic acid, ephedrine, norephedrine, phenylpropanolamine (norpseudoephedrine) and pseudoephedrine are controlled substances (as in the 1988 United Nations Convention against illicit traffic in narcotic drugs and psychotropic substances).
All the other potential precursors (alpha-acetylbenzylcyanide, benzene, bromobenzene, allylbenzene, benzyl cyanide, benzyl chloride, benzaldehyde, 2-phenylpropanal, phenyl-2-nitropropene and alpha-methylstyrene) are commercially available and not under international control. However, in Switzerland, allylbenzene, benzaldehyde, benzyl chloride and benzyl cyanide are on the Alert List of the Chemical Industry [OFSP, 1997]. This means that any purchase of these chemicals is recorded and the report to authorities is on a voluntary basis.
In other words, if the vendor suspects the chemical will be used for illicit drug manufacture, he is encouraged to report it to the authorities but this is not compulsary.

The synthetic routes to BMK described above vary in terms of cost, simplicity and skill. However, it is important to mention that route C (benzene, chloroacetone and aluminium chloride) was used in one of the biggest amphetamine clandestine laboratory found in Europe which was dismantled in 2000 [Bakouri and van Rijn, 2001].

1.3 The Leuckart route

This route is named after R. Leuckart who first published his method of aminating ketones into amines in 1885 [Leuckart, 1885]. The two-step reaction is the following :

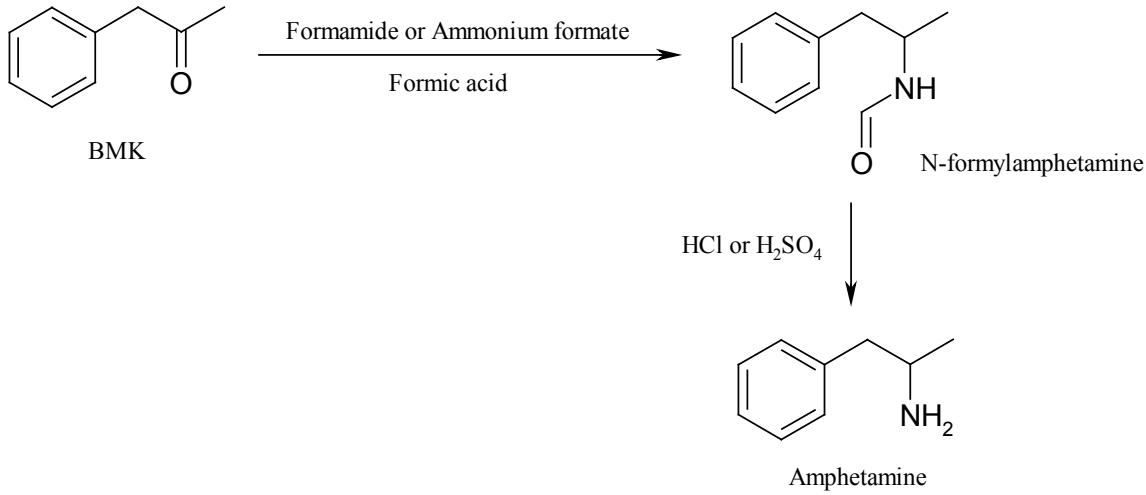


Figure 2 : the Leuckart route

Originally, Leuckart and subsequently Wallach [Wallach, 1891] used formamide or ammonium formate and heated the reaction mixture in sealed tubes at 210-240°C. Later one, Ingersoll and others showed that refluxing the mixture was an excellent alternative and that the use of formamide gave better yields [Ingersoll et al., 1936, Crossley and Moore, 1944, Moore, 1949].
In this reaction, BMK is refluxed with formamide at temperatures between 150 and 170°C. Formic acid is added to improve the yield of the intermediate N-formylamphetamine.
For the hydrolysis step, sulfuric acid or hydrochloric acid are generally used. Boiling times can vary a lot. Generally, 3 to 6 hours refluxing is the rule for the first step while 1 to 3 hours are typically performed for the hydrolysis step.

1.4 The reductive amination route

This synthesis is a more straightforward reaction and is performed in one step. The precursor is the same as for the Leuckart route (BMK). Ammonia or ammonium acetate are used as the amine reactants and sodium borohydride ($NaBH_4$) or sodium cyanoborohydride ($NaBH_3CN$) [Borch et al., 1971, Braun et al., 1980, Schulgin, 1991] are used as the reducing agents :

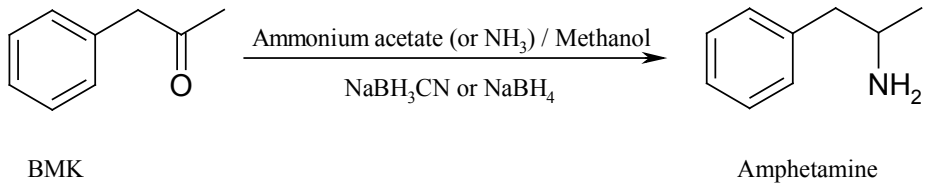


Figure 3 : the reductive amination route

There are various alternatives to reduce ketones to primary or secondary amines and most of them have been reviewed by Allen and Cantrell [Allen and Cantrell, 1989]. It is interesting to note that, although much easier (the reaction can be carried out at room temperature) and much cleaner (higher purity) than the Leuckart method, it has not been found to be a popular method for the manufacture of amphetamine. On the other hand, this synthetic route is widely used for the production of MDMA (MethyleneDioxyMethylAmphetamine, the active ingredient of ecstasy).

One explanation could be the lack of publications with regard to primary amines. Indeed, the main applications of this synthetic route have been described for secondary amines rather than primary amines such as amphetamine or MDA (MethyleneDioxyAmphetamine).

1.5 The nitrostyrene route

In this case, it's also a reductive amination but the precursor is phenyl-2-nitropropene. This compound can be purchased (Aldrich Chemicals) or synthesised from benzaldehyde [Schulgin, 1991, Noggle et al., 1994]. Reduction of phenyl-2-nitropropene to amphetamine can be carried out with various reducing agents such as Red-Al [Butterick and Unrau, 1974], sodium borohydride with BH_3-THF [Mourad et al, 1984] or lithium aluminium hydride ($LiAlH_4$) [Noggle et al., 1994, Kabalka and Varma, 1990]

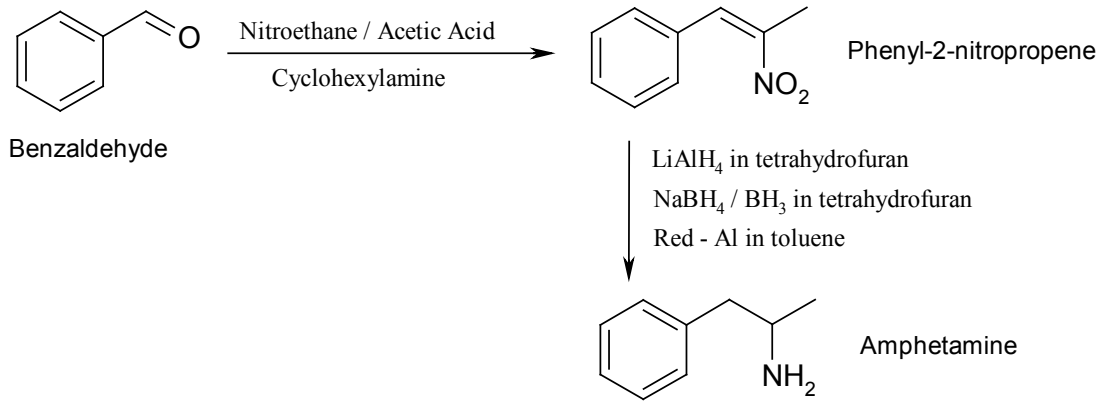


Figure 4 : the nitrostyrene route

1.6 Miscellaneous

Of course, many other synthetic routes can be found in the open literature. Amphetamine is a rather simple molecule and there are various ways to synthesise it. Again, Allen and Cantrell [Allen and Cantrell, 1989] have reviewed most of them as well as Dal Cason where various synthetic routes were described for the manufacture of MDA, the methylenedioxy derivative of amphetamine [Dal Cason, 1990]. However, it was considered useful to illustrate three others :

The Ritter reaction :

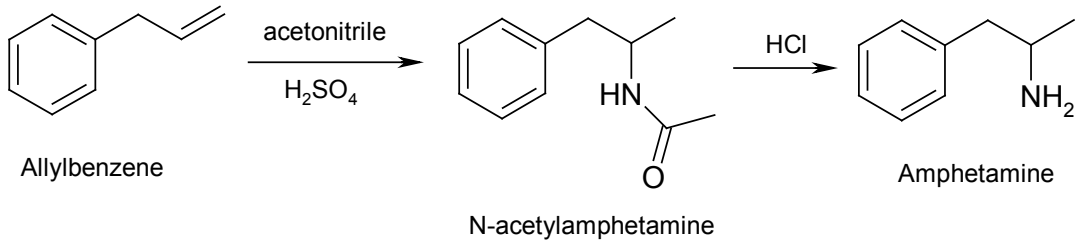


Figure 5 : the Ritter route

Allylbenzene is simply mixed with acetonitrile and sulfuric acid in an ice-bath. The intermediate N-acetylamphetamine will be formed. Then, it is hydrolysed with hydrochloric acid to produce amphetamine [Ritter and Kalish, 1948, Noggle et al., 1995]. It is important to mention that this reaction will not work if safrole is used instead of allylbenzene to produce MDA (safrole being the corresponding methylenedioxy derivative of allylbenzene) [Ritter and Murphy, 1952, Noggle et al., 1995, Robak, 1995].

Direct and one-pot reaction from benzyl cyanide [Rinehart et al., 1987]

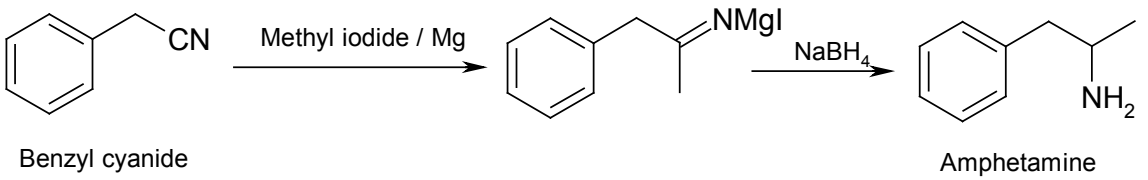


Figure 6 : synthesis of amphetamine from benzyl cyanide

This new method has the advantage of being performed at room temperature and is a one-step synthesis. Methyl iodide and magnesium turnings are added to dry tetrahydrofuran to form a solution of methylmagnesium iodide. This was added to a cooled solution of benzylcyanide in tetrahydrofuran. The reaction mixture is then stirred at room temperature and cooled in an ice-bath. The solution is then diluted with methanol and sodium borohydride added. The reaction mixture is further stirred at room temperature. In theory, this very easy synthesis should give 80 % yield.
This uncommon route is one illustration of the various possibilities to synthesise amphetamine from uncontrolled and very cheap chemicals. Indeed, the example here uses benzyl cyanide (also known as phenylacetonitrile) which costs approximately 12 Euro per litre.

Synthesis from norephedrine or norpseudoephedrine (phenylpropanolamine).

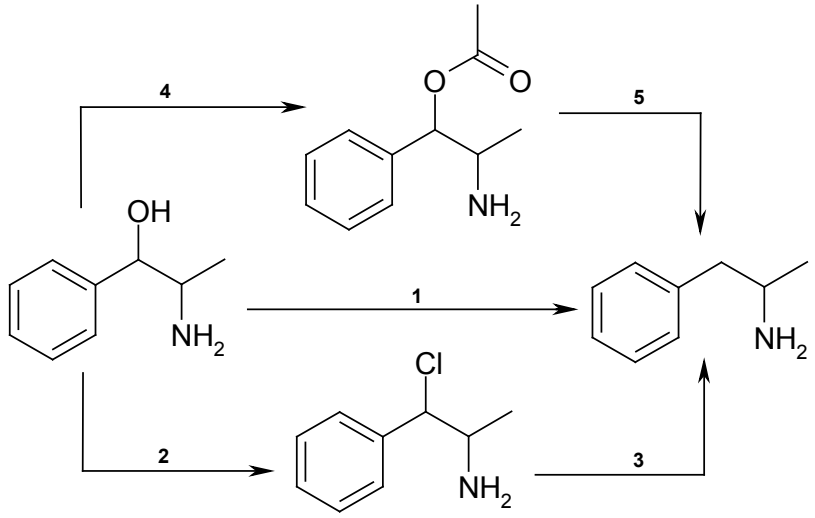


Figure 7 : synthesis pathways from norephedrine or norpseudoephedrine to amphetamine

This route is derived from several popular methods used to synthetise methamphetamine [Remberg and Stead, 1999] where ephedrine (or pseudoephedrine) is the starting material instead of norephedrine or norpseudoephedrine. Boswell has decribed three different ways to synthetise amphetamine from norephedrine or norpseudoephedrine [Boswell et al., 2002]. Norephedrine is reacted 22 hours with hydriodic acid and red phosphorous to yield amphetamine in a relatively low yield of 38 % (route **1**). This route is very well known in methamphetamine synthesis [Skinner, 1990] and can be adapted as well for amphetamine by substituting the starting material. The second method consists of reacting norephedrine with thionyl chloride to form the chlorinated intermediate (route **2**). The latter was then submitted to catalytic hydrogenation (palladium on carbon) to obtain amphetamine as an oil with a yield of 72 % (route **3**). This method is also used in methamphetamine synthesis [Allen, 1987] and again can be adapted to amphetamine.

In the third method, norephedrine is reacted with acetic acid and acetic anhydride to form O-acetylnorephedrine (route **4**). The latter is then submitted to catalytic hydrogenation (10 % palladium on carbon) or catalytic transfer hydrogenation (10 % palladium on carbon and ammonium formate) to give amphetamine in, respectively, 65 % and 89 % yield (route **5**).
It should be noted that the use of 1S, 2S-(+)-norpseudoephedrine or 1R, 2S-(-)-norephedrine will produce the optical isomer d-amphetamine [Noggle et al., 1987]. If d,l-norephedrine is used as the starting material, then the racemic amphetamine will be obtained. The latter will also be obtained with the aforementioned synthesis methods (Leuckart, reductive amination, nitrostyrene, Ritter).

1.7 Experimental

First, the synthesised amphetamines were analysed by gas chromatography – mass spectrometry (GC-MS) to identify the synthesis products, intermediates and by-products. It is self-evident that amphetamine synthesis produces numerous products that cannot all be identified. Indeed, a typical amphetamine profile can contain more than 200 peaks (see figure 8 below). Therefore, identification and separate synthesis of all these compounds is impossible. For these reasons and because of the short time available for this task, only a limited number of compounds (21) were chosen for further investigations.

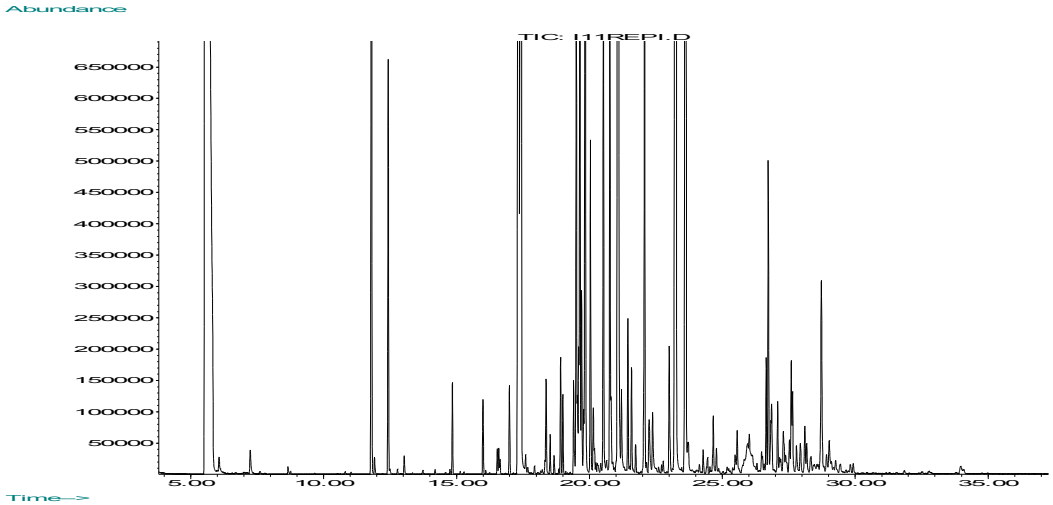


Figure 8 : typical amphetamine profile of a synthesised sample (Leuckart route)

Syntheses of the chosen impurities, i.e. target compounds were carried out. The synthesis methods used are described in **Annex 1**. **Table 1** (see next page) lists the compounds produced by each Partner. The reader is noted that abbreviated names of the target compounds have been used in the later parts of this text.

GC-MS was used to identify and determine the purity of the synthesised impurities. The latter was estimated on the basis of the total ion chromatogram (TIC), which was recognised as non-ideal technique but sufficient for the intended use. The GC-MS method was based in part, but not wholly, on published methods and the method was reached by empirical experimentation by the group. It was not considered as the final optimised method, but well-suited for the identification and purity analysis of the synthesis products. The GC-MS method (Method 1) has been described in detail in **Annex 2**.

Deeper structural information on the synthesised products was obtained using FTIR, UV and NMR spectroscopy. Infra-red spectra (450 to 4000 cm^{-1}) were obtained using KBr, i.e. each of the compounds as thin films on KBr, or mixed with KBr. Alternatively, IR spectra was recorded using the Attenuated Total Reflection Fourier Transform Infra Red Spectroscopy (ATR-FTIR) directly from the synthesised product or using the Diffuse Reflectance Infrared Fourier Transform Spectroscopy (DRIFTS), between 600 to 4000 cm^{-1}.

In respect of NMR data, ^{1}H NMR data were obtained at 250 MHz (400 MHz Partner 4) with the sample dissolved in $CDCl_3$. ^{13}C NMR data were obtained at 90 MHz (62.9 MHz Partner 3) with the sample again dissolved in $CDCl_3$.

Ultraviolet spectra were obtained for the compounds as synthesised in (i) methanol, (ii) 0.1M sodium hydroxide in methanol and (iii) 0.1M sulphuric acid in methanol.

All the chemical structures and spectroscopic data can be found in **Annex 3** and the various instruments used in this task have been summarised in **Table 2**.

Table 1 Compounds synthesised by each Partner during the course of Task 1.

Number	Trivial name	Abbreviation	Molecular formula	MW	Responsible Partner
1	Nitrostyrene		$C_9H_9NO_2$	163.17	1
2	Benzyl methyl ketoxime	ketoxime	$C_{10}H_{14}NO$	164.22	1
3	N-(β-phenylisopropyl)benzaldiimine	benzaldimine	$C_{17}H_{21}N$	239.36	1,2
4	2-methyl-3-phenylaziridine	aziridine	$C_9H_{11}N$	133.19	1
5	N-(β-phenylisopropyl)benzyl methyl ketimine	ketimine	$C_{18}H_{21}N$	251.37	2
6	N-acetylamphetamine		$C_{11}H_{15}NO$	177.24	2
7	Benzoylamphetamine		$C_{16}H_{17}NO$	239.31	2
8	Benzylamphetamine		$C_{16}H_{19}N$	225.33	2
9	phenyl-2-propanol		$C_9H_{12}O$	136.19	2
10	1-oxo-1-phenyl-2-(β-phenylisopropylimino)propane	1-oxo	$C_{18}H_{19}NO$	265.35	2
11	2-oxo-1-phenyl-2-(β-phenylisopropylamino)ethane	2-oxo	$C_{17}H_{19}NO$	253.34	2
12	N,β-hydroxy-N,N-di(β-phenylisopropyl)amine	cathinol	$C_{20}H_{31}NO$	301.47	2
13	N-(β-phenylisopropyl)cathinone	cathinone	$C_{20}H_{29}NO$	299.45	2
14	4-methyl-5-phenylpyrimidine		$C_{11}H_{10}N_2$	170.21	3
15	1,3-diphenyl-2-propylamine	DPPA	$C_{15}H_{17}N$	211.30	3
16	4-benzylpyrimidine		$C_{11}H_{10}N_2$	170.21	3
17	N-formylamphetamine		$C_{10}H_{13}NO$	163.21	4
18	N,N-di-(β-phenylisopropyl)amine	DPIA	$C_{18}H_{23}N$	253.38	4
19	N,N-di-(β-phenylisopropyl)methylamine	DPIMA	$C_{19}H_{25}N$	267.41	4
20	N,N-di-(β-phenylisopropyl)formamide	DPIF	$C_{19}H_{23}NO$	281.39	4
21	2,4-dimethyl-3,5-diphenyl pyridine		$C_{19}H_{18}N$	260.35	4

Table 2 Instruments used by each Partner for obtaining the spectroscopic data.

Technique	Partner			
	1	2	3	4
GC-MS	HP6890/5973 (Agilent)	HP6890/5973 (Agilent)	HP6890/5973 (Agilent)	HP6890/5973 (Agilent)
FTIR	ATI Mattson Genesis Series	Spectrum 1600 (Perkin-Elmer)	Spectrum 2000 (Perkin-Elmer)	Mattson Cygnus 100
^{1}H and ^{13}C NMR	Bruker DPX 40	Varian Inova 300 and Bruker Avance DRX 500	Bruker AF250	Bruker DPX 400
UV	Nicam UV2	HP1090 DAD (Hewlett-Packard)	HP 8453 (Hewlett-Packard)	Nicam UV2

The 21 synthesised compounds are briefly discussed below (IUPAC names are in brackets) :

(1) Nitrostyrene [(2-Nitroprop-1-enyl)benzene]

Nitrostyrene, also known as phenyl-2-nitropropene or trans-beta-methyl-beta-nitrostyrene is the intermediate compound in the so-called nitrostyrene route. It was therefore considered important to confirm identity by synthesising it and to use it as target compound. It is also commercially available from Sigma-Aldrich chemicals.

(2) Benzyl methyl ketoxime [1-Phenylpropan-2-one oxime]

Benzyl methly ketoxime, also abbreviated ketoxime through out this text, is thought to be the result of uncomplete reduction of nitrostyrene to amphetamine. It is therefore considered as an impurity specific to the nitrostyrene route. However, controlled syntheses showed that it could also be found in amphetamine synthesised by the reductive amination and Leuckart routes (although in lesser amounts). Thus, it is not a route-specific impurity. In addition, this impurity occurs as two peaks in the chromatogramme because of the presence of the two isomers (E and Z).

(3) *N*-(*β*-Phenylisopropyl)benzaldiimine [1-Phenyl-N-(phenylmethylidene)propan-2-amine]

This impurity, abbreviated benzaldiimine or aldiimine, is an intermediate compound from the reductive amination of BMK to amphetamine. It is thought to be the result of a reaction between amphetamine and benzaldehyde, the latter being an impurity of commercial or synthesised BMK [Theuween and Verweij, 1980]. However, this impurity has been found in amphetamine synthesised via the three synthetic routes. Thus, it is not a route-specific impurity.

(4) 2-Methyl-3-phenylaziridine

This compound seems to be the result of reduction of the ketoxime. This impurity is therefore not theoretically route-specific. However, high amounts of this impurity was only found in nitrostyrene amphetamine whereas only traces were detected in reductive amination amphetamine. No traces of this impurity was found in Leuckart amphetamine. Synthesis of this impurity was carried out according to Kotera [Kotera et al., 1968].

(5) *N*-(*β*-Phenylisopropyl)benzyl methyl ketimine [N-(1-Methyl-2-phenylethylidene)-1-phenylpropan-2-amine]

This impurity, abbreviated ketiimine, is an intermediate compound from the reductive amination of BMK to amphetamine. It is thought to be the result of a reaction between amphetamine and BMK. However, this impurity has been found in amphetamine synthesised via the three synthetic routes [Sinnema and Verweij, 1981]. Thus, it is not a route-specific impurity. Isolation of this compound was difficult as it was found to be unstable.

(6) *N*-Acetylamphetamine [N-Benzyl-1-phenylpropan-2-amine]

This impurity has been found in amphetamine synthesised via the three synthetic routes [Theeuwen and Verweij, 1981]. The origin of this impurity remains unclear. One hypothesis is the acetylation of amphetamine from the following sources :

- Acetamide present as an impurity in commercial formamide (Leuckart).
- Acetic acid present as an impurity in commercial formic acid (Leuckart).
- Ammonium acetate in the reductive amination of BMK and the reduction of nitrostyrene.
- Traces of formaldehyde / acetaldehyde in methanol

(7) Benzoylamphetamine [N-(1-Methyl-2-phenylethyl)benzamide]

This impurity has been found in amphetamine synthesised via the three synthetic routes. The origin of this impurity remains also unclear.

(8) Benzylamphetamine [N-Benzyl-1-phenylpropan-2-amine]

This impurity has been found in amphetamine synthesised via the three synthetic routes. The origin of this impurity seems to be the same as for the aldiimine. In this case, the aldiimine is further reduced to produce benzylamphetamine.

(9) 1-Phenyl-2-propanol [1-Phenylpropan-2-ol]

When BMK is reduced to amphetamine, a competing reaction is the reduction to phenyl-2-propanol. Therefore, this impurity is often detected in high amounts in reductive amination amphetamine [Van den Ark et al., 1978b]. However, it is also observed in Leuckart and nitrostyrene amphetamine. Hence, it is not considered as a route-specific impurity.

(10) 1-Oxo-1-phenyl-2-(*β*-phenylisopropylimino)propane [2-[(1-Methyl-2-phenylethyl)imino]-1-phenylpropane-1-one]

This impurity has been found in reductive amination amphetamine [Theeuwen and Verweij, 1981]. The origin of this impurity is thought to be the result of the reaction between amphetamine and phenyl-1,2-propanedione, the latter being an impurity of commercial or synthesised BMK. Isolation of this compound was difficult as it was found to be unstable.

(11) 2-Oxo-1-phenyl-(β-phenylisopropylamino)ethane [N-(1-Methyl-2-phenylethyl)-2-phenylacetamide]

This impurity has been found in amphetamine synthesised via the three synthetic routes. The origin of this impurity remains unclear.

(12) N-β-Hydroxy-N,N-di(β-phenylisopropyl)amine [2-[(1-methyl-2-phenylethyl)amino]-1-phenylpropan-1-ol]

This impurity has been found in reductive amination amphetamine. The origin of this impurity is thought to be the result of a further reduction of 1-oxo via cathinone (compound 13).
It contained two diastereoisomers and recristallisation gave only one pure diastereoisomer.

(13) N-(β-Phenylisopropyl)cathinone [2-[(1-Methyl-2-phenylethyl)amino]-1-phenylpropan-1-one]

This impurity has been found in reductive amination amphetamine. The origin of this impurity is thought to be the result of a further reduction of 1-oxo. For these three impurities (1-oxo, cathinol and cathinone), it has not been determined if they are route-specific or not. However, they have not been observed in the nitrostyrene and Leuckart amphetamines.

(14) 4-Methyl-5-phenylpyrimidine

This impurity has always been considered as a route-specific impurity for Leuckart amphetamine. It is thought to be a specific product of a reaction between one molecule of BMK and two molecules of formamide [Van den Ark et al., 1977b, Van den Ark et al., 1978d]. However, trace amounts have been found in synthesised reductive amination amphetamine. Nevertheless, if a fair amount of this impurity is detected, it is reasonable to assume that the synthetic route used was Leuckart.
Moreover, higher amounts of this impurity is produced when no formic acid is added during the Leuckart reaction [Huizer et al., 1985]. Synthesis of this impurity was carried out according to Koyama [Koyama et al., 1975].

(15) 1,3-Diphenyl-2-propylamine

This impurity is the product of the reaction between amphetamine and dibenzylketone [Noggle et al., 1985], the latter being an impurity of commercial or synthesised BMK [Kram, 1977]. Theoretically, it is therefore an impurity that could be found in Leuckart and reductive amination amphetamine. This was confirmed by controlled syntheses. Moreover, it was not detected in synthesised nitrostyrene amphetamine.

(16) 4-Benzylpyrimidine

Same remarks as compound 14. Synthesis of this impurity was carried out according to Abbotto [Abbotto et al., 1991].

(17) N-Formylamphetamine [1-Methyl-2-phenylethylformamide]

This compound is the intermediate product which leads to amphetamine in the Leuckart reaction (see paragraph 2.3). Its presence in the final product therefore results from incomplete hydrolysis.
For these reasons, it was believed (and still is) to be specific to the Leuckart route. However, the controlled syntheses showed us that this compound is also present in reductive amination amphetamine as well as nitrostyrene amphetamine (although in lesser amount).

(18) N,N-di-(β-Phenylisopropyl)amine [N-(1-Methyl-2-phenylethyl)-1-phenylpropan-2-amine]

This impurity is the product of the reaction between amphetamine and BMK. The unstable ketiimine (compound 5) is first produced which is further reduced to form this dimer also abbreviated DPIA. In the Leuckart reaction, it is the main impurity when formic acid is added in the first step of the reaction [Huizer et al., 1985].
However, as for the ketiimine, it is not route-specific and is detected in amphetamine synthesised via the three different routes.

(19) N,N-di-(β-Phenylisopropyl)methylamine [N-Methyl-N-(1-methyl-2-phenylethyl)-1-phenylpropan-2-amine]

This impurity is thought to be the result of the reduction of impurity 20 [Huizer et al., 1985] and has been identified first as an impurity in methamphetamine [Bailey et al., 1974]. As impurity 20 is a result of the formylation of DPIA (compound 18), it is therefore assumed that this impurity, together with impurity 20, could be specific to the Leuckart reaction. Indeed, formylation could not, theoretically, occur in reductive amination or nitrostyrene amphetamine as no formamide or formic acid are present in these reactions. Further, this impurity was not found in amphetamine synthesised via reductive amination or via the nitrostyrene route.
Synthesis of this impurity was carried out according to Schmitt [Schmitt et al., 1966].

(20) N,N-di-(β-Phenylisopropyl)formamide [bis-(1-methyl-2-phenylethyl)-formamide]

As mentioned above, this impurity is thought to be the result of the formylation of DPIA (impurity 18).Synthesis was carried out by boiling DPIA with formamide [Huizer et al., 1985].

(21) 2,4-Dimethyl-3,5-diphenylpyridine

This more complex impurity is thought to be the result of the reaction between two molecules of BMK with one molecule of formamide [Van der Ark et al., 1978]. Many pyridine impurities are detected in amphetamine synthesised via the Leuckart route. The differences reside in the various methyl and / or phenyl positions in the chemical structure. Unfortunately, partner 4 was only able to synthesises one of these pyridines. However, this is already quite an achievement as the synthesis of these pyridines has never been described in the forensic literature. Nevertheless, synthesis was possible by following the procedures described by Hauser [Hauser and Eby, 1956] and Wajon [Wajon and Arens, 1957].

The pyridine impurities are considered to be specific to the Leuckart route. However, trace amounts were found in reductive amination amphetamine. Still, the amounts detected were at the ultra trace level. Therefore, these pyridines (as the pyrimidines 14 and 16) remain very strongly indicative of the Leuckart route.

1.8 Preliminary conclusions from task 1

All together 21 compounds found as impurities in street amphetamine have been successfully synthesised. Full spectroscopic data of these compounds was recorded. This data set is unique and offers a huge potential for future developments in chemical profiling of amphetamine.
From these 21 impurities, few are considered to be route-specific. However, the data obtained in task 6 (see section 6.6.5) provide more clues as to the impurities which enable the distinction between the three synthetic routes studied in this project.

2. Task 2 – Stability of impurities in different solvents

2.1 Introduction

In gas chromatography it is essential that the analytes are stable in the solvent chosen for the introduction of the sample into the gas chromatograph. This is even more important when routine profiling analyses are performed. Indeed, 50 to 100 samples can be prepared on a given day and put on the sample tray of the gas chromatograph autosampler awaiting injection. Given the run time of a typical analysis (30 to 45 minutes), the time difference between the first injection and the last injection can be up to 100 hours. Therefore, it is of utmost importance that during this time delay no reaction occurs in the sample vial. Otherwise, the comparison between the first and last profile will be biased.

For these reasons, the stability of the synthesised impurities in various solvents as a function of time and temperature was investigated in Task 2. Also, the experiments were repeated with synthesised amphetamine in order to determine the stability of the impurities after liquid-liquid extraction.
The objective was to find the best solvent in terms of stability of the impurities.

The subtasks within Task 2 were the following :

- Evaluate the linearity of the detector response for each of the synthesised impurities.
- Examine the stability of the impurities at two different temperatures (8°C and 25°C) over time (0 - 96 hours) as a total mixture in six different solvents.
- Examine the stability of the impurities in extracts obtained from liquid-liquid extraction (LLE).

2.2 Results and discussion

2.2.1 Determination of linearity of FID and MSD response to target compounds

Linearity was evaluated by least squares regression analysis in the concentration range of 0.1 – 500 µg/mL in isooctane using Method 1 (**Annex 2**). Briefly, this method consisted of a GC-FID-MSD method, with splitless injection into two similar columns to produce simultaneously both the MSD and the FID trace. This dual-column assembly was applied by Partners 1, 2 and 4, whereas Partner 3 used two separate GC units for FID and MSD.

The linear regression equations have been described in **Annex 4**.

The linearity of the FID was studied at each participating laboratory prior to the stability study to validate the GC method. Partner 2 and Partner 3 used the calibration range 0.1 - 20 µg/mL. Partner 1 and Partner 4 used the ranges 0.1 - 500 and 0.1 - 250 µg/mL, respectively. All labs used tetracosane as the internal standard. The concentration was however different. Partner 2 and 3 used 10 µg/mL, while Partner 4 and Partner 1 used 100 µg/mL. This explains the difference in the calibration function.

All data yielded r^2 values greater than 0.994. Full results can be found in **Annex 5**.

Thus, all compounds tested produced linear detector responses from the GC – FID over the concentration range in which the forthcoming experimentation was to be undertaken. Linearity of the MSD was not investigated at this stage since it was applied to qualitative use only.

2.2.2 Determination of stability of impurities in synthetic mixtures and in synthesised amphetamine

The stability of the synthesised impurities, each at 10 µg/mL, was examined as a mixture (synthetic mixture) in six different solvents namely iso-octane (2,2,4-trimethylpentane), toluene, dichloromethane, diethyl ether, ethyl acetate and ethanol. The stability was evaluated over 0, 4, 12, 24, 48 and 96 hours at 25 ºC. Some of the experiments were also carried out at 8 ^{o}C if decomposition was observed at 25°C.

An internal standard (tetracosane : $C_{24}H_{50}$; 10 µg/mL) was added to all mixtures of compounds and each injection was made in duplicate.
Additionally, the stability of the impurities in extracts, prepared from the amphetamine synthesised as a part of this study, was investigated by the liquid-liquid extraction (LLE) method used routinely over the years in Partner 2 and 3 laboratories [Kärkkäinen et al., 1994, Jonson and Strömberg, 1993]. This consists of dissolving amphetamine (300 mg) in 3.0 mL phosphate buffer (63.2 mM, pH 7.00 ± 0.03) and extracting the solution with 1.2 mL of solvent. Synthesised impurities were added to the extract in cases they were otherwise absent in the extract. Ethanol was discarded from this experiment as it could not have been separated in the LLE process.

A thermostatic bath equipped with a liquid circulation system was attached to the GC autosampler tray to provide precise temperature control of the sample vials stored in the tray.

The results of the stability study can be found in **Annex 6.**

An example of how the calculations have been made is shown below :

$$\text{Relative response factor (RRF)} = \frac{\text{Peak area of impurity}}{\text{Peak area of internal standard}}$$

$$\text{Ratio} = \frac{\text{Relative response factor of time delay (0, 4, 12, 24, 48 or 96 h)}}{\text{Relative response factor at time 0}}$$

Example for 2,4-dimethyl-3,5-diphenylpyridine in isooctane at 25°C

	At T = 0	At T = + 4h	At T = + 12h	At T = + 24h	At T = + 48h	At T = + 96h	RSD
RRF	0.847	0.839	0.839	0.851	0.837	0.837	0.7 %
Ratio	1.00	0.991	0.991	1.005	0.988	0.988	0.7 %

As can be seen in the example above, no significant change was observed for this impurity in isooctane at 25°C (less than 1 % relative standard deviation). However, this was not the case with all impurities. The following paragraph describes the various problems encountered :

2.2.3 Unstable impurities in synthetic mixtures and in synthesised amphetamine

The stability of the **cathinol** in the Partner 2 synthetic mixture was poor as the compound was sometimes found in increasing concentration and sometimes the compound was completely lost. This phenomenon was most probably caused by the instability of the compound in the analytical system and thus its actual stability in different solvents could not be established. The stability of **N-(β-phenyl-isopropyl)benzyl methyl ketiimine** and **1-oxo-1-phenyl-2-(β-phenylisopropylimino)-propane** in the Partner 2 samples was poor causing nearly complete loss of these analytes. This observation was further confirmed by preparing and analysing a separate solution of only these two compounds.
DPPA in the synthetic mixture turned out to be unstable to some extent in dichloromethane, diethyl ether and in ethyl acetate.
Benzaldiimine seem to start to decompose after a while in ethyl acetate, ethanol and dichloromethane. The same remark is valid for **nitrostyrene** in ethanol and to a lesser extent in dichloromethane whereas the **ketoxime** seem to decompose slightly in ethanol.

Data at 8 ºC was collected only for the reductive amination impurities. No significant differences were found and thus full dataset was not collected. Moreover, it was also found rather difficult in practice to operate at low temperatures, i.e. the sample vials cannot be continuously maintained at 8 °C during the analysis without specially designed laboratory facilities. Thus the results obtained at 25 °C illustrate the stability that can be achieved in practice.

As for impurities extracted from synthesised amphetamine, they mirrored the results obtained in synthetic mixtures. However, a few outlier results were observed (no real trend) in dichloromethane, diethyl ether and ethyl acetate. Ethanol was obviously not used for liquid-liquid extraction experiments but it was studied as it could later be considered for solid-phase extraction experiments (see section 4).

Table 3 below summarises the results obtained by the four partners in terms of relative standard deviation (6 injections at 6 different times) :

Table 3 Relative standard deviations (in %) for six injections at time t = 0, 4, 12, 24, 48 and 96 hours

Synthetic mixture	isooctane	toluene	dichloromethane	diethyl ether	ethyl acetate	ethanol
Benzyl methyl ketone*	0.8	1.1	0.8	1.3	1.0	0.5
2-methyl-3-phenyl aziridine	0.6	1.0	0.6	1.6	0.5	0.8
Benzyl aziridine*	2.2	1.1	2.4	6.4	n/a	n/a
Benzyl methyl ketoxime (isomers 1+2)	1.5	1.9	2.4	4.0	1.2	4.0
Nitrostyrene	0.7	0.7	9.3	1.6	0.7	5.9
Benzaldiimine***	0.9	2.1	9.5	1.4	19.5	6.1
N-acetylamphetamine	2.3	4.6	3.8	1.8	2.4	0.9
Benzylamphetamine	1.3	3.1	2.0	3.7	3.9	1.1
Benzoylamphetamine	0.6	1.2	1.8	2.4	0.5	0.5
2-oxo	0.9	1.0	2.2	2.4	0.5	0.9
N-formylamphetamine	2.0	1.2	1.1	1.4	0.9	1.7
4-methyl-5-phenylpyrimidine	0.9	1.1	1.9	2.6	2.3	2.8
4-benzylpyrimidine**	1.2	1.0	1.8	1.8	1.5	2.4
DPPA**	1.7	2.0	7.2	6.4	5.3	1.5
DPIA** (isomers 1+2)	1.1	0.8	1.6	1.7	0.9	1.1
DPIMA** (isomers 1+2)	2.3	3.2	2.1	1.7	4.3	2.8
DPIF** (isomers 1+2)	1.0	1.1	1.8	7.8	0.6	0.6
2,4-dimethyl-3,5-diphenylpyridine	0.7	1.4	1.6	2.7	0.5	0.6
Mean	**1.3**	**1.6**	**3.0**	**2.9**	**2.7**	**2.0**

Amph extract	isooctane	toluene	dichloromethane	diethyl ether	ethyl acetate
Benzaldiimine	10.0	8.5	n/a	11.7	10.7
N-acetylamphetamine	n/a	6.3	2.8	13.5	6.6
Benzylamphetamine	1.9	4.0	8.0	8.7	1.6
Benzoylamphetamine	7.1	5.4	n/a	1.6	1.5
N-formylamphetamine	n/a	1.1	1.4	6.5	1.6
4-methyl-5-phenylpyrimidine	1.4	2.9	3.6	3.0	1.3
4-benzylpyrimidine	1.3	2.1**	4.9	1.9	1.2
DPIA** (isomers 1+2)	1.0	1.3	1.2	5.1	1.2
DPIMA (isomers 1+2)	1.9	n/a	1.2	7.3	2.3
DPIF** (isomers 1+2)	0.8	0.7	1.0	2.2	1.3
2,4-dimethyl-3,5-diphenylpyridine	2.8	2.3	4.7	2.5	4.2
Mean	**3.1**	**3.5**	**3.2**	**5.8**	**3.0**
Total mean	**2.2**	**2.6**	**3.1**	**4.4**	**2.9**

* BMK and benzyl aziridine were present as impurities and studied as well. Their identities were checked with available standards, benzyl aziridine being synthesised by partner 1 although not present in the task 1 list.

** Mean values taken from partners 3 and 4 (some impurities were studied in both labs).

*** Mean value taken from partner 1 and 2 (impurity studied in both labs).

n/a : not available. Generally because of coelution problems.

In table 3, cathinol, N-(β-phenyl-isopropyl)benzyl methyl ketiimine and 1-oxo-1-phenyl-2-(β-phenylisopropylimino)-propane were not taken into account given their unstability in all solvents.
Excluding these three unstable impurities, the results show that iso-octane, followed by toluene, are the best solvents in terms of stability of the analytes. However, good results were obtained for the other solvents as well but the relative standard deviations were somehow a little bit higher. Also, the use of diethyl ether highlighted some practical problems. Indeed, even with crimped sealed vials, it is not seldom that the solvent evaporates if the vial is kept for a long period of time on the sample tray. This is somehow not surprising given the very high volatility of this solvent.

2.3 Conclusion

Whilst some impurities are more stable in amphetamine extracts and others more stable in a synthetic mixture, the overall results seem to indicate that iso-octane should be the solvent of choice for the subsequent Task 3 and method development. This did not preclude the use of toluene in other stages of the project (for example, optimisation of the extraction protocol) as it behaved similarly to iso-octane in terms of stability. At this stage, it may not as well preclude the use of any of the other solvents, except maybe for diethyl ether whose high volatility gives rise to practical problems.

In general, the relative standard deviations (RSD) values obtained for iso-octane and toluene (which are somehow smaller compared to the other solvents) are in the order of magnitude typically met in valid GC applications. This fact strongly supports the hypothesis that the target compounds can be reliably analysed in these solvents with the exception of cathinol, N-(β-phenyl-isopropyl)benzyl methyl ketiimine and 1-oxo-1-phenyl-2-(β-phenylisopropylimino)propane. These three compounds cannot be reliably quantified.

3. Task 3 - Optimisation of gas chromatographic system

3.1 Introduction

Optimisation of the GC system was carried out such that each operating parameter was individually investigated. The task was divided into three subtasks:

Subtask 3.1	Sample introduction technique and injector operating conditions.
Subtask 3.2	Column stationary phase and temperature programme.
Subtask 3.3	Detector type and operating conditions.

3.2 Injection technique

3.2.1 Experimental

In this subtask, the injection port temperature was investigated using both split and splitless injection techniques. Extracts of Leuckart, nitrostyrene and reductive amination amphetamine were prepared according to the method described in Task 2. The solvent used was iso-octane. The extractions were performed in triplicate, the extracts combined and divided for subsequent method development to guarantee reproducible samples for all experiments.

In the initial studies, reference values for relative response factors (RRF's) for impurities found in the amphetamine synthesised by each laboratory were determined by Partner 2 using cool on-column (COC) injection. These results were required as they represent an "absolute" sample introduction technique, since the sample is not heated in the injection block and all of it enters the column directly. The analyses were performed using the oven track mode of the cool on-column inlet, which constantly maintains the temperature of the inlet 3 °C higher than that of the column oven. Other parameters were the same as used in Task 2.

In the second step the test samples were analysed using splitless and split injection techniques by all Partners. For the splitless mode a deactivated glass liner was installed (HP Part No. 18740-80220). Additionally, a retention gap pre-column was connected between the injection port and the analytical column. The reason for this is that splitless injection produces symmetric peaks for target compounds of various boiling points only if so-called solvent effect is fully utilised. Pre-columns are known to improve the solvent effect especially for the low-boiling point compounds. It was not necessary to study this phenomenon as it has been thoroughly described in the literature [Grob, 1993]. For the split mode the standard split method as applied in Task 2 was employed. However, in order to be able to detect the peaks when using the split injection, a lower split ratio of 1:20 and a higher injection volume of 2 µL were required.

Both the split and splitless modes were evaluated in each laboratory using the same amphetamine extracts that had been used previously for the COC injection studies carried out by Partner 2. A number of different injector temperatures (220, 240, 260 and 280°C) were evaluated to determine the effects of temperature on responses from the compounds in the extracts when using split and splitless injections. Measurement of RRF values and examining both systematic and random errors were carried out to find the most suitable injection technique and optimum injection temperature.

The percentage relative deviation between the COC and the split and splitless techniques were determined by measuring the RRF's, i.e., peak area of a certain impurity per peak area of an internal standard, from the split and splitless injections and comparing them with those obtained with the COC technique. The relative deviation can be considered as a measure for systematic error and calculated as:

$$\text{Relative deviation}\,(\%) = \frac{\text{RRF(inj. technique)} - \text{RRF(cool on column)}}{\text{RRF(cool on column)}} \times 100\% \qquad \textbf{(1)}$$

Each RRF value is the mean value calculated from three replicate injections. Values for the relative deviation can be positive or negative, depending on the type of discrimination the inlet causes to the analyte. Systematic error can be derived by summing the absolute values of the relative deviation:

$$\text{Absolute value} = |\text{Relative deviation}| \quad \textbf{(2)}$$

$$\text{Systematic error (\%)} = \frac{\sum_{i}^{n} \text{absolute value}}{\text{n}} \times 100\%, \quad \textbf{(3)}$$

where n is the number of target compounds.

Random error of each injection technique and temperature was estimated by investigating the RSD's of the RRF's in three replicate analyses. Average of the relative standard deviations of the RRF's illustrate the random error :

$$\text{Random error (\%)} = \frac{\sum_{i}^{n} \text{RSD}}{\text{n}} \times 100\%, \quad \textbf{(4)}$$

where n is the number of target compounds.

3.2.2 Results and discussion

Random and systematic error :

Random error cause replicate results to differ from one another and will affect the precision, or reproducibility, of an experiment. If the random error is small, then the result will be precise.

Systematic error cause the results to be in error in the same sense (for example, all too high or all too low). However, in a given experiment, there may be several sources of systematic error, some positive and others negative. This is why, in equation 3, we are taking the absolute value of the relative deviation in order to get the total systematic error, also called the bias.

The results illustrating the random and systematic errors are summarised in **table 4 a**nd **table 5.**

Table 4 Random error using different sample introduction techniques and conditions. Only compounds studied in Task 2 and found to be stable were used for this study.

RANDOM ERROR		Splitless				Split			
	On-column	220 ºC	240 ºC	260 ºC	280 ºC	220 ºC	240 ºC	260 ºC	280 ºC
Ketoxime (1)	2.86 %	1.35 %	4.98 %	11.00 %	0.82 %	2.12 %	6.37 %	2.36 %	2.77 %
Ketoxime (2)	2.97 %	2.47 %	2.89 %	8.57 %	0.51 %	10.20 %	1.10 %	1.57 %	3.05 %
Formylamphetamine	2.38 %	2.15 %	7.29 %	7.66 %	3.38 %	3.48 %	3.20 %	0.43 %	21.78 %
Acetylamphetamine	2.67 %	4.26 %	22.55 %	16.61 %	17.59 %	9.50 %	10.29 %	6.25 %	1.82 %
Benzaldiimine	3.09 %	4.53 %	24.03 %	7.73 %	9.42 %	2.06 %	1.85 %	2.26 %	0.35 %
Average (Partner 1)	**2.79 %**	**2.95 %**	**12.35 %**	**10.31 %**	**6.34 %**	**5.47 %**	**4.56 %**	**2.57 %**	**5.95 %**
Benzaldiimine	2.59 %	2.83 %	0.99 %	4.99 %	4.58 %	3.56 %	1.48 %	1.02 %	0.96 %
Benzylamphetamine	1.30 %	1.60 %	2.19 %	2.17 %	0.88 %	1.02 %	0.39 %	1.40 %	1.40 %
DPIA (1)	1.05 %	1.89 %	1.33 %	1.80 %	0.40 %	0.94 %	0.47 %	1.55 %	1.54 %
DPIA (2)	1.34 %	1.33 %	0.84 %	1.75 %	0.80 %	0.88 %	0.64 %	1.59 %	1.58 %
Benzoylamphetamine	0.69 %	1.88 %	1.95 %	4.21 %	1.09 %	1.43 %	0.77 %	2.68 %	2.65 %
Average (Partner 2)	**1.39 %**	**1.91 %**	**1.46 %**	**2.98 %**	**1.55 %**	**1.57 %**	**0.75 %**	**1.65 %**	**1.63 %**
4-Methyl-5-phenylpyrimidine	0.16 %	5.25 %	1.72 %	6.09 %	6.10 %	1.47 %	0.86 %	1.45 %	0.55 %
4-benzylpyrimidine	0.20 %	4.99 %	1.97 %	6.37 %	6.49 %	4.05 %	2.81 %	2.21 %	3.12 %
Benzaldiimine	1.96 %	5.27 %	2.38 %	6.31 %	2.79 %	6.89 %	3.51 %	5.21 %	10.88 %
Benzylamphetamine	0.12 %	3.42 %	1.45 %	3.90 %	3.76 %	1.45 %	0.17 %	2.67 %	1.31 %
DPIA (1)	2.00 %	1.23 %	1.63 %	4.29 %	2.92 %	0.82 %	0.71 %	0.93 %	0.39 %
DPIA (2)	3.96 %	7.62 %	2.40 %	5.61 %	3.59 %	0.44 %	0.51 %	1.12 %	0.54 %
DPIMA	0.41 %	2.25 %	2.37 %	4.34 %	2.70 %	4.59 %	5.97 %	1.14 %	2.50 %
DPIF (1)	0.05 %	0.46 %	0.97 %	1.80 %	1.38 %	1.56 %	3.85 %	3.38 %	0.94 %
DPIF (2)	0.41 %	0.22 %	1.10 %	1.68 %	1.43 %	2.15 %	1.21 %	1.69 %	3.57 %
Average (Partner 3)	**1.03 %**	**3.41 %**	**1.78 %**	**4.49 %**	**3.46 %**	**2.60 %**	**2.18 %**	**2.20 %**	**2.64 %**
4-Methyl-5-phenylpyrimidine	0.35 %	2.04 %	0.93 %	1.27 %	1.11 %	0.45 %	0.29 %	0.91 %	1.24 %
Formylamphetamine	0.69 %	2.11 %	0.96 %	1.22 %	0.61 %	0.90 %	0.18 %	0.26 %	1.95 %
4-Benzylpyrimidine	0.27 %	1.86 %	1.03 %	1.34 %	0.93 %	0.69 %	0.24 %	0.49 %	1.40 %
Benzaldiimine	1.15 %	4.89 %	1.45 %	0.92 %	3.37 %	0.10 %	0.23 %	1.14 %	4.81 %
Benzylamphetamine	0.47 %	5.13 %	1.19 %	1.45 %	0.51 %	2.31 %	3.45 %	0.56 %	5.06 %
DPIA (1)	1.52 %	1.64 %	2.02 %	1.06 %	0.73 %	0.38 %	0.20 %	0.78 %	0.42 %
DPIA (2)	1.37 %	1.33 %	0.99 %	1.06 %	1.16 %	0.30 %	0.25 %	0.54 %	0.29 %
DPIMA	0.33 %	1.30 %	1.05 %	0.64 %	0.36 %	0.37 %	0.31 %	0.64 %	0.39 %
Benzoylamphetamine	2.92 %	1.67 %	1.06 %	0.72 %	1.64 %	0.49 %	0.31 %	0.78 %	1.14 %
2,4-dimethyl-3,5-diphenylpyridine	4.14 %	3.63 %	26.86 %	24.79 %	32.66 %	0.31 %	0.60 %	0.89 %	1.44 %
DPIF (1)	0.54 %	0.55 %	0.36 %	0.24 %	0.18 %	0.15 %	0.15 %	0.37 %	0.28 %
DPIF (2)	0.64 %	0.00 %	0.36 %	0.20 %	0.21 %	0.12 %	0.07 %	0.33 %	0.29 %
Average (Partner 4)	**1.20 %**	**2.18 %**	**3.19 %**	**2.91 %**	**3.62 %**	**0.55 %**	**0.52 %**	**0.64 %**	**1.56 %**
Average (all Partners)	**1.44 %**	**2.62 %**	**3.98 %**	**4.57 %**	**3.68 %**	**2.10 %**	**1.69 %**	**1.57 %**	**2.59 %**

With exception of the results from Partner 1 the random error (repeatability) is less than 5%. High deviation in Partner 1 results is most probably due to instrumental problems. The most repeatable injection technique is split at 260 °C.

In general, the random error is smaller in split injection compared to splitless injection.

Table 5 Systematic error using different sample introduction techniques and conditions. Partner 1 data was considered as outlier and not included in the average values.

SYSTEMATIC ERROR	Splitless				Split			
	220 ºC	240 ºC	260 ºC	280 ºC	220 ºC	240 ºC	260 ºC	280 ºC
Ketoxime (1)	20.72 %	9.64 %	0.25 %	1.04 %	62.04 %	65.48 %	65.65 %	66.31 %
Ketoxime (2)	62.92 %	57.80 %	53.20 %	54.19 %	4.82 %	6.15 %	6.08 %	9.13 %
Formylamphetamine	20.58 %	12.73 %	2.85 %	2.80 %	70.08 %	70.27 %	67.75 %	50.87 %
Acetylamphetamine	81.29 %	78.56 %	136.12 %	146.32 %	17.62 %	4.09 %	11.23 %	11.02 %
Benzaldiimine	43.12 %	48.33 %	39.13 %	17.56 %	251.52 %	235.80 %	236.76 %	231.04 %
Average (Partner 1)	**45.73 %**	**41.41 %**	**46.31 %**	**44.38 %**	**81.22 %**	**76.36 %**	**77.49 %**	**73.67 %**
Benzaldiimine	68.11 %	80.91 %	108.36 %	126.22 %	56.79 %	51.65 %	49.98 %	59.58 %
Benzylamphetamine	1.75 %	4.32 %	3.07 %	2.63 %	15.25 %	6.92 %	3.71 %	3.60 %
DPIA (1)	10.12 %	4.08 %	4.78 %	4.36 %	14.25 %	5.73 %	4.50 %	3.90 %
DPIA (2)	8.37 %	2.01 %	3.12 %	3.27 %	19.04 %	9.94 %	0.04 %	0.17 %
Benzoylamphetamine	15.62 %	10.23 %	7.03 %	0.57 %	12.86 %	8.45 %	2.51 %	3.87 %
Average (Partner 2)	**20.79 %**	**20.31 %**	**25.27 %**	**27.41 %**	**23.64 %**	**16.54 %**	**12.15 %**	**14.22 %**
4-methyl-5-phenylpyrimidine	22.76 %	21.02 %	25.03 %	21.36 %	12.47 %	11.42 %	9.29 %	13.23 %
4-benzylpyrimidine	21.41 %	21.02 %	25.14 %	21.36 %	14.97 %	12.34 %	13.37 %	13.09 %
Benzaldiimine	15.18 %	14.35 %	23.59 %	36.97 %	9.87 %	0.76 %	0.51 %	3.50 %
benzylamphetamine	7.14 %	5.34 %	7.27 %	5.41 %	5.36 %	1.49 %	0.44 %	1.32 %
DPIA (1)	0.58 %	1.38 %	3.62 %	2.97 %	34.82 %	36.41 %	37.30 %	36.95 %
DPIA (2)	23.77 %	15.08 %	15.32 %	13.64 %	92.89 %	87.62 %	84.85 %	86.02 %
DPIMA	3.17 %	4.21 %	3.00 %	1.03 %	3.15 %	1.58 %	5.27 %	3.27 %
DPIF (1)	2.68 %	1.38 %	0.20 %	0.71 %	5.57 %	6.41 %	1.96 %	4.88 %
DPIF (2)	3.64 %	1.86 %	0.20 %	0.28 %	5.43 %	8.96 %	1.67 %	3.44 %
Average (Partner 3)	**11.15 %**	**9.52 %**	**11.49 %**	**11.53 %**	**20.50 %**	**18.55 %**	**17.18 %**	**18.41 %**
4-Methyl-5-phenylpyrimidine	26.93 %	14.23 %	6.02 %	4.87 %	45.69 %	34.02 %	25.29 %	16.16 %
Formylamphetamine	9.28 %	0.89 %	7.73 %	7.72 %	19.82 %	10.77 %	3.70 %	0.77 %
4-Benzylpyrimidine	19.92 %	9.27 %	2.11 %	1.41 %	43.54 %	31.98 %	22.99 %	13.89 %
Benzaldiimine	13.09 %	22.82 %	26.75 %	23.43 %	12.01 %	7.01 %	1.04 %	7.11 %
Benzylamphetamine	6.19 %	17.66 %	25.83 %	26.21 %	4.99 %	0.58 %	6.85 %	12.18 %
DPIA (1)	16.78 %	5.06 %	5.14 %	6.49 %	16.11 %	9.89 %	4.09 %	0.13 %
DPIA (2)	13.83 %	1.05 %	8.05 %	10.43 %	20.06 %	13.60 %	7.51 %	3.48 %
DPIMA	6.91 %	3.52 %	12.65 %	15.08 %	4.47 %	0.86 %	3.22 %	6.47 %
Benzoylamphetamine	3.35 %	11.58 %	16.54 %	17.79 %	37.99 %	33.61 %	27.61 %	23.37 %
2,4-dimethyl-3,5-diphenylpyridine	0.44 %	18.19 %	30.53 %	40.65 %	3.77 %	6.37 %	11.15 %	13.00 %
DPIF (1)	0.44 %	2.85 %	7.77 %	9.94 %	10.97 %	13.00 %	15.89 %	18.26 %
DPIF (2)	3.86 %	6.08 %	9.93 %	11.73 %	13.33 %	15.13 %	17.82 %	20.14 %
Average (Partner 4)	**10.09 %**	**9.43 %**	**13.25 %**	**14.65 %**	**19.40 %**	**14.74 %**	**12.26 %**	**11.25 %**
Average (Partners 2 - 4 only)	**12.5%**	**11.6%**	**15.0%**	**16.0%**	**20.6%**	**16.4%**	**13.9%**	**14.3%**

The systematic errors which mainly arise from discrimination problems of the vaporising inlets are all fairly high compared with the random errors. Partner 1 results must be excluded because of their inexplicably high deviation. According to Partners 2, 3 and 4 results the best condition is splitless at 240°C. However, split injection is not significantly different from the splitless technique although the lowest temperatures with splitless injection give the smallest systematic errors.

In order to take into account both errors, a total error U was calculated. This error can be estimated with the formula often used for calculating combined measurement uncertainty :

$$U = \sqrt{\left(u_{sys}\right)^2 + \left(u_{ran}\right)^2} \qquad \textbf{(5)}$$

where u_{sys} and u_{ran} are bias (systematic error) and precision (random error), respectively [Eurachem, 2000].

Table 6 concludes total error. The smallest total error is achieved with splitless injection at 240 °C. Almost the same results can be obtained with split injection at 260 °C.

Table 6 Summary of errors affecting vaporising sample introduction techniques.

TOTAL ERROR	Splitless				Split			
	220 °C	240 °C	260 °C	280 °C	220 °C	240 °C	260 °C	280 °C
Random error, all Partners	2.62 %	3.98 %	4.57 %	3.68 %	2.10 %	1.69 %	1.57 %	2.59 %
Systematic error, Partners 2 - 4	12.51 %	11.55 %	14.95 %	16.02 %	20.60 %	16.40 %	13.94 %	14.30 %
Total error	12.78 %	**12.22 %**	15.63 %	16.44 %	20.71 %	16.49 %	**14.03 %**	14.53 %

3.2.3. Conclusions

Optimisation of the injection technique was difficult. The best injection technique, the cool on-column sample introduction, was excluded as it was considered not to be robust enough for everyday work. Thus the choice had to be made between different vaporising techniques. The results show that the split and splitless technique work similarly at different temperatures. The technique causing the smallest total error, i.e. splitless at 240 °C, probably offers the best performance especially if low concentration samples are to be analysed. Practically, the same results can be obtained using split injection at a higher temperature (260 °C).

Split injection at 260 °C was chosen for Subtask 3.2 as this technique provides better chromatographic performance under various conditions than splitless technique. This was considered necessary because the aim of Subtask 3.2 was the optimisation of chromatographic separation. Also, split injection at 260°C gave a much better random error than splitless at 240°C (1.6 % vs 4.0 %). However, this did not preclude the use of splitless injection at 240°C or even 250°C at a later stage in the project.

Although splitless injection at 240 °C gave the best overall performance, 250 °C was used in all other tasks as this temperature is commonly used in many laboratories performing amphetamine profiling. It is expected that this 10 °C difference does not have a significant impact on the results.

3.3 Choice of Column and Oven Temperature

3.3.1 Experimental

It was decided that in the framework of the current study only columns of standard dimensions would be used. Thus the length, the internal diameter and the film thickness were fixed to 25 – 30 m, 0.20 – 0.25 mm and 0.25 – 0.33 μm, respectively. In addition, potential stationary phases were limited to those that can be operated up to 300 °C. Taking these restrictions into consideration each Partner initially evaluated three phenyl methyl silicone stationary phase columns with different stationary phase ratios, namely Ultra 1, Ultra 2 and HP50+. All columns were 25 m x 0.20 mm, d_f 0.33 μm (HP50+ : 0.31 μm) from the Agilent company. An additional column, HP-1701, also from the Agilent company was studied by Partner 2 although the maximum temperature of this column was only 280°C. This was because the HP-1701 column is widely used for the analysis of polar compounds.

In addition to the choice of column, column temperature programme was also evaluated and optimised. Temperature program profiles were limited to only linear ramps to ensure the best possible reproducibility of retention times (t_R) and retention factors (k). Six different column temperature programme rates, ranging from 2 to 12 ºC/min, were employed, all starting at 60ºC and ending at 300°C. Injections were performed in split mode with an injection temperature of 260ºC according to the decision made in subtask 3.1. Simultaneous FID/MSD detections was applied such that chromatographic performance was calculated for the FID trace and peak purity/co-elution was monitored using the MSD trace. Samples of home-made amphetamine, prepared as in the previous subtask, were used as test samples.

3.3.2 Results and discussion

3.3.2.1 Separation power, resolution and inertness

The performance of each column was assessed by measuring separation power, resolution and inertness. Overall separation power was estimated on the basis of the number of peaks that could be integrated in the chromatogram. Sensitivity of the integrator was individually calibrated for each chromatogram relative to the size of the internal standard peak. Resolution of the target compounds was investigated through the concept of graphical resolution.

Graphical resolution can determined by the following equation

$$R = \frac{2\,\Delta t_R}{W_{b1} + W_{b2}} \qquad \textbf{(6)}$$

Where Δt_R is the difference between retention times of two peaks and W_b is the width of the peak at its base.

Example :

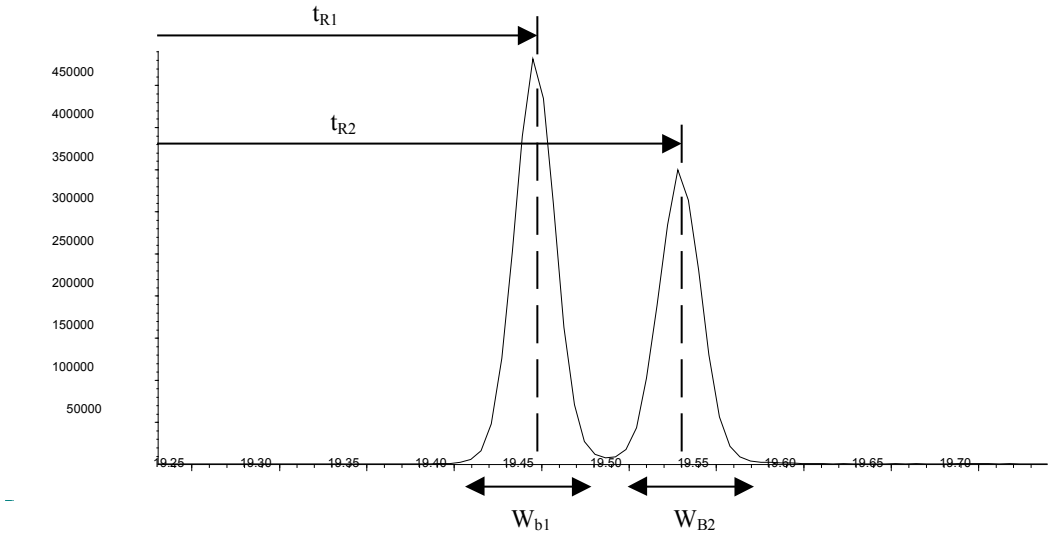


Figure 9 : example illustrating the concept of graphical resolution

In this case, t_{R1} is 19.45 min and t_{R2} is 19.53 min. W_{b1} and W_{b2} are 0.07 min. Therefore the resolution is :

R = 2 x (19.53 – 19.45) / (0.07 + 0.07) = 0.16 / 0.14 = **1.14**

In this subtask, the resolution between target compounds were calculated. In table 8, results are given as a percentage of target compounds which are separated with a resolution > 1.

Inertness was estimated on the basis of peak asymmetry, calculated as a ratio of second and first half-widths (at 10% height) of each peak, respectively.

Example :

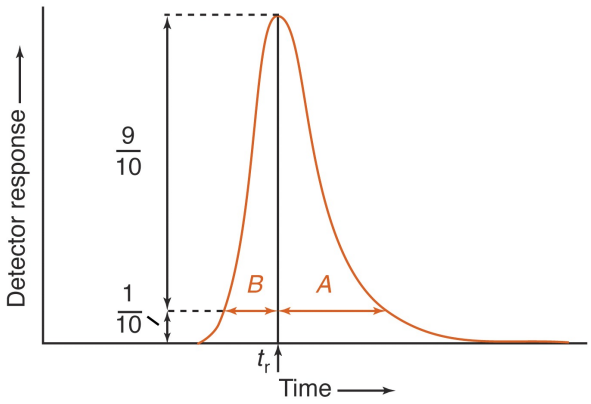


Figure 10 : graphical representation for the calculation of inertness

In this case, if B is 0.08 min and A is 0.16 min, the inertness will be calculated as 0.16 / 0.08 = **2**.
A value above 1 will mean that the peak is tailing while a value below 1 will correspond to peak fronting. Of course, the ideal value is one (perfect symmetry).

Resolution and asymmetry were calculated using the peak performance functions of the macro tools in the Agilent GC Chemstation (Rev. A06.03) software. At this stage of the project, it was decided that the target compounds would be divided in Classes A and B on the basis of their stability (Task 2 results), presence in street samples and based on experience from previous profiling internal studies [Kronstrand, 1990, Johansson, 1991, Lindberg 1991, Alm et al., 1992]. Class A compounds are presumed to be more important and reliable. The list of the target compounds is given in **Table 7**.

Table 7 **Identified amphetamine impurities synthesised by Leuckart, reductive amination and nitrostyrene methods. Compounds printed with strike-out font have been excluded from all calculations due to their instability.**

No.	Class	Compound	New compound and type of identification
1	B	Benzyl methyl ketone	-
2	B	4-methyl-5-phenylpyrimidine*	-
3	B	N-formylamphetamine*	-
4	B	4-benzylpyrimidine*	-
5	A	1,3-diphenyl-2-propylamine*	-
6	B	N,N-di(β-phenylisopropyl)amine (1)*	-
7	B	N,N-di(β-phenylisopropyl)amine (2)*	-
8	A	N,N-di(β-phenylisopropyl)methylamine (1)*	-
9	A	N,N-di(β-phenylisopropyl)methylamine (2)*	-
10	A	1-benzyl-3-methylnaphthalene*	Id. based on a RM from UN[1]
11	A	1,3-dimethyl-2-phenylnaphthalene*	Id. based on a RM from UN[1]
12	A	2,6-dimethyl-3,5-diphenylpyridine*	Id. based on literature data[2]
13	A	2,4-dimethyl-3,5-diphenylpyridine*	-
14	A	2,6-diphenyl-3,4-dimethylpyridine*	Id. based on a RM from NFI[3]
15	A	N,N-di(β-phenylisopropyl)formamide (1)*	-
16	A	N,N-di(β-phenylisopropyl)formamide (2)*	-
17	A/B	2-benzyl-2-methyl-5-phenyl-2,3-dihydropyrid-4-one*	Id. based on literature data[4]
18	A	Pyridine 14	Id. based on MSD data
19	A	Pyridine 7	Id. based on MSD data
20	A/B	Pyridine X	Id. based on MSD data
21	A	Phenyl-2-propanol	-
22	A	Acetylamphetamine	-
23	A	N-(β-phenylisopropyl)benzaldimine*	-
24	A/B	Benzylamphetamine*	-
25	-	~~N-(β-phenyl-isopropyl)benzyl methyl ketimine~~	-
26	A	1-oxo-1-phenyl-2-(β-phenylisopropylimino)propane	-
27	A	Benzoylamphetamine*	-
28	A	2-oxo-1-phenyl-(β-phenylisopropylamine)ethane	-
29	B	1-hydroxy-N,N-di(β-phenylisopropyl)amine (Cathinol)	-
30	B	N-(β-phenylisopropyl)cathinone	-
31	A	2-methyl-3-phenylaziridine	-
32	A	dimethyl-3-phenylaziridine	Id. based on MSD data
33	A	2-phenylmethylaziridine	Id. based on MSD data
34	A	Benzyl methyl ketoxime (1)	-
35	A	Benzyl methyl ketoxime (2)	-
36	B	Phenyl-2-nitropropene	-

[1] Reference material (RM) from the United Nations Office of Drugs and Crime (scientific section)
[2] [Van den Ark et al., 1978a]
[3] Reference material (RM) from the Netherlands Forensic Institute
[4] [Van den Ark et al., 1977a]
* Compounds studied by partner 4 in this subtask

The results obtained by Partner 4 are summarised in **Table 8.** The reader is advised to note that the results are based on only 19 target compounds (see asterisks in table 7).

Table 8 Performance of each column at each temperature programme. The results are averages of 19 compounds in one sample analysed in IPSC, n=3. "n.a." stands for "not analysed".

Column	T-program ° C/min	Separation power (no. of integrated peaks)	Target peaks with resolution ≥ 1	Target peak asymmetry (absolute value)
Ultra 1	**2**	64	84 %	1.60
	4	67	84 %	1.45
	6	66	76 %	1.55
	8	64	82 %	1.52
	10	65	71 %	1.35
	12	62	71 %	1.39
Ultra 2	**2**	67	76 %	1.46
	4	**70**	66 %	1.58
	6	69	76 %	1.38
	8	66	79 %	1.48
	10	66	84 %	1.44
	12	63	82 %	1.42
HP 50+	**2**	62	92 %	1.39
	4	58	89 %	1.29
	6	52	**95 %**	1.25
	8	52	**95 %**	**1.21**
	10	52	87 %	1.27
	12	55	87 %	1.36

From these results, it can be seen that slow temperature programmes give, in general, better separation power. The best results were obtained with the Ultra 1 and Ultra 2 columns with slow temperature programmes (2, 4 and 6°C per min). In terms of resolution, the best result was obtained with the HP 50+ column at 6 and 8°C per min. Also, this same column and temperature programmes gave the best inertness.
The following chromatogrammes illustrate the resolution results from table 8 (best resolution for each column). Only part of the chromatogramme where resolution problems were occuring are shown :

Ultra 1, 2 deg / min

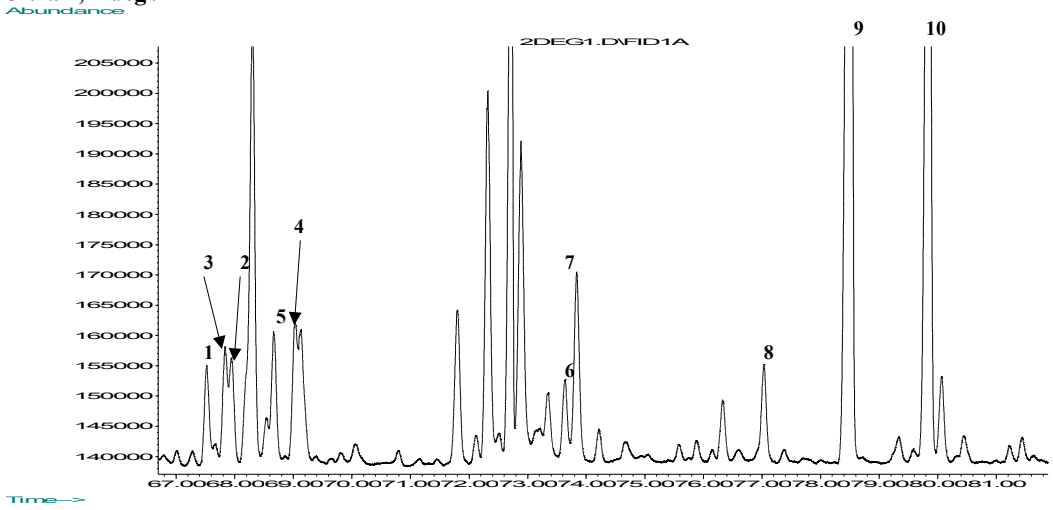


Figure 11 : Chromatogramme illustrating separation of Leuckart impurities on a Ultra 1 column at 2 deg / min. Identification of peaks: 1: DPIMA 1, 2: DPIMA 2, 3: 1-benzyl-3-methylnaphthalene, 4: 1,3-dimethyl-2-phenylnaphthalene, 5: Benzoylamphetamine, 6: 2,6-dimethyl-3,5-diphenylpyridine, 7: 2,4-dimethyl-3,5-diphenylpyridine, 8: 2,6-diphenyl-3,4-dimethylpyridine, 9: DPIF 1, 10: DPIF 2.

Ultra 2, 10 deg / min

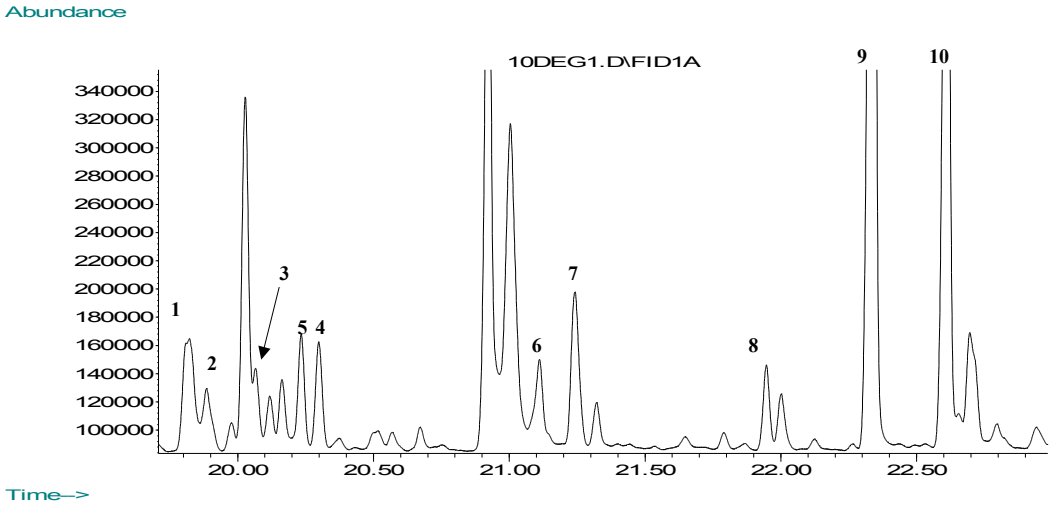


Figure 12 : Chromatogramme illustrating separation of Leuckart impurities on a Ultra 2 column at 10 deg / min. Identification of peaks: same as figure 11.

HP 50+, 8 deg / min

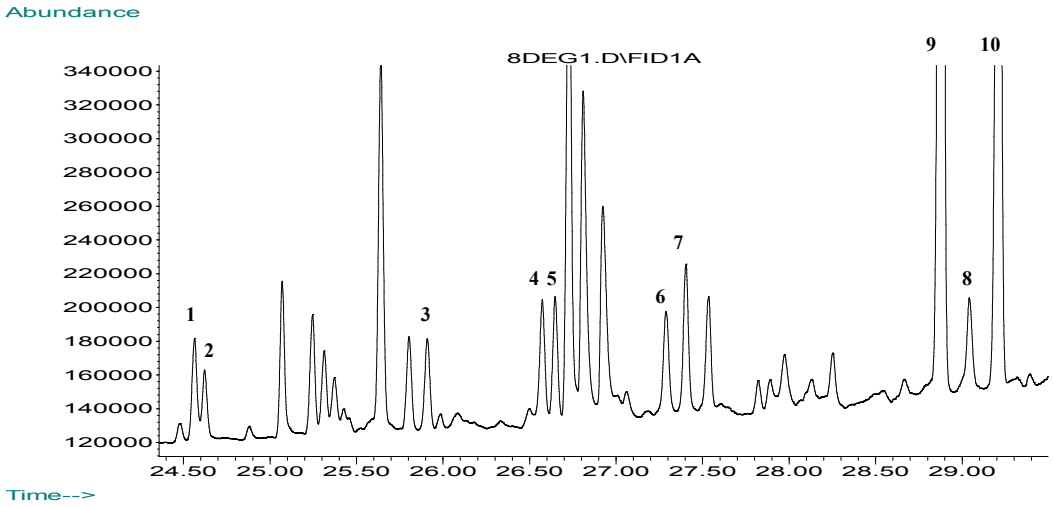


Figure 13 : Chromatogramme illustrating separation of Leuckart impurities on a HP 50+ column at 8 deg / min. Identification of peaks: same as figure 11.

The combined results for all partners are summarised in **Table 9.** The reader is advised to note that the results are based on all 35 target compounds (table 7).

Table 9 Performance of each column at each temperature programme. The results are averages of 35 compounds in four different samples analysed in four different laboratories, n=3. "n.a." stands for "not analysed".

Column	T-program ° C/min	Separation power (no. of integrated peaks)	Target peaks with resolution ≥ 1	Target peak asymmetry (absolute value)
Ultra 1	**2**	125	81 %	2.37
	4	128	79 %	2.18
	6	125	74 %	2.23
	8	113	74 %	2.23
	10	112	73 %	1.95
	12	107	64 %	1.96
Ultra 2	**2**	**141**	81 %	2.51
	4	140	74 %	2.26
	6	129	79 %	2.18
	8	126	75 %	2.24
	10	126	77 %	1.97
	12	114	78 %	1.92
HP 50+	**2**	130	**91 %**	1.99
	4	117	85 %	1.93
	6	100	**91 %**	1.75
	8	102	88 %	1.75
	10	103	89 %	**1.60**
	12	99	84 %	1.65
HP 1701	**2**	75	n.a.	n.a.
	4	75	n.a.	n.a.
	6	68	n.a.	n.a.
	8	64	n.a.	n.a.
	10	63	n.a.	n.a.
	12	62	n.a.	n.a.

HP 1701 column was not further investigated due to its inferior separation power.

Again, the combined results confirm that slow temperature programmes give better results in terms of separation power. It also confirms that the HP 50+ column gives somehow better resolution and better inertness compared to the two other columns.

At this stage of the project, it was decided to continue with a HP 50+ column due to its better performance in terms of inertness and resolution. Also, visual comparisons showed that it was probably the best column for satisfactory separation of all target compounds (as illustrated in figures 11, 12 and 13).
Therefore, this column was used in Task 4, but at a later stage the noise level of the column caused by excessive bleeding was found to be unacceptable. The bleeding seemed to vary from column to column but overall it was much higher than could be accepted (see figure 14) :

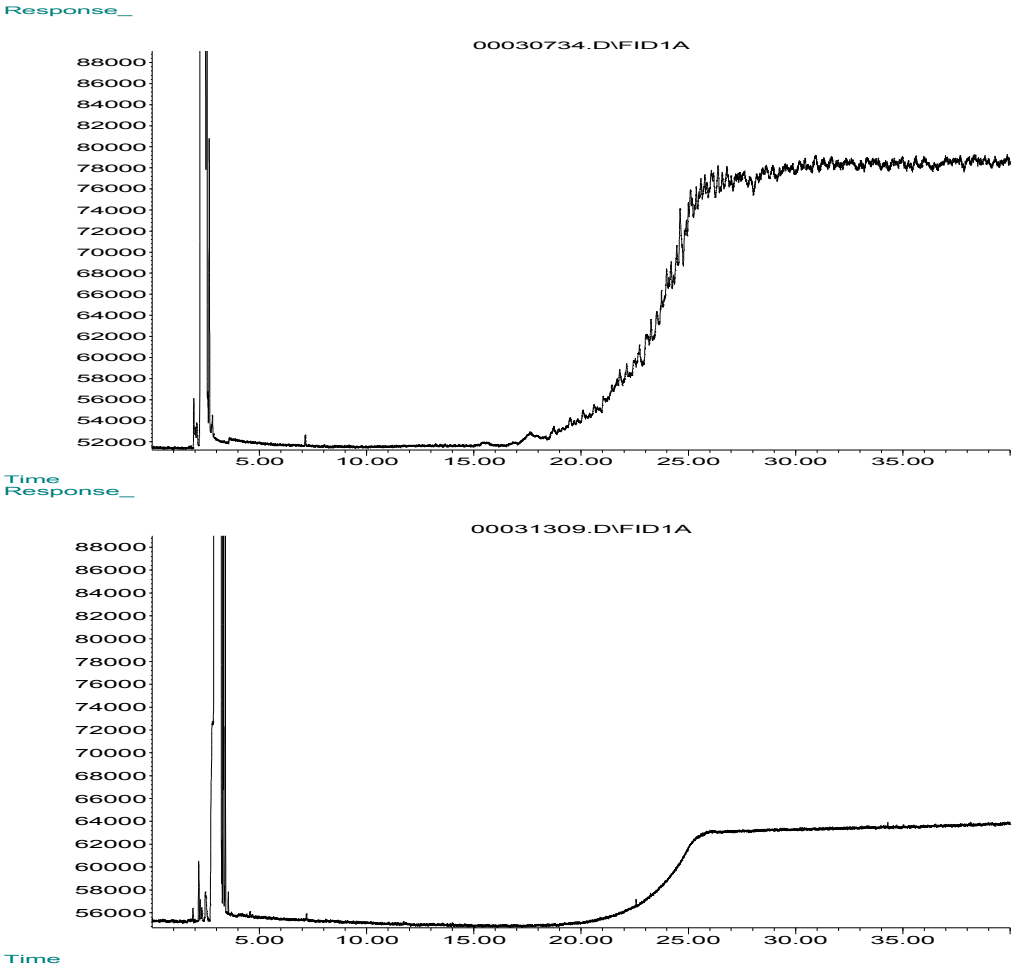


Figure 14 Illustration of the bleeding of HP50+ column (top) compared to that of the Ultra 2 column (bottom). Temperature programme: 60 °C, 8 °C/min => 300 °C (10 min). FID detection. Chromatograms have been recorded by Partner 2.

To overcome this problem other columns of similar or nearly similar stationary phase were investigated. HP-35, DB-35MS and DB-17MS were chosen for the study. The number 35 meaning that the stationary phase is made of 35 % poly(diphenylsiloxane) and 65 % poly(dimethylsiloxane) whereas the DB-17MS contains the same stationary phase as the HP50+ column, namely 50 % poly(diphenylsiloxane) and 50 % poly(dimethylsiloxane). Therefore, the DB-35MS and HP-35 are slightly less polar than the HP 50+ and DB-17MS.
J&W columns (i.e., DB columns) were chosen on the basis that the Agilent company was merged with the J&W company and thus the columns were available from the same source. It was considered an advantage that only one manufacturer would represent the entire analytical system.

The same experiments were carried out to evaluate the target compounds resolution and inertness of the J&W columns. The data was calculated for Class A compounds only as they were recognised as having the main priority. Moreover, the data was recorded only for temperature programme rates providing analysis time of 30 min or less, i.e. 8, 10 and 12 °C/min. **Table 10** summarises the performance of each column.

Note : These further experiments were carried out by partners 2 and 3 only.

Table 10 Performance of each column at each temperature programme. The results are based on the Class A compounds only.

Column	T-program °C/min	Target peaks (Class A) with resolution > 1.0	Asymmetry Average (absolute value)
Ultra 1	2	74 %	2.74
	4	72 %	2.49
	6	65 %	2.57
	8	62 %	2.54
	10	66 %	2.23
	12	65 %	2.29
Ultra 2	2	75 %	2.98
	4	66 %	2.43
	6	74 %	2.51
	8	72 %	2.62
	10	75 %	2.19
	12	79 %	2.11
HP 50+	2	**89 %**	2.36
	4	85 %	2.22
	6	88 %	2.00
	8	85 %	1.84
	10	85 %	1.76
	12	82 %	1.76
HP-35	*8*	*87 %*	*1.91*
	10	*87 %*	*1.43*
	12	***89 %***	*1.53*
DB-35MS	*8*	*87 %*	***1.28***
	10	*87 %*	*1.42*
	12	*72 %*	*1.33*
DB-17MS	*8*	*88 %*	*1.78*
	10	*84 %*	*1.43*
	12	*83 %*	*1.50*

The best overall resolution for the Class A compounds was obtained on HP-35 column at 12°C/min, although DB-35MS gave nearly the same performance at 8°C/min. Also DB-17MS performed well in terms of overall resolution.

The DB-35MS was the most inert column and bleeding was negligible compared to the other columns. Thus, taking all aspects into consideration, DB-35MS was found to be the best compromise to meet all the requirements. The column was chosen for the following tasks (tasks 5 and 6).

3.3.3 Conclusions

The non-polar columns Ultra 1 and Ultra 2 had the highest overall separation power which is commonly known as these columns have the highest efficiency. For the current application however other features had to be emphasised. Resolution of the targeted analytes, i.e. the synthesis impurities, was the most important selection criterion. Inertness is nearly as important since it illustrates the ability of the column to separate compounds of varying chemical characteristics. HP 50+ offered great performance but column to column reproducibility was insufficient, as clearly demonstrated by the varying stationary phase bleed. Besides, the Agilent company has acknowledged the excessive bleeding of this column. Taking all these factors into consideration the DB-35MS column was the best compromise in terms of chromatographic performance.

Temperature programme rate was shown to have a great impact on the performance of each column. Finally, 8 °C/min was chosen as it provided an acceptable compromise between analysis time and chromatographic performance.

3.4 Selection of the detection technique

3.4.1 Introduction

Evaluation of different detection techniques was performed using FID and MSD in Scan and SIM (Single Ion Monitoring) modes. The reader is advised to note that some MSD results of this subtask have been obtained using 'Ultra' ion source parts in the HP5973 mass spectrometer. At some stage, the standard ion source caused excessive tailing, especially of the hydrocarbon peaks, caused by contaminated and thus activated metal surface in the ion source. This phenomenon occured almost at the same time in all four laboratories indicating a common problem directly related to the standard ion source. Illustrations will be shown in section 4.4.3. Meanwhile, the part numbers (Agilent Technologies) of the new ion source parts can be found below :

1) G2589-20043 Ultra Ion Chamber
2) G2589-20044 Ultra Repeller
3) G2589-20045 Drawout Plate

In this subtask, splitless injection was used. One of the reasons was to determine the lowest concentrations which can be detected with each detector. Also, as mentioned in section 4.2.3, the injection port was set at 250°C. Finally, the oven temperature programme started at 90°C. It was determined that this increased the speed of analysis without affecting the chromatographic performance.
For MS data (Scan and SIM), ion response was measured instead of peak area using the Chemstation software.
Additionally, Partner 3 evaluated performance of nitrogen-phosphorus selective detection (NPD).

3.4.2 Experimental

A modified Grob test mixture was used in this subtask. The following chromatogramme shows the various compounds used in this subtask by partner 4 (all compounds were commercially purchased):

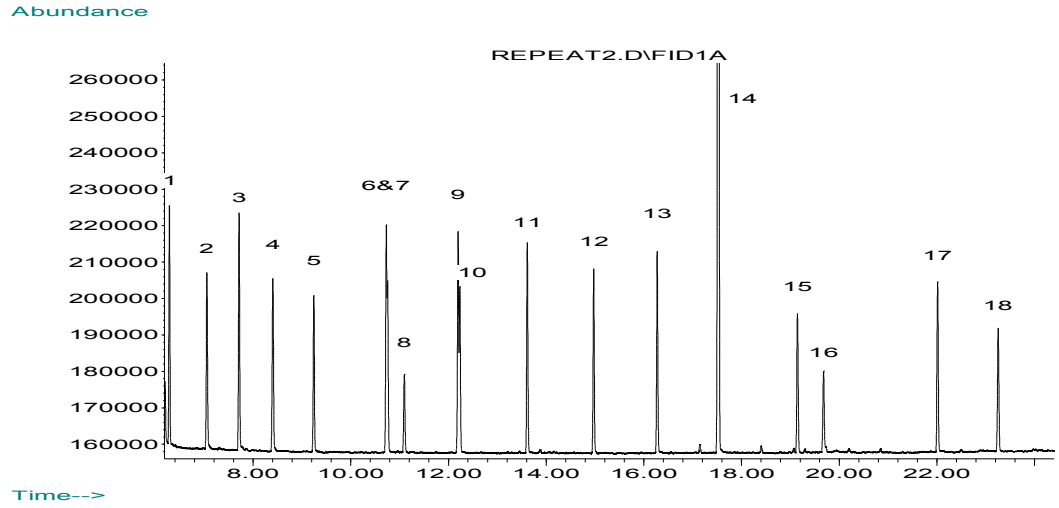


Figure 15 : FID chromatogramme containing the 18 compounds used for this subtask by partner 4. Run acquired on a DB-35MS column (see annex 2 for full method). . Identification of peaks: 1: dodecane, 2: 2,6-dimethyl phenol, 3: tridecane, 4: 2,6-dimethyl aniline, 5: Decanoic acid methyl ester, 6: pentadecane, 7: undecanoic acid methyl ester, 8: dicyclohexylamine, 9: hexadecane, 10: dodecanoic acid methyl ester, 11 : heptadecane, 12: octadecane, 13: nonadecane, 14: eicosane, 15: N-methyldiphenethylamine, 16: ketamine, 17: tetracosane, 18: trimipramine.

A dilution series of the test mixture was prepared for the evaluation of sensitivity and linearity. Different performance tests were carried out as follows :

Repeatability : The modified Grob mixture was injected repeatedly twenty times and the relative standard deviation (RSD) calculated for all relative peak areas (relative to the internal standard). This data was produced for FID and for MSD in Scan and SIM modes.

Reproducibility : The modified Grob mixture was injected once every day for twenty days. Calculations were performed as for repeatability.

Sensitivity : Different concentrations of the Grob mixture were injected in three replicates. Concentrations producing a signal-to-noise ratio of 10:1 was then extrapolated. Detection limits as well as limits of determination were calculated.

Linearity : The test mixture was run at five different concentrations covering a range of five orders of magnitude (but having the internal standard at constant concentration). The correlation coefficient was calculated and linearity checked.

3.4.3 Results and discussion

Tailing problem :

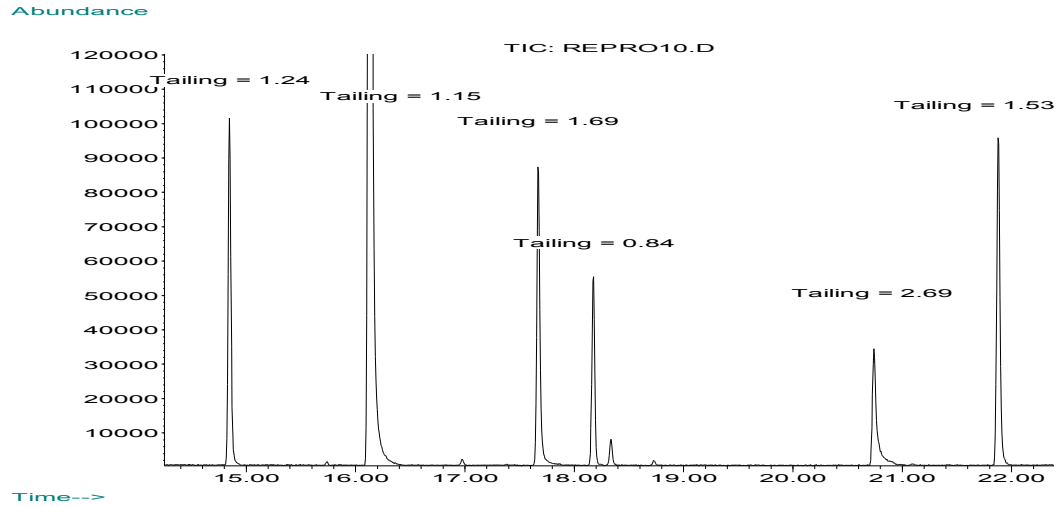


Figure 16 : MS chromatogramme showing the tailing problem in a Grob mixture. From left to right : nonadecane, eicosane, N-methyldiphenethylamine, ketamine, tetracosane, and trimipramine

As can be seen in figure 16, tailing begins to occur as from nonadecane and particularly affects the highest boiling alkane, namely tetracosane. Also, a loss of sensitivity was observed for this compound. The tailing values were calculated with the Chemstation software (see section 4.3.2.1, figure 10).

Figure 17 below shows the same Grob mixture after installation of the new ultra ion source MS parts :

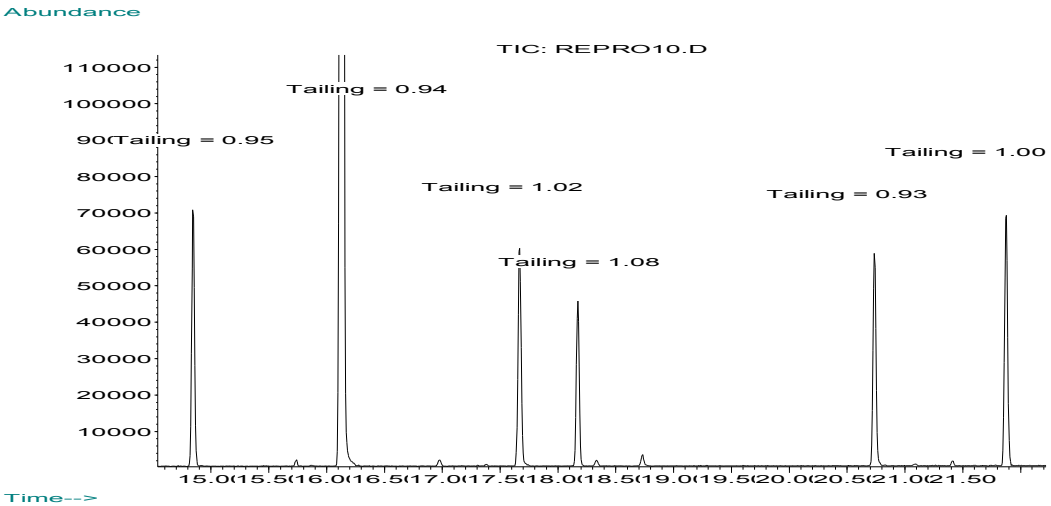


Figure 17 : Same as figure 16 but after installation of the new MS parts.

As can be seen in figure 17, the peaks are now almost perfect and there is no loss of sensitivity for the high boiling alkane (tetracosane).

As will be shown below, the direct effect of using the new MS parts was much better overall repeatability and reproducibility. However, as mentioned by the manufacturer (Agilent Technologies), the small drawback is a slight loss in sensitivity. Nevertheless, this was found to be not so obvious as the improvement in peak shape somehow compensated for the slight weaker response of the ultra ion source.

Repeatability

The modified Grob mixture was injected repeatedly twenty times and the relative standard deviation (RSD) calculated for all relative peak areas (relative to the internal standard). This data was produced for FID and for MSD in Scan and SIM modes.
All compounds were prepared in toluene at a concentration of 0.001 mg / ml, except the internal standard (eicosane) which had a concentration of 0.01 mg / ml.

Table 11 Repeatability of the Grob mixture with FID, MS in SCAN and SIM mode

	IPSC				
	FID	**SCAN OLD MS**	**SCAN NEW MS**	**SIM OLD MS**	**SIM NEW MS**
Dodecane (C12)	1.6 %	1.8 %	1.7 %	0.6 %	0.7 %
2,6-dimethylphenol	1.8 %	2.0 %	1.4 %	0.9 %	0.7 %
Tridecane (C13)	1.3 %	1.9 %	1.5 %	1.1 %	1.0 %
2,6-dimethyl aniline	1.3 %	1.9 %	1.5 %	0.8 %	0.7 %
Decanoic acid Me-ester	0.9 %	2.3 %	1.0 %	1.2 %	0.5 %
Pentadecane *	1.0 %	4.0 %	2.7 %	-	-
Undecanoic acid Me-ester	2.4 %	4.3 %	5.9 %	5.1 %	2.0 %
Dicyclohexylamine	14.7 %	11.2 %	14.8 %	11.8 %	20.9 %
Hexadecane*	0.9 %	3.9 %	2.5 %	-	-
Dodecanoic acid Me-ester	1.0 %	5.7 %	1.6 %	8.2 %	2.7 %
Heptadecane (C17)	0.6 %	4.1 %	1.5 %	1.3 %	0.5 %
Octadecane (C18)	0.5 %	4.3 %	2.1 %	1.4 %	0.5 %
Nonadecane (C19)	0.4 %	3.6 %	2.2 %	1.6 %	0.7 %
N-methyl-diphenethylamine	3.1 %	3.2 %	2.0 %	3.3 %	2.1 %
Ketamine	4.1 %	3.9 %	2.2 %	4.5 %	1.0 %
Tetracosane (C24)	0.7 %	11.9 %	3.5 %	6.7 %	2.1 %
Trimipramine	2.1 %	3.9 %	2.7 %	4.2 %	2.6 %
Average	**2.3 %**	**4.3 %**	**3.0 %**	**3.5 %**	**2.6 %**

*) In SIM mode, pentadecane and undecanoic acid methyl ester could not be separated as they coelute and selected ions were only chosen for undecanoic acid methyl ester. The same for hexadecane and dodecanoic acid methyl ester where only the latter was measured.

The results show that the repeatabilities of different detectors are rather similar and that the MSD performs better after installation of the new MS parts.

Reproducibility

The modified Grob mixture was injected once every day for twenty days. Preparation of samples and calculations were performed as for repeatability.

Table 12 Reproducibility of the Grob mixture with FID, MS in SCAN and SIM mode

	IPSC				
	FID	**SCAN OLD MS**	**SCAN NEW MS**	**SIM OLD MS**	**SIM NEW MS**
Dodecane (C12)	1.3 %	7.6 %	3.5 %	6.3 %	2.3 %
2,6-dimethylphenol	1.5 %	3.9 %	2.4 %	2.2 %	1.5 %
Tridecane (C13)	1.8 %	7.3 %	3.3 %	6.6 %	2.3 %
2,6-dimethyl aniline	1.0 %	3.2 %	2.7 %	1.6 %	1.4 %
Decanoic acid Me-ester	1.3 %	5.2 %	3.3 %	3.2 %	2.2 %
Pentadecane *	2.5 %	9.0 %	10.6 %	-	-
Undecanoic acid Me-ester	2.4 %	11.6 %	10.0 %	6.4 %	7.6 %
Dicyclohexylamine	6.9 %	11.2 %	7.7 %	9.7 %	9.7 %
Hexadecane*	1.3 %	8.9 %	9.0 %	-	-
Dodecanoic acid Me-ester	1.0 %	11.4 %	8.4 %	6.1 %	4.7 %
Heptadecane (C17)	1.1 %	5.4 %	3.1 %	3.4 %	2.2 %
Octadecane (C18)	1.0 %	4.4 %	3.1 %	2.8 %	1.9 %
Nonadecane (C19)	0.5 %	3.6 %	2.7 %	2.9 %	1.6 %
N-methyl-diphenethylamine	2.5 %	13.3 %	2.6 %	13.7 %	2.5 %
Ketamine	6.7 %	20.5 %	3.8 %	21.7 %	3.8 %
Tetracosane (C24)	3.3 %	26.8 %	5.1 %	26.7 %	5.0 %
Trimipramine	1.8 %	15.8 %	3.2 %	17.4 %	3.2 %
Average	**2.2 %**	**9.9 %**	**5.0 %**	**8.7 %**	**3.5 %**

The results show that the reproducibility of FID is slightly better. This was however expected as this detector is known to be very stable. However, the MSD performed quite well (in SCAN and SIM mode) and it is here evident that the "old" MSD showed reproducibility problems with the later eluting compounds.

Sensitivity

Limits of determination :

Limits of determination are regarded as the lower limits for precise quantitative measurements, as opposed to qualititative detection [Miller and Miller, 2000]. For this matter, the RSD values for three replicates were taken into account and the limit of determination was defined as the lower limit where RSD values were still below 10 %.
Unfortunately, the limits of determination could only be calculated for FID and the MS modes (Scan and SIM) with the standard ion source. Replicates were not performed with the new ion source, thus limits of determination could not be calculated. However, partners 2 and 3 did perform these experiments and were able to calculate the limits of determination for the new ion source.
Table 13 below shows the separate results regarding limits of determination :

Table 13 **Results of the limits of determination study (n = 3). N.D. stands for not detected. RSD are in % and are average values calculated from all compounds in the modified Grob mixture.**

Partner 2	**FID**	**NPD**	**MS SCAN (NEW)**	**MS SIM (NEW)**	**MS SCAN (OLD)**	**MS SIM (OLD)**
1 µg / ml	-	-	-	-	-	-
0.1 µg / ml	**0.1**	-	**6**	2	-	-
0.05 µg / ml	16	-	18	2	-	-
0.01 µg / ml	21	-	20	**6**	-	-
0.005 µg / ml	25	-	N.D.	11	-	-
Partner 3		-				
0.5 µg / ml	2	2	3	3	-	-
0.1 µg / ml	7	6	3	**5**	-	-
0.05 µg / ml	**8**	**9**	**9**	11	-	-
0.01 µg / ml	N.D.	N.D.	N.D.	34	-	-
0.005 µg / ml	N.D.	N.D.	N.D.	50	-	-
Partner 4 (IPSC)						
1 µg / ml	2	-	-	-	6	2
0.1 µg / ml	4	-	-	-	**10**	**9**
0.05 µg / ml	**7**	-	-	-	23	12
0.01 µg / ml	N.D.	-	-	-	N.D.	17
0.005 µg / ml	N.D.	-	-	-	N.D.	9

The table shows the average RSD values of the target compounds at various concentrations. The lowest concentration with acceptable repeatability is considered as the limit of determination. This concentration is obviously difficult to define, but, in this context, between days reproducibility values have been used as a reference and thus RSD values less than 10% are considered acceptable.
From this table, it can be seen that there are slight differences between labs. However, it shows that the limit of determination for FID is probably around 0.05 µg / ml (as for NPD) while it's more like 0.1 µg / ml for MS in the SCAN mode. As for MS in the SIM mode, results from different labs seem to be a little contradictory and the true limit of determination is difficult to define. However, the absolute sensitivity of SIM is definitely ten times better compared to the other detection methods as it's still possible to detect compounds at a concentration of 0.005 µg / ml (or 5 ng / ml).

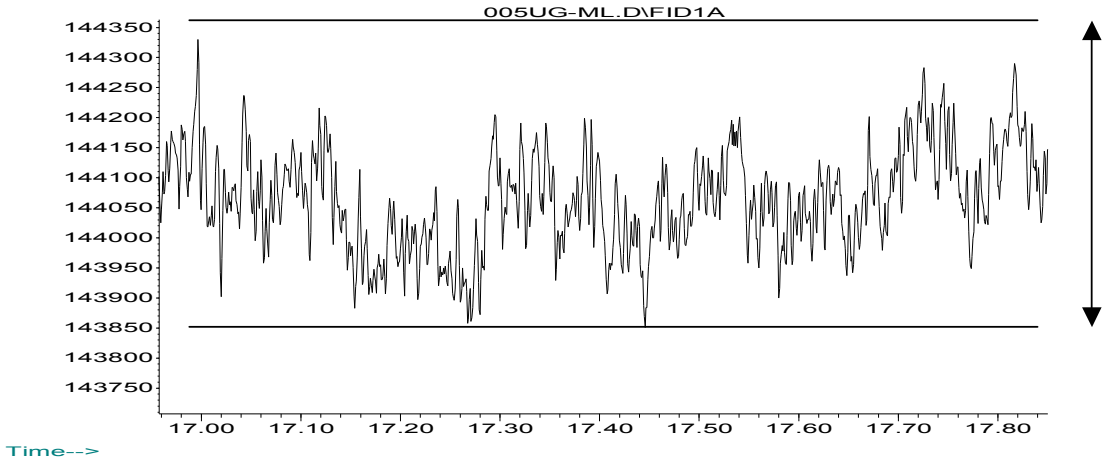
Detection limits :

The limit of detection can be described as the concentration which gives an instrument signal significantly different from the blank or background signal [Miller and Miller, 2000]. The general trend is to define the limit of detection as the analyte concentration giving a signal equal to the blank signal + three standard deviations of the blank.
In this subtask, detection limits were calculated as follows :

$$\frac{C_{dl}}{\frac{S_{dl}}{N_{dl}}} = \frac{C_{exp}}{\frac{S_{exp}}{N_{exp}}} \qquad (7)$$

Where S_{dl}, N_{dl} and C_{dl} are, respectively, the signal, noise and concentration at the detection limit. S_{exp}, N_{exp} and C_{exp} are, respectively, the signal, noise and concentration for experimental values.
The ratio S_{dl} / N_{dl} was defined as 3 (signal to noise ratio of 3) and N_{exp} was defined as the amplitude noise (see figure 18) :

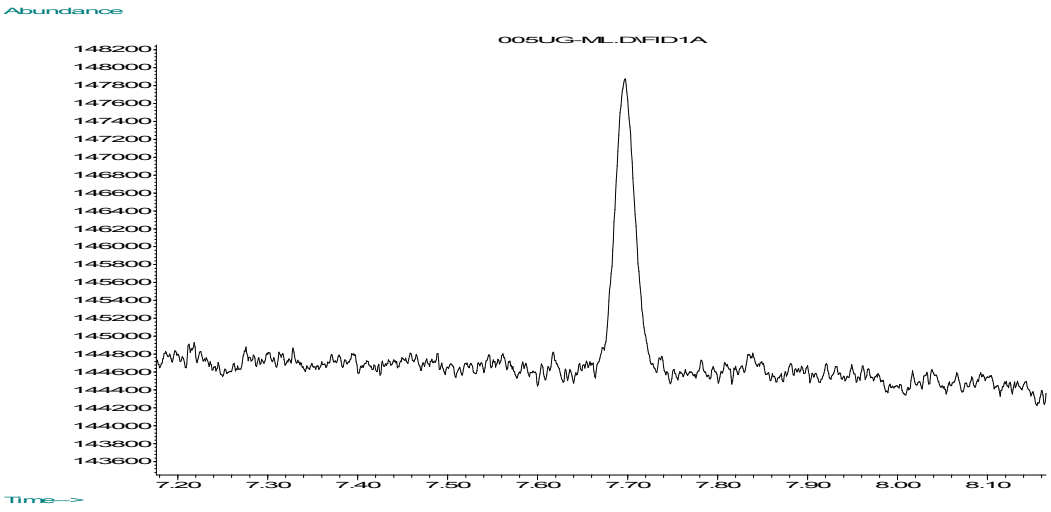
Figure 18 : FID chromatogramme illustrating the amplitude noise.



From figure 18, the amplitude noise can be estimated by looking at the Y scale. In this case, N_{exp} would be : (144350-143850) = 500.

From figure 19 below, the peak height (S_{exp}) can now be obtained visually or by using the Chemstation software :

Figure 19 : FID chromatogramme showing tridecane at a concentration of 0.05 µg / ml



In this case, the peak height is 3400. Thus, the detection limit can be calculated as follows (from equation 7) :

$$C_{dl} = \frac{C_{exp}}{\frac{S_{exp}}{N_{exp}}} \times \frac{S_{dl}}{N_{dl}} = \frac{0.05}{\frac{3400}{500}} \times 3 = 0.022\ \mu g / ml$$

The following table (table 14) summarises the results obtained (partner 4 only) :

Table 14 Results of the detection limits. Concentrations are in ng / ml (partner 4 only).

Partner 4	FID	MS SCAN (NEW)	MS SIM (NEW)	MS SCAN (OLD)	MS SIM (OLD)
Dodecane	20.7	10.1	0.7	7.4	0.6
2,6-dimethylphenol	30.1	15.9	0.7	11.7	0.7
Tridecane	22.2	11.8	1.5	9.6	1.5
2,6-dimethylaniline	31.2	16.7	0.3	10.8	0.3
C10 ester	36.3	16.6	0.4	15.0	0.5
Pentadecane	22.4	15.4	-	18.9	-
C11 ester	31.4	16.8	0.5	14.1	0.6
Dicyclohexylamine	62.9	31.8	0.3	48.0	1.2
Hexadecane	22.8	17.0	-	20.3	-
C12 ester	31.7	17.7	0.6	17.4	0.8
Heptadecane	28.8	31.4	14.3	42.6	13.6
Octadecane	28.9	31.1	10.5	43.4	7.3
Nonadecane	28.6	32.5	11.1	45.5	9.7
N-methyl-di-PEA	42.4	37.8	0.6	53.8	1.1
Ketamine	63.7	91.8	1.1	123.1	2.0
Tetracosane	30.8	37.7	4.3	83.0	3.4
Trimipramine	42.0	49.5	1.1	95.7	3.0
Mean	**33.9**	**28.3**	**3.2**	**38.8**	**3.1**

This table shows that, in terms of qualitative detection limits, the MS is as sensitive as the FID detector. Moreover, it has to be noted that, although the new MS ion source was believed to be less sensitive compared to its standard counterpart, this is not the case. As mentioned earlier, this is probably due to the better chromatographic performance which is obtained with the new MS ion source. Finally, this table confirms that the SIM mode is approximately ten times more sensitive than the SCAN mode.

Linearity

The test mixture was run at five different concentrations covering a range of five orders of magnitude (but having the internal standard at constant concentration). The correlation coefficient was calculated and linearity checked.
The linearity of different detectors has been calculated as explained in **Annex 4**. The concentration range studied by Partners 2, 3 and 4 was 1 – 100 µg/mL, 1 – 500 µg/mL and 1 – 500 µg/mL, respectively. A range of 1 – 10 000 µg/mL was investigated for FID and NPD by Partner 3. The results have been summarised in **Annex 7**.
As for partner 4, the experiments were carried out with the standard and new MS ion source parts. The range of concentrations were 10 to 500 µg/mL with the standard ion source and 1 to 500 µg/mL for the new ion source. The reader should note that linearity was not calculated for the SIM mode. However, the following figure shows the difference in linearity between the standard ion source and new ion source.

Figure 20 : graphs showing linearity of tetracosane and dicyclohexylamine with different detectors

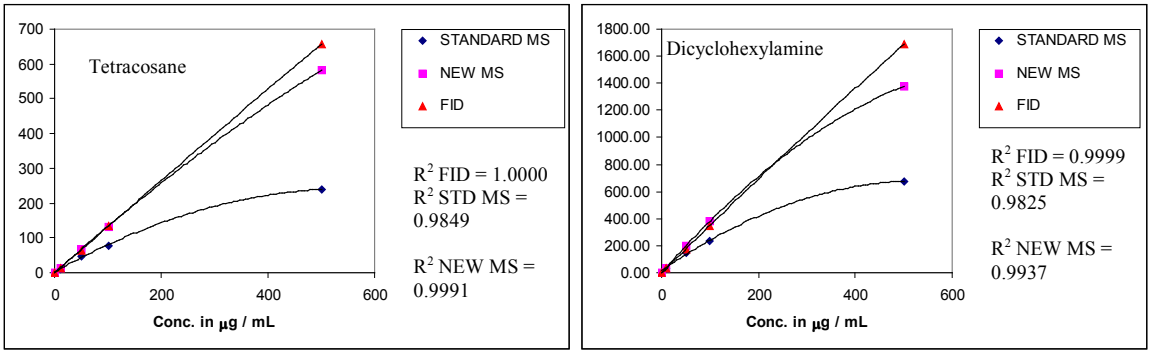


Figure 20 clearly shows that the standard ion source suffers from linearity problems already as from 100 µg / mL. The new ion source gave excellent linearity for tetracosane, even over five orders of magnitude. The same remark is valid for all other compounds in the Grob mixture. However, for an amine compound (dicyclohexylamine), 500 µg / mL seems to be the limit of linearity.
As for FID, the linearity is excellent. This was expected as this detector is known to have excellent linearity, even up to seven orders of magnitude. NPD, studied by partner 3, also showed good linearity.

3.4.4 Conclusions

Overall, all detectors performed well in terms of repeatability, reproducibility, sensitivity and linearity. The only exception being the MSD with the standard ion source. Indeed, this short study clearly demonstrated that, if the tailing phenomenon occurs, as it was the case in the four laboratories, the detector will loose in sensitivity and produce poor results in terms of reproducibility and linearity, especially for high boiling compounds which elute later in the chromatogram.

MSD in SCAN and SIM mode, with the ultra ion source, has almost equal performances compared to FID detection. The main advantage of MS detection is, of course, the ability to identify compounds as a full mass spectrum which is retrievable for all chromatographic peaks. Moreover, it is capable of quantifying coeluting peaks (by measuring the ion responses separately instead of the peak areas). This very important feature, also described as selectivity, is of course not available with FID detection. NPD is also selective as it will detect only compounds containing nitrogen and phosphorous atoms. However, although most of amphetamine impurities contain nitrogen, some do not and most of them only contain one atom of nitrogen.

Finally, the good performance of the MSD with the new ultra ion source indicated that it might become the choice of detection for the final method. The reader is however noted that FID was used in parallel to MSD in scan mode for Tasks 4, 5 and 6 to evaluate the long-term stability of the MSD technique.

3.5 Quality control

During the course of the study, quality control criteria were investigated. A Grob mixture was used to control the performance of the system. The final Grob mixture was the same as the one used in subtask 3.3 with the following modifications : pentadecane and hexadecane were removed and n-octanol and 2-ethylhexanoic acid added. Peak asymmetry values for 2-ethyl hexanoic acid are typically low because the peak is strongly fronting due to the acidity of the compound. The presence of this compound is only visually evaluated as it gives an indication of the activity of the column. If this compound is not detected, it is a strong indication that the column is becoming active, i.e. the stationary phase is deteriorating.
Concentrations of the individual compounds in the Grob mixture should be 1.0 µg/mL except for 2-ethyl hexanoic acid which should be at 10 µg/mL concentration (for splitless injection).

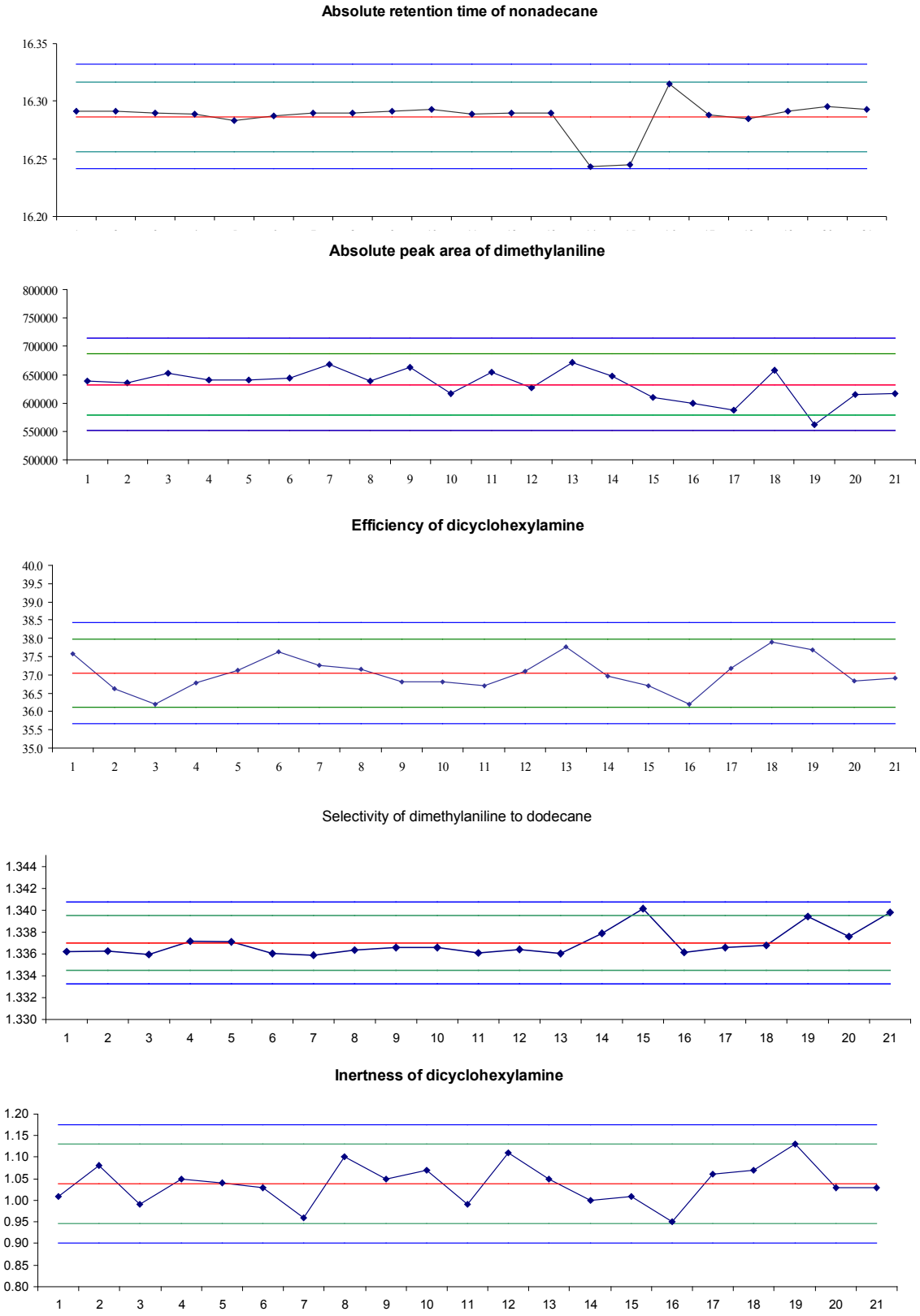
The reader should note that control charts provide a good means to control the parameters in practice. Moreover, new acceptance range may need to be calculated for peak area each time a new Grob stock solution is prepared.
Very narrow retention time range could be used because the final method applies the retention time locking system (Agilent ChemStation software), feature that locks the retention time of the internal standard. However the retention times of other compounds may fluctuate e.g. as a result of ageing of the column or after maintenance actions. Therefore, quality control procedures with clearly defined acceptance criteria are required.

Rather high deviation can be accepted for absolute peak areas since relative peak areas are used in this study.

The following graphs are examples which illustrate the criteria that should be checked :

The red line represents the average value.
The green lines represent two times the standard deviation and are considered as the warning limits.
The blue lines represent three times the standard deviation and are considered as the action limits.

Figure 21 : examples of control charts for quality control



As can be seen in figure 21, two additional criteria are applied : efficiency and selectivity. Efficiency is a term indicating the ability of the column to elute in a narrow chromatographic peak. In our case, it has been approximately calculated by multiplying the absolute peak area by two and divide by the peak height :

$$\text{Efficiency} = \frac{2 \times \text{peak area}}{\text{peak height}} \qquad \textbf{(8)}$$

Therefore, if peaks get broader (loss of efficiency), the peak area will remain constant while the peak height will decrease. Thus, the overall value will increase.

Selectivity is defined as the degree by which the stationary phase differentiates solutes. Separation in gas chromatography is based on different selectivities for components present in a sample. In our case, selectivity has been estimated by dividing the retention times of the compounds by the retention time of dodecane.

The quality control criteria need to be precisely monitored in order to check the instrument performance. It is also essential that immediate corrective action is taken if any significant deviation from the acceptable range is observed. This is particularly important if exchange of data is to be carried out between laboratories.

In figure 21, an example can be seen in the top chart (retention time). Runs 14 and 15 showed a faster retention time on the border of the action limit although the first 13 runs showed perfect stability. As a corrective action the method was relocked (Retention Time Locking) and the deviation corrected.

3.6 Additional liner study

During task 3, almost all injection and detection parameters were studied. However, the influence of the choice of liner was not studied. A preliminary study performed by partner 2 showed that tapered liners gave generally better results in terms of recovery and repeatability compared to straight liners. Although this study was done on a limited number of amphetamine impurities, partner 4 decided to further study three different tapered liners and compare their efficiency in terms of recovery and repeatability. Both a Grob mixture and a synthesised amphetamine extract were used in order to study the influence on Grob compounds and amphetamine impurities.

Sample preparation

A Grob sample was used at a concentration of 0.001 mg / ml in toluene. Three different volumes were injected : 0.2, 1 and 2 microlitres. Three replicates were analysed for each volume.

GC method

The same GC method as in subtask 3.3 was used (see **Annex 2**). FID data was used for the calculations.

Liners

Three liners were studied (all purchased from Agilent Technologies) :

a) 5183-4647 : tapered liner packed with glass wool (middle). Volume : 870 microlitres.
b) 5181-3316 : tapered liner without glass wool. Volume : 900 microlitres.
c) 5062-3587 : tapered liner packed with glass wool (bottom). Volume : 900 microlitres.

Results for Grob compounds

First, the absolute peak areas obtained with the three injection volumes were compared between each liner. Three replicates were injected for each volume and the mean peak area calculated. Table 15 below illustrate the results obtained :

Table 15 Ratios in % of peak response between the three chosen liners

5062-3587 vs 5181-3316	Peak area vs peak area in %		
	0,2 µl	1 µl	2 µl
2,6-dimethylphenol	119%	99%	113%
Tridecane	99%	109%	115%
2,6-dimethylaniline	104%	105%	114%
Tetradecane	105%	109%	117%
Undecanoic acid methyl ester	104%	110%	118%
Dicyclohexylamine	89%	100%	111%
Dodecanoic acid methyl ester	103%	109%	119%
Heptadecane	105%	108%	120%
Octadecane	105%	109%	120%
Nonadecane	108%	109%	119%
Tetracosane	109%	105%	112%
Mean	**105%**	**107%**	**116%**

5062-3587 vs 5183-4647	Peak area vs peak area in %		
	0,2 µl	1 µl	2 µl
2,6-dimethylphenol	109%	120%	123%
Tridecane	106%	116%	119%
2,6-dimethylaniline	112%	120%	128%
Tetradecane	105%	112%	119%
Undecanoic acid methyl ester	109%	115%	121%
Dicyclohexylamine	109%	111%	114%
Dodecanoic acid methyl ester	111%	110%	118%
Heptadecane	107%	107%	112%
Octadecane	104%	105%	110%
Nonadecane	109%	105%	107%
Tetracosane	104%	100%	99%
Mean	**108%**	**111%**	**115%**

5181-3316 vs 5183-4647	Peak area vs peak area in %		
	0,2 µl	1 µl	2 µl
2,6-dimethylphenol	92%	121%	108%
Tridecane	107%	107%	103%
2,6-dimethylaniline	107%	114%	112%
Tetradecane	100%	103%	102%
Undecanoic acid methyl ester	105%	104%	102%
Dicyclohexylamine	122%	111%	103%
Dodecanoic acid methyl ester	109%	101%	99%
Heptadecane	103%	98%	93%
Octadecane	99%	96%	91%
Nonadecane	100%	96%	90%
Tetracosane	96%	95%	89%
Mean	**104%**	**104%**	**99%**

Table 15 shows that there are significant differences between the 5062-3587 liner and the two others, particularly if 1 or 2 microlitres are injected. Thus, these results indicate that the 5062-3587 liner performs better in terms of recovery for Grob compounds. Secondly, the repeatability for each liner was calculated (three replicates for three injection volumes). The mean values for all Grob compounds were taken into account. Table 16 below illustrate the results obtained :

Table 16 Repeatability in % for each liner (N = 3)

	5062-3587	5181-3316	5183-4647
0,2 µl	3.7 %	3.1 %	4.5 %
1 µl	1.7 %	8.3 %	1.4 %
2 µl	1.3 %	9.2 %	0.9 %

These results show that both liners, 5062-3587 and 5183-4647 gave similar repeatability whereas 5181-3316 gives somehow high deviation, particularly when 1 or 2 microlitres are injected.

Results for amphetamine extract

200 mg of synthesised amphetamine were dissolved in 4 ml Tris buffer 1M (ph 8.10). 200 microlitres of toluene containing nonadecane at 0.01 mg /ml were added.
An extract of 100 µl was taken with a pipette and diluted with the internal standard solution to 1 ml. 2 microlitres were injected in triplicate. The same GC method as above was used. The relative peak areas (to internal standard) were compared between each liner. Table 17 below illustrate the results obtained :

Table 17 Ratios in % of relative peak response between the three chosen liners

	5062-3587	5183-4647	5181-3316
4-methyl-5-phenylpyrimidine	100 %	89 %	94 %
4-benzylpyrimidine	100 %	91 %	94 %
1,3-diphenyl-2-propylamine	100 %	98%	92 %
N,N-di(β-phenylisopropyl)amine (1)	100 %	98 %	90 %
N,N-di(β-phenylisopropyl)amine (2)	100 %	98 %	90 %
N,N-di(β-phenylisopropyl)methylamine (1)	98 %	100 %	90 %
N,N-di(β-phenylisopropyl)methylamine (2)	97 %	100 %	90 %
1,3-dimethyl-2-phenylnaphthalene	100 %	90 %	87 %
2,6-dimethyl-3,5-diphenylpyridine	100 %	98 %	91 %
2,4-dimethyl-3,5-diphenylpyridine	100 %	98 %	91 %
pyridine 7-14	100 %	100 %	92 %
pyridine 272	100 %	100 %	93 %
pyridine X	100 %	99 %	93 %
2,6-diphenyl-3,4-dimethylpyridine	100 %	100 %	93 %
N,N-di(β-phenylisopropyl)formamide (1)	99 %	100 %	94 %
N,N-di(β-phenylisopropyl)formamide (2)	99 %	100 %	94 %
2-benzyl-2-methyl-5-phenyl-2,3-dihydropyrid-4-one	98 %	87 %	100 %
Mean	**99.4 %**	**96.8 %**	**92.2 %**

These results confirm the previous ones obtained with the Grob mixture. Thus, the 5062-3587 liner is also the best choice in terms of recovery for amphetamine impurities. Finally, the repeatability for each liner was calculated (three replicates). The mean values for all impurities were taken into account. Table 18 below illustrate the results obtained :

Table 18 Repeatability in % for each liner (N = 3)

5062-3587	5181-3316	5183-4647
1.2 %	1.8 %	0.8 %

As for the Grob compounds, the 5183-4647 liner gave the best result for amphetamine impurities in terms of repeatability and the 5181-3316 the worst.

Conclusions

On the basis of this short study, it is clear that the liner containing no glass wool (5181-3316) is not as good as the two others in terms of recovery and repeatability. The two other liners seem to be equivalent in terms of repeatability but the 5062-3587 liner which contains a thin plug of glass wool at the bottom of the liner seems to give better peak response, hence better recovery. This liner was only introduced into the final GC method in task 6. However, as mentioned in the general conclusions below, this liner may cause some tailing when used in a single-column system (which was not the case in this short study as MS and FID detection was used simultaneously with a two-column system). However, this has only been confirmed by partner 2 and concerns only Grob compounds. Besides, later studies showed that this tailing only concerned the first eluting compounds in the Grob mixture and that it could be solved by using a shorter splitless time (30 seconds instead of 60).

3.7 General conclusions of task 3

Task 3 established that excellent performance could be obtained using the following conditions.

Instrument:	HP 6890 gas chromatograph, HP 5973 mass selective detector with HP MS Chemstation rev. B.01.00
Column:	35% phenyl methyl silicone column 30 m (L) x 0.25 mm (i.d.), d_f 0.25 µm (DB-35MS, HP part no. 122-3832) attached to: Thin-film column 35% phenyl methyl silicone pre-column, 2 - 3 m (L) x 0.25 mm (i.d.), d_f 0.10 µm (DB-35MS). The thin-film column can be ordered as a custom-made product from the Agilent/J&W company. Alternatively a deactivated retention gap can be used, but this type of pre-column has shorter lifetime.
Column connector:	Non-deactivated press-fit connector (HP part no. 5181-3395) was used to connect the retention gap pre-column onto the analytical column
Carrier gas:	Helium, ca. 41 cm/s at 90 °C at MSD, constant flow (see retention time locking).
Sample introduction:	1 µL splitless, 60 mL/min total flow after 1 min (gas saver 20ml/min after 1.5 min), single-tapered glass wool packed liner (HP part no. 5062-3587). Injection volume may be varied from 0.2 to 4.0 µL depending on the concentration of the sample.
Temperatures:	
Injector:	250°C
Oven T-programs:	90 °C (1 min), 8 °C/min, 300 °C (10 min)
GC-MS interface:	310 °C
FID:	310 °C
Retention time locking:	The retention time of internal standard (nonadecane) should be locked to 15.00 min (MSD).
MS information:	
Solvent delay:	4 min
Mass range:	40 - 300 a.m.u. (0-30 min), then 30 to 500 amu.
Sample rate #:	3, A/D samples 8
MS quad temp:	150 °C
MS source:	230 °C

4 Task 4 – optimisation of extraction procedure

4.1 Introduction

Liquid-liquid extraction (LLE) and solid phase extraction (SPE) methods were evaluated to establish the best sample preparation method. Home-made amphetamines synthesised by the nitrostyrene (partner 1), reductive amination (partner 2) and Leuckart methods (partners 3 and 4) were used as the main test samples (TEST-1). Another set of these samples (TEST-2) containing lactose and caffeine was prepared to study matrix effects. Additionally, the efficiency of the extraction methods was assessed using standard impurities made by each Partner. The efficiency of the extraction was measured using relative peak areas for the target compounds and the overall selectivity.

Before starting this Task, all Partners had to prepare a large homogenised sample of amphetamine (40-50 g) so that the amount was sufficient to complete all studies. Method development was carried out with the aim of obtaining a robust and straightforward method having excellent repeatability, reproducibility, accuracy and sensitivity.

This Task was divided into Subtasks with the first four covering the LLE method development work and the fifth concentrating on the SPE:

Subtask 4.1	Screening study for the optimisation of type, pH and concentration of buffer and type of solvent
Subtask 4.2	Further optimisation of type, pH and concentration of buffer using the best solvent
Subtask 4.3	Optimisation of extraction procedure
Subtask 4.4	Study of matrix effects
Subtask 4.5	Optimisation of SPE procedure.

All data presented in this task have been obtained with MS detection in the SCAN mode (ion quantitation).

4.1.1 Preparation of buffers and test samples

TEST-1 sample :

Six different batches were synthesised via the Leuckart route and synthesis conditions varied in order to produce a maximum of impurities. These were mixed together and homogenised with a pestle and a mortar. Then, the powder was transferred to a 1L round bottom flask. Petroleum ether was added until a slurry was obtained. This was mixed on a Rotavapor for 3 hours. The solvent was then let to evaporate at room temperature overnight. Once dried, the powder was recovered with a spatula and mixed again with a pestle and a mortar. A total of 57 grams were available.

TEST-2 sample :

7 grams of TEST-1 sample were mixed together with 14 grams of anhydrous caffeine (Fluka) and 14 grams of D (+) lactose monohydrate (Fluka). The mixture was thoroughly homogenised with a pestle and mortar.

BUFFERS :

The pH meter was calibrated before preparation of every new buffer and a magnetic stirrer used during pH adjustment.

1. Preparation of 0.1 M citrate buffer, pH 6.20

Solution A: 21.0 g of citric acid were dissolved in 1 litre of deionised water.
Solution B: 29.4 g of sodium citrate ($C_6H_5O_7Na_3 \cdot 2H_2O$) were dissolved in 1 litre deionised water. 140 ml of solution A and 856 ml of solution B were mixed. pH was adjusted exactly to pH 6.20 by adding dropwise at first 1.0 M and thereafter 0.1M NaOH or HCl.

2. Preparation of 0.1 M phosphate buffer, pH 7.00

Solution A: 13.8 g of $NaH_2PO_4 \cdot H_2O$ were dissolved in 1 litre deionised water. Solution B: 26.8 g of $Na_2HPO_4 \cdot 7H_2O$ were dissolved in 1 litre distilled water. 390 ml of solution A and 610 ml of solution B were mixed. pH was adjusted exactly to pH 7.00 by adding dropwise at first 1.0 M and thereafter 0.1M NaOH or HCl.

3. Preparation of 0.1 M Tris buffer, pH 7.9

Solution A: 12.11 g of Trizma base (Tris(hydroxymethyl)aminomethane, $C_4H_{11}NO_3$) were dissolved in 1 litre distilled water.
Solution B: 0.1 M hydrochloric acid solution was prepared. 50 ml of solution A and 32.0 ml of solution B were mixed. pH was adjusted exactly to pH 7.90 by adding dropwise at first 1.0 M and thereafter 0.1M NaOH or HCl.

The corresponding 1.0 M solutions were prepared accordingly by using 10 times higher concentrations. 0.5 M solutions can be made by diluting the 1.0 M solutions.

Extraction :

200 mg of TEST-1 (respectively TEST-2) samples are weighed and transferred in a test tube. 2 ml of buffer are added and the test tube shaken vigorously for 30 min. The pH is then measured and adjusted to the pre-defined value (pH 6.20 or 7.00 or 7.90) using 0.1M NaOH or HCl.
200 µl of solvent are added (isooctane, toluene and dichloromethane) or 400 µl (diethyl ether and ethyl acetate) and again the test tube is shaken vigourously for 30 min. The test tube is centrifuged at 3000 rpm for 5 min.
50 – 80 µl of the organic phase is then collected, placed in an autosampler vial (with insert vial) and injected.
The reader should note that 400 µl was the minimum possible volume in order to have phase separation with diethyl ether and ethyl acetate.

4.1.2 Buffer Capacity and dissolution power

It was considered of the utmost importance that the buffer of choice had (i) high dissolution power and (ii) high buffer capacity. The former is required in order to dissolve an amount of amphetamine which is large enough to allow sufficient sensitivity for trace level concentrations of synthesis impurities. This is extremely important since a partly dissolved sample may have a different profile compared to the same sample which is entirely dissolved [Jonson and Artizzu, 1998]. Recent trends in the characteristics of street amphetamine have shown that very pure amphetamine containing very few impurities occur more and more often and therefore a method with high sensitivity and selectivity is required.

Street amphetamine can be very acidic or basic, depending on the batch of drug and also on the eventual adulterants which are used to cut the drug. For example, the use of aspirin (acetylsalicylic acid) as a cutting agent will render the powder slightly acidic. High buffer capacity is required to avoid the need for laborious adjustment of pH to the designated value. The ideal situation would be one in which no pH adjustment is required after dissolving the amphetamine in the buffer.

The dissolution power and buffer capacity of different buffers were studied for the TEST-1 and TEST-2 amphetamine samples of different concentrations. Solubilities of these "model" samples in each buffer were investigated using citrate, phosphate and TRIS buffer solutions at concentrations of 0.1 and 1.0 M. The former concentration represents buffers commonly used in the existing profiling methods (0.1 M) and the latter is a high concentration which represents the almost maximum concentration possible for these buffers.
The results for dissolution power were as expected. TEST-2 samples were difficult to dissolve in all buffers. This is due to the high amount of caffeine (40 %) which is not very soluble in aqueous solutions. On the other hand, TEST-1 samples were dissolved in all buffers. However, the phosphate buffer 1 M was the one that required the most time to dissolve all the amphetamine. In general, no significant differences were observed between 0.1 and 1 M buffers, indicating that the use of more concentrated buffers is possible without loss in dissolution power. Finally, from a subjective point of view (visual observations), it seems that TRIS buffers were the best in terms of dissolution power.

As for buffer capacity, each buffer was studied by measuring the pH after the amphetamine had been added to the buffer and by the amount of 1M sodium hydroxide that had to be added to return to the nominal pH of the buffer. These results, which were obtained by partners 2 and 3 only, are presented in **table 19 :**

Table 19 : Buffer capacity. The nominal pH values of the citrate, phosphate and Tris buffers were 6.2, 7.0 and 7.9, respectively. The reader should be noted that the TRIS-buffer is harmful to gel based KCL electrode. Therefore, the measurements in this buffer were made quickly and the measurements were less accurate. It was found out later on during the study that electrode manufacturers recommend special electrodes for organic buffers. It was also found out that pH adjustment of different buffers at different concentrations was found to be very difficult, hence pH was adjusted to target pH±0.05.

Samples	Buffers	Nominal pH	Partner 2 pH after addition of amph.	Partner 3 pH after addition of amph.	Mean delta pH
TEST-1	0.1 M citrate	6.20	5.62	6.06	0.36
	1.0 M citrate	6.20	6.17	6.25	0.04
TEST-1	0.1 M phosphate	7.00	6.38	6.71	0.46
	1.0 M phosphate	7.00	6.87	6.98	0.07
TEST-1	0.1 M TRIS *	7.90	3.94	7.95	2.01
	1.0 M TRIS	7.90	7.81	7.88	0.06
TEST-2	0.1 M TRIS	7.90	7.99	7.67	0.16
	1.0 M TRIS	7.90	8.02	7.82	0.10
TEST-2	0.1 M citrate	6.20	6.01	6.17	0.11
	1.0 M citrate	6.20	6.22	6.18	0.02
TEST-2	0.1 M phosphate	7.00	6.78	6.88	0.17
	1.0 M phosphate	7.00	6.99	7.02	0.01

* It is unknown whether the unexpectedly high delta pH values is due to incompatibility of the electrode for Tris buffer.

As expected, the results clearly show that 1.0 M buffers have much better buffer capacity in all cases compared to 0.1 M buffers. The applicability of buffers at various concentrations for extraction purposes was studied in more detail in the next Subtasks.

4.2 Type, pH and concentration of buffer and type of solvent

4.2.1 Experimental

In the preliminary screening study buffers were prepared at 0.1 and 1.0 M concentrations, and at pH 6.2 (citrate), pH 7.0 (phosphate) and pH 7.9 (Tris). All these pH values deviate 0.2 pH units from the pKa value of their corresponding acids. Iso-octane, toluene, dichloromethane, diethyl ether and ethyl acetate were used as the solvents. Matrix interferences were studied by using pure home-synthesised amphetamine and the same amphetamine mixed with lactose and caffeine in 20:40:40 (%, w/w) proportions.
The parameters such as type, pH and concentration of buffer, type of solvent and influence of matrix are very probably dependent on each other and they all have an influence on the partition coefficient of each target compound. Table 20 below summarises the parameters studied and the number of experiments performed :

Table 20 Variables used and number of experiments

Parameters						N° of experiments
Buffer type pH	citrate 6.20	phosphate 7.00	Tris 7.90			3
Concentration (mol/L)	0.1	1.0				2
Buffer volume	2 mL					1
Solvent type	iso-octane	toluene	dichloromethane	diethyl ether	ethyl acetate	5
Solvent volume	200 µL					1
Matrix effect	none	lactose + caffeine				2
Replicates	1					1

Overall, the number of experiments is 3 x 2 x 1 x 5 x 1 x 2 x 1, which equals to 60.

4.2.2 Results and discussion

In order to generate an overview of the results, relative response factors (RRF) were calculated in both TEST-1 and TEST-2 samples (peak area of target compound / peak area of internal standard). Z-scores were then calculated, i.e. the RRF values were normalised by substracting the mean and dividing by the standard deviation according to equation 9 :

$$Z_i = \frac{RRF_i - \frac{\sum_i^n RRF}{n}}{(\text{Standard Deviation})_n} \quad \textbf{(9)}$$

Where Z_i is the z-score and i is the target compound and n = 30 (n = 30 for TEST-1 samples and 24 for TEST-2 samples). Thus, the mean and standard deviation are calculated for one target compound in all conditions.
Then, the average z-score was calculated for all target compounds in each condition according to equation 10 :

$$Z_{tot} = \frac{\sum_i^n Z_i}{N} \quad \textbf{(10)}$$

Where i is the target compound and N is the number of target compounds studied. In this subtask, N = 15 because the following impurities were studied by partner 4 :

1. Benzyl methyl ketoxime 1
2. Benzyl methyl ketoxime 2
3. Benzylamphetamine
4. 1,3-diphenyl-2-propylamine (DPPA)
5. DPIA
6. DPIMA 1
7. DPIMA 2
8. 1-benzyl-3-methylnaphtalene
9. 1,3-dimethyl-2-phenylnaphtalene
10. Benzoylamphetamine
11. 2,6-dimethyl-3,5-diphenylpyridine
12. 2,4-dimethyl-3,5-diphenylpyridine
13. 2,6-diphenyl-3,4-diphenylpyridine
14. DPIF 1
15. DPIF 2

These impurities were detected in the amphetamine batch synthesised by partner 4. Twelve of them are considered Class A compounds (see table 7, section 4.3.2). Although DPIA (the two isomers put together) was considered as a class B compound in section 4.3.2, it was added to the list as it is now considered an important impurity especially in reductive amination amphetamine. The two phenyl-2-propanone oxime isomers were also added to the list as they were found to be important impurities, especially for nitrostyrene amphetamine. Nevertheless, it can also be detected in Leuckart amphetamine and, as it was detected in this amphetamine batch, it was decided to include it in the list of impurities.

The results were separately calculated for TEST-1 (100 % amphetamine) and TEST-2 (20 % amphetamine, 40 % caffeine, 40 % lactose) samples. They are shown in **table 21** and were sorted in downward order, the highest value indicating the best condition :

Table 21 : Z-scores for 15 amphetamine impurities with various buffers and solvents

TEST-1				TEST-2			
Solvent	**pH**	**Buffer**	**Average**	**Solvent**	**pH**	**Buffer**	**Average**
Toluene	7	Phosphate 1M	1.333	Toluene	7	Phosphate 1M	1.540
Toluene	7.9	TRIS 1M	1.197	Toluene	7.9	TRIS 0.1M	1.275
Dichloromethane	7	Phosphate 1M	1.001	Toluene	7.9	TRIS 1M	1.145
Dichloromethane	7.9	TRIS 0.1M	0.972	Toluene	7	Phosphate 0.1M	0.956
Toluene	7.9	TRIS 0.1M	0.955	Toluene	6.2	Citrate 0.1M	0.913
Toluene	6.2	Citrate 1M	0.914	Toluene	6.2	Citrate 1M	0.782
Dichloromethane	7.9	TRIS 1M	0.902	Isooctane	7.9	TRIS 0.1M	0.765
Dichloromethane	7	Phosphate 0.1M	0.839	Isooctane	7.9	TRIS 1M	0.592
Dichloromethane	6.2	Citrate 0.1M	0.803	Isooctane	7	Phosphate 1M	0.416
Toluene	7	Phosphate 0.1M	0.712	Isooctane	7	Phosphate 0.1M	0.267
Dichloromethane	6.2	Citrate 1M	0.694	Isooctane	6.2	Citrate 0.1M	0.218
Toluene	6.2	Citrate 0.1M	0.501	Isooctane	6.2	Citrate 1M	0.121
Isooctane	7.9	TRIS 1M	0.236	Ethyl acetate	7.9	TRIS 1M	-0.291
Isooctane	7.9	TRIS 0.1M	0.108	Ethyl acetate	7.9	TRIS 0.1M	-0.508
Isooctane	6.2	Citrate 1M	0.033	Ethyl acetate	7	Phosphate 1M	-0.513
Isooctane	7	Phosphate 1M	0.032	Diethyl ether	7	Phosphate 1M	-0.536
Isooctane	6.2	Citrate 0.1M	-0.126	Diethyl ether	7.9	TRIS 0.1M	-0.640
Isooctane	7	Phosphate 0.1M	-0.131	Diethyl ether	7.9	TRIS 1M	-0.713
Ethyl acetate	7	Phosphate 1M	-0.727	Ethyl acetate	6.2	Citrate 1M	-0.775
Ethyl acetate	7.9	TRIS 1M	-0.742	Ethyl acetate	6.2	Citrate 0.1M	-0.821
Diethyl ether	7	Phosphate 1M	-0.812	Diethyl ether	7	Phosphate 0.1M	-0.826
Ethyl acetate	6.2	Citrate 1M	-0.854	Ethyl acetate	7	Phosphate 0.1M	-0.834
Ethyl acetate	7.9	TRIS 0.1M	-0.887	Diethyl ether	6.2	Citrate 1M	-0.922
Ethyl acetate	7	Phosphate 0.1M	-0.898	Diethyl ether	6.2	Citrate 0.1M	-0.935
Diethyl ether	7.9	TRIS 1M	-0.930				
Diethyl ether	7.9	TRIS 0.1M	-0.938				
Ethyl acetate	6.2	Citrate 0.1M	-1.018				
Diethyl ether	6.2	Citrate 1M	-1.085				
Diethyl ether	7	Phosphate 0.1M	-1.109				
Diethyl ether	6.2	Citrate 0.1M	-1.223				

First of all, it was not possible to perform extractions with the TEST-2 samples and dichloromethane. Indeed, the first problem is that dichloromethane lies beneath the aqueous phase, thus making the collection of the organic layer more difficult. Moreover, it was impossible to separate the two phases with this type of sample and with the chosen extraction method. This is unfortunate as dichloromethane showed to be a good extraction solvent. Even doubling the volume of dichloromethane (200 to 400 µl) did not help as the other partner laboratories were not able to separate the two phases with 400 µl.
Also, this double volume of solvent probably explains why diethyl ether and ethyl acetate gave poor results compared to toluene and isooctane. Indeed, their extraction power could not compensate for the volume used. It is probable that the same remarks would be valid if dichloromethane could have been extracted when using a volume of 400 µl.

4.2.3 Conclusions

Table 21 clearly shows that toluene is the most suitable solvent, followed by isooctane. It also shows that the type of solvent was the most influential parameter in this subtask. Regarding concentration of buffer, it is difficult to tell if it has a significant influence on the extraction power. In terms of pH, it is also difficult to draw a definitive conclusion. However, the results indicate that phosphate at pH 7.0 and TRIS at pH 7.9 give better results than citrate at pH 6.2. This is probably due to the fact that some impurities are slightly basic and the remaining are rather neutral, hence the small overall difference of extraction power between pH 7.0 and 7.9. Results from other partners were in agreement with these findings.

4.3 Further optimisation, multiple extractions, buffer and solvent volumes

4.3.1 Introduction

The next stage was to compare one and multiple (3) step extractions and buffer and extraction volumes. Total recovery and relative standard deviation of results obtained with different methods were studied with the aim of finding conditions providing the highest sensitivity and the best repeatability.

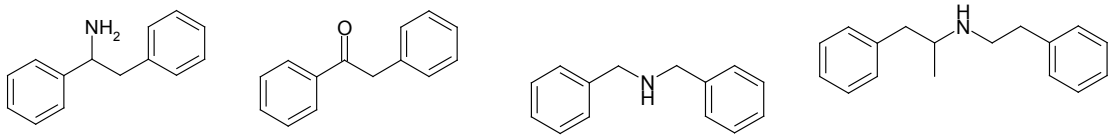
Based on subtask 4.1 results, TRIS and phosphate buffers were used in this Subtask. The pKa values of the buffers were chosen as their pH values to maximise buffer capacity. The variables were investigated according to **table 22** :

Table 22 Parameters for subtask 4.2

Parameters	TEST-2 sample (20 % amphetamine, 40 % caffeine, 40 % lactose)				N° of experiments
Buffer volume	2 mL	8 mL			2
Buffer type Solvent type	Tris, pH 8.1 toluene	Phosphate, pH 7.2 iso-octane			2
Solvent volume	200 µL	3 x 200 µL	600 µL	3 x 600 µL	4
Replicates	3				3
				Total	**48**

4.3.2 Nitrostyrene amphetamine

At this stage of the project, there was a lack of data and results for nitrostyrene amphetamine. Partner 1 who was responsible for studying nitrostyrene impurities encountered various problems which hampered their work. Therefore, it was decided that, at least for this subtask, partner 4 would synthesise a batch of nitrostyrene amphetamine and perform the required analyses.
For this purpose, a batch of nitrostyrene amphetamine was synthesised according to the recipe described in ANNEX 1. Some new impurities were identified and included in the data analysis. These are described below :



1,2-diphenethylamine | 1,2-diphenyl ethanone (Benzylphenylketone) | Dibenzylamine | α-methyl-diphenethylamine

Figure 22 : chemical structures of new nitrostyrene impurities

As 1,2-diphenyl ethanone and dibenzylamine are commercially available, these two compounds were purchased. The identity of these two nitrostyrene impurities were then confirmed after comparison of their retention times and mass spectra with those of the reference compounds. Regarding 1,2-diphenethylamine, it was simply synthesised by reductive amination of the purchased 1,2-diphenylethanone. Again, its identity was further confirmed in the same way. As for α-methyl-diphenethylamine, it is not absolutely certain that the impurity is indeed this compound as no reference material was available. The identification is only based on its mass spectrum which perfectly matches the mass spectrum of α-methyl-diphenethylamine found in the NIST library. However, further identification could be performed by synthesising this compound, synthesis that could be performed by reacting BMK and 2-phenethylamine. For time reasons, this synthesis was not carried out.

4.3.3 Results and discussion

Relative response factors values (RRF) were calculated by dividing the peak areas of the impurities by the peak area of the internal standard (nonadecane in this subtask). The following impurities were studied :

1. 2-methyl-3-phenylaziridine
2. Benzyl methyl ketoxime 1
3. Benzyl methyl ketoxime 2
4. 1,2-diphenethylamine
5. Dibenzylamine
6. 1,2-diphenyl ethanone
7. Benzylamphetamine
8. α-methyl-diphenethylamine

Sensitivity was calculated by dividing the RRF value of a certain impurity under a specified condition by the maximum value obtained for that compound. For each condition an average value was calculated for all compounds to illustrate the overall performance. The highest average value was used to indicate the best sensitivity.

Recovery was calculated in the same way. However, the volume of solvent was taken into consideration, i.e. values of samples extracted three times with 200 µl were multiplied by three, values of samples extracted with 600 µl were multiplied by three and values of samples extracted three times with 600 µl were multiplied by 9. The highest average value was used to identify the best recovery.

The lowest relative standard deviation value indicated the best repeatability (three replicates).

The results obtained for the sensitivity, repeatability and recovery for the nitrostyrene TEST-2 sample are shown in table 23 **:**

Table 23 : Sensitivity, recovery and repeatability for nitrostyrene impurities

	Sensitivity	**Recovery**	**Repeatability (n=3)**
8mL Tris+200µL toluene	77%	58%	8%
8mL Tris+600µL toluene	31%	68%	4%
8mL Tris+3x200µL toluene	37%	80%	9%
8mL tris+3x600µL toluene	13%	82%	4%
8mL phosphate+200µL iso-octane	27%	21%	13%
8mL phosphate+600µL iso-octane	16%	36%	5%
8mL phosphate+3x200µL iso-octane	19%	44%	5%
8mL phosphate+3x600µL iso-octane	8%	55%	**2%**
2mL Tris+200µL toluene	**100%**	75%	8%
2mL Tris+600µL toluene	36%	79%	8%
2mL Tris+3x200µL toluene	44%	**97%**	6%
2mL Tris+3x600µL toluene	14%	89%	10%
2mL phosphate+200µL iso-octane	36%	28%	8%
2mL phosphate+600µL iso-octane	18%	39%	7%
2mL phosphate+3x200µL iso-octane	23%	50%	5%
2mL phosphate+3x600µL iso-octane	9%	59%	4%
Toluene all, average	**44%**	**79%**	7%
Iso-octane all, average	20%	42%	6%
One-step 200 all, average	**60%**		9%
One-step 600 all, average	25%	56%	6%
Three-steps 200 all, average	31%	**68%**	6%
Three-steps 600 all, average	11%		5%
2 mL all, average	**35%**	**65%**	7%
8 mL all, average	29%	56%	6%

The results in **table 23** can be summarised in the following way :

In general, toluene gave better sensitivity than iso-octane. One-step extraction with 200 µL solvent was the most sensitive method whereas three-steps extraction 600 µL solvent was the worst method. This is somehow expected as sensitivity in this case is a matter of concentration.
Regarding the volume of buffer, 2 mL was better than 8 mL.

Toluene gave far better recovery than iso-octane. Three-steps extraction with 600 µL gave the best recovery and one-step extraction with 200 µL the worst. Finally, the use of 2 mL of buffer enabled a better recovery compared to 8 mL.

Regarding repeatability, no significant differences were observed between the various conditions. Only the one-step extraction with 200 µL seem to be a little worse.

These results, which are only for nitrostyrene impurities, were combined with those from other partners. Partner 2 studied the reductive amination impurities and partner 3 the Leuckart impurities. As can be seen in table 24, the overall results do not deviate much from the results obtained in table 23.

Table 24 : Sensitivity, recovery and repeatability for all impurities. Combined results from partners 2,3 and 4.

	Sensitivity				**Recovery**				**Repeatability (n=3)**			
	Amination	Leuckart	Nitrostyr.	**All**	Amination	Leuckart	Nitrostyr.	**All**	Amination	Leuckart	Nitrostyr.	**All**
8mL Tris+200µL toluene	84%	97%	77%	86%	60%	77%	58%	65%	5%	7%	8%	7%
8mL Tris+600µL toluene	25%	35%	31%	30%	49%	84%	68%	67%	23%	6%	4%	11%
8mL Tris+3x200µL toluene	41%	38%	37%	39%	84%	90%	80%	85%	3%	9%	9%	7%
8mL tris+3x600µL toluene	16%	13%	13%	14%	84%	90%	82%	85%	4%	13%	4%	7%
8mL phosphate+200µL iso-octane	40%	75%	27%	47%	29%	60%	21%	37%	15%	7%	13%	12%
8mL phosphate+600µL iso-octane	11%	24%	16%	17%	24%	58%	36%	39%	4%	9%	5%	6%
8mL phosphate+3x200µL iso-octane	9%	31%	19%	20%	63%	73%	44%	60%	8%	13%	5%	9%
8mL phosphate+3x600µL iso-octane	12%	10%	8%	10%	70%	69%	55%	65%	4%	10%	2%	**5%**
2mL Tris+200µL toluene	100%	97%	100%	**99%**	68%	77%	75%	73%	5%	4%	8%	6%
2mL Tris+600µL toluene	42%	36%	36%	38%	76%	84%	79%	80%	5%	5%	8%	6%
2mL Tris+3x200µL toluene	48%	40%	44%	44%	87%	95%	97%	**93%**	2%	6%	6%	**5%**
2mL Tris+3x600µL toluene	21%	13%	14%	16%	97%	90%	89%	92%	3%	10%	10%	8%
2mL phosphate+200µL iso-octane	68%	71%	36%	58%	50%	56%	28%	45%	2%	18%	8%	9%
2mL phosphate+600µL iso-octane	28%	27%	18%	24%	60%	64%	39%	54%	3%	7%	7%	6%
2mL phosphate+3x200µL iso-octane	40%	30%	23%	31%	88%	73%	50%	70%	4%	6%	5%	**5%**
2mL phosphate+3x600µL iso-octane	29%	10%	9%	16%	60%	69%	59%	63%	8%	9%	4%	7%
Toluene all, average				**46%**				**80%**				7%
Iso-octane all, average				28%				54%				7%
One-step 200 all, average				**73%**				55%				8%
One-step 600 all, average				27%				60%				7%
Three-steps 200 all, average				33%				**77%**				**6%**
Three-steps 600 all, average				14%				76%				7%
2 mL all, average				**41%**				**71%**				**6%**
8 mL all, average				33%				63%				8%

From the overall results illustrated in table 24, It is very clear that the most sensitive extraction method is 2 mL TRIS buffer with 200 µl of toluene. This is valid for all impurities regardless of the synthetic route. The repeatability is also quite good with this extraction method.

Regarding recovery, it is evident that three-step extraction will recover a higher percentage of impurities. However, the dilution that results from this operation causes a significant loss in sensitivity. Nevertheless, the recovery results show again that toluene is a much better solvent than isooctane and that 2 mL of buffer is to be preferred to 8 mL.

4.3.4 Conclusions

The best performing liquid-liquid extraction method for all impurities was the Tris / toluene system, 2 mL buffer volume and one-step extraction with 200 µL of solvent. This method did not give the best recovery, but this could only have been improved by using more solvent, either in one step or in three steps. However, such an increase in solvent volume seriously decreases the sensitivity. Moreover, three-steps extractions were considered far too laborious and time-consuming for routine use in forensic laboratories.

4.4 Study on influence of the matrix

4.4.1 Introduction

Matrix effects were studied using amphetamine at various concentrations. Synthesised amphetamine (via the Leuckart route) was cut with caffeine (the most common adulterant found in street amphetamine) in order to have concentrations of 15 and 50 % amphetamine. Two buffers and solvents were evaluated also in this subtask to verify their performance for different types of samples. Therefore, Tris and phosphate buffers were evaluated with both isooctane and toluene. This time 4 mL buffer was chosen as the lowest buffer volume as 2 mL was found to be insufficient in terms of dissolution power for heavily cut samples. Additionally, the phosphate buffer was chosen as 0.5 M by concentration as the phosphate salt tended to crystallise at higher concentrations. The parameters studied are summarised in **table 25 :**

Table 25 Variables used for the study on the influence of the matrix

Parameter	**LEVEL**		
Type of buffer pH Concentration	phosphate 7.2 0.5M	TRIS 8.1 1.0M	
Solvent	iso-octane	toluene	
Solvent volume	200 µL		
Buffer volume	4 mL	8 mL	
Sample: amount of amphetamine amount of caffeine	30 mg 170 mg	100 mg 100 mg	200 mg 0 mg
Replicates	3		

4.4.2 Results and discussion

The results of this subtask are summarised in **table 27.** The RRF values of all impurities were normalised against the amount of amphetamine (15%, 50% and 100%). RRF values were calculated by dividing the peak area of the impurity by the peak area of the internal standard. For the 15 % amphetamine, RRF values were therefore multiplied by 100 / 15 and for 50 % amphetamine, they were multiplied by 2.
Averages were then calculated for the figures obtained for the three concentrations. Sensitivity was calculated by dividing the RRF value of a certain impuritiy under a specified condition by the maximum value for that compound. The sensitivity illustrates ability of the method to compare samples with low concentration impurities.

In order to illustrate the stability of the method against matrix effects, the deviations between RRF values at different concentrations of amphetamine were calculated. For example, if no matrix effects are present, the normalised RRF value of an impurity in 15 % amphetamine should be the same as the RRF value in 100 % amphetamine. **Table 26** provides an example for one impurity. In this case, 2,4-dimethyl-3,5-diphenylpyridine :

Table 26 : example of calculation for the deviation of RRF values

Tris buffer and toluene	RRF values of 2,4-dimethyl-3,5-diphenylpyridine
4 ml and 15 % amphetamine	31.80
4 ml and 50 % amphetamine	34.52
4 ml and 100 % amphetamine	34.67
Relative Standard Deviation (RSD)	**4.8 %**

The same calculations were made for each conditions and all impurities. The final RSD's shown in table 27 are the averages for all impurities in each condition.

Table 27 : sensitivity and deviations for the study on the influence of matrix effects

Extraction system	**Sensitivity**	**RSD between different dilutions**
TRIS, 4 ml, toluene	**98 %**	**4 %**
TRIS, 4 ml, isooctane	90 %	7 %
TRIS, 8 ml, toluene	94 %	5 %
TRIS, 8 ml, isooctane	84 %	9 %
Phosphate, 4 ml, toluene	83 %	17 %
Phosphate, 4 ml, isooctane	78 %	17 %
Phosphate, 8 ml, toluene	86 %	9 %
Phosphate, 8 ml, isooctane	78 %	8 %

4.4.3 Conclusions

Overall, it is clear that the best extraction system is 4 ml TRIS buffer with toluene. Indeed, this system not only gives the best sensitivity, but also is the most robust against matrix effects.
Results obtained from partner 3 (also Leuckart amphetamine) confirmed these observations. As for reductive amination impurities (partner 2), the best sensitivity was given by 4 ml phosphate buffer and toluene. However, the deviations were quite high with this extraction system. 4 ml TRIS buffer with toluene was second best and gave almost the same sensitivity. Also, the deviations were very small with this system confirming that it is robust against matrix effects.
Regarding nitrostyrene impurities, unfortunately no results are available for this subtask.

4.5 Optimisation of solid phase extraction (SPE)

4.5.1 Introduction

Solid-phase extraction (SPE) resembles liquid-liquid extraction (LLE) since both are based on partition mechanism, although the former is a dynamic process whereas LLE is based on static equilibrium. The partition mechanism, however, enables application of the best buffer found for the optimised LLE method also in SPE. This is also a prerequisite for obtaining interchangeable results between these two extraction techniques. On the other hand, the elution solvent used in SPE has a different function than the organic solvent used in LLE.

In LLE the solvent acts as one of the phases participating in the partition process. The SPE stationary phase such as octadecyl silica has the same function. In SPE, the organic solvent is used to elute all target compounds out of the SPE column for subsequent GC analysis. In theory, the elution solvent should have as good elution power (see "eluotropic values" or "solvent strength values" in HPLC literature) as possible although solubility in water may also play an important role: residual water in an SPE column may hinder elution of the analytes if an apolar solvent is used. In conclusion, selection criteria for the organic solvent used in LLE and in SPE are rather different.

In this study two SPE columns were evaluated. The best buffer found in Subtasks 4.1 - 4.3 (TRIS), two buffer volumes and two solvents with different eluotropic values were studied as well.

4.5.2 Experimental

Fourteen different SPE columns (100 mg/1mL) obtained from IST (International Sorbent Technology) were considered. These include the following various types : non-endcapped, endcapped and monofunctional octadecyl (C18), non-endcapped and endcapped octyl (C8), non-endcapped, and endcapped cyanopropyl (CN), non-endcapped, and endcapped phenyl (PH), aminopropyl (NH_2), ethylenediamine-N-propyl (PSA) and 2,3-dihydroxypropoxypropyl (DIOL).
Additionally, mixed-mode extraction columns, namely Confirm HCX containing both non-polar (C8) and strong cation exchange ($-SO_3^-$) functional groups and Multimode containing non-polar (C18), strong cation exchange and strong anion exchange ($-NR_3^+$) functional groups were studied. Finally, a polymer-based Oasis HLB (Waters Inc.) column was evaluated.

From some initial studies performed by partner 2 and based upon extraction efficiencies, the HCX and Oasis HLB columns were selected for further investigation. The parameters used for the comparison of these columns are presented in **table 28 :**

Table 28 Variables used for the optimisation of solid phase extraction

Parameter		**LEVEL**	
SPE columns		HCX	Oasis HLB
Type of buffer		TRIS	
Solvent	type	ethyl acetate	toluene
	volume	200 µL	
Buffer	volume	4 mL	8 mL
	concentration	1.0 M	
Number of eluate fractions		3	

The SPE column was conditioned with 1mL of methanol and 1 mL of 1M Tris buffer. 200 mg of amphetamine (TEST-2 : 20% amphetamine, 40% caffeine, 40% lactose) were weighed into a test tube. The powder was dissolved in 4 mL of 1M Tris buffer (pH 8.1) by shaking for 30 min. The sample was loaded into the SPE column using a flow rate of 1 mL/min. The column was washed with 2 mL of water and dried with nitrogen gas using a flow rate of approx. 60 mL/min for 10min. The analytes were eluted using three times 200 µL of solvent containing nonadecane as an internal standard at a concentration of 10 µg / ml. Each fraction was collected separately in an appropriate test tube. The extracts were finally placed in GC vials. The samples were analysed using the GC method described in **Annex 2**.

4.5.3 Results and discussion

First, the HCX columns were evaluated as the OASIS HLB columns were not yet available. The target compounds were identified and relative response factors (RRF's) calculated for each target compound, i.e. ratio between the analyte and the internal standard peak areas. The values were normalised by calculating RRF / maximum RRF for each condition. The recovery was calculated for the sum of the RRF's of the three different fractions at each condition. An average of the recoveries of different target compounds was calculated to estimate the overall performance of the extraction. The highest value indicates the best recovery. The recoveries as well as the repeatabilities (for three replicates) are summarised in table 29 :

Table 29. Relative recoveries and repeatability of the target compounds obtained with the HCX columns.

	RECOVERY				REPEATABILITY			
	HCX, 4mL Tris+et.ac.	HCX, 8mL Tris+et.ac.	HCX, 4mL Tris+tolu	HCX, 8mL Tris+tolu	HCX, 4mL Tris+et.ac,	HCX, 8mL Tris+et.ac.	HCX, 4mL Tris+tolu	HCX, 8mL Tris+tolu
DPPA	100 %	95 %	0 %	0 %	2 %	3 %	-	-
DPIA 1 + 2	100 %	84 %	21 %	22 %	8 %	2 %	15 %	5 %
DPIMA 1	100 %	84 %	40 %	41 %	10 %	3 %	11 %	11 %
Naphtalene 1	85 %	100 %	47 %	56 %	4 %	4 %	9 %	15 %
Benzoylamph.	100 %	93 %	48 %	50 %	8 %	4 %	3 %	4 %
2,6-3,5 pyridine	93 %	100 %	53 %	69 %	7 %	8 %	10 %	11 %
2,4-3,5 pyridine	91 %	100 %	25 %	40 %	1 %	5 %	10 %	6 %
Pyridine 7	95 %	100 %	58 %	74 %	3 %	7 %	3 %	9 %
Pyridine 14	100 %	93 %	43 %	45 %	13 %	6 %	7 %	14 %
DPIF 1	98 %	100 %	42 %	47 %	6 %	5 %	4 %	6 %
DPIF 2	97 %	100 %	42 %	47 %	7 %	4 %	5 %	6 %
Average	**97 %**	**94 %**	**35 %**	**41 %**	**6 %**	**5 %**	**8 %**	**9 %**

Table 29 clearly shows that ethyl acetate is by far superior in terms of recovery with this type of SPE column. Repeatability is also better with ethyl acetate. Regarding the buffer volume, there is no significant difference between the use of 4 and 8 mL.
In the next phase, the OASIS HLB column should have been studied in the same way and the results compared to the HCX column. However, for time reasons, it was only possible to study one condition. As 4 mL Tris and ethyl acetate give the best recovery for the HCX column, this condition was chosen. The values were calculated in the same way as table 29 and are presented in table 30 below :

Table 30. Relative recoveries and repeatability of the target compounds obtained with the OASIS and HCX columns (4 mL TRIS, ethyl acetate).

	RECOVERY		REPEATABILITY	
	HCX, 4mL Tris+et.ac.	OASIS, 4mL Tris+et.ac.	HCX, 4mL Tris+et.ac.	OASIS, 4mL Tris+et.ac.
DPPA	83 %	100 %	2 %	3 %
DPIA 1 + 2	100 %	92 %	8 %	3 %
DPIMA 1	100 %	63 %	10 %	12 %
Naphtalene 1	100 %	99 %	4 %	13 %
Benzoylamph.	79 %	100 %	8 %	14 %
2,6-3,5 pyridine	100 %	76 %	7 %	12 %
2,4-3,5 pyridine	77 %	100 %	1 %	3 %
Pyridine 7	100 %	87 %	3 %	8 %
Pyridine 14	36 %	100 %	13 %	3 %
DPIF 1	100 %	100 %	6 %	5 %
DPIF 2	99 %	100 %	7 %	5 %
Average	**89 %**	**92 %**	**6 %**	**7 %**

Table 30 show that very similar results are found between the OASIS HLB column and the HCX column.

However, after observation of the reductive amination amphetamine, it was noticed that the OASIS HLB column extracted more of the polar compounds. This was confirmed by the results of partner 2 who studied reductive amination amphetamine. Their results show a far better recovery for their corresponding impurities which are more polar than Leuckart impurities. Table 31 below illustrate their results :

Table 31. Relative recoveries of reductive amination target compounds obtained with the OASIS and HCX columns.

RECOVERY	HCX 4mL AcOEt	HCX 8 ml AcOEt	HCX 4mL toluene	HCX 8 ml toluene	Oasis 4mL AcOEt	Oasis 8 ml AcOEt	Oasis 4mL toluene	Oasis 8 ml toluene
Acetylamphetamine	100%	16%	16%	6%	42%	32%	26%	23%
Benzylamphetamine	77%	65%	99%	82%	82%	93%	98%	100%
DPIA (1)	75%	63%	100%	83%	75%	83%	88%	89%
DPIA (2)	73%	61%	100%	83%	71%	78%	85%	85%
Cathinol (1)	81%	67%	83%	67%	96%	100%	99%	93%
Cathinol (2)	67%	55%	76%	62%	87%	91%	100%	92%
Benzoylamphetamine	15%	8%	11%	7%	85%	100%	81%	84%
2-oxo	21%	13%	15%	8%	87%	100%	79%	82%
Mean	**64%**	**44%**	**63%**	**50%**	**78%**	**85%**	**82%**	**81%**

As can be seen in table 31, the Oasis column performs much better in terms of recovery for reductive amination impurities. It also shows that there doesn't seem to be any significant difference between the use of ethyl acetate or toluene with the Oasis column. This has been confirmed for Leuckart impurities by partner 3.

Finally, the HCX and OASIS columns were briefly compared to liquid-liquid extraction. Two extracts from two different Leuckart amphetamines (20 % amphetamine and 50 % amphetamine) were extracted with 4 ml TRIS 1M and 200 microlitres of toluene (optimised LLE procedure). The results were compared to those obtained by SPE with 4 ml TRIS 1M and 200 microlitres ethyl acetate (HCX and OASIS columns). Table 32 illustrates the results obtained :

Table 32. Comparison of recoveries between LLE (4ml TRIS, 200 µl toluene), SPE with HCX and OASIS (4ml TRIS, 200 µl ethyl acetate).

	HCX vs LLE 20% amph.	HCX vs LLE 50% amph.	OASIS vs LLE 20% amph.	OASIS vs LLE 50% amph.	OASIS vs HCX 20% amph.	OASIS vs HCX 50% amph.
DPPA	127%	121%	226%	170%	178%	141%
DPIA 1	142%	124%	146%	138%	103%	112%
DPIMA 1	227%	132%	142%	143%	63%	109%
Naphtalene 1	40%	78%	26%	65%	65%	84%
Naphtalene 2	45%	71%	30%	56%	66%	79%
Benzoylamphetamine	178%	165%	182%	169%	102%	102%
2,6-3,5-pyridine	65%	84%	52%	80%	80%	96%
2,4-3,5-pyridine	64%	83%	63%	81%	99%	97%
pyridine 7	74%	80%	59%	77%	80%	97%
pyridine 14	85%	-	72%	-	85%	-
DPIF 1	129%	120%	127%	126%	98%	105%
DPIF 2	131%	121%	125%	124%	95%	103%
Mean	**109%**	**107%**	**104%**	**112%**	**93%**	**102%**
Mean 2 samples	**108 %**		**108 %**		**98 %**	

This table confirms the previous results where no significant difference was found between the HCX and Oasis columns in terms of recovery for Leuckart impurities. It also shows that SPE gives a slightly better recovery compared to the optimised LLE method. This also has been confirmed by other partner results. Indeed, SPE gives slightly better recovery not only for Leuckart but also for reductive amination impurities.

Still, it was expected that recovery for SPE would be far better to the value shown in table 32 (i.e. 108 % compared to LLE).
The probable explanation was found later as it was determined that the non-polar impurities were somehow not totally transferred to the SPE column from the test tube containing the dissolved amphetamine.

Indeed, it seemed that these « oily » compounds were sticking to the walls of the test tube and were therefore not entirely transferred to the SPE column.
To improve the transfer, the Tris buffer was modified in such a way that it contained 10 % methanol. This indeed removed a fair amount of the non-polar impurities from the test tube and thus improved the transfer.

However, adding methanol is a risk in the further elution of the SPE column as the impurities could be partially lost in the loading phase of the SPE column (i.e. elute with the buffer / methanol and not be retained in the column).
Unfortunately, for time reasons it was not possible to carry out further investigations in order to determine the exact influence of these modifications.
Moreover, it was also found out that this « sticking » effect is dependent upon the test tubes which are used for dissolving the amphetamine. Indeed, this phenomena was observed for polypropylene tubes (used by partner 4) and for used glass tubes (used by partner 2). However, for partner 3, who used brand new Kimax® test tubes (made of borosilicate glass), this effect was not significant.

4.5.4 Conclusions

In conclusion, SPE gives good results. Recovery and repeatability are quite similar to liquid-liquid extraction. However, there are some concerns regarding the future inter-laboratory results if SPE is chosen as the extraction method. Indeed, standardization of the equipment would be of paramount importance as the same SPE equipment should be purchased as well as the same test tubes in order to guarantee comparable data. We also have no information regarding the reproducibility of SPE columns in time.
For these reasons, it was finally decided to choose liquid-liquid extraction as the final extraction method.

Outside of the studies shown above the dissolving and extraction times in the LLE method were separately optimised. As an outcome of this study it was found that dissolving and extraction times could be reduced to 10 minutes without any loss in the performance.

The final optimised LLE method is described in detail below.

- Buffer: 1M Tris buffer, pH 8.10.

- Organic solvent: Toluene (HPLC quality) containing nonadecane as internal standard at 10µg/mL concentration.

- Balance: Analytical balance with minimum 0.1mg resolution.

- Test tubes: New (or as good as new) glass test tubes (ca. 10mL) with Teflon lined screw caps.

- Shaking system: Horizontal or rotating system.

- LLE procedure: Dissolve 200 ± 5 mg amphetamine in 4 mL Tris buffer and shake vigorously for 10 minutes. Add 200 µL toluene and shake again for another 10 min. Centrifuge the tubes for 2-3 min at ca. 2500 rpm to separate the phases. Take an aliquot of the toluene layer and place it in a GC vial containing 100 µL insert vial (Agilent part no. 5181-1270). Analyse the samples using the GC method described in **Annex 3**.

- Calibration : Calibrated pipettes are used for all liquid measurements. Accuracy for 4 mL measurement (buffer) is ± 0.04 mL and ± 0.02 mL for 0.2 mL (organic solvent). Water is used for calibration of the large pipette (4 mL) and toluene for the small pipette (0.2 mL) taking into account that the density of toluene (0.867 g/mL).

  Example : put a GC vial on an analytical balance, set to zero, add 0.2 mL of toluene, the weight should be 173.4 mg (0.2 * 0.867 * 1000). Calibrate the pipette accordingly.

5 Task 5 – determination of the variability of the optimised method

5.1 Introduction

The aim of this task was to investigate the stability of the optimised method developed in the previous tasks. Variations within a day, between days and between laboratories were investigated. For this task, twelve batches of amphetamine were prepared and homogeneised. Each batch was split into four equal parts.
One part stayed at the « manufacturing » laboratory, the three others were sent to Partner's laboratories. Each laboratory had then 3 x 4 = 12 samples. In addition, a 13th sample was prepared in each laboratory by cutting one of the samples by a factor ten in order to study the influence of dilution.
At this stage of the project, the final choice of detector was not made. Therefore, FID and MS detection were recorded simultaneously and both were studied. Moreover, one laboratory also studied within day and between days variations with NPD detection.

5.2 Experimental

5.2.1 Preparation of samples

For this task, twelve batches of amphetamine were prepared. Six of them were synthesised via the Leuckart route, three via the nitrostyrene route and three via the reductive amination route.
For the Leuckart amphetamine, phenyl-2-propanone (Fluka®) was refluxed with formamide and formic acid, then hydrolysed with sulfuric or hydrochloric acid. Amphetamine base was then isolated and cristallised as the sulphate salt.
For reductive amination amphetamine, phenyl-2-propanone was stirred at room temperature with ammonium acetate and sodium cyanoborohydride. Amphetamine base was then isolated and cristallised as the sulphate salt.
For nitrostyrene amphetamine, phenyl-2-nitropropene (Sigma-Aldrich®) was stirred at room temperature with sodium dihydrobis(2-methoxyethoxy)aluminate (Fluka®) according to Butterick [Butterick and Unrau, 1974]. Amphetamine base was then isolated and cristallised as the sulphate salt.

5.2.2 Composition of samples

Red 1, 2 and 3 had to be spiked with target compounds previously synthesised because of their absence or too low concentrations in these batches.
Most of the synthesised batches were cut with various adulterants and / or diluents in order to reflect amphetamine samples seized in the illicit market. Table 33 describes the composition of the studied amphetamines :

Table 33. Composition of the sixteen amphetamine batches.

Sample	Amphetamine (%)	Caffeine (%)	Lactose (%)	Mannitol (%)	Koffisal® (%)*	Sucrose (%)
Leuckart 1	10	-	70	-	20	-
Leuckart 2	10	25	65	-	-	-
Leuckart 3	15	-	-	41		44
Leuckart 4	100	0	0	-	-	-
Leuckart 5	50	25	25	-	-	-
Leuckart 6	20	40	40	-	-	-
Nitro 1	6	16	78	-	-	-
Nitro 2	6	12	-	82	-	-
Nitro 3	6	-	47	47	-	-
Red 1	20	40	40	-	-	-
Red 2	20	40	40	-	-	-
Red 3	15	-	85	-	-	-
Leu 1 diluted	1	-	97	-	2	-
Leu 5 diluted	5	22,5	72,5	-	-	-
Leu 6 diluted	2	4	94	-	-	-
Red 1 diluted	2	4	94	-	-	-

* Koffisal ® is a Danish preparation used as adulterant in illicit amphetamine (in Denmark) containing phenazone salicylamide, phenazone salicylate and caffeine.

5.2.3 Integration and target compounds

Fixed integration parameters were used to enable data comparability. When small peaks were not automatically integrated using these parameters and / or when obvious deviations occurred for some compounds, manual re-integration was performed.
Relative Response Factors (RRF) values were calculated by normalising the peak areas of the target compounds to the peak area of the internal standard.
For MS quantitation, ion responses were used. A target ion was chosen for each target compound as well as two qualifiers. The target compounds as well as their target ions chosen for MS quantitation are shown in table 34 :

Table 34. Target compounds and their target ions used in task 5.

	Leuckart 1	Leuckart 2	Leuckart 3	Leuckart 4	Leuckart 5	Leuckart 6	Red 1	Red 2	Red 3	Nitro1	Nitro2	Nitro3	Target ions
Benzaldehyde oxime [e]										X	X	X	121
2-methyl-3-phenylaziridine [a]										X	X	X	132
Benzyl methyl ketoxime (isomer 1) [a]										X	X	X	149
Benzyl methyl ketoxime (isomer 2) [a]										X	X	X	131
N-acetylamphetamine [a]							X	X	X				118
1,2-diphenethylamine [e]										X	X	X	106
Benzenemethanamine, N-(phenylmethyl)- [d] (commercial name : dibenzylamine)										X	X	X	106
Benzylamphetamine [a]			X	X	X	X	X	X	X	X	X	X	134
DPPA : 1,3-diphenyl-2-propylamine [a]	X	X	X										120
Diphenethylamine, α-methyl- [e]										X	X	X	148
DPIA 1 : N, N-di-(β-phenylisopropyl)amine (isomer 1) [a]							X	X	X				162
DPIA 2 : N, N-di-(β-phenylisopropyl)amine (isomer 2) [a]							X	X	X				162
DPIMA 1 : N,N-di(β-phenylisopropyl)methylamine 1 (isomer 1) [a]	X		X	X									176
DPIMA 2 : N,N-di(β-phenylisopropyl)methylamine 1 (isomer 2) [a]	X		X	X									176
Cathinol 1 : 1-hydroxy-N,N-di(β-phenylisopropyl)amine (isomer 1) [a]							X	X	X				162
Cathinol 2 : 1-hydroxy-N,N-di(β-phenylisopropyl)amine (isomer 2) [a]							X	X	X				162
1,3-dimethyl-2-phenylnaphthalene [b]	X			X	X	X							232
Benzoylamphetamine [a]		X			X	X	X	X	X				105
2-oxo-1-phenyl-(β-phenylisopropylamine)ethane [a]							X	X	X				162
2,6-dimethyl-3,5-diphenylpyridine [c]	X	X	X	X	X	X							259
2,4-dimethyl-3,5-diphenylpyridine [a]	X	X	X	X	X	X							259
Pyridines 7 and 14 [f]	X	X		X	X	X							258
Pyridine 272 [g]	X				X								272
Pyridine X [h]	X												258
2,6-diphenyl-3,5-dimethylpyridine [c]	X	X	X	X	X								258
DPIF 1 : N,N-di(β-phenylisopropyl)formamide (isomer 1) [a]	X	X	X	X	X	X							190
DPIF 2 : N,N-di(β-phenylisopropyl)formamide (isomer 2) [a]	X	X	X	X	X	X							190

[a] Synthesised within the SMT project.

[b] Compounds of which standards were provided by the United Nations Office for Drugs and Crime (UNODC).

[c] Compounds of which standards were provided by the Netherlands Forensic Institute (NFI).

[d] Other compounds for which standards were available.

[e] Other compounds for which standards were not available. Identification based only upon comparison with Wiley or NIST MS library.

[f] Two compounds not separated with the GC method. Their mass spectra indicate that they are pyridine derivatives. The names pyridine 7 and pyridine 14 are those used by partner 3 in their laboratory (SKL, Sweden).

[g] Pyridine derivative tentatively identified as 2,4-dimethyl-3-phenyl-6-(phenylmethyl)-pyridine by van den Ark [van den Ark et al., 1978c].

[h] Compound with the same mass spectrum as identified pyridines. The name "pyridine x" was given as the exact chemical structure could not be determined.

5.2.4 Time frame of analyses

Each test sample was analysed at day 0 (T_0), after 7 days (T_7) and after 14, 28, 42 and 56 days (T_{14}, T_{28} , T_{42} and T_{56}). For the determination of the within day variation, six extracts were prepared and analysed on a chosen day for each batch of amphetamine.
The between days variation was calculated using the values for each day and the average value of the day where six replicates were analysed, thus n = 6.
Additionally, each laboratory had to choose one batch of amphetamine and dilute it 10 times. Table 35 summarises the analyses carried out :

Table 35. Time frame of analyses

Samples	**Number of analyses**						
	Day 0	**Day 7**	**Day 14**	**Day 28**	**Day 42**	**Day 56**	**Total**
Leuckart 1 [a]	6	1	1	1	1	1	11
Leuckart 2 [a]	1	6	1	1	1	1	11
Leuckart 3 [a]	1	1	6	1	1	1	11
Red 1 [b]	1	1	1	6	1	1	11
Red 2 [b]	1	1	1	1	6	1	11
Red 3 [b]	1	1	1	1	1	6	11
Leuckart 4 [c]	6	1	1	1	1	1	11
Leuckart 5 [c]	1	6	1	1	1	1	11
Leuckart 6 [c]	1	1	6	1	1	1	11
Nitro 1 [d]	6	1	1	1	1	1	11
Nitro 2 [d]	1	6	1	1	1	1	11
Nitro 3 [d]	1	1	6	1	1	1	11
dilute sample [*]	1	1	1	6	1	1	11
Total	**28**	**28**	**28**	**23**	**18**	**18**	**143**

[a] Samples synthesised via the Leuckart route in laboratory n° 4 (IPSC, Lausanne, Switzerland).
[b] Samples synthesised via the reductive amination of benzylmethyl ketone in laboratory n° 2 (NBI, Finland).
[c] Samples synthesised via the Leuckart route in laboratory n° 3 (SKL, Sweden).
[d] Samples synthesised via the nitrostyrene route in laboratory n° 4 (IPSC, Lausanne, Switzerland).
[*] The last sample in this table corresponds to the diluted sample. Leuckart 1 was used by Laboratory n° 4, Red 1 was used by Laboratory n° 2, Leuckart 5 by Laboratory n° 3 and Leuckart 6 by Laboratory n° 1.

Extractions of the amphetamine samples were performed with the method optimised in task 4 and described in section 4.5.4. GC methods are described in **Annex 2**.

5.3 Results and discussion

5.3.1 Within day variations

In this section, the results of Laboratory n° 1 have been presented separately because this laboratory had some chromatographic problems and was not able to meet the requirements of the quality control sample before the beginning of this task. This is the reason why only FID data is available for this laboratory.
The within day variations of different laboratories when analysing the synthesised batches using FID and MS are summarized in tables 36 and 37 respectively.

Table 36. Within day variation of different laboratories given as the average RSD (%) of all target compounds for each sample. Samples were analysed with GC-FID. The relative standard deviations (RSD's) have been calculated on the basis of relative response factor (RRF) values.

Sample	Lab 2	Lab 3	Lab 4	Mean of labs 2 - 4	*lab 1*	*Mean of all labs*
Leuckart 1	10 %	4 %	7 %	7 %	7 %	7 %
Leuckart 2	6 %	6 %	8 %	7 %	12 %	8 %
Leuckart 3	5 %	4 %	9 %	6 %	10 %	7 %
Leuckart 4	3 %	3 %	6 %	4 %	4 %	4 %
Leuckart 5	6 %	6 %	8 %	7 %	12 %	8 %
Leuckart 6	3 %	2 %	6 %	4 %	8 %	5 %
Red 1	2 %	2 %	3 %	2 %	8 %	4 %
Red 2	4 %	4 %	5 %	4 %	11 %	6 %
Red 3	6 %	4 %	4 %	5 %	4 %	5 %
Nitro 1	5 %	4 %	5 %	5 %	15 %	7 %
Nitro 2	9 %	7 %	5 %	7 %	9 %	8 %
Nitro 3	7 %	8 %	6 %	7 %	8 %	7 %
Dilute sample	3 %	6 %	8 %	-	6 %	-
Average	**5 %**	**5 %**	**6 %**	**5 %**	**9 %**	**6 %**

Table 37. Same as table 36 but for GC-MS

Sample	Lab 2	Lab 3	Lab 4	Mean of labs 2 - 4
Leuckart 1	5 %	2 %	4 %	4 %
Leuckart 2	5 %	5 %	9 %	6 %
Leuckart 3	5 %	4 %	7 %	5 %
Leuckart 4	3 %	3 %	8 %	5 %
Leuckart 5	8 %	5 %	9 %	7 %
Leuckart 6	4 %	2 %	5 %	4 %
Red 1	4 %	5 %	2 %	4 %
Red 2	3 %	5 %	4 %	4 %
Red 3	5 %	4 %	2 %	4 %
NITRO 1	7 %	7 %	6 %	7 %
NITRO 2	8 %	9 %	6 %	8 %
NITRO 3	8 %	8 %	6 %	7 %
Dilute sample	4 %	18 %	4 %	-
Average	**5 %**	**6 %**	**6 %**	**5 %**

Finally, the within day variations for NPD detection are presented in table 38. Relative Response Factors (RRF) values were calculated by normalising the peak area of the target compound to the peak area of the internal standard which, in this case, was 2,6-dimethyl aniline. Only one laboratory (lab n° 3) performed the analyses with NPD detection.

Table 38. Same as tables 36 and 37 but for GC-NPD

NPD detection	Within day variation
Leuckart 1	3 %
Leuckart 2	6 %
Leuckart 3	5 %
Leuckart 4	3 %
Leuckart 5	7 %
Leuckart 6	4 %
Red 1	4 %
Red 2	4 %
Red 3	5 %
Nitro 1	4 %
Nitro 2	8 %
Nitro 3	10 %
Dilute sample	6 %
Average	**5 %**

As can be seen from the results illustrated in tables 37, 38 and 39, the within day variations are in the order of 5 to 6 % with three different detectors. It seems that the detection method does not have any significant influence on the results.

5.3.2 Between days variations

Again, the results of Laboratory n° 1 have been presented separately for the same reasons as mentioned previously. The between days variations of different laboratories when analysing the synthesised batches using GC-FID, GC-MS and GC-NPD are summarized in tables 39 to 41 respectively.

Table 39. Between days variations of different laboratories given as the average RSD (%) of all target compounds for each sample. Samples were analysed with GC-FID.

Sample	Lab 2	Lab 3	Lab 4	Mean of labs 2 - 4	Lab 1	Mean of all labs
Leuckart 1	9 %	8 %	6 %	8 %	8 %	8 %
Leuckart 2	7 %	9 %	8 %	8 %	16 %	10 %
Leuckart 3	13 %	6 %	11 %	10 %	21 %	13 %
Leuckart 4	5 %	7 %	8 %	7 %	16 %	9 %
Leuckart 5	10 %	6 %	11 %	9 %	16 %	11 %
Leuckart 6	9 %	6 %	10 %	8 %	15 %	10 %
Red 1	4 %	6 %	3 %	4 %	13 %	7 %
Red 2	9 %	10 %	6 %	8 %	16 %	10 %
Red 3	8 %	9 %	7 %	8 %	11 %	9 %
Nitro 1	10 %	9 %	7 %	9 %	14 %	10 %
Nitro 2	6 %	8 %	7 %	7 %	14 %	9 %
Nitro 3	8 %	8 %	8 %	8 %	21 %	11 %
Dilute sample	13 %	22 % *	7 %	-	11 %	-
Average	**9 %**	**9 %**	**8 %**	**8 %**	**15 %**	**10 %**

* Exceptionally high deviation due to inhomogeneity of the sample.

Table 40. **Same as table 39 but for GC-MS.**

Sample	**Lab 2**	**Lab 3**	**Lab 4**	**Mean of labs 2 - 4**
Leuckart 1	4 %	7 %	6 %	6 %
Leuckart 2	5 %	11 %	5 %	7 %
Leuckart 3	10 %	10 %	8 %	9 %
Leuckart 4	4 %	10 %	8 %	7 %
Leuckart 5	8 %	4 %	7 %	6 %
Leuckart 6	11 %	8 %	6 %	8 %
Red 1	7 %	10 %	4 %	7 %
Red 2	6 %	10 %	4 %	7 %
Red 3	5 %	7 %	3 %	5 %
Nitro 1	11 %	11 %	7 %	10 %
Nitro 2	11 %	12 %	7 %	10 %
Nitro 3	12 %	11 %	8 %	10 %
Dilute sample	4 %	11 %	4 %	-
Average	**8 %**	**9 %**	**6 %**	**8 %**

Table 41. **Same as tables 39 and 40 but for GC-NPD.**

NPD detection	Between days variation
Leuckart 1	5 %
Leuckart 2	9 %
Leuckart 3	6 %
Leuckart 4	8 %
Leuckart 5	6 %
Leuckart 6	7 %
Red 1	6 %
Red 2	10 %
Red 3	8 %
Nitro 1	12 %
Nitro 2	8 %
Nitro 3	10 %
Dilute sample	18 %
Average	**9 %**

As can be seen from the results illustrated in tables 39, 40 and 41, the between days variations (over a period of two months) are in the order of 8 % both with FID and MS and 9 % for NPD. Again, it seems that the detection method does not have any significant influence on the results.

5.3.3 Effects of dilution

Four samples were diluted 10 times with lactose. The aim was to determine the effect of dilution on the extraction and GC analyses in terms of variation. Each laboratory used one sample for dilution.
The average of the relative response factors for each target compound was calculated. The values for the diluted samples were then multiplied by ten. The variation was then calculated for each target impurity according to equation 11 :

$$\text{Variation target impurity in \%} = \frac{\text{absolute value}\left(\bar{x} - \bar{y}\right)}{\left(\bar{x} + \bar{y}\right)} \div 2 \qquad \textbf{(11)}$$

Where $\bar{x}$ is the mean value for the sample and $\bar{y}$ is the mean value for the diluted sample.
For the total variation, the average of all target impurities was calculated and results are shown in table 42 :

Table 42. Deviation in % between diluted and undiluted samples (factor 10)

	Lab 2	Lab 3	Lab 4	Mean of labs 2 - 4	Lab 1	Mean of all labs
FID detection	7 %	10 %	9 %	**9 %**	17 %	**11 %**
MS detection	15 %	13 %	18 %	**15 %**	-	-

5.3.4 Inter-laboratory variation

As in sections 5.3.1 and 5.3.2, results from laboratory n°1 are presented separately. In order to calculate the variations between laboratories, both mean values for within day and between days were taken into account for each laboratory (n = 8). Table 43 below shows an example on how the relative standard deviations were calculated and overall results are presented in table 44.

Table 43. Example of calculation for determination of the inter-laboratory variation

sample Red 1	Lab 2 within day	Lab 2 betw. day	Lab 3 Within day	Lab 3 betw. day	Lab 4 within day	Lab 4 betw. day	Lab 1 within day	Lab 1 betw. day	Mean	RSD
N-acetylamphetamine	3.45	3.72	3.80	3.94	4.12	4.14	3.07	3.46	3.71	10%
benzylamphetamine	39.00	38.95	38.29	38.42	37.44	36.94	28.31	35.86	36.65	10%
DPIA 1	2.53	2.51	2.48	2.52	2.51	2.51	1.81	2.38	2.41	10%
DPIA 2	5.58	5.54	5.69	5.66	5.42	5.39	4.31	5.40	5.37	8%
Cathinol 1	21.54	21.45	18.72	19.15	20.96	20.88	13.31	19.48	19.44	14%
Cathinol 2	1.06	1.01	0.94	0.99	1.10	1.09	0.70	1.06	1.00	13%
Benzoylamphetamine	7.38	7.26	6.70	6.82	6.66	6.70	5.53	6.89	6.74	8%
2-oxo	15.81	15.51	14.65	14.58	14.14	14.19	11.52	14.59	14.37	9%

Mean 10%

Table 44. Inter-laboratory variations given as the average RSD's (%) of all target compounds for each sample.

	Inter-laboratory (FID) Mean labs 2, 3 and 4	Inter-laboratory (FID) Mean all labs	Inter-laboratory (MS) Mean labs 2, 3 and 4
Leuckart 1	9 %	10 %	9 %
Leuckart 2	10 %	14 %	9 %
Leuckart 3	6 %	13 %	14 %
Leuckart 4	7 %	9 %	10 %
Leuckart 5	8 %	10 %	7 %
Leuckart 6	11 %	11 %	9 %
Red 1	4 %	10 %	13 %
Red 2	12 %	14 %	9 %
Red 3	7 %	10 %	6 %
Nitro 1	10 %	15 %	15 %
Nitro 2	8 %	12 %	11 %
Nitro 3	9 %	14 %	12 %
Mean	**8 %**	**12 %**	**10 %**

As can be seen in table 44, inter-laboratory variation is in the same order of magnitude as the between days variation (8 %). If laboratory n° 1 is taken into account, this value raises to 12 %. Regarding MS detection, good results were obtained as the variation was determined to be 10 % in average. However, some chromatograms had to be re-evaluated in order to present the results shown in table 44. Indeed, some integration problems (especially for FID detection) were observed. The purpose of the next paragraph (5.3.5) is to illustrate some examples of these potential problems.
Figure 23 is an illustration of a « Leuckart » sample analysed in four laboratories :

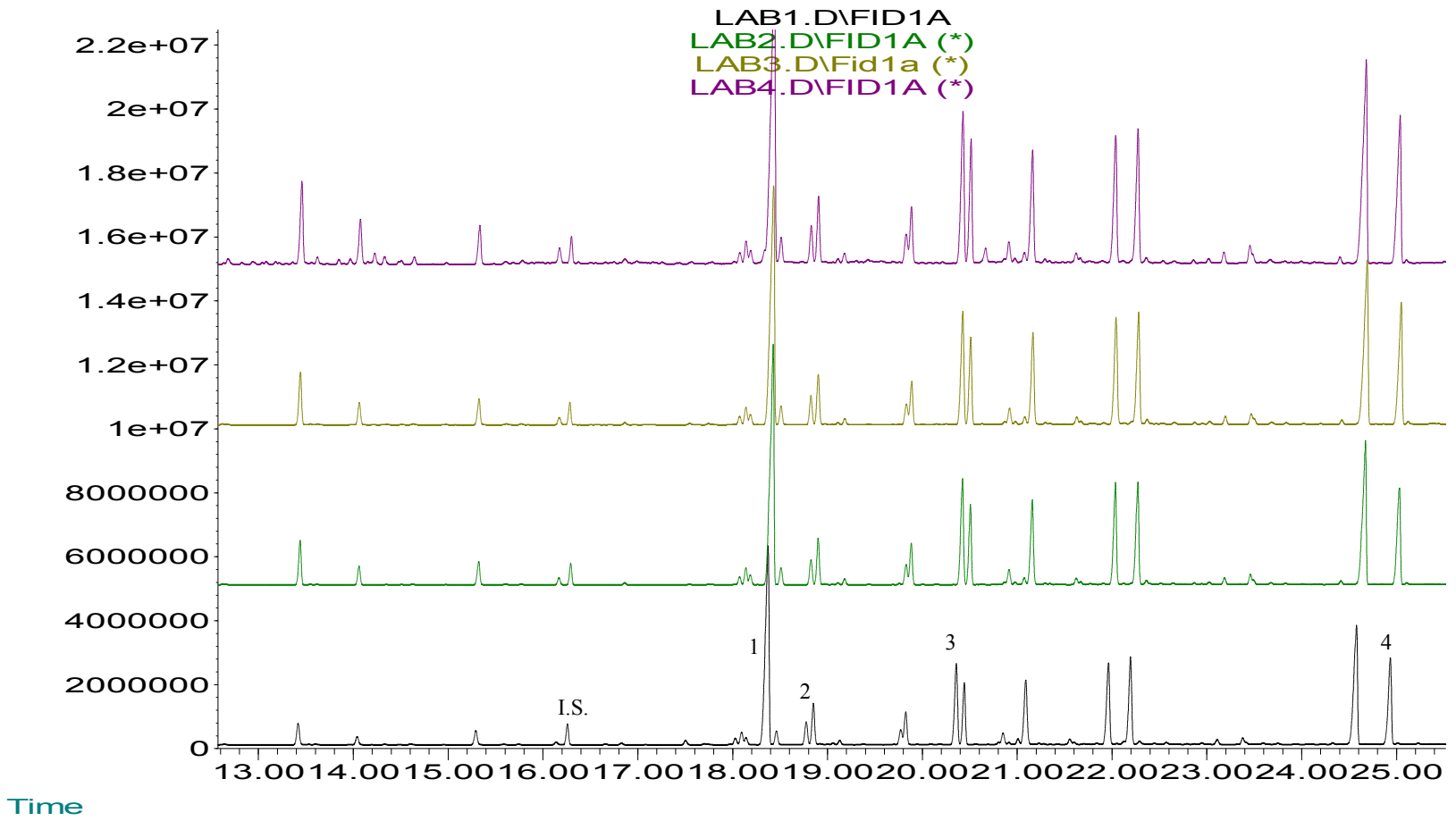


Figure 23 : FID chromatograms of sample Leuckart 3 analysed in four laboratories. Major impurities are annotated : I.S. : internal standard (nonadecane), 1. 1,3-diphenyl-2-propylamine, 2. DPIA isomers, 3. DPIMA isomers, 4. DPIF isomers.

5.3.5 Factors affecting integration

During the course of this study, it was observed that the combination of polypropylene test tubes (for liquid-liquid extraction) with toluene caused some contamination of the extracts due to co-extracted oligomers. This is easily illustrated by chromatograms of blank extracts in figure 24 :

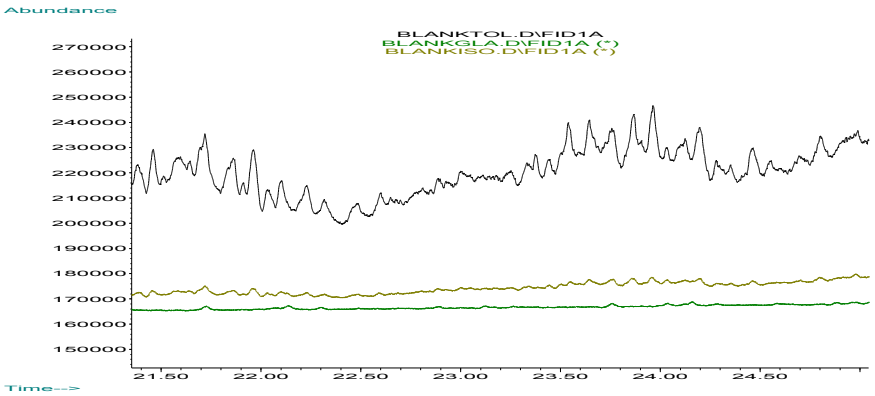


Figure 24 : FID chromatogrammes of blank extracts. Top : blank extract of toluene with polypropylene tube. Middle : blank extract of isooctane with polypropylene tube. Bottom : blank extract of toluene with new glass tube (Pyrex).

Therefore, some deviations occured between laboratories when polypropylene tubes were used with toluene, especially when integrating very small peaks. An example of integration errors due to this problem is shown in figure 25 :

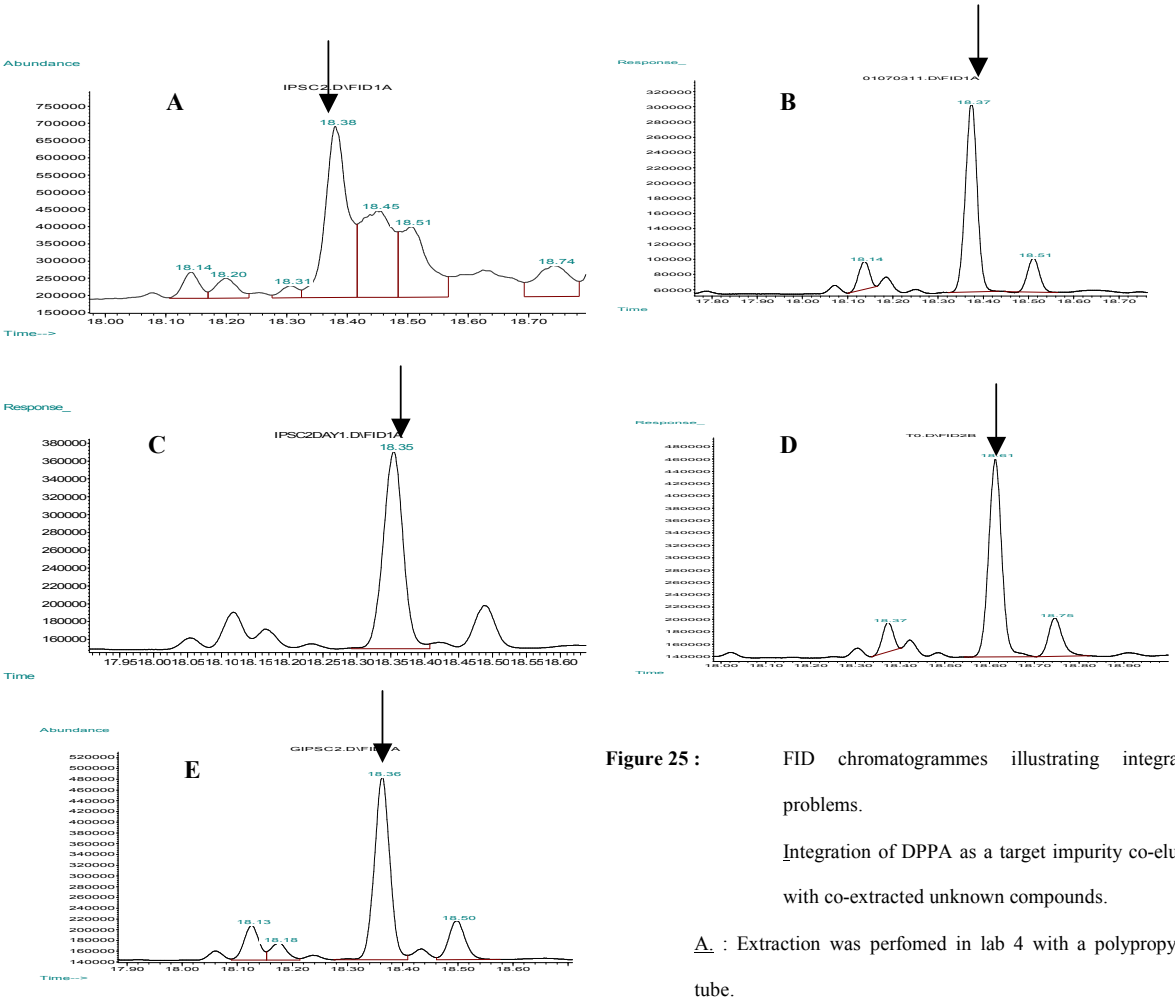


Figure 25 : FID chromatogrammes illustrating integration problems.

Integration of DPPA as a target impurity co-eluting with co-extracted unknown compounds.

A. : Extraction was perfomed in lab 4 with a polypropylene tube.

B. : same sample analysed in lab 2 extracted with a glass tube.

C. : same sample analysed in lab 1 extracted with a glass tube.

D. : same sample analysed in lab 3 extracted with a glass tube (Kimax).

E. : same sample analysed in lab 4, this time with a new glass tube (Pyrex).

Because of coelution with unknown compounds present in the sample extract (toluene + polypropylene, see figure 25, chromatogram A), the integration gave a higher relative response factor for this target impurity and this laboratory. After having prepared a new extract in a glass test tube, these interfering compounds disappeared and the chromatograms from the three labs became almost identical.
Relative standard deviation between laboratories for this target impurity was reduced from 17 to 8 %. Similar phenomena were also encountered with other target impurities and appropriate corrections were made.

5.3.6 Comparison of toluene and isooctane for Leuckart amphetamine

Three samples from the list shown in table 33 were chosen to be extracted with isooctane and analysed. As mentioned earlier, toluene is definitely a better solvent for nitrostyrene and reductive amination amphetamines as many of the target impurities in these amphetamines are more polar compounds. However, for Leuckart amphetamine, no significant differences were yet observed. Therefore, the variability (within day, between days, between laboratories) was studied for these three samples extracted with both isooctane and toluene. Results are summarised in table 45 below :

Table 45. Summary of variations for three Leuckart samples extracted with both isooctane and toluene and both analysed with FID and MS (within day, between days, between laboratories).

FID	Toluene Within day	Isooctane Within day	Toluene Between days	Isooctane Between days	Toluene Between 3 labs*	Isooctane Between 3 labs*	Toluene Between 4 labs	Isooctane Between 4 labs
IPSC 1	7%	4%	8%	5%	9%	8%	10%	16%
SKL 1	4%	7%	9%	7%	7%	7%	9%	11%
SKL 2	8%	5%	11%	10%	8%	9%	10%	16%
Mean	**6%**	**5%**	**9%**	**7%**	**8%**	**8%**	**10%**	**14%**

MS	Toluene Within day	Isooctane Within day	Toluene Between days	Isooctane Between days	Toluene Between 3 labs*	Isooctane Between 3 labs*
IPSC 1	4%	*5%*	6%	*7%*	9%	?
SKL 1	5%	*4%*	7%	*7%*	10%	?
SKL 2	7%	5%	6%	8%	7%	9%
Mean	**5%**	**5%**	**6%**	**7%**	**9%**	**9%**

* Only laboratories 2, 3 and 4.

5.4 Conclusions

From the results obtained in this task, the following conclusions could be drawn :

In general, within day variation for a same sample was in the order of 5 to 6 %. Between days variation was in the order of 8 to 10 %. As for inter-laboratory variation, it was in the order of 8 to 12 % depending on the detection method (FID or MS) and the number of laboratories (three or four). The results emphasised the importance of quality control as the only laboratory who failed to meet the quality control requirements gave expected higher deviations.
In addition, homogeneity of studied samples also have a big influence on the results. Therefore, it was of the utmost importance to thoroughly homogeneise samples before analysis in order to get as minimal variations as possible.

Dilution of samples also had an influence on variations. The limited experiments showed that an increase in deviation is to be expected (in the order of 11 % according to table 43). However, concentration effects will be further studied in task 6.

MS quantitation gave similar results to FID in terms of repeatability and reproducibility. However, higher deviations occured when diluted samples were compared to original samples. This may be due to a linearity problem as FID is by far a more linear detector compared to MS. Moreover, for some samples, variations between laboratories seemed to be slightly higher when using MS values instead of FID. As for NPD which was studied separately by laboratory n° 3, it gave similar results as MS and FID in terms of repeatability and reproducibility.
Nevertheless, a more thorough comparison of the detection techniques can be found in a previous task (see chapter 3.4).

Also, MS quantitation will be further investigated in task 6 as new MS parts were used in this task. Indeed, the standard ion source was replaced by an Ultra ion source (Agilent part number G2589-20043), the standard repeller was replaced by an Ultra repeller (Agilent part number G2589-20044) and a new draw out plate was installed which has a hole with a larger diameter (Agilent part number G2589-20045). Preliminary results have shown that this change considerably improves the linear range and stability of the MS detector.
Moreover, the selectivity of the MS detector has the advantage of eliminating co-elution problems. This important feature in integration might outweigh the disadvantage of a less linear detector compared to FID and NPD.

Regarding impurities having deviations higher than 10 % the source of deviation was not always found. However, almost all of these high deviations concerned very small peaks or peaks coeluting with unidentified matrix compounds (especially in FID detection).

It was observed that new glass tubes should be used in order to ensure a stable baseline and reduce the risks of extracting other interfering compounds with toluene. This improved significantly the results between laboratories.

Regarding the choice of solvent it could be concluded that iso-octane gave very similar results compared to toluene for Leuckart amphetamine. However, inter-laboratory variation (FID, four laboratories) seems to be slightly better with toluene (10 vs. 14 % for isooctane). This confirms that toluene is a very stable solvent and is the solvent of choice for the optimised method.

Although not mentioned previously in this task, peak height was tested for quantitation. However, studies on a few samples immediately showed that relative standard deviations were much higher compared to peak area.

Relative response factors were used as a measure of the peak size in this study. It could, however, be expected that use of different peak size normalisation procedures would reduce the variation. This will be studied in more detail in the following task.

6 Task 6 – Evaluation of methods for the comparison and classification of amphetamine profiles

6.1 Introduction

There were many aims in Task 6. We wanted to evaluate different methods for comparison and classification of amphetamine profiles in order to :

- Identify which synthetic route was used (Leuckart, reductive amination or nitrostyrene).
- Discriminate between amphetamine samples synthesised with different recipes within the same synthetic route.
- Identify amphetamine samples synthesised with the same or similar recipes.
- Identify amphetamine samples from the same batch of synthesis.

In order to evaluate the extent to which samples of different degrees of similarity could be discriminated from each other, we had to focus our attention on the intra-variability (samples from the same batch) and the inter-variability (samples from different batches). A number of syntheses were carried out for this matter.

The task was divided in three subtasks. In the first, amphetamine samples were synthesised and street samples collected. It was recognised that the quality and number of these samples would have a big influence on the final conclusions. That is, the data set may not be representative enough to validate the conclusions. However, the maximum input was applied in the time frame allowed for this part of the project.

In the second subtask, the samples were analysed by the three partner laboratories using the optimised sample preparation procedure and GC method. A control sample (synthesised amphetamine) was used in order to ensure that the three partner laboratories continuously gave comparable data.

Finally, in the third subtask, the generated data was used to evaluate the numerical classification and comparison methods.

6.2 Synthesis of amphetamine and collection of street samples

Amphetamine synthesised using different recipes

Several batches of amphetamine were synthesised using the reductive amination route (NBI 1, NBI 2 and NBI 3), the Leuckart route (SKL 1, SKL 2, SKL 3, IPSC 1, IPSC 2 and IPSC 3) and the nitrostyrene route (ST 1 and ST 2). In order to vary the profiles as much as possible the two laboratories responsible for the Leuckart synthesis co-operated in the choice of recipes. The conditions employed in the Leuckart synthesis are summarised in table 46. The recipes used in the synthesis of benzyl methyl ketone can be found in annex 8.
Regarding reductive amination amphetamine, more or less the same recipe was used for all three batches, since it was found to be hard to vary the synthetic conditions. The conditions used were similar to those used in Task 1 (see annex 1). Partner 1, who did not participate in Task 6, provided two batches of nitrostyrene amphetamine. Unfortunately, no detailed information on the synthetic conditions was available.

Table 46. Different conditions used in the synthesis of Leuckart amphetamine. The numbers given in the columns of BMK, formamide and formic acid represent the molar proportions used in the syntheses.

Batch	BMK	Formamide	Formic acid	Boiling temp. (ºC)	Boiling time (h)	Hydrolysis temp. (ºC)	Hydrolysis time (h)	Precipitation (pH)
SKL 1	1	4	4	150	5	110	2	6
SKL 2	1	6	6	160-180	4	110	2	3
SKL 3	1	2.5	2.5	140-150	6	110	2	5
IPSC 1	1	7	3.5	175-180	6	150-170	2	neutral
IPSC 2	1	17	0	175-180	4	150-170	4	neutral
IPSC 3	1*)	10	2	175-180	3.5	150-170	5	neutral
Repeated synthesis	1	6.7	3.5	160	2.5	110	1.5	7
Control sample	1	2.4	2.7	150-160	4	110	2	7

*) Synthesised BMK was used.

Part of these amphetamine batches were further diluted using caffeine and lactose to provide samples with different concentrations. Therefore, in addition to 100 % Leuckart and reductive amination amphetamines, we also had 40 % and 5 % amphetamines. The original concentrations of the nitrostyrene batches were found to be only 16 % (ST 1) and 7 % (ST 2). Their impurity content, however, was very high, and thus these samples could be further diluted. The concentrations of these samples are, for simplicity, hereafter referred to as 20 and 100 %, although their concentrations in reality were 3.2 and 16 % (ST 1) and 1.4 and 7 % (ST 2), respectively.

Repeated synthesis

Repeated syntheses using a Leuckart recipe were carried out by Partners 3 and 4 (six batches each). The recipe employed is summarised in table 46 and a detailed description of the synthesis is presented in annex 8. It has to be emphasised that this recipe was kindly provided by the Netherlands Forensic Institute and was representative of the current recipes used in illicit laboratories in the Netherlands.
The aim was to produce batches of amphetamine and hence impurity profiles which represented the highest degree of similarity that could be achieved by applying the same recipe (within a laboratory by one operator and between two laboratories with two operators). To achieve this, the syntheses were carried out under tightly controlled conditions.

Control sample

Partner 3 synthesised a large batch of Leuckart amphetamine to be used as a control sample by all Partners. The recipe used (table 46) was chosen such that a rich impurity profile was obtained. A total amount of 246 g of amphetamine sulphate was produced and distributed to the participating Partners.

Other synthesised samples

Nine Leuckart amphetamine samples precipitated from the same batch of amphetamine base were taken from the reference collection of partner 3. Three samples were precipitated at each of pH 3, 5 and 7. Additionally, six Leuckart and three nitrostyrene batches synthesised in Task 5 were made available for Task 6. The reductive amination samples from Task 5 were not used, since they had been spiked with additional impurities.

Street samples

The aim was to collect a representative number of samples of all synthetic routes. However, it should be noted that the true synthetic route of these samples was unknown before analysis. In total, 361 street samples were collected by the three partners (100 by partner 2, 149 by partner 3 and 112 by partner 4). By street samples, we meant samples that were seized by law enforcement agencies (police, customs, etc.). In general, this represents small seizures (typically a few grams). However, big seizures (a few hundred grams to a few kilograms) could be included as well.

As for the samples obtained by the Swiss partner, they are summarized in **Annex 9**. The Zurich Canton Police and the City of Zurich Police (WD, Wissenschaftlicher Dienst) are greatly acknowledged for providing the majority of the Swiss amphetamine samples. A few samples were obtained from various Canton Police forces (Neuchâtel, Genève, Jura, Valais). Their contribution is acknowledged as well.

As for the whole data set of samples used in task 6, it is summarized in table 47 :

Table 47 Synthesised and collected samples analysed in Task 6.

Samples	Synthetic route	Amphetamine concentration	No. of analyses per concentration, batch and Partner		
			Partner 2	Partner 3	Partner 4
NBI 1 - 3	Reductive amination	5, 40 and 100 %	3	3	3
SKL 1 - 3	Leuckart	5, 40 and 100 %	3	3	3
IPSC 1 - 3	Leuckart	5, 40 and 100 %	3	3	3
ST 1 - 2	Nitrostyrene	20 and 100 %	3	3	3
Repeated recipe SKL (6 batches)	Leuckart	100 %	0	3	0
Repeated recipe IPSC (6 batches)	Leuckart	100 %	0	0	3
Precipitated from same oil (9 batches)	Leuckart	100 %	0	1	0
Control sample	Leuckart	100 %	2/sequence	2/sequence	2/sequence
Task 5 samples	6 Leuckart, 3 Nitrostyrene	20-100 %	1	1	1
100 street samples	-	Unknown	1	0	0
149 street samples	-	Unknown	0	1	0
112 street samples	-	Unknown	0	0	1
15 street samples *	-	Unknown	1	1	1

* These were blind samples and were taken from the data set of 361 street samples.

6.3 Selection of target compounds and their analysis

Target compounds :

The target compounds for task 6 were selected based on the experience and knowledge gained in previous tasks. The number of amphetamine impurities synthesised during the course of task 1 was limited, therefore a number of other compounds found in amphetamine extracts were added to the target compound list. In task 3, a group of class A compounds was established, which was used for evaluation in tasks 4 and 5. During the course of these tasks it was established that some of the class A compounds were commonly absent, whereas some other new compounds could often be identified. The name and structure of some of these substances are unknown, although recorded mass spectra can be used for tentative identification. After tasks 4 and 5, the target list of impurities contained altogether 51 compounds. The list of 51 ompounds and mass spectra of the new compounds are given in **Annex 10**. However, during the processing of GC data it became obvious that collecting data for all 51 peaks was very time consuming. Moreover, a number of the 51 compounds were considered troublesome, i.e. some of them were seldom present or otherwise hard to identify, while others showed high standard deviation when samples were repeatedly analysed. Due to the above mentioned reasons the number of target compounds for task 6 was reduced to 33 (table 48) by removing the troublesome compounds, i.e. impurities 35 – 51 in **Annex 10** (note that the two isomers of N,N-di(β-phenylisopropyl)amine (compounds 14 and 15) were combined and treated as one compound).

Table 48 List of the 33 amphetamine target compounds used in the profiling method. N,N-di(β-phenylisopropyl)amine 1 and 2 were combined.

#	Compound
1	2-methyl-3-phenylaziridine
2	Benzylmethyl ketoxime 1
3	Benzyl methyl ketoxime 2
4	4-Methyl-5-Phenylpyrimidine
5	Possibly N-propylbenzamide *
6	4-Benzylpyrimidine
7	N-Acetylamphetamine
8	N-Formylamphetamine
9	1,2-Diphenylethylamine
10	N,N-Dibenzylamine
11	1,2-Diphenylethanone
12	Benzylamphetamine
13	1,3-diphenyl-2-propylamine
14	N,N-di(β-phenylisopropyl)amine 1
15	N,N-di(β-phenylisopropyl)amine 2
16	α-methyldiphenethylamine
17	N,N-di(β-phenylisopropyl)methylamine 1
18	N,N-di(β-phenylisopropyl)methylamine 2
19	Unknown A2
20	1-benzyl-3-methylnaphthalene
21	Unknown A3
22	1-hydroxy-N,N-di(β-phenylisopropyl)amine 1 (cathinol 1)
23	1,3-dimethyl-2-phenylnaphthalene
24	Unknown A4
25	Benzoylamphetamine
26	Unknown B2
27	2-oxo-1-phenyl-(β-phenylisopropylamino)ethane
28	2,6-dimethyl-3,5-diphenylpyridine
29	2,4-dimethyl-3,5-diphenylpyridine
30	Pyridine 7 and 14
31	Pyridine 272
32	2,6-diphenyl-3,5-dimethylpyridine
33	N,N-di(β-phenylisopropyl)formamide 1
34	N,N-di(β-phenylisopropyl)formamide 2

* Compound № 5 has a very similar mass spectrum compared to N-propylbenzamide (according the Wiley MS library). Purchase of this compound from Sigma – Aldrich (after the end of the project) confirmed the similarity of the mass spectrum but also showed that its retention time was slighly different (around 0.4 min) compared to the target compound found in our synthesised amphetamine. Although the identity of the purchased chemical was not guaranteed by Sigma – Aldrich, it is reasonable to assume that target compound № 5 is probably not N-propylbenzamide. Still, for sake of simplicity, this name is used throughout the remaining text in this chapter.

A few compounds in the above list were not evaluated in task 5. Therefore, their variability (within and between laboratories) were somehow unknown. However, partner 3 re-evaluated the data for four of them, namely the unknowns A2, A3, A4 and B2. No significant variations were found. Indeed, the within and between laboratories reproducibilities were found to be in the order of magnitude of the other studied compounds.

Analysis :

The amphetamine samples were prepared and analysed according to the optimised liquid-liquid extraction procedure and gas chromatographic method, respectively. In IPS, the divider dual column system was used (see annex 2). Partner 2 used the same system while partner 3 used separate instruments for MSD and FID analyses. The Agilent Retention Time Locking system (RTL) was used to lock the retention time of the internal standard (nonadecane; C_{19}) to 16.30 min on the FID.

In the dual column system the retention time of nonadecane on the MSD column was shorter, i.e. approximately 15.00 min, due to the lower pressure at the capillary outlet. One target ion and two qualifier ions were used for identification and quantification of the 33 target compounds on the MSD. For the FID the number of variables was 32 instead of 33 as a consequence of the coelution of unknown A4 and benzoylamphetamine, which accordingly were treated as one peak.
As mentioned earlier, the two isomers of DPIA were combined both in FID and MS detection. The number of analysis per sample is summarised in table 47. However, following our findings from tasks 3 and 5 regarding the comparison between FID and MS detection, it was decided to only use the MS data in task 6.

Quality control :

The quality of the gas chromatographic analyses was controlled by use of a blank sample, a modified Grob mixture and an amphetamine control sample. The blank samples were prepared using the optimised LLE procedure except that no amphetamine was added (blank extracts). The amphetamine control sample was prepared and analysed in the same way as the other amphetamine samples. The analytical sequence always started with two blank samples to clean the system followed by a Grob sample and the amphetamine control sample. Then, the amphetamine samples were analysed with a blank sample inserted after every fifth amphetamine sample. At the end of each sequence there was a blank sample followed by a Grob sample and again an amphetamine control sample.
Control charts, as described in section 3.5, were plotted for retention time, peak area and peak asymmetry. Peak widths were only inspected visually due to the limitations of the Chemstation software. A visual inspection of the profile of the amphetamine test sample was also useful, although a bit subjective.

6.4 Pre-treatment methods

The aim of this subtask was to find a suitable pre-treatment method for our data and also for our subsequent mathematical algorithms. Indeed, certain algorithms that will be presented later require certain normalisation procedures. Also, in amphetamine profiles and especially in Leuckart amphetamine, it is often the case that one target compound (namely DPIA) is 100 to 1'000 times bigger than the other target compounds. This poses problems in the further mathematical comparison as this big peak will have a huge influence in the numerical results (see closure effect below).

There are many methods that can be employed for pre-treatment of data. These can be used alone or in combination with each other. It would be unrealistic to test all of them and all possible combinations. So we limited ourselves to the following which seemed to be well suited to our purpose :

Normalisation :

Each target peak in a chromatogram is divided by the area sum of all target peaks. This enables comparison of samples of different amphetamine concentration. A typical problem associated with this method is referred to as the closure effect. This effect appears when a huge peak makes up a significant part of the area sum and therefore reduces the influence of the other peaks. One way to avoid this problem is to exclude large peaks in the peak area sum that is used in the normalisation. Another way is to perform weighing before the normalisation or by applying the logarithm or fourth square root functions to the normalised data.

Weighing :

The area of each peak is divided by its standard deviation calculated from the whole data set. The procedure will give small peaks the same influence as large ones (if the relative variation is the same). When weighing the data there is, however, a risk that small peaks, where the risk of integration error is higher, will have too much an influence. This could mean that noise peaks will have an influence in the profile.

Logarithm :

This is a common method used to reduce the influence of large peaks while still allowing large peaks to have a greater influence than small peaks. With this method, noise is not a problem. However, problems do occur when there are zeroes in the data set because $\log_{10}(0)$ can not be calculated. This can result in important information being lost. However, replacing the zeroes with a low value (e.g. a value below the detection limit) can overcome this problem.

Fourth square root :

This method is sometimes used instead of the logarithm when there are zeroes in the data. Large peaks get a somewhat higher influence when using the fourth square root (4root) as compared to the logarithm [Sjöström, 2003]. An advantage with this method is that it can be applied to zeroes.

Handling of zeroes :

Handling of zeroes is an important issue in the mathematical calculations because it influences the calculated results. Two approaches were used for handling of zeroes. These are referred to as Alternatives 1 and 2.

In Alternative 1, zeroes were left unchanged in the database and then omitted in the calculations. For example, if two profiles are compared, all peak areas corresponding to zeroes will be considered as non-existent.

In Alternative 2, zeroes were replaced with a peak area corresponding to an estimate of half the detection limit of the method. These values were determined to be 200 for MS and 1000 for FID.

Example :

Alternative 1	Peak 1	Peak 2	Peak 3	Peak 4	Peak 5
Sample A	150000	269000	**0**	389000	**0**
Sample B	143000	256000	**0**	401000	47000

Alternative 2	Peak 1	Peak 2	Peak 3	Peak 4	Peak 5
Sample A	150000	269000	**200**	389000	**200**
Sample B	143000	256000	**200**	401000	47000

In this fictitious example, two samples with only five peaks are compared. With alternative 1, the zeroes are considered as blanks. Therefore, only peaks 1, 2 and 4 will be compared to each other.
With alternative 2, the zeroes are replaced by the value 200 (for MS data), hence all five peaks are compared to each other.

6.5 Distance methods

Once the data is acquired and treated, mathematical calculations come into play in order to evaluate the distance between two or more samples. Various algorithms are available for this purpose. In this project, the distance methods evaluated were : Euclidean distance, Manhattan distance, Pearson correlation, Similarity index, Quotient method, Canberra index and the Squared sinus function. Although they are not technically all distance methods (Pearson calculates a correlation, squared sinus calculates an angle between two vectors, etc.), they were called as such for simplicity. The choice of these methods was made for the following reasons : three of them were already in use in the partner's laboratories as part of their own profiling programs (partner 2 : Similarity index [Kärkkäinen et al., 1994], partner 3 : Quotient method [Jonson and Strömberg, 1993], partner 4 : squared cosinus which is the reverse of the squared sinus [Esseiva et al., 2003]). As for the Euclidean distance, it has been used in relation to methamphetamine profiling [Tanaka et al., 1994, Perkal et al., 1994] and to heroin profiling [Klemenc, 2001]. Pearson correlation is being used in Poland for amphetamine profiling [Krawczyk, 2001]. Finally, Manhattan distance has been used in relation to heroin [Myors, 2001] whereas the Canberra distance was used in physical evidence comparisons [Thornton et al., 1975].

The minimal and maximal values given by these methods are not all the same. Therefore, some normalisation was applied in order to have a range between 0 and 100, 0 being the perfect match whereas 100 would be the complete mismatch. This approach allowed us to directly compare the performance of these methods.
The distances were calculated using a home-made software programmed by Partner 3 or an Excel macro provided by Partner 4. After verification, both systems could be used as they gave the same results. The distance methods employed are briefly described below.

Euclidean distance :

The most commonly known distance method is probably the *Euclidean distance*. Graphically, it can be visualised as the distance between two points (samples) in space. The Euclidean distance is calculated according to equation 12,

$$\text{Euclidean distance} = \sqrt{\sum_{i=1}^{n}(x_i - y_i)^2} \quad (12)$$

where x_i denotes the area of the i:th peak in profile X and y_i the peak area of the i:th peak in profile Y. The total number of peaks is n. In order to get values between 0 and 100, each distance had to be divided by the largest distance in the data set and then multiplied by 100.

Pearson correlation :

In this method the correlation coefficient (r) between peak areas in two profiles is calculated. Two very similar samples will have a high correlation with a r value close to 1 and two very dissimilar (negatively correlated) samples will have a value close to –1. Uncorrelated samples will have a r value close to 0. The correlation coefficient is calculated according to equation 13 ,

$$r_{kl} = \frac{\sum_{j=1}^{n}\left(x_{kj} - \bar{x}_k\right)\left(x_{lj} - \bar{x}_l\right)}{\sqrt{\sum_{j=1}^{n}\left(x_{kj} - \bar{x}_k\right)^2 \sum_{j=1}^{n}\left(x_{lj} - \bar{x}_l\right)^2}} \quad (13)$$

where x_{kj} and x_{lj} are the j:th peaks of profiles K and L, respectively, and $\bar{x}_k$ and $\bar{x}_l$ are the means of the peaks of profiles K and L, respectively. Conversion of distances to the range 0 - 100 was obtained using equation 14.

$$\text{Pearson value} = \frac{(1-r)}{2} \times 100 \quad (14)$$

Similarity index :

Similarity index (SI) is a metric where a high number corresponds to a high similarity. The formula is given in equation 15,

$$SI = \frac{100 \times m}{n} \times \sum_{i=1}^{n}\left(\left(w \times \frac{x_i}{y_i} - k_2\right)^k - 1 + m\right)^{-1} \quad (15)$$

where k , k_2 , m and w are constants. The x_i is chosen as the bigger of x_i and y_i, which gives $x_i/y_i \geq 1$.
According to Eerola and Lehtonen [Eerola and Lehtonen, 1988], the constants k, k_2, m and w are given the values 6, 0.25, 50 and 1, respectively, which results in equation 16,

$$SI = \frac{100 \times 50}{n} \times \sum_{i=1}^{n}\left(\left(\frac{x_i}{y_i} - 0.25\right)^6 - 1 + 50\right)^{-1} \quad (16)$$

The highest value that can be obtained from this formula is 101.67. Conversion of distances to the range 0 - 100 was obtained using equation 17,

$$\text{Distance} = 100 - \frac{(SI)}{1.0167} \quad (17$$

Quotient method :

In the quotient method the quotients of peak areas of corresponding impurities in the two profiles to be compared are calculated. This means that there will be as many quotients as there are target impurities. The quotient calculation is illustrated mathematically by equation 18,

$$q_i = \frac{x_i}{y_i} \quad (\mathbf{18})$$

where q_i is the i:th quotient of x_i and y_i, which are the i:th impurity in profiles X and Y, respectively. If the two profiles are similar the calculated quotients will be similar. The next step is to calculate the distances between each quotient and the other quotients. These calculations are made according to equation 19,

$$r_{ik} = \left| \frac{(q_i - q_k)}{(q_i + q_k)} \right| \quad (\mathbf{19})$$

where q_i and q_k are quotients of peaks *i* and *k*, respectively. Since there is always a random variation present a value, r_{max}, has been introduced. By experience it is known that a r_{max} value of 20 % is useful in amphetamine profiling [ref]. This value gives the spread within which the quotients are considered to be similar. For each quotient the number of quotients where $r_{ik} < r_{max}$ is calculated. The maximum of these numbers (i.e. the number of peaks within r_{max}) is then transformed to a distance. This is made by dividing the number of quotients where $r_{ik} > r_{max}$ with the total number of peaks and multiplying this value with 100, which will provide distances in the range 0-100.

Example :

If 33 target compounds are used and the number of quotients where $r_{ik} < r_{max}$ is 32, the number of quotients where $r_{ik} > r_{max}$ is 1. Therefore, the distance will be equal to (1 / 33) * 100 = 3.03.
One characteristic of this method is that it is discontinuous. Indeed, if the number of quotients where $r_{ik} < r_{max}$ is 31, we jump to a distance of 6.06. This feature had to be taken into account in the interpretation of the results.
One important other characteristic is that a r_{max} value has to be chosen before proceeding to the calculations.

It should be noted that alternative 1 (see section 6.4) was used a bit differently with the quotient method. Indeed, zeroes were left in the database as such and when two corresponding target compounds in two profiles had the value zero it was considered as a match. In other words, the quotient of these two target compounds (although absent) was considered as 1.
On the other hand, if one of the two corresponding target compounds had a value (and the other a zero) it was considered as a non-match. Both the home-made software (partner 3) and the Excel macro (partner 4) were set in order to take care of these considerations.
Moreover, in some cases, partner 3 have included some missing values. This was the case when a peak area could not be determined although the target compound was thought to be present. Therefore, the peak area was given a missing value defined as « m ». For all distance methods, these « m » values were treated as zeroes with one exception : when a zero was compared with a « m », it was considered as non-match with the quotient method.
It should be noted that partners 2 and 4 avoided the use of this supplementary variable by giving either a value or a zero when confronted to such a problem.

Canberra index :

Canberra index is a kind of block distance where the difference in each variable is weighed by the sum of the two values of the variable. This means that the contribution from each variable / target compound can not be larger than 1/n. The equation of the Canberra index is given in equation 20,

$$Canberra\ Index = \frac{1}{n} \sum_{i=1}^{n} Abs \frac{(x_i - y_i)}{(x_i + y_i)} \quad (\mathbf{20})$$

where x_i and y_i are the area of the i:th peak in profiles X and Y, respectively. The total number of peaks is *n*. In order to get distances in the range 0-100 the Canberra index is multiplied by 100.

Manhattan :

Manhattan is related to the Canberra index. The difference is that Manhattan does not take the size of peaks into consideration in the comparison. The Manhattan distance is calculated according to equation 21,

$$D_{kl} = \sum_{j=1}^{n} \left| x_{kj} - x_{lj} \right| \quad (21)$$

where x_{kj} and x_{lj} are the areas of the j:th peak in profiles K and L, respectively.

In order to get values between 0 and 100, each distance had to be divided by the largest distance in the data set and then multiplied by 100.

Squared sinus [Keto, 1989, Esseiva et al., 2003] :

The sinus of the angle between two profiles in space is calculated and the obtained value is squared. When the angle is 0º the distance is 0 (sinus 0º =0), which means that the profiles are identical. When the angle is 90º the distance is 1 (sinus 90º =1) and means that the profiles are completely different.

$$\text{squared sinus} = 1 - \frac{\left(\sum_{j=1}^{n} \left(x_{kj} \times x_{lj} \right) \right)^2}{\sum_{j=1}^{n} x_{kj}^{\,2} \times \sum_{j=1}^{n} x_{lj}^{\,2}} \quad (22)$$

where x_{kj} and x_{lj} are the j:th peaks of profiles K and L, respectively and n is the number of target compounds. Conversion of distances to the range 0 - 100 was obtained by multiplying the value by 100.

6.6 Evaluation of pre-treatment methods

6.6.1 The data set

The correct choice of pre-treatment method is an extremely important decision since it will affect the quality of the extracted information. For this purpose, it was decided to study a relatively small data set and its composition is shown in table 49. This data set also had to be representative and contain various amphetamine profiles. This is why amphetamines from different synthetic routes were included as well as related samples (from controlled syntheses) and unrelated samples (street samples from police seizures). The different pre-treatment methods (one at a time or in combination) were applied to this data set of 44 samples. Only MSD data were evaluated for this purpose with 33 target compounds (see table 48).

Table 49 Samples included in the data set used for distance calculations and evaluation of pre-treatment methods. In total, 44 samples were used.

Type of sample	Leuckart	Reductive amination	Nitrostyrene	All methods
Different recipe	12	6	4	22
Repeated synthesis	2	0	0	2
Precipitated from same oil but at different pH	2	0	0	2
Task 5 samples	6	0	3	9
Street samples	3	6	0	9
Sum	25	12	7	44

6.6.2 The pre-treatment and distance combinations

The various algorithms described in section 6.5 were applied to this data set after various pre-treatment data combinations. An exception was made for the quotient method. Indeed, this method, used by partner 3 for many years in their laboratory, was mainly designed to be used with raw data. Therefore, other comparisons with the quotient method were carried out at a later stage. The various combinations are summarized in table 50 :

Table 50. Combinations of pre-treatment and distance methods

Pre-treatment method *	Manhattan	Canberra	Euclidean	Pearson	Similarity index	Squared Sinus
	Alternative 1					
N	x	x	x	x	x	x
N + W	x	x	x	x	x	x
W + N	x	x	x	x	x	x
N + log	x	x	x	x	x	x
N + 4root	x	x	x	x	x	x
W + N + log	x	x	x	x	x	x
W + N + 4root	x	x	x	x	x	x
	Alternative 2					
N	x	x	x	x	x	x
N + W	x	x	x	x	x	x
W + N	x	x	x	x	x	x
N + log	x	x	x	x	x	x
N + 4root	x	x	x	x	x	x
W + N + log	x	x	x	x	x	x
W + N + 4root	x	x	x	x	x	x

* N = Normalisation. N+W = Normalisation + Weighing. W+N = Weighing + Normalisation. N+log = Normalisation + logarithm. N+4root = Normalisation + 4^{th} square root. W+N+L = Weighing + Normalisation + Logarithm. W+N+4root = Weighing + Normalisation + 4^{th} square root.

6.6.3 Principal Component Analysis (PCA)

Principal Component Analysis (PCA) is a bilinear modeling method that is used to simplify interpretation of large data matrices. A bilinear modeling method is designed for situations where collinearity exists among the original variables. The information carried by the original variables is projected onto a smaller number of underlying variables called principal components (PC's). The latter are linear functions of the original variables. PCA is thus a data reduction or compression method.
The first principal component covers as much of the variation in the data as possible. The second principal component is orthogonal to the first and covers as much of the remaining variation as possible, and so on.
By plotting the principal components, one can view interrelationships between different variables, and detect and interpret sample patterns, groupings, similarities or differences.
In this section, two types of plots will be illustrated : score plots and loading plots. Score plots show the locations of the samples according to the model, and can be used to detect sample patterns, groupings, similarities or differences. Loading plots show how well a variable is taken into account by the model. They can be used to understand how much each variable contributes to the meaningful variation in the data, and to interpret variable relationships.
Finally, it should be remembered that PCA is an exploratory (or unsupervised) method.

6.6.4 Evaluation of false positives in the data set

The first aim was to investigate how well various pre-treatment methods performed in combination with distance methods. As a measure of the performance, the idea was to determine the ability of each combination to discriminate between linked and unlinked samples.
In this data set of 44 samples, 18 links were present. 11 of them were between original samples and diluted samples. 6 were between samples synthesised with the same recipe by the same operator and the last one was between two samples that came from the same batch of amphetamine. These 18 links, translated into distance values, consisted our linked population.For the unlinked population, distances were calculated between unrelated samples. This gave us a number of 617 distances.

The first measure of the performance of these 84 combinations was to determine the percentage of false positives. For this, the maximum distance in the linked population was calculated (for each combination) and considered as the threshold value. Then, all distances between unlinked samples that were inferior to this threshold value were considered as false positives. In other words, the distance between two unrelated samples which is smaller that the maximum distance between two linked samples is a false positive.
As for false negatives, it was not considered useful to calculate them in this subtask as the percentage would be based only on 18 distances (from 18 links). The results are shown in table 51 :

Table 51. Percentage of false positives in the data set of 44 samples (out of 617 distances)

Pre-treatment method *	Manhattan	Canberra	Euclidean	Pearson	Similarity index	Squared Sinus
	Alternative 1					
N	5.3	0.8	4.4	11.0	1.9	7.5
N + W	9.2	0.8	7.1	2.4	1.9	1.3
W + N	4.1	1.5	8.9	4.5	1.3	2.1
N + log	6.3	**0.2**	2.9	**0.8**	**1.0**	**0.6**
N + 4root	**1.9**	1.0	**1.5**	**1.0**	1.9	**1.1**
W + N + log	20.1	1.0	4.7	2.1	1.1	1.0
W + N + 4root	6.5	1.3	2.9	1.5	1.9	1.0
	Alternative 2					
N	3.9	14.3	2.8	7.0	9.1	6.5
N + W	4.4	14.3	4.1	1.1	9.1	1.1
W + N	4.2	19.6	47.2	10.2	10.7	13.6
N + log	6.3	**2.1**	3.4	6.5	**1.1**	3.4
N + 4root	**1.8**	7.0	**0.6**	**0.6**	11.2	**0.8**
W + N + log	12.6	2.3	6.6	7.9	2.6	6.2
W + N + 4root	1.8	14.1	3.2	6.5	22.4	4.5

From the results in table 51, it was not possible to draw any conclusion as to the best combination. Even the choice between alternatives 1 and 2 seemed not possible. Therefore, it was decided to keep each distance method and both alternatives. However, it was judged possible to choose the best pre-treatment method for each distance method from the results above. Thus, normalisation followed by the fourth square root was the best compromise with Manhattan, Euclidean, Pearson and the squared sinus whereas normalisation followed by the logarithm was the best compromise for the Similarity and Canberra indexes.
Thus, a further investigation will be carried out with a larger data set in a next section. Meanwhile, the same data set was evaluated with principal component analysis in order to study the influence of the pre-treatment methods in the classification of the samples into the three synthetic routes present in this data set.

6.6.5 Evaluation with PCA

The data concerned 33 variables and 44 samples. Both alternatives 1 and 2 were considered together with the seven pre-treatment methods, hence 14 models. This data was introduced into the Unscrambler®. This is a commercial software for multivariate analysis which was kindly made available for the last 8 months of the project by Camo Process AS, Oslo, Norway. The results are illustrated and explained below :

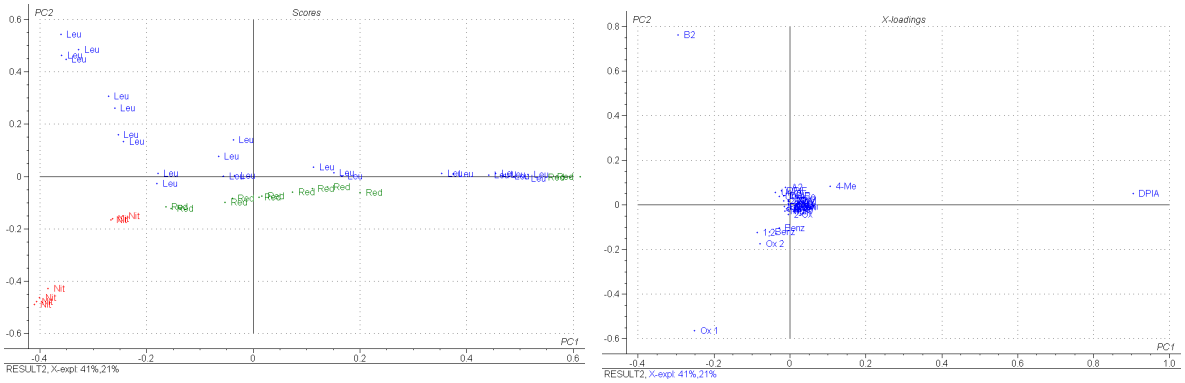


Figure 26 : Normalisation to the sum with alternative 1. Left : scores plot. Right : loadings plot.

The plot on the left hand side shows the scores plot. It can be seen that the three synthetic routes are not well separated. The explanation is found in the loadings plot on the right hand side and is directly related to the closure effect described in section 6.4. Indeed, three huge peaks (DPIA, phenyl-2-propanone oxime and unknown B2) are making up a significant part of the area sum in the samples of our data set and are responsible for this poor model. All other target compounds have almost no influence (they are all in the center of the loadings plot).
As mentioned before, one way of avoiding this problem is to exclude large peaks in the peak area sum that is used in the normalisation. However, this was considered unwise as DPIA has a major influence in the discrimination between Leuckart and reductive amination samples and phenyl-2-propanone oxime has the same role in the discrimination between the nitrostyrene route and the two others.
Another solution is to apply the other pre-treatment combinations. The best results was obtained with normalisation followed by the 4th square root (with both alternatives) and are shown below. The plots from the other combinations can be found in annex 11.

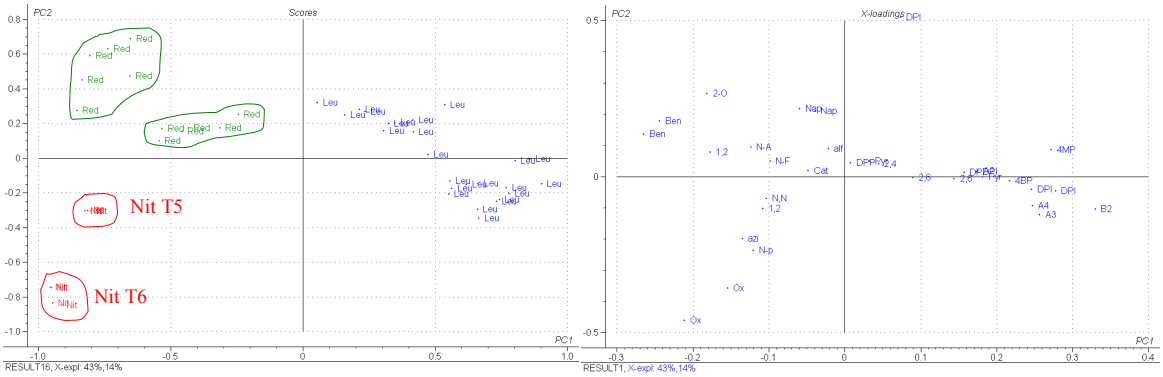


Figure 27 : Normalisation + 4th square root. Alternative 2 only. Left : scores plot. Right : loadings plot.

With this pre-treatment method, the three synthetic routes are well separated. The Leuckart samples are on the right hand side whereas the reductive amination samples are on the top left and the nitrostyrene in the bottom left. In addition, The nitrostyrene samples from task 5 and task 6 could be differentiated. This was to be expected as the task 5 samples were synthesised by partner 4 and the task 6 samples by partner 1 and through a totally different recipe. Also, the seized reductive amination samples (top left) could be discriminated from the synthesised ones (middle left).
These results are quite in accordance with those obtained in section 6.6.4 where normalisation followed by the 4th square root gave the lowest percentage of false positives together with normalisation and logarithm.
As for PCA, normalisation and logarithm also gave satisfactory results (second best) although not as good as the 4th square root.

In conclusion, based on the results obtained from this small data set, normalisation followed by the 4th square root is the best pre-treatment method and will be used in the subsequent task.
However, normalisation followed by the logarithm will still be considered with the Similarity index and the Canberra index as it gave the better results with these distance methods.
As for alternatives 1 or 2, there are still both under consideration as they seem to give both similar results.

6.7 Further reduction of target compounds

6.7.1 Introduction

At this stage of the project, it was thought necessary to decrease the number of target compounds. Indeed, the idea was to test whether the same results could be obtained with fewer variables.
At first, 27 target compounds were used instead of 33 : The two isomers of the phenyl-2-propanone oxime were combined whereas cathinol 1, pyridine 272, unknown A4, 4-methyl-5-pyrimidine and 4-benzylpyrimidine were removed.
The two isomers of phenyl-2-propanone oxime were combined as it was difficult to integrate them separately when this compound is present in high amounts. Cathinol 1 was removed as it was found in very few samples and in very small proportions. Pyridine 272 is generally a small peak and as a number of pyridine derivatives are already present in the list, it was thought that its removal would not be harmful. The same applies to unknown A4. Unknown A2 and A3, which are isomers of unknown A4, are still present in the list.
As for the two pyrimidines, although they are considered specific to the Leuckart route, especially if they are present in relatively high proportions, they were removed because of their well known volatility. Indeed, they are known to evaporate relatively quickly depending on the storage conditions of the amphetamine powder. Therefore, it was considered preferable to seek a profiling method without these two compounds.
Further, depending on the results, the number of target compounds was even further reduced or increased.

6.7.2 Evaluation of false positives in the data set

According to the conclusions of section 6.6, normalisation followed by the fourth square root seemed to be the best compromise. In order to reduce the amount of work, it was therefore decided to use this pre-treatment method in this subtask and to use only one distance method. Based on the results displayed in table 52 (section 6.6.4), the choice was made for the Pearson correlation. Still, both alternatives (1 and 2) were tested.

In table 52, it could be seen that only 1.0 % false positives was found with alternative 1 and 0.8 % with alternative 2. This corresponds to 6 and 5 false positives respectively (N = 617). By reducing the number of target compounds to 27, these numbers were further reduced. Indeed, only two false positives were found with alternative 1 and one false positive with alternative 2. Thus, even better results were obtained with less target compounds in terms of false positives.

Therefore, three additional target compounds were removed : phenyl-2-propanone oxime (isomers 1 + 2), N-propylbenzamide and the aziridine leaving us with 24 target compounds. These three compounds were tentatively removed as they were eluting first in the chromatogram and were therefore presumed to be the most volatile.
The same results were obtained compared to 27 compounds. That is : two false positives with alternative 1 and one with alternative 2. However, these three compounds are mainly found in nitrostyrene amphetamine and the following PCA analysis will show that they can not be all removed. Indeed, they were found important in the discrimination between nitrostyrene and the two other synthetic routes.

6.7.3 Evaluation with PCA

PCA models with 33, 27 and 24 compounds were made with both alternatives. Normalisation followed by the fourth square root was used. Results are shown below :

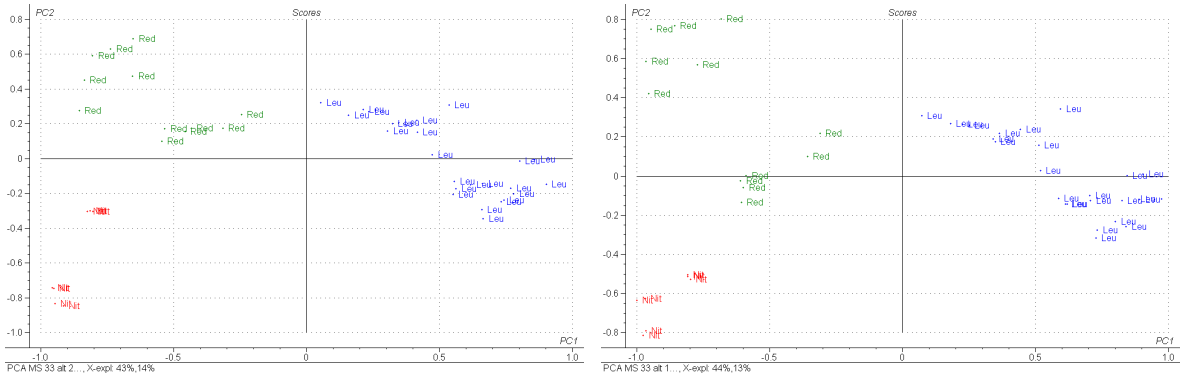


Figure 28 : Normalisation + 4th square root. 33 compounds. Left : alternative 2. Right : alternative 1.

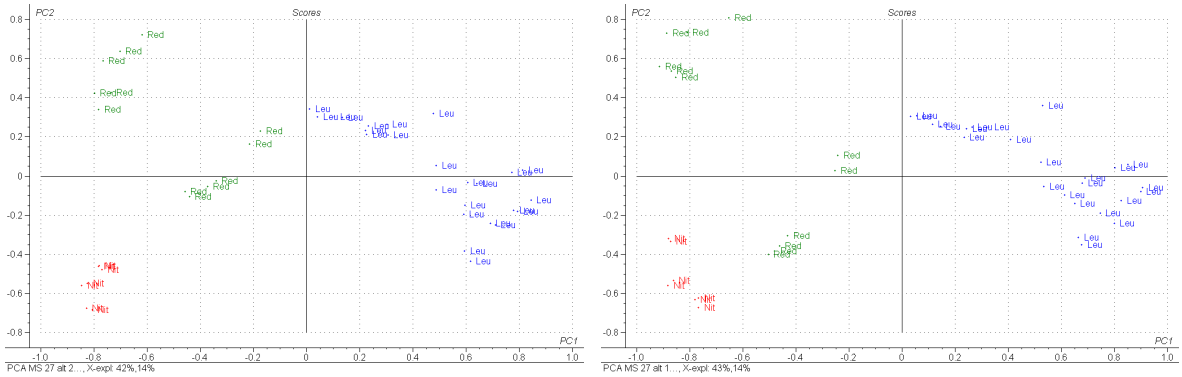


Figure 29 : Normalisation + 4th square root. 27 compounds. Left : alternative 2. Right : alternative 1.

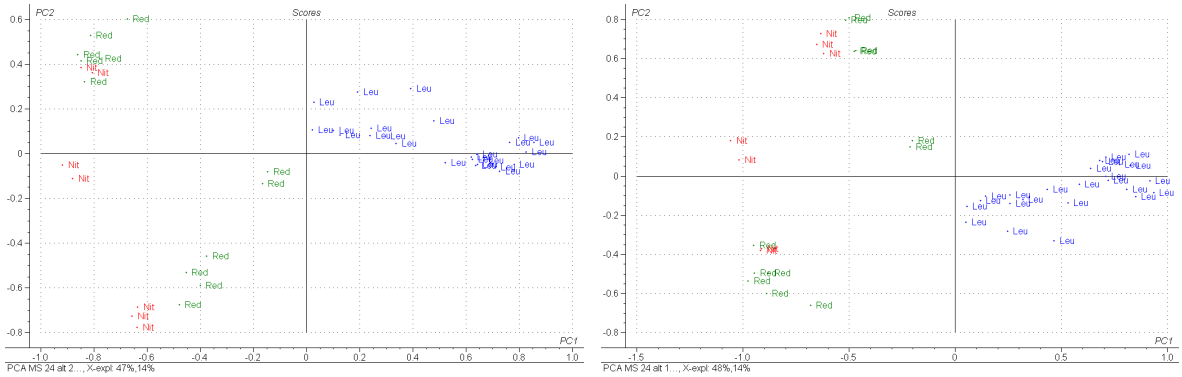


Figure 30 : Normalisation + 4th square root. 24 compounds. Left : alternative 2. Right : alternative 1.

As can be seen in figure 30, some nitrostyrene samples could not be differentiated from reductive amination samples when only 24 compounds are used. This confirms that the three removed compounds are important for discriminating the nitrostyrene route. However, the use of 27 target compounds give similar PCA models compared to 33 compounds.

Still, an attempt was made to reduce the number of target compounds from 27 to 25 or 26 by removing one or two of these nitrostyrene compounds, i.e. phenyl-2-propanone oxime, 2-methyl-3-phenylaziridine and N-propylbenzamide. According to the loadings plot of the PCA models, phenyl-2-propanone oxime is a very important variable in order to discriminate the nitrostyrene route. It was therefore kept. After some fine tuning, it was determined that 2-methyl-3-phenylaziridine could be removed leaving us with 26 compounds. With these 26 compounds, the number of false positives was the same as with 27 (two with alternative 1 and one with alternative 2). The PCA model with 26 compounds is shown below :

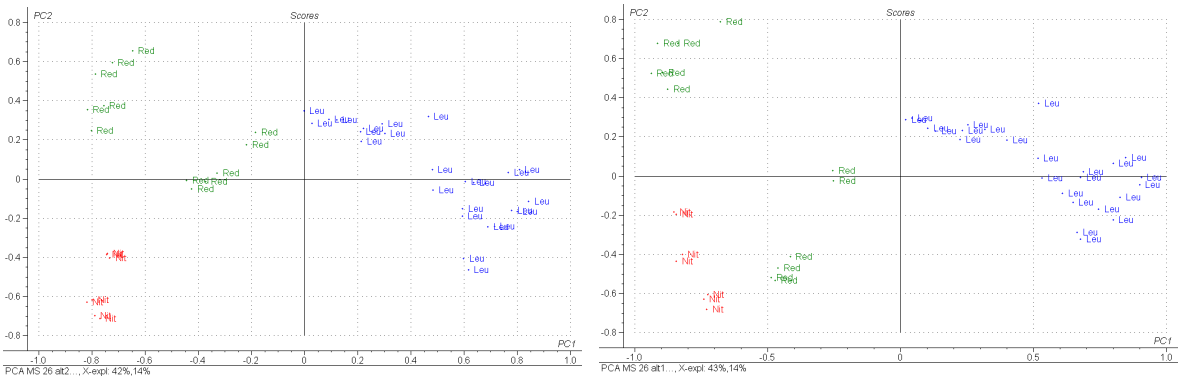


Figure 31 : Normalisation + 4th square root. 26 compounds. Left : alternative 2. Right : alternative 1.

Although a bit subjective, observations of these PCA models tend to indicate that alternative 2 provides a better separation of the three synthetic routes compared to alternative 1 (especially between nitrostyrene and reductive amination). The following chapter will actually show that alternative 2 is indeed probably better suited than alternative 1.

6.8 Final evaluation and choice of the distance method

6.8.1 The new data set

The outcome of the previously performed experiments was not sufficient to decide which distance method to use in the final method. Consequently, a new and bigger data set was designed to further compare the different distance methods. For this purpose, two populations were created : the first consisted of distances calculated between samples known to be from the same batch of amphetamine and the second consisted of distances between samples known not to be from the same batch. The two sets of data were compared and the number of distances being in the overlapping zone were determined and used as a measure of the performance of each distance method in discriminating between linked and unlinked samples. The number of false positives and false negatives were calculated from this overlapping region. The following figures illustrates graphically the idea :

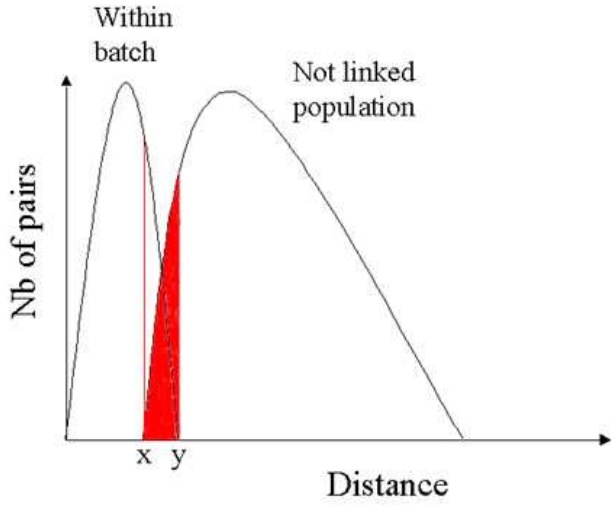


Figure 32a : schematic illustration of the overlapping region in the evaluation of the performance of the distance methods. The coloured zone indicates the region of false positives.

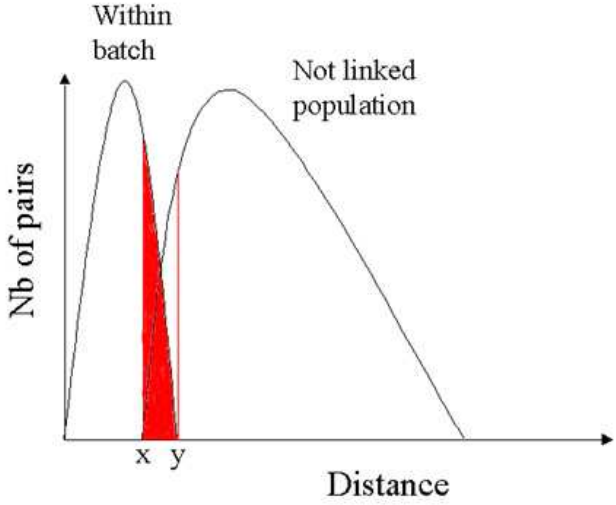


Figure 32b : schematic illustration of the overlapping region in the evaluation of the performance of the distance methods. The coloured zone indicates the region of false negatives.

In figures 32a and 32b, the overlapping region is situated between x and y, x being the minimum distance between unlinked samples and y being the maximum distance between linked samples (same batch). If x and y are chosen as the thresholds, the coloured region in figure 32a corresponds to the number of false positives and the coloured region in figure 32b to the number of false negatives.
Of course, in the ideal situation, there would be no overlap. Moreover, in the data set used in this section, there should be no overlap as we are comparing synthesised samples (within batch population) with street samples (samples seized on the illicit market). Thus, the aim was to determine the distance method which would give the smallest overlap, i.e. the smallest number of false positives and negatives.

Within batch population :

The samples used for calculation of distances between samples of the same batch were made up of those synthesised within the project. Where applicable, different concentrations (5, 40 and 100 %), were included in the calculations. Moreover, all replicates and analyses in different laboratories were included. In total this generated 3901 within batch distances. The detailed composition is as follows :

-	Reductive amination sample n°1 synthesised by partner 2 :	27 analyses →	number of pairs : 351
-	Reductive amination sample n°2 synthesised by partner 2 :	27 analyses →	351
-	Reductive amination sample n°3 synthesised by partner 2 :	27 analyses →	351
-	Leuckart sample n°1 synthesised by partner 3 :	24 analyses →	276
-	Leuckart sample n°2 synthesised by partner 3 :	24 analyses →	276
-	Leuckart sample n°3 synthesised by partner 3 :	27 analyses →	351
-	Leuckart sample n°1 synthesised by partner 4 :	27 analyses →	351
-	Leuckart sample n°2 synthesised by partner 4 :	27 analyses →	351
-	Leuckart sample n°3 synthesised by partner 4 :	27 analyses →	351
-	Nitrostyrene sample n°1 synthesised by partner 1 :	18 analyses →	153
-	Nitrostyrene sample n°2 synthesised by partner 1 :	18 analyses →	153
-	Control sample synthesised by partner 3 :	34 analyses →	561
-	Leuckart sample n°1 (partner 3, task 5) :	3 analyses →	3
-	Leuckart sample n°2 (partner 3, task 5):	3 analyses →	3
-	Leuckart sample n°3 (partner 3, task 5):	3 analyses →	3
-	Leuckart sample n°1 (partner 4, task 5):	3 analyses →	3
-	Leuckart sample n°2 (partner 4, task 5):	3 analyses →	3
-	Leuckart sample n°3 (partner 4, task 5):	3 analyses →	3
-	Nitrostyrene sample n°1 (partner 2, task 5) :	3 analyses →	3
-	Nitrostyrene sample n°2 (partner 2, task 5) :	3 analyses →	3
-	Nitrostyrene sample n°3 (partner 2, task 5) :	2 analyses →	1

Total : 3901

Not linked population :

In order to create the unlinked population described in figures 32a and 32b, 20 synthesised samples (from 20 different batches, 13 Leuckart, 3 reductive amination and 4 nitrostyrene) were compared to street samples (samples seized on the illicit market). Including a few replicates, this represented 390 samples (about 40 % from Sweden, 30 % from Finland and 30 % from Switzerland). Thus, 20 x 390 = 7'800 distances were generated.

6.8.2 Evaluation of false positives and false negatives

As mentioned in section 6.6.4, only the following combinations were studied with this new data set :

- Pearson (normalised + 4^{th} square root). Alternatives 1 and 2.

- Squared sinus (normalised + 4^{th} square root). Alternatives 1 and 2.

- Similarity index (normalised + logarithm). Alternatives 1 and 2.

- Canberra index (normalised + logarithm). Alternatives 1 and 2.

- Euclidean distance (normalised + 4^{th} square root). Alternatives 1 and 2.

- Manhattan distance (normalised + 4^{th} square root). Alternatives 1 and 2.

- Quotient (raw data). Zeroes as such. With r_{max} at 5, 10, 15 and 20 %.

Once the distances calculated, the data was plotted as histograms in order to get graphics similar to those shown in figures 32a and 32b. All histograms can be found in annex 12.
Only the two best results are illustrated here in figures 33a and 33b :

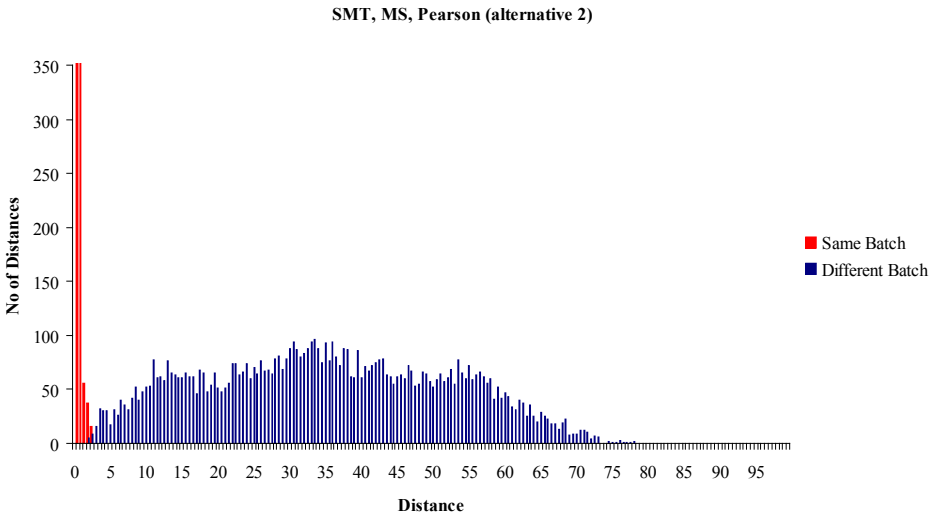


Figure 33a : schematic illustration of the overlapping region with Pearson correlation after normalisation + 4th square root and alternative 2.

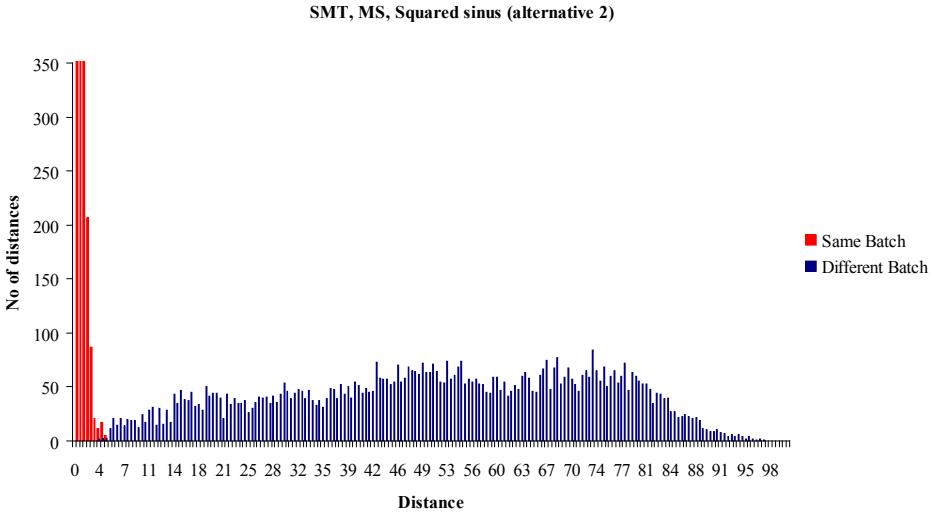


Figure 33b : schematic illustration of the overlapping region with squared sinus after normalisation + 4th square root and alternative 2.

In terms of numbers, table 52 summarizes the percentages of false positives and false negatives for each distance method :

Table 52 **Percentages of false positives and false negatives for each distance method. N = 7800 for the calculation of false positives and N = 3901 for the calculation of false negatives.**

Distance method	Alternative	False positives in %	False negatives in %
Pearson	1	9.0	93.4
Pearson	2	**0.1**	**1.2**
Squared sinus	1	0.3	4.3
Squared sinus	2	**0.1**	**0.9**
Euclidean	1	1.2	19.0
Euclidean	2	0.9	3.4
Manhattan	1	2.2	42.5
Manhattan	2	1.5	2.5
Similarity Index	1	1.6	7.4
Similarity Index	2	0.3	4.3
Canberra	1	0.6	3.5
Canberra	2	1.3	8.2
Quotient 20 %	-	1.1	4.6
Quotient 15 %	-	1.7	8.3
Quotient 10 %	-	5.8	19.4
Quotient 5 %	-	16.0	42.6

Note: The percentage values given for the Quotient method corresponds to the r_{max} value used.

6.8.3 Discussion and conclusions

First of all, table 52 shows an interesting result which illustrates quite well the main disadvantage of alternative 1 with some distance methods. Indeed, the most outstanding value is 93.4 % of false negatives with Pearson and alternative 1. This is explained by the fact that two unrelated samples (a reductive amination synthesised sample and a reductive amination street sample) had only two target compounds in common. Some other target compounds were present in one sample and absent in the other and vice-versa. With alternative 1 only the peak areas of the two compounds are taken into consideration for the comparison. This is of course not sufficient for discriminating the two samples. Thus, a very low correlation value was obtained (close to 0) and was used as the threshold value for the calculation of false negatives. As this value was very low, the majority of the within batch distances were over this threshold, hence a high percentage of false negatives.
However, in this kind of situation, not all distance methods will give a very low value. For example, the squared sinus will accentuate the difference between two samples if target compounds are present in a sample and absent in the other. This is directly related to the mathematical algorithm (see equation 22, section 6.5).

Nevertheless, these results clearly indicate that alternative 2 with Pearson or squared sinus are the most powerful methods in discriminating between two populations of linked (same batch) and unlinked samples, thus minimizing the risks of false positives and false negatives. Of course, these results are valid only for the data set tested. However, they confirm the results obtained in section 6.6.4 and it is therefore a strong indication of the advantages of these comparison methods over the others. Moreover, these results are in total accordance with those obtained by Esseiva [Esseiva et al., 2003] who found that Pearson and the cosine function (the inverse of the sinus) were the best methods in relation to the profiling of heroin. Therefore, it is an additional indication that these methods are indeed the most suited for the purpose.

Finally, it should be mentioned that the replacement of zeroes by the value 200 (alternative 2) is not that critical with these two methods. Indeed, zeroes could be left as such as this does not interfere with the fourth square root (the 4^{th} square root of 0 is 0) and has little influence in the algorithms of Pearson or the squared sinus. Therefore, this alternative that we could call alternative 3 would give almost the same results as alternative 2. A few subsequent calculations made by partner 3 confirmed this assumption. However, it was decided to stick to the decision of replacing zeroes by 200 as the conclusions are based on this alternative. Moreover, it was considered too much time consuming and useless to perform again all calculations with this third alternative which would not bring any improvement.

6.9 Estimation of useful thresholds

6.9.1 Thresholds for determination of samples from the same batch

At this time, Pearson correlation and the squared sinus function were both considered as the best distance methods for comparing pairwise amphetamine samples in a database. Before that, the GC-MS raw data (with zeroes replaced by the value 200) should be normalised to the sum and each normalised peak submitted to the fourth square root. After comparison of two or more samples, numerical values between 0 and 100 will be obtained where 0 is the perfect match.

If the profiling method is to be applied in routine work, it is important to determine an estimate of one or more thresholds which would help the analyst in the interpretation. For this purpose, calculations were made between samples coming from the same batch which were at the same or different concentrations and which were analysed in the same laboratory or in different laboratories. The synthesised batches from task 6 were used in this case as their origin are known. The results are shown in tables 53a and 53b and in figures 34a and 34b :

Table 53a **Mean, min, max and standard deviation values for distances between samples coming from the same batch. Pearson correlation (N+4root, alternative 2).**

	N° of distances	Mean	Std deviation	Min	Max
Within batch, within lab and same concentration	174	0.013	0.027	0.0001	0.200
Within batch, between lab and same concentration	792	0.257	0.274	0.010	1.876
Within batch, within lab and diff. concentration	243	0.278	0.206	0.011	0.733
Within batch, between lab and diff. concentration	747	0.370	0.279	0.009	1.894

Table 53b **Mean, min, max and standard deviation values for distances between samples coming from the same batch. Squared sinus (N+4root, alternative 2).**

	N° of distances	Mean	Std deviation	Min	Max
Within batch, within lab and same concentration	174	0.022	0.045	0.00005	0.297
Within batch, between lab and same concentration	792	0.480	0.491	0.022	2.759
Within batch, within lab and diff. concentration	243	0.638	0.441	0.067	1.768
Within batch, between lab and diff. concentration	747	0.766	0.487	0.020	3.002

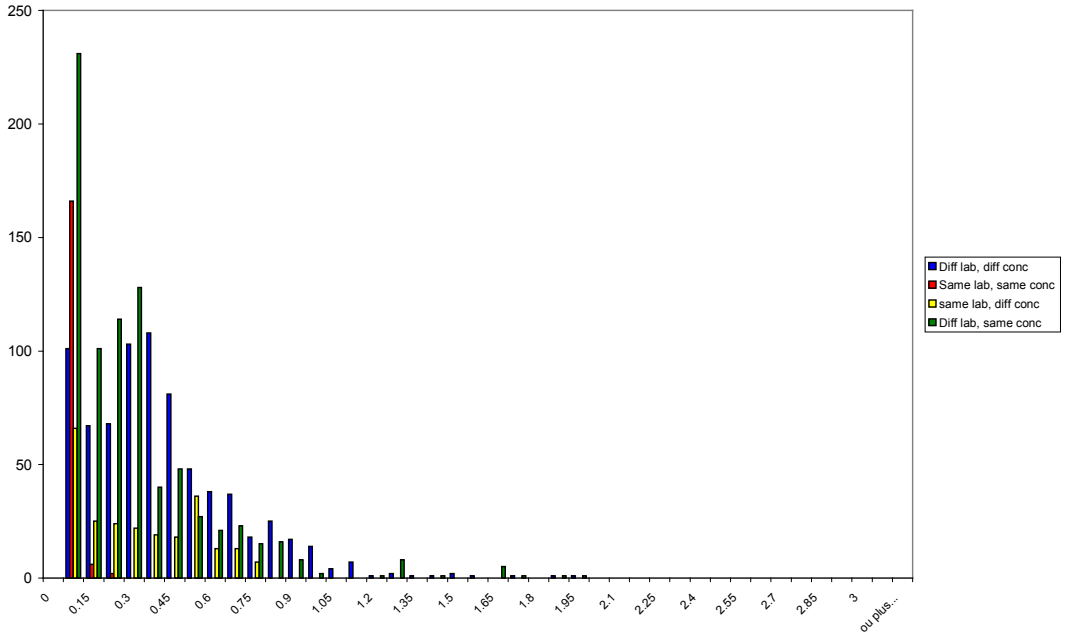


Figure 34a : schematic illustration of the distances of samples from the same batch. Pearson after normalisation + 4th square root and alternative 2.

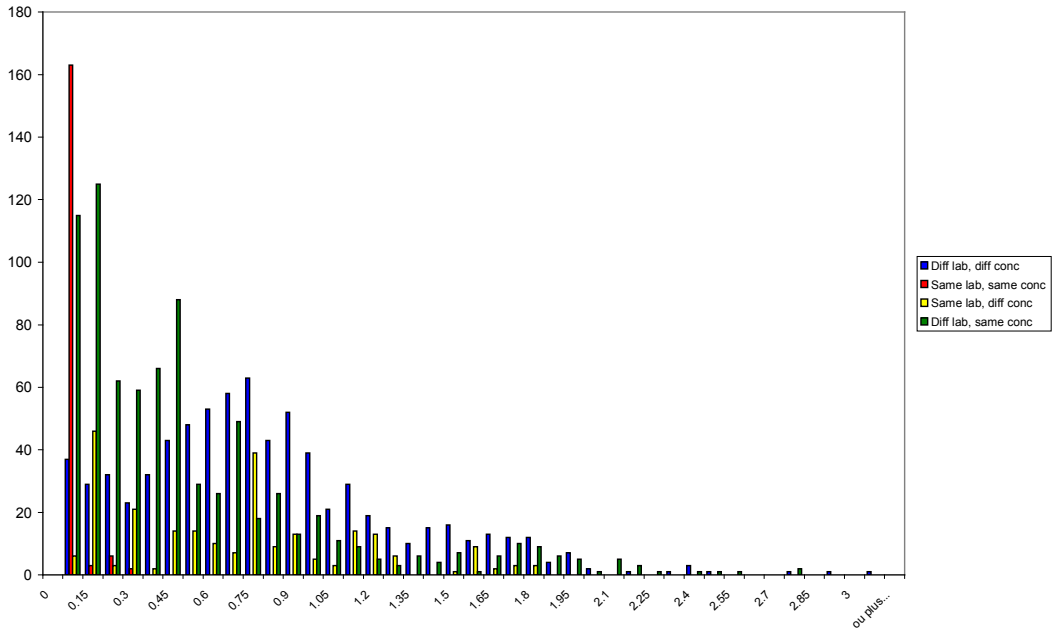


Figure 34b : schematic illustration of the distances of samples from the same batch. Squared sinus after normalisation + 4th square root and alternative 2.

From table 53a and especially from figure 34a, it can be seen that the majority of distances for samples from the same batch are in the region 0 to 0.5 for Pearson. As for the squared sinus, table 53b and figure 34b show us that the majority of distances are in the region from 0 to 1.
Of course, the distances are smaller if the samples are analysed in the same laboratory. Also, the distances are smaller if the compared amphetamine samples are of the same concentration.
Full detailed mean, standard deviation, min and max values describing all combinations can be found in annex 13.

6.9.2 Thresholds for samples synthesised with the same recipe

As mentioned in section 6.2, both partners 3 and 4 synthesised six batches of amphetamine using the same recipe. The syntheses were thoroughly controlled in order to get as reproducible profiles as possible. The idea was to determine the degree of variation of the profiles when one person in one laboratory synthesised multiple batches. In addition, it was also considered interesting to investigate the degree of variation between batches synthesised by two different people in two different laboratories but following exactly the same recipe.
For each batch, three replicates (three extracts) were prepared and analysed. Results are illustrated in figure 35a for Pearson and in figure 35b for the squared sinus :

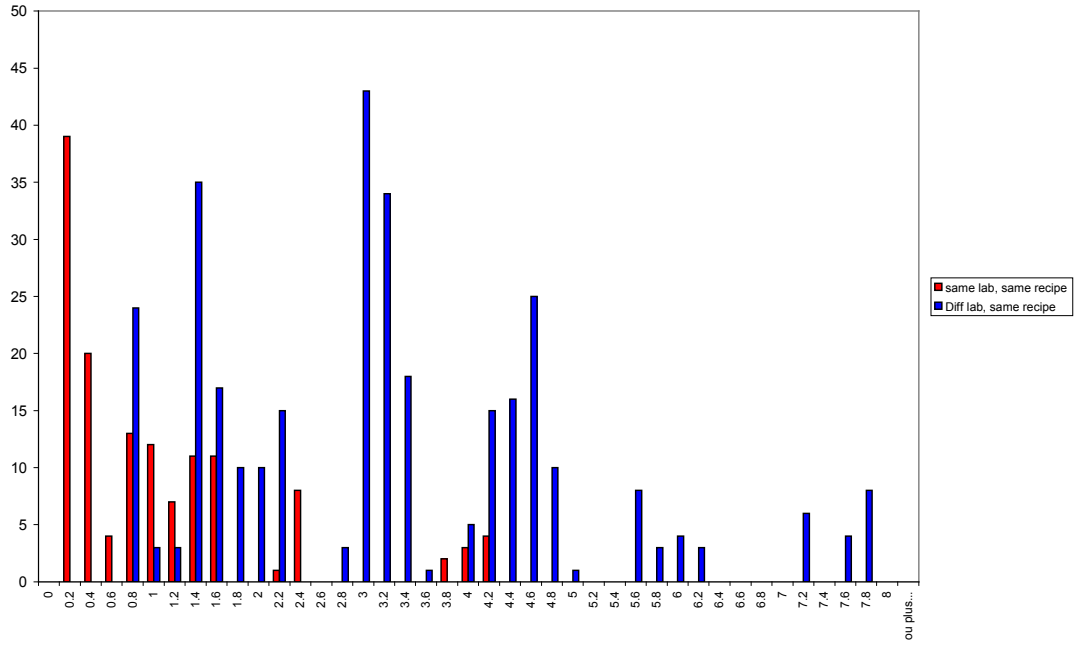


Figure 35a : schematic illustration of the Pearson distances between samples from multiple batches synthesised in one lab and one operator (in red). In blue, distances between samples from batches synthesised in two laboratories by two operators following exactly the same recipe.

As expected, the distances increase when different batches are compared although they were synthesised with the same recipe in one laboratory. Figure 35a shows us that the majority of distances are situated between 0 and 1.5 for Pearson whereas they were between 0 and 0.5 for samples coming from the same batch.
Regarding the distances between samples of batches synthesised in two different laboratories using the same recipe, they increase expectedly as well although some batches are very close. Indeed, the majority of distances are between 3.0 and 8.0 although a few distances are between 0.8 and 2.2.

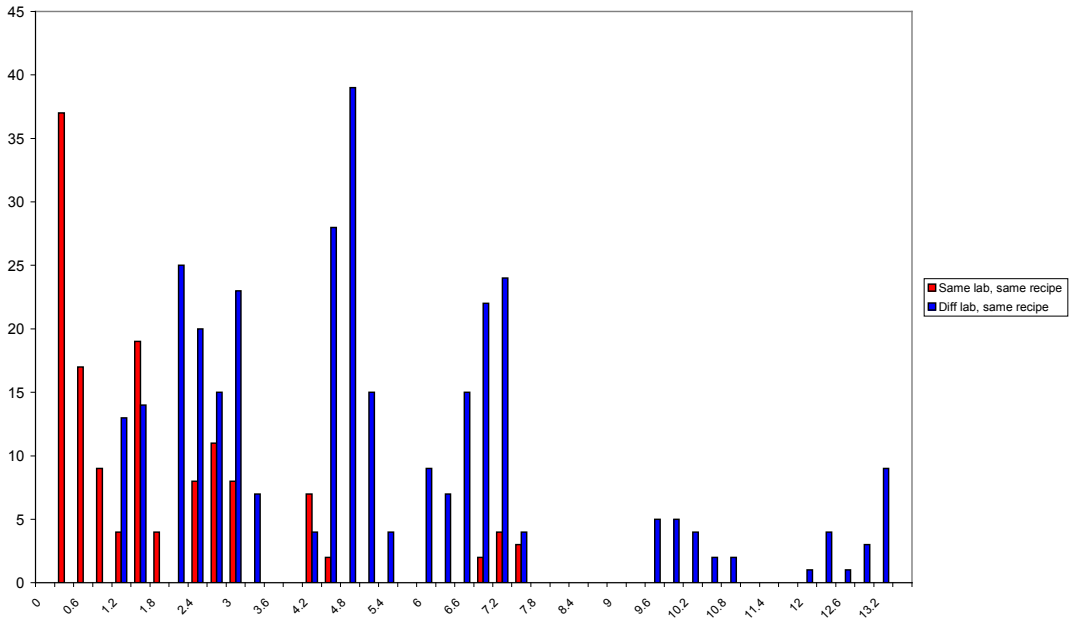


Figure 35b : schematic illustration of the squared sinus distances between samples from multiple batches synthesised in one lab and one operator (in red). In blue, distances between samples from batches synthesised in two laboratories by two operators following exactly the same recipe.

The same remarks are valid for the distances calculated with the squared sinus. The only difference is that the values are slightly shifted to the right with the squared sinus (if zero is considered to be the further left). Indeed, figure 35b shows us that the majority of distances are situated between 0 and 3.0 whereas they were between 0 and 1.0 for samples coming from the same batch.
Regarding the distances between samples of batches synthesised in two different laboratories using the same recipe, the majority of distances are between 4.0 and 13.5 although a few distances are between 1.2 and 3.8.

Overall results are summarized in table 54 :

Table 54 Mean, min, max and standard deviation values for distances between samples coming from different batches. Pearson and Squared sinus (N+4root, alternative 2).

	N° of distances	Mean	Std deviation	Min	Max
Same recipe, same lab. Pearson.	135	0.92	1.03	0.02	4.09
Same recipe, two labs, Pearson.	324	3.12	1.72	0.70	7.74
Same recipe, same lab. Squared sinus.	135	1.65	1.87	0.03	7.33
Same recipe, two labs, Squared sinus.	324	4.98	2.92	1.12	13.15

Finally, in order to visualise the distribution of distances, figures 36a and 36b were drawn from table 55 values as well as from the values shown in tables 54a and 54b. The within batch values used here are those from the analyses made in different laboratories for batches of the same concentration. This represents the closest situation to the batches made from the same recipe as the latter were analysed in two different laboratories and were of the same concentration (approx. 100 % amphetamine).

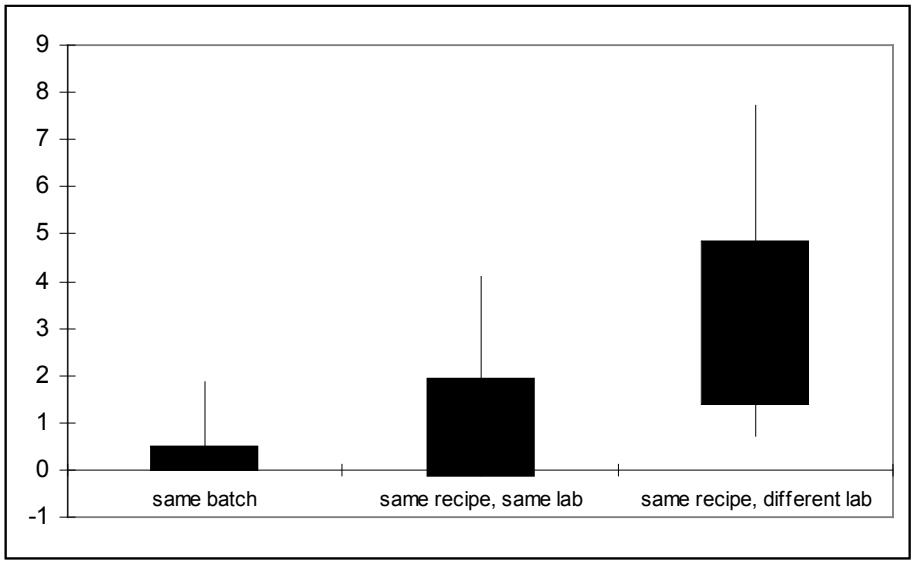


Figure 36a : graph of the Pearson distances summarizing the results from tables 54a and 55. The black areas represent the mean ± standard deviation. The extremities of the black lines represent the minimum and maximum values.

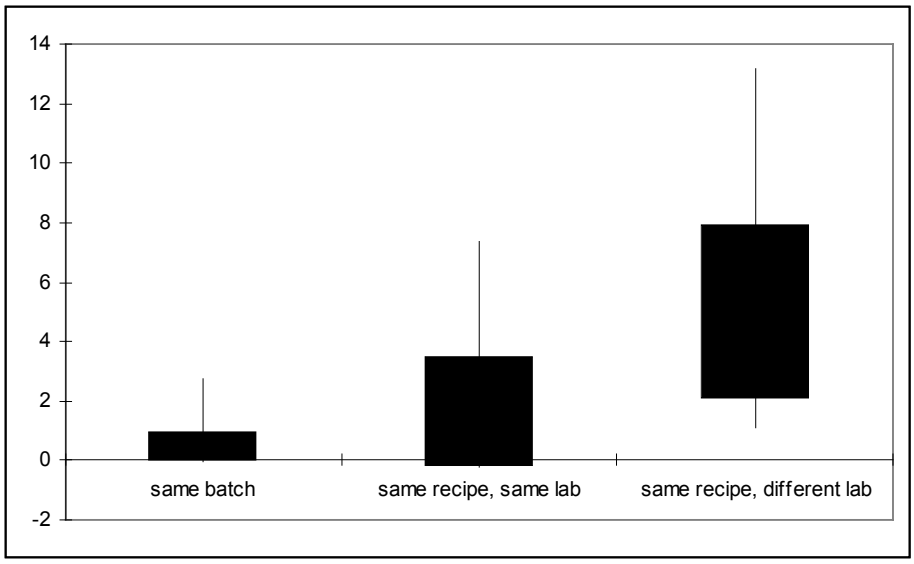


Figure 36b : graph of the squared sinus distances summarizing the results from tables 54b and 55. The black areas represent the mean ± standard deviation. The extremities of the black lines represent the minimum and maximum values.

6.9.3 Conclusions

From section 6.9.1, it could be estimated that Pearson distances between 0 and 0.5 are representative of samples coming from the same batch. For the squared sinus, the estimate is between 0 and 1.0.
Figures 36a and 36b, which is the summary of this small experiment of repeated syntheses, indicate to us that several batches synthesised successively in one laboratory gave Pearson distances between 0 and 2.0. For the squared sinus, the estimate is between 0 and 3.5.
Finally, when the batches synthesised with the same recipe by partners 3 and 4 were compared, the Pearson distances varied between 1.4 and 4.8. For the squared sinus, the values varied between 2.0 and 8.0.
Although these estimates don't take into account the minimum and maximum values (only mean ± standard deviation), they still give an idea of the thresholds to use in further interpretation.
For example, if two unknown samples are compared and a Pearson distance value of 0.1 is found, it is a strong indication that they probably come from the same batch. If a value of 1.0 is found, it is rather an indication that they were synthesised by the same laboratory with the same recipe but belong to a different batch. However, if a value of 1.5 is found, no conclusion could be drawn as they could originate from two different sources. Still, in this situation, it is very probable that the exact same recipe was used.

Nevertheless, these values are based only on a small data set and on a limited number of controlled syntheses. Only extensive future work will enable us to determine if these values are correct and could be used in further interpretation. A much larger number of samples from known sources will have to analysed for this purpose.
Also, the distances between batches synthesised with the same recipe in two laboratories are probably too pessimistic (or optimistic depending on how you look at them). Indeed, the synthetic conditions were really too carefully controlled and are probably not at all representative of the synthetic conditions used in clandestine laboratories. Therefore, it is expected that the distances values would be much higher for illicit samples coming from multiple batches of one laboratory as well as from batches originating from different laboratories using the same recipe.

Finally, it should be remembered that the results in section 6.9.2 are valid for Leuckart amphetamine only as it was not possible to repeat these experiments for reductive amination and nitrostyrene amphetamine.

6.10 Analysis of blind samples

6.10.1 Introduction

In order to determine if the final method was performing correctly, it was decided to check the ability of the method to find correct links in the database. Therefore, each partner sent a few of their samples to the others without any labels or indications about their identities. However, these samples were already analysed previously and the corresponding data stored in a common database. Thus, the analyses and the subsequent calculations should enable us to correctly identify these so-called blind samples.
For this matter, partner 4 sent five samples (I1 to I5) to partners 2 and 3. Unfortunately, sample I3 could not be analysed by partner 2 as the glass recipient broke during transportation. Alternatively, partner 4 received five blind samples from partner 2 (N1 to N5) and also five blind samples from partner 3 (S1 to S5).
To avoid partners from sending by accident the same samples to each other, it was decided that partners should only send five of their « street samples ». In this way, each partner would analyse a blind sample which was not analysed previously in their laboratory. Nevertheless, all partners would be using the same database which consists of 768 samples (synthesised + street samples) and try to find the identity of the blind samples by comparing them to the whole common database.

6.10.2 Results

The results are summarized in table 55. Pearson and squared sinus gave the same results. However, for sake of simplicity, only Pearson distances are given here :

Table 55 **Results from the blind test study. In brackets, Pearson distances obtained. Each laboratory put their best match as the possible candidate.**

		Results		
Sample number	Expected results	Partner 2	Partner 3	Partner 4
I1	B1643	✓ *(0.24)*	✓ *(0.09)*	-
I2	GEJU	✓ *(2.22)*	✓ *(1.08)*	-
I3	18A	*Not analysed*	✓ *(0.87)*	-
I4	5012B	✓ *(0.32)*	✓ *(0.02)*	-
I5	7922	✓ *(0.39)*	✓ *(0.08)*	-
S1	S015	✓ *(0.19)*	-	✓ *(0.09)*
S2	S047	***S035 (0.31)*** ✓ *(0.32)*	-	***S035 (0.25)*** ✓ *(0.26)*
S3	S081	✓ *(0.47)*	-	✓ *(0.37)*
S4	Not in database	***S087 (0.41)***	-	***S087 (0.44)***
S5	S003	✓ *(0.31)*	-	✓ *(0.19)*
N1	NC3545	-	✓ *(0.07)*	✓ *(0.32)*
N2	NC12662	-	✓ *(1.06)*	✓ *(0.14)*
N3	NC19078A	-	✓ *(0.04)*	✓ *(0.07)*
N4	NC3911	-	✓ *(0.17)*	✓ *(0.08)*
N5	NC15906A	-	***NC15906B (0.16)*** ✓ *(0.35)*	***NC15906B (0.11)*** ✓ *(0.56)*

6.10.3 Discussion and conclusions

As can be seen in table 55 above, they were three false positives (for samples S2, S4 and N5) but they could all be explained quite simply.
First, subsample S2 was taken from sample S047. However, comparison between samples S035 and S047 showed a Pearson correlation of 0.001, which means that it is almost impossible to discriminate between these two samples, at least with our 26 target compounds, and very strongly indicates that these two « street » samples are coming from the same batch of amphetamine. Therefore, when analysing this sample as blind, it is almost impossible to determine if it belongs to sample S035S or S047S. Indeed, the Pearson distances are almost exactly the same between the blind samples and samples S035S and S047S, independently of which laboratory is analysing the samples.
Secondly, sample S4, which was sent by partner 3, was actually a sample which was not in our database. Therefore, no correspondence was supposed to be found. Still, a good match was found with sample S087S (with a Pearson value of less than 0.5). In this case, we can not exclude that this sample belongs in fact to the same batch of amphetamine as the real sample S4.
Finally, subsample N5 came from sample NC15906A sent by partner 2. But a better match was found with the B sample. Again, it is probable that the subsamples A and B from sample NC15906 are from the same batch of amphetamine and that the reason for a better match with sample B is just a result of small random variation.

Otherwise, all other blind samples were correctly identified and were always found to be the best match in the database. Also, the distances were generally quite small and below the 0.5 threshold which was determined in section 6.9.1 (within batch distances).
One rather big exception is sample I2. Partners 2 and 3 correctly identified the blind sample but with rather big Pearson values. This could be explained by the probable inhomogeneity of this sample. Indeed, sample GEJU consisted of about a thousand capsules filled with amphetamine powder (two or three of these capsules have to be emptied in order to have enough powder for analyses). Thus, partners 2 and 3 received powdered samples from different capsules. So the relatively large Pearson values could be explained by the inhomogeneity of the amphetamine in these capsules. In a ideal situation, all capsules should have been emptied beforehand, the powder homogeneised and then the samples sent to other partners.

In general, table 55 shows very good results. However, this outcome was not quite straightforward. Indeed, a number of corrections had to be made in the integration of the 26 target compounds. This topic, which is a very important one, will be further discussed in the final conclusions. Still, the main drawback of analysing samples in different laboratories was found to be the integration and not the analysis itself. That is, when especially confronted to very small peaks, different operators will make different decisions. Some will discard the peak if its shape is not adequate and a qualifier ion is missing, others will still integrate the peak as they believe that the compound is still there although the peak is not perfect. The MS Chemstation software could take the decision to avoid the human factor but, unfortunately, its possibilities are quite limited and also often lead to integration errors.
Therefore, a number of rules have to be set and applied, particularly if new inexperienced operators have to perform the integration of MS chromatograms. For the three partners in this task, it was absolutely necessary in order to obtain the results in table 55. Thus, if in the future such a method would be used in routine in different laboratories and the data exchanged, a solution to this problem has to be found.

6.11 General conclusions for task 6

In task 6, the optimised method developed in the project was used to analyse a number of new amphetamine samples. Although the instrument was still set with a dual column system (one column to the FID detector and the other to the MS), only the MS data was used for the evaluation as GC-MS was found to be the method of choice. In a first stage, amphetamine batches were synthesised through different recipes and different synthetic routes and 361 "street samples" were collected by the three partners. The synthetised samples were further diluted 2.5 and 20 times (generally with caffeine and lactose) and all samples synthesised in task 6 were analysed in triplicate (three extracts). In total, this represented a database with 768 entries.
As for the data analysis, 33 target compounds were selected. Application of Principal Component Analysis (PCA) to a smaller data set helped us to reduce the number of target compounds to 26 without affecting the discrimination power of the method. Indeed, it was still possible to discriminate samples synthesised via the three different synthetic routes (Leuckart, reductive amination and nitrostyrene). Moreover, the number of false positives was minimised.
Pre-treatment of the data before application of a numerical comparison method was found to be a crucial step. Normalisation has to be applied in order to be able to compare amphetamine samples of different concentrations without changing the sample preparation. Weighing (or standardization) is necessary to reduce the influence of one (or more) huge peaks. These can be one hundred to a thousand times bigger than the remaining target compounds and can bias the comparison. Dealing with zeroes was also an important issue with some numerical methods.
Finally, the best pre-treatment method was found to be the following :

- All zeroes are replaced by 200 (value correspoonding to the half detection limit of the MS system).
- Each peak response is divided by the sum of all peak responses (normalisation).
- Each normalised value is square rooted twice (fourth square root).

Once the data is pre-treated, it can be used to calculate distances between amphetamine samples. Seven different methods were evaluated : Euclidean distance, Manhattan distance, Pearson correlation, Similarity index, Quotient method, Canberra index and the Squared sinus function.
Pearson correlation and the squared sinus were found to be the best suited numerical methods for comparison of amphetamine profiles. After discussion with all partners, it was finally decided to carry out future work with the Pearson correlation as it is an integrated function in Microsoft Excel and might be more convenient to use, especially if special computer programs are not available.

When varying the recipe within a synthetic route, it was found very easy to discriminate the resulting amphetamine profiles. However, if exactly the same recipe is used, the discrimination is more difficult, especially if the same operator has performed the syntheses. To help in the interpretation, some thresholds values are proposed which have to be confirmed and updated in the future. These values give an idea of the intra-variability and inter-variability in amphetamine production but a much larger number of amphetamine samples from known sources (such as samples from clandestine laboratories) need to be analysed in order to confirm or negate these values.
For the time being, the proposed thresholds are the following (Pearson values normalised from 0 to 100) :

0 to 0.5 : Samples probably originate from the same batch or, at least, originate from the same laboratory (using one recipe).

0.5 to 1.0 : Samples probably originate from the same laboratory. They could be from the same batch but are more likely to be from different batches (always using the same recipe).

1.0 to 2.0 : Samples possibly originate from the same laboratory. However, they could be from another laboratory which is using exactly the same recipe.

2.0 to … : Samples were synthesised with a different recipe or even a different synthetic route.

These values are, of course, only rough guidelines. In practice, they are used to sort samples in a database. All potential links are then carefully checked visually. Indeed, Total Ion Chromatograms (TIC) and even Extracted Ion Chromatograms (EIC)* are compared and special attention is paid to the similarities and dissimilarities of the profiles. This is where the experience of the forensic chemist comes into play and also some subjectivity. This is why only "certain" links are generally reported to the law enforcement agencies where the correlation value is almost a perfect match and the chromatograms are almost mirror images. In these cases, there is a very little place for error and it is almost certain that the link is true, meaning that the two (or more) samples come from the same batch or, at least, from the same clandestine laboratory.
The grey area is when the match is not perfect, for example, when Pearson values are between 1.0 and 2.0 as described above. In these cases, it is almost impossible to draw a conclusion unless some special similarities or dissimilarities can be found in the chromatograms. Then, the forensic chemist might have some more indications that would confirm or negate the link or he might be unable to draw any further conclusions.

As mentioned in section 6.10.3, the main problem in the final optimised method is the integration of peaks. It also has to be harmonised although the MS chemstation software does the integration automatically with fixed parameters. Indeed, the operator still has to check the integration manually in order to verify that the software has correctly integrated each target compound. Although, this verification can be very quick (30 seconds per chromatogram for an experienced operator), many corrections are often necessary.
The most common dilemma is to integrate a very small peak or not. This occurs when the software has integrated a very small peak and the operator thinks it should not be or vice versa. Depending on the operator and its experience, the decision will be different. This can lead to inconsistencies when comparing data between laboratories. In a ideal world, all data could be centralised in one laboratory and only one operator would perform the integration of all chromatograms. However, this is, of course, inconceivable. Therefore, a number of rules have to be set and applied or another solution has to be found.
In the last few years, Agilent Technologies® in collaboration with the National Institute of Standards and Technology (NIST) have commercialised an automated mass spectrometry deconvolution and identification system (AMDIS) which works with the Agilent Chemstation [ref. application note]. This system is more powerful than the usual MS Chemstation in identifying compounds in complex MS chromatograms and could be a future solution to our problem.
The MS Chemstation identifies target compounds by integrating the target ion as well as two or up to three qualifier ions. It also compares the ion ratios to known ratios. However, complex matrices affect ion ratios and background noise can do the same with ion ratios of very small peaks. This can cause numerous false positives (the software integrates a peak when it shouldn't) and false negatives (the software does not integrate the peak when it should). Therefore, to be certain of the results, manual verification is performed and, as mentioned above, leads to inconsistencies if many operators are integrating the same samples.
AMDIS fully deconvolutes the MS data file by using mathematical algorithms [Colby, 1992] and « cleans » the contaminated mass spectra. For all targeted compounds, the full mass spectrum is cleaned from matrix interferences and background noise. It is then compared to the mass spectra of an user-constructed library (for special applications such as ours) or to a NIST library. This comparison uses full mass spectra and not just two or three ion ratios and is retention time independent. However, after spectral identification, it can use the Retention Time Locking (RTL) system in order to accept or reject peaks based on a time-window.
If successful, this system could totally eliminate the human factor in integration as it will decide consistently if the target compound is present or not.
Still, this will hopefully be tested in a future project called CHEDDAR. This project will be discussed in the final conclusions (section 8).

* An extracted ion chromatogram is a chromatogram of a particular mass or mass range from data acquired in scan mode. Multiple ions can be chosen and extracted. In this project, a macro was developed by Kaisa Jalava (partner 2) which extracts 14 ions representative of the Leuckart target compounds (5 for reductive amination and 5 for nitrostyrene). This allows us to obtain « clean » chromatograms where all matrix and interfering compounds are removed. Thus, the visual comparison becomes much easier, especially with complex samples or samples of different concentration.

7 Application of the profiling method to real samples

7.1 Analysis of powdered samples

At this stage of the project a number of powdered samples had been analysed with the new optimised profiling method. As mentioned in task 6, 112 samples coming from various police seizures were analysed in IPS. These were compared with the help of the new numerical comparison method and the potential links were recorded. Chromatograms of these potential links were visually compared in order to evaluate the correctness of the numerical result. These comparisons were solely used for research purposes and for somehow validating the analytical method. There was no aim to report links to the concerned police forces for operational or strategic purposes. Still, in order to illustrate the methodology of the comparison, an example is presented below :

A new sample consisting of a white capsule containing amphetamine powder was taken from the IPS collection. This sample (internal number 1071) was not used during the project and was therefore not in the project database. It was analysed with the new profiling method and compared to the whole database (N = 768 entries).
Only one possible match came out of the comparison with a good Pearson correlation of 0.39. It concerned the sample 3343 / 00 from the WD Zürich (Scientific Section, city of Zürich police). At first, the total ion chromatograms (TIC) were compared and are shown below in figure 37 :

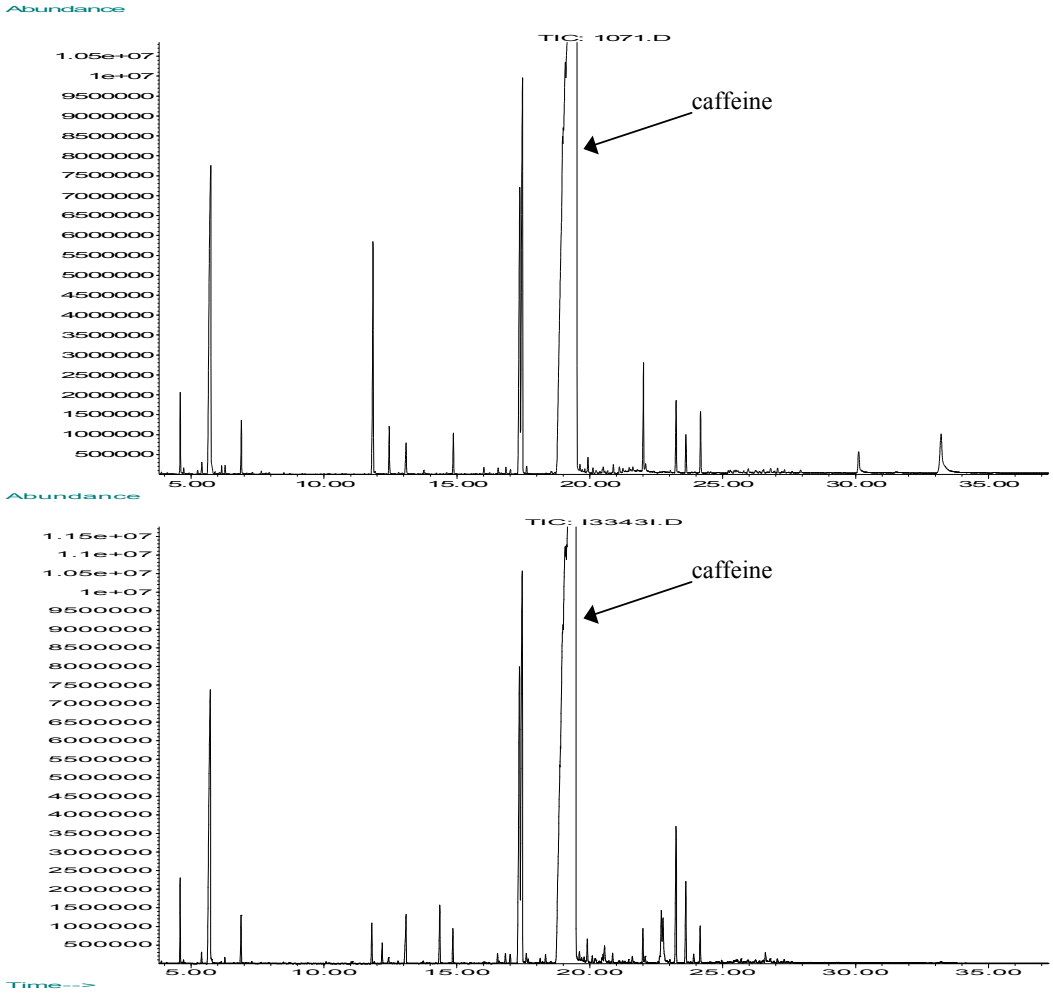


Figure 37 : *Top : total ion chromatogram of sample 1071.*
Bottom : total ion chromatogram of sample 3343 / 00 from project database.

As can be seen in figure 37, TIC chromatograms are not always ideal for direct comparison as many matrix peaks can be present as well as peaks from volatile compounds which can be present in one sample and absent (or present in much lower amounts) in the other sample. Still, TIC chromatograms can be useful as most cutting agents or other compounds can be identified (for example, caffeine, lidocaine, 1-phenethylamine, etc.).
Therefore, extracted ion chromatograms (EIC) are considered much more practical for direct comparison as they provide « cleaner » chromatograms and the peaks present are generally those of the target compounds. Figure 38 below shows the corresponding EIC chromatograms of the samples described above :

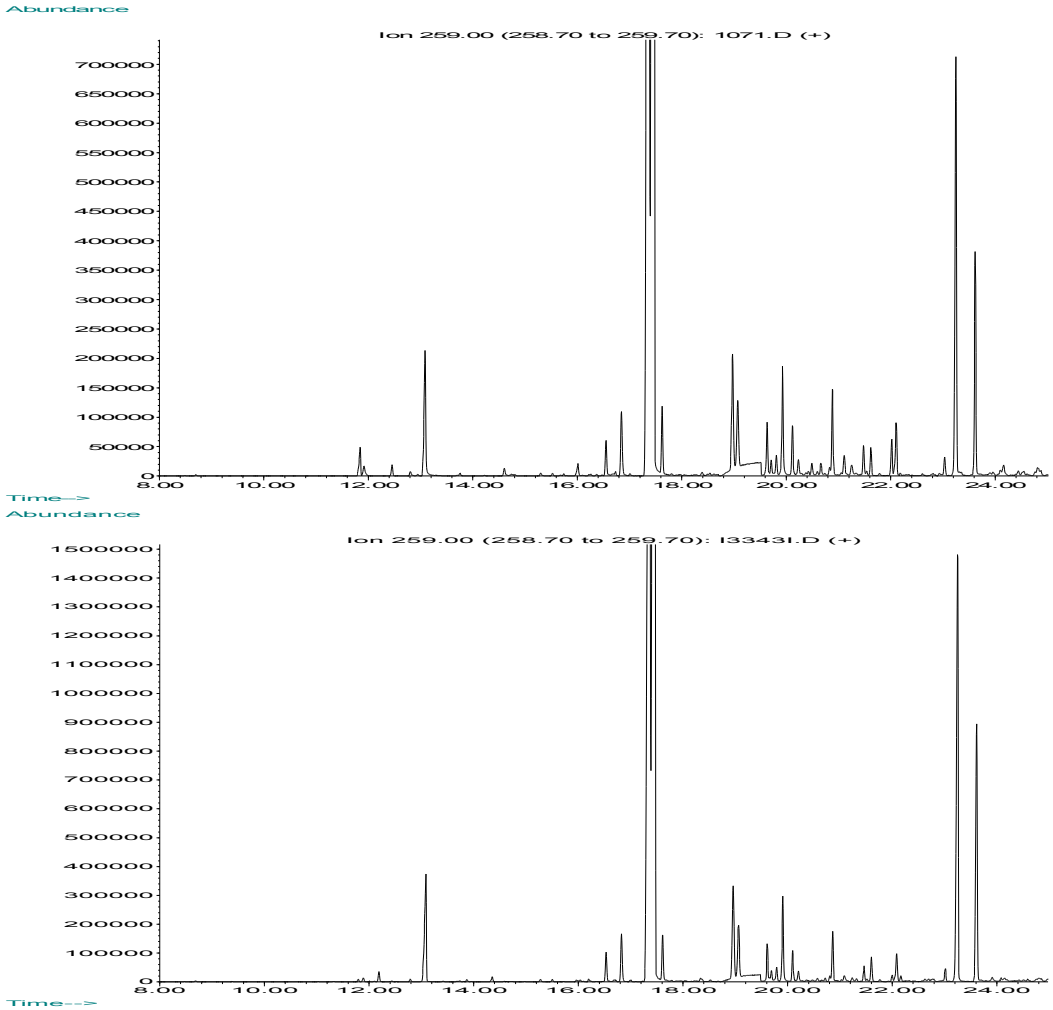


Figure 38 : *Top : extracted ion chromatogram of sample 1071.*
Bottom : extracted ion chromatogram of sample 3343 / 00 from project database.
Ions extracted (in this case for Leuckart amphetamine) : 105, 106, 118, 120, 134, 143, 148, 160, 162, 176, 190, 232, 258, 259.

The big advantage of EIC is also that the chemstation macro will automatically normalise the chromatogram to the highest target compound peak. Therefore, amphetamines of different concentrations can be directly compared without any manual adjustment of the y axis.
Figure 38 above show that these two samples are indeed very close and confirm the Pearson correlation result. It is therefore very probable that these two samples belong to the same batch of amphetamine.

Another way of comparing two samples is to draw a graph of the normalised and standardised data where the numerical values are represented on the y axis and the 26 target compounds are on the x axis. This kind of illustration is especially useful for determining which target compound is responsible for small (or maybe big) differences. Figure 39 is the graph regarding the two samples 1071 and 3343/00 :

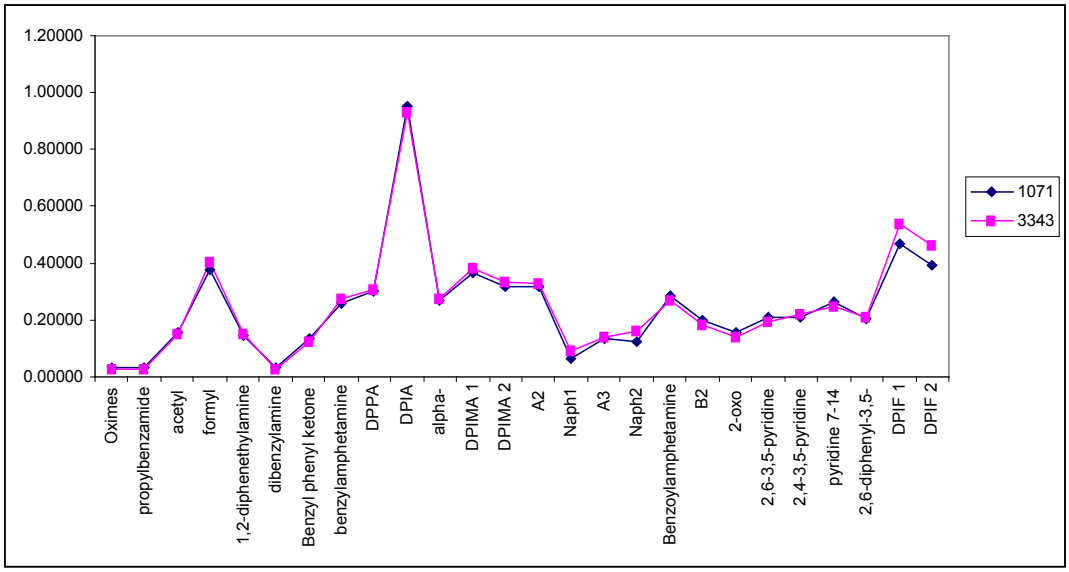


Figure 39 : graphical representation of the 26 target compounds values for samples 1071 and 3343 / 00.

Figure 39 confirm the very strong similarities between samples 1071 and 3343 / 00. Finally, the link was confirmed indirectly. Indeed, 1071 is just an internal number and further investigation into the sample collection of IPS indicated that this sample originated from the WD Zürich and that the corresponding police number was 3343 / 00 !
Therefore, the two samples came in fact from the same case but they were obtained through two different channels at two different times : sample 1071 was received in 2000 from the WD Zürich whereas sample 3343 / 00 was sampled in 2002 in the WD Zürich and taken back to IPS for the purpose of the project.

7.2 Analysis of tabletted samples

IPS has a collection of illicit tablets which started in 1995. The majority of the tablets contain MDMA (MethyleneDioxyMethylAmphetamine) and are sold as « Ecstasy » on the illicit market. However, a number of these contain amphetamine as the active ingredient. At the time, around 10 % of seized tablets received by IPS contained amphetamine as the active ingredient [Anglada et al., 2002]. It was possible to analyse 87 of such tablets in order to test the new profiling method on tablets.

However, there are three problems when analysing tablets : i) the amount of amphetamine present in a tablet is generally low, ii) in the sample preparation, an emulsion can form between the aqueous and organic layer making the pipetting of the organic solvent very difficult, iii) palmitates and stearates present in tablets can interfere with the chromatographic analysis.
Indeed, the tablets analysed contained an average of 25 mg amphetamine with a standard deviation of ± 20 mg.
Therefore, in order to obtain a suitable profile a whole tablet has to be crushed and analysed.
Regarding emulsions, they are probably due to certain additives which are used in the tablet manufacture. Still, no tablets in this study showed intense emulsions. As a result, it was possible to sufficiently separate the aqueous and organic layer in all cases.
But the main problem is the presence of fatty acids which can cause major interferences in the chromatogram depending on the amount present. In most cases, their concentrations were low and target compounds could still be integrated thanks to the selectivity of the mass spectrometer. However, in some cases, their concentrations were extremely high and the amphetamine target compounds were consequently quite difficult to identify and integrate. Figure 40 illustrates an example of a sample where high concentrations of fatty acids were detected :

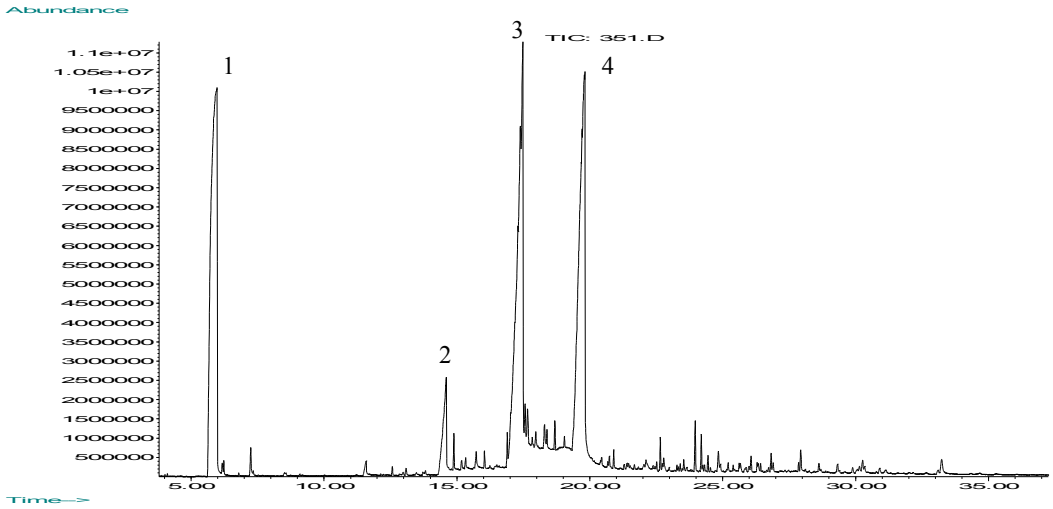


Figure 40 : Total ion chromatogram of the profile of an amphetamine tablet sample. Peak 1 : amphetamine, peak 2 : myristic acid, peak 3 : palmitic acid, peak 4 : stearic acid.

In the example shown in figure 40, target compounds situated in the time interval from 17 to 20 minutes were quite difficult to identify and integrate. In some cases, the concentration of fatty acids was even greater increasing the peak width of these compounds and causing major, if not fatal, interferences in the chromatogram.
However, there is a way of removing these fatty acids prior to analysis. Indeed, after dissolution in the aqueous buffer, the test tube can be centrifuged and the supernatant transfered to a syringe fitted with a PTFE disk filter (Schleicher & Schuell, diameter 30 mm, pore size 0.45 µm). After filtration, The organic solvent is added to the recovered aqueous solution. Experience has shown that this is an efficient method of removing almost 100 % of the fatty acids with very little loss of target compounds. It also reduces the emulsion formation if present without filtering. Unfortunately, for time and availability reasons, the amphetamine tablets analysed in this study could not be prepared and analysed again with this method.
However, the majority of tablets contained very low amounts of fatty acids and despite the fact that a few tablets contained very high concentrations of fatty acids, all 87 samples could be profiled using the method developed in the previous tasks. A number of interesting results have been found and are presented below :

7.2.1 Similar / different external characteristics and similar organic profiles

Figure 41 : front pictures of three different tablets coming from the same case

Figure 41 shows three different tablets. The first tablet (left in figure 41) has a different logo and a different colour compared to the two other tablets. There were also differences between the other physical characteristics but these were very small and probably not significant (see further details in Annex 14).
However, they all displayed very similar organic profiles (see Annex 14A) which indicated a possible common origin (same batch of amphetamine). In addition, the probable link was somehow confirmed by the fact that the first two tablets (left and middle in figure 41) were part of the same police seizure. Regarding the third tablet (right in figure 41), although it was seized in a different part of Switzerland and a year earlier, it displayed almost identical visual and physical characteristics compared to the second tablet.

Thus, the hypothesis is that the two « smiling sun » tablets have a common source of manufacture. The similarity of the organic profile pushes the hypothesis further and indicates a common source of the amphetamine itself.
In conclusion, the organic profiling of tablets can be useful in two ways : i) it can reveal links between tablets bearing different visual and physical characteristics, ii) it can confirm links which are based only on visual and physical characteristics. In the latter case, differences in the organic profiles would not automatically negate the link but would rather indicate that different batches of amphetamine were used by the same tablet manufacturer (see section 7.2.2).
In Annex 14, three other cases are illustrated where very similar organic profiles were found between tablets bearing various visual and physical characteristics. All corresponding chromatograms can be found in Annex 14A.

7.2.2 Similar external characteristics and different organic profiles

Figure 42 : front pictures of two similar tablets coming from two different cases

This is an example of a case where two tablets with almost identical visual, physical and also chemical characteristics (same cutting agents and same concentration of amphetamine, see Annex 15) displayed quite different organic profiles (see Annex 15A). As the two seizures are more than a year apart, the first hypothesis would be that different batches of amphetamine were used in the manufacture of these tablets and even that two distinct illicit amphetamine laboratories are the source of these two batches of amphetamine. However, the fact that the two amphetamine batches came from the same illicit amphetamine laboratory can not be excluded. Indeed, the difference in the organic profile may be the result of a high variation in the production of one illicit laboratory. Still, this remains a hypothesis as very little knowledge of the variations within illicit laboratories is available at this time.
In conclusion, when identical visual, physical and even chemical characteristics are observed between tablets, it does not guarantee that the amphetamine contained in these tablets come from the same batch or even have the same origin, especially if there is a large time difference between the seizures.

In Annex 16, another similar case concerning three seizures is presented. In that case, two out of three tablets showed almost identical visual, physical and chemical characteristics including their organic profiles. However, the third tablet, which was visually and physically indistinguishable from the two others, contained lactose as an extra cutting agent. It should be noted that it was seized two, respectively three years later.
Observation of its organic profile showed small differences but also strong similarities (see Annex 16A). In that case, the more likely hypothesis is that the amphetamine contained in the third tablet comes from a different batch but from the same illicit amphetamine laboratory. But again, this remains a hypothesis. Indeed, the variations observed in Annex 16A could be the result of batch inhomogeneity. Thus, the amphetamine contained in these three tablets could still come from the same batch despite the fact that the seizures were made in three different years (1998, 1999 and 2001).
Annex 17 shows five tablets with similar visual and physical characteristics. However, slight differences are found in the chemical composition. Indeed, the first tablet contains 28 mg of amphetamine and is cut with lactose. The fifth tablet contains 44 mg of amphetamine and is cut with caffeine. Regarding the three other tablets, they contain similar amounts of amphetamine (13, 15 and 19 mg) and are cut with both caffeine and lactose.
Observation of the organic profiles show strong similarities between the three tablets with similar chemical composition whereas the two other tablets show very different organic profiles. This case is an example where tablets with similar visual and physical characteristics can have a different chemical composition and also different organic profiles. However, in this case, time may have an influence as these tablets were seized over a period of four years (1995, 1997, 1997, 1998 and 1999). Therefore, a possible hypothesis is that the same tablet manufacturer has varied his tablet composition over time (amount of amphetamine and cutting agents) and used different batches of amphetamine.

7.2.3 Similar external characteristics and similar profiles

In general, tablets with almost identical visual, physical and primary chemical characteristics have also very similar organic profiles. This was the most common observation in this study as all contemporary tablets (from different cases) which beared the same visual, physical and primary chemical characteristics showed as well very similar organic profiles (with the exception of non-contemporary cases discussed in section 7.2.2). Therefore, only one example among others is illustrated in Annexes 18 and 18A.
This raises the question whether organic profiling is really useful in cases of amphetamine tablets for determining common sources of the amphetamine itself. Indeed, if the organic profiling always confirms the links already established by the visual and physical characteristics as well as the primary chemical information (amount of amphetamine and cutting agents), then these latter characteristics may be sufficient for linking tablets also at the amphetamine level. Thus, the investment into organic profiling analyses may be unnecessary, at least on a routine basis.
Nevertheless, as shown in section 7.2.1, organic profiling has the potential of linking tablets which are different from a visual and physical point of view. This could be especially useful in the two following situations : i) linking tablets where one tablet manufacturer has used one large batch of amphetamine but different punches (to produce different logos) or different dyes (to produce different colours) or different tabletting machines (to produce different tablets) or any combination of the above ; ii) determining common sources of amphetamine in tablets produced by different tablet manufacturers.
But the great majority of tablets found on the illicit market and sold as « ecstasy » contain MDMA (MethyleneDioxyMethylAmphetamine) as the active ingredient. Therefore, the above remarks are more relevant in relation to MDMA profiling and the reader is referred to the work of Zingg [Zingg, 2004] for further discussions in relation to MDMA tablets.

8 Final conclusion and future activities

This work dealt with the development of a gas chromatographic method for the profiling of amphetamine powder. Existing methods were already in use in some countries, especially in Sweden and Finland where amphetamine is still the number one illicit drug. However, the existing methods were never thoroughly optimised and did not take into account synthetic routes other than the Leuckart route. Although this synthesis method remains by far the most popular way of synthesising illicit amphetamine in Europe, this may change in the future. For example, in Finland almost half of the seized amphetamine is now produced via the reductive amination method. This figure was by far much lower at the beginning of this project. Also, in the case of Finland, there are strong indications that the amphetamine is smuggled from Estonia and even produced there. With the expansion of the EU to 25 member states including the baltic countries and Poland which are known to be amphetamine producers, who knows what the future helds for us in terms of amphetamine production methods.
In addition, the existing methods were not harmonised and thus not adapted for data exchange. Therefore, if comparisons of samples were required, these had to be sent to one and only forensic laboratory which would carry out the analyses. This system of course implies many administrative barriers as it is not easy to send samples through borders even if efforts are being made to simplify the paperwork and transmission of samples. Further, the main drawback of sending drug samples is time. Indeed, as profiling results are forwarded to law enforcement agencies, these may not be interested in obtaining results months after the beginning of the corresponding investigation. The sooner the results can reach the concerned law enforcement agency, the more chances the information will be helpful and useful.
This is why the ultimate aim of this work was to develop a harmonised method that could be used in many different forensic laboratories and where the data generated would be reliable enough for comparison purposes. Thus, samples analysed in different laboratories could be directly compared and the sending of samples to a central laboratory would no longer be required. This would result in a significant gain in response time and efficiency and enhance collaboration between different forensic laboratories in Europe.
A previous attempt was made in using a common profiling method in different laboratories but it concerned a non-synthetic drug, namely heroin [Strömberg et al., 2000]. It was somehow unsuccessful as the reproducibility between the three participating different laboratories was not considered good enough. According to their conclusions, the undesired variations originated from difficulties in harmonizing integration of FID chromatograms (the method was GC-FID) and poor chromatography of some impurities which could not be ignored as they were considered essential for discrimination between samples. However, their relative lack of success may be explained by the fact that very little optimisation studies were performed prior to the use of the chosen method.

So, the challenge began in February 1999 and was divided in a number of subtasks. The first was to synthetise a number of known impurities in order to confirm their presence in illicit samples and use them as references in further tasks. Unfortunately, as mentioned in section 1.7, an amphetamine profile can contain more than 200 peaks and it is impossible to identify and synthetise all compounds. Therefore, a selection was made based on prior knowledge, the literature available and the allocated time frame. 21 target compounds were successfully synthesised and full spectroscopic data is now available which makes this data set quite unique (mass spectra, infrared spectra, ultraviolet spectra, ^{1}H-NMR and ^{13}C-NMR spectra).

In the second task, the stability of these target compounds were studied in various solvents. This enabled us to detect three unstable target compounds which could not be reliably quantified. It also demonstrated the difficulty of use of diethyl ether due to its very high volatility. Finally, it showed that isooctane and toluene were very inert solvents and therefore were probably the most suitable solvents for our application.

The third task dealt with the optimisation of the sample introduction technique, the choice of column and temperature programme as well as the choice of detector and operating conditions. The results showed that split and splitless injection techniques work similarly at different temperatures. In order to make a final choice, random and systematic errors were calculated and splitless injection was found to be giving the smallest total error. Also, splitless injection is more suited if low concentration samples have to be analysed.
Regarding the choice of column, separation power, resolution and inertness were the key factors in selecting an appropriate column. Finally, the choice was made for a DB-35 MS column with a temperature programme of 8 degrees per minute.
In terms of detection, this study enabled us to find out some limitations of the standard ion source of the Agilent 5973 mass spectrometer. Replacement of the standard ion source with a so-called Ultra ion source gave much better results in relation to linear range, repeatability and reproducibility. The only drawback being a slight loss in sensitivity. This weakness in performance has been later acknowledged by the Agilent company and nowadays the new Agilent GC-MS systems are equipped with the Ultra ion source with a small modification in order to preserve maximum sensitivity (also known as Inert MS).
MS (with the Ultra ion source) was finally chosen as the most appropriate detector for our application. It gave very similar results compared to FID and NPD in terms of repeatability, reproducibility and sensitivity. As for linearity, the linear range of the mass spectrometer will probably never be as large as the FID. Still, it was considered sufficient for our application. But the main advantage of the MS compared to other detectors is its selectivity. That is : its ability to quantitate two different compounds even if they are coeluting. This is performed by recording the response of ions specific to each compound. This also allowed the overall integration of target compounds to be much more reliable, easy and fast.

The fourth task was dedicated to the optimisation of the sample preparation. In the case of liquid-liquid extraction, many different variables were studied such as the type of buffer, pH, concentration and volume of buffer, type and volume of organic solvent and even the influence of cutting agents such as lactose and caffeine. For solid phase extraction, 14 different types of cartridges were first of all tested by partner 2 and two of them were retained for further comparison.
Buffer capacity, dissolution power of the buffer, repeatability, recovery and sensitivity were all important factors that were taken into account during this task. Practical aspects also partly influenced our final decision. Finally, a liquid-liquid extraction with a TRIS buffer at pH 8.1 and toluene was determined to be the best compromise.

In the fifth task, the optimised method was tested on synthetised samples and the variations within and between laboratories were determined. Within laboratory and within day, the average relative standard deviation of the method was determined to be in the order of 5 to 6 %. Within laboratory and within a period of two months, this value was determined to be in the order of 8 to 10 %. Finally, as the same samples were sent to the various laboratories, it was possible to determine an inter-laboratory value. In this case, it was determined to be in the order of 8 to 12 %. It should be emphasised here that one of the main outcomes of this task was the realisation of the importance of quality control. Indeed, the only laboratory which failed to meet the quality control requirements gave the most deviating results. Therefore, the inter-laboratory value is rather closer to 8 % if all quality control requirements are met. These results were quite encouraging before tackling the sixth task which dealt with the study of numerical methods for the comparison of amphetamine profiles.

For the purpose of this final task of the project, 768 samples were available. Almost half were street samples collected by partners 2, 3 and 4 and the rest were synthesised samples (analysed in triplicate) which were used as such or further diluted (usually with caffeine and / or lactose). At first, 33 target compounds were selected. However, it was finally possible to reduce the number of target compounds to 26 without affecting the discrimination power of the method.

Pre-treatment of the data before application of a numerical comparison method was found to be critical. It was finally determined that replacement of zeroes by a value of 200 (which corresponded at the time to half the detection limit of the MS detector) and normalisation to the sum of peaks was the most adapted to our application. Moreover, the obtained values had to be weighed in order to reduce the influence of dominating peaks. The most appropriate method was determined to be the application of the fourth square root to the normalised values.
Various distance methods were evaluated : Euclidean distance, Manhattan distance, Pearson correlation, Similarity index, Quotient method, Canberra index and the Squared sinus function.
Pearson correlation and the squared sinus were found to be the best suited numerical methods for comparison of amphetamine profiles. After discussion with all partners, it was finally decided to carry out future work with the Pearson correlation as it is an integrated function in Microsoft Excel and might be more convenient to use, especially if special computer programs are not available.

Unfortunately, no time was available to further study other interesting numerical methods such as neural networks [Kingston, 1992, Casale and Watterson, 1993, Welsh et al., 1996] or even to evaluate continuous likelihood ratios based on the Bayes theorem as described by Dujourdy for heroin samples [Dujourdy, 2003]. However, a little time was found to test the method on amphetamine tablets. As described in section 7, preliminary results were encouraging and indicate that the method could easily be applied to tablets considering that filtering would be highly recommended in the sample preparation process. The information provided by the organic profile can then be combined with the visual, physical and other chemical characteristics of the tablet for interpretation purposes. However, it is at present not quite clear if results from amphetamine powders can be directly compared to results from tabletted amphetamine.

At this stage, corresponding to October 2002, the project ended and discussions started in order to give a future to this new optimised method for profiling amphetamine. At the time, another project named CASE was already underway and concerned only EU member states and Norway. It was, and still is, financed by the Swedish government and consists of sending amphetamine samples (from large seizures only) to a central laboratory for profiling, namely the Swedish National Forensic Science Laboratory (SKL). For this purpose, SKL were, and still are, using the same profiling method they have been using for decades. Therefore, any further work would have to run parallel to this project, at least until the end of 2005, date of the ending of the CASE project.

In this matter, Finland were the first to act as they developed a database for storing data acquired with the new optimised method and enable comparisons of profiles as part of their national profiling program. It was developed in the Crime Laboratory of the National Bureau of Investigation in Vantaa, Finland (partner 2) and runs under Microsoft Access. Excel macros were subsequently developed in order to automatically transfer data from the Chemstation to the database. The database can then display the fifty best matches when a new sample is compared to the rest of the database and also displays graphical representations of the chromatograms which are quite helpful for direct visual comparisons. It should be noted here that the program is extremely fast as thousands of calculations are made within a matter of seconds.
This database is now also in use in the Netherlands Forensic Institute where this new method is now applied on a routine basis as part of a new European research project which was given the acronym CHEDDAR (Collaborative and Harmonised European Database for the Determination of Amphetamine Relations). Indeed, funding was obtained for a two year project through the AGIS program (Directorate General of Justice and Home Affairs) of the EU commission and the project started officially in December 2003 under the coordination of SKL.
This project is somehow the continuation of the SMT project and many aspects that were not addressed previously will be, if possible, studied in this new project. First of all, the aim is to develop a database, similar to the Microsoft Access database, on a Microsoft SQL server. This server would be physically located in one laboratory and other partners would be able to upload their data directly through the internet. They could also consult the database from their local computer.
A second aim is to improve our knowledge on the correlations between recipes used in the clandestine manufacture of amphetamine and the corresponding profiles. It is believed that this further knowledge will be of valuable help in the interpretation of eventual links between samples. For this matter, a number of syntheses according to illicit recipes are planned as well as the study of critical steps in the synthesis such as hydrolysis, cristallisation and the influence of the starting material.
The third aim is to fully document the method in order to simplify the learning process for eventual new laboratories who would wish to apply the method. This also includes the development of macros for quality control purposes, the determination of quality control criteria for potential new partners, the problems of archiving and audit trail, the sending of control samples again for quality control purposes, the harmonisation of integration as discussed in section 6.11, etc.

Two other important issues which were unfortunately not addressed in the SMT project will be further studied : i) the influence of drying and ii) the influence of storage.
Indeed, it is possible to encounter wet samples as clandestine manufacturers do not always wash and / or dry their product. Thus, depending on the manufacturing process, the packaging and the time interval between the production and the seizure, the sample will be more or less « wet » when arriving at the forensic laboratory for analysis. Some laboratories choose to not dry the sample, some others will apply different methods to dry the sample. Thus, the aim is to evaluate the influence of these processes on the organic profile.
Regarding storage, little information is presently available in relation to the « ageing » of amphetamine powder. Therefore, stability and storage studies are planned to investigate this matter.
Finally, there is an aim to study the variability in clandestine production as well as the variation in large seizures. As mentioned in the introduction (section A.2), this is directly related to the concept of inter-batch and intra-batch variation. If sufficient samples can be obtained from such sources, this would provide invaluable information and be of great help in further interpretation of links between samples.

Despite these remaining tasks, the development of a harmonised method for the profiling of amphetamine was a complete and successful project. The best illustration of this success could be the fact that already three laboratories are now applying this method to their amphetamine samples : the National Bureau of Investigation, Finland, the Netherlands Forensic Institute and the Forensic Science Service, England and Wales. Moreover, Sweden (SKL) have decided to switch to this method as from 2006 (after the end of the CASE project) in spite of their existing method that has been in routine use for the last 25 – 30 years.

Still, one issue that has yet to be addressed is the relationship and cooperation with law enforcement agencies whether these are local, national or international. Indeed, there is very little use in keeping profiling results within one or more forensic laboratories. The information is ultimately destined for the law enforcement agencies in order to help them in their respective investigations (intelligence purpose). For this matter, a close and structured collaboration is necessary between the various protagonists in order to ensure the usefulness of profiling and also to guarantee a mutual understanding of the purpose. This is probably the biggest challenge to date.

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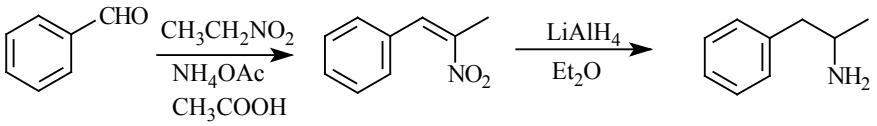
ANNEX 1 SYNTHESIS OF AMPHETAMINE AND SPECIFIC IMPURITIES

1 SYNTHESIS OF AMPHETAMINE

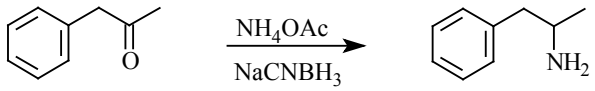
1.1 Synthesis of amphetamine from nitrostyrene with reduction by LiAlH4



Ammonium acetate (14.46 g, 0.188 mol), benzaldehyde (17.93 g, 0.169 mol), nitroethane (35 mL, 0.443 mol) and glacial acetic acid (140 mL) were mixed and refluxed at 110°C for 2 hours. The reaction mixture was subsequently poured onto crushed ice. The resultant nitrostyrene was removed by filtration and re-dissolved in warm ethanol and recrystallised. Nitrostyrene was obtained in 46% yield (12.8 g, 0.079 mol).

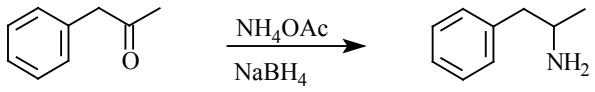
$LiAlH_4$ (0.24 g, 0.006 mol) was suspended in sodium dry diethyl ether and cooled to –78°C in a dry ice / acetone bath. Nitrostyrene (0.377g, 0.002 mol) in 10 mL of dry diethyl ether was added to the $LiAlH_4$ and the temperature of the reaction mixture slowly allowed to rise to ambient. After the reaction appeared complete (30 minutes) 35 mL 20% aqueous sodium tartrate was added whilst the mixture was stirred, to quench the reaction. A biphasic system formed. The ether layer containing the amphetamine free base was removed and the free base analysed by GC – MSD. The final yield was 0.078 g amphetamine (0.0006 mol, 29%).

1.2 Synthesis of amphetamine by reductive amination using sodium cyanoborohydride



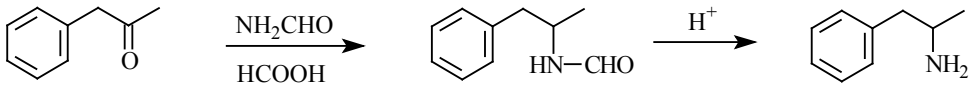
Benzyl methyl ketone (5.0 g, 0.037 mol) and ammonium acetate (28.2 g, 0.37 mol) were stirred in MeOH at room temperature for 3 hours. $NaCNBH_3$ (2.4 g, 0.037 mol) was added and stirring was continued another 4 hours. The reaction was quenched by evaporation of the solvent. The residue was dissolved in water/acetone and acidified with concentrated HCl. The mixture was washed with dichloromethane. The water phase was alkalised with NaOH and extracted with dichloromethane. The organic solvent was evaporated and amphetamine (2.5 g, 0.019 mol, 50%) was obtained as an oil. The oil was crystallised to amphetamine sulphate by addition of 37% sulphuric acid and MeOH.

1.3 Synthesis of amphetamine by reductive amination using sodium borohydride



The mixture of benzyl methyl ketone (2.02 g, 0.015 mol) and ammonium acetate (12.51 g, 0.162 mol) in 100 mL of 2-propanol was stirred at room temperature for 3 hours. $NaBH_4$ (0.07 g, 0.0018 mol) was added and stirred for another 3 hours. The reaction was quenched by addition of water and concentrated HCl. The reaction mixture was stirred overnight. The organic solvent was evaporated, and the residue acidic water layer was washed with dichloromethane. The water phase was basified with NaOH pellets and extracted with dichloromethane. The solvent was evaporated under reduced pressure yielding amphetamine oil (0.64 g, 0.0047 mol, 32%). The oily product was crystallised to amphetamine sulphate by addition of 37% sulphuric acid and MeOH.

1.4 Synthesis of amphetamine by the Leuckart route



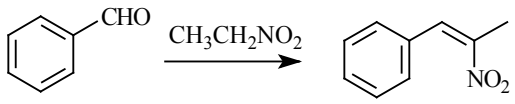
Benzyl methyl ketone (360 g, 2,6829 mol), formamide (309 g, 6,7130 mol) and formic acid (304 g, 6,749 mol) were refluxed for 4 hours. The mixture was allowed to cool to room temperature, and washed once with distilled water (800 mL). NaCl was added to facilitate the phase separation.

32% HCl (520 mL) was added to the organic layer and the mixture was refluxed for 1,5 hours. The mixture was allowed to cool and washed once with hexane (800 mL). The solution was basified with NaOH, and steam distilled under continuous addition of distilled water until all amphetamine oil had been removed from the reaction vessel. The distilled amphetamine oil was dissolved in 99.5% ethanol (1:4; w/v). 33% Sulphuric acid (diluted with 99.5% ethanol) was added to the solution of amphetamine under continuous stirring. At pH ~ 6.5, the addition was stopped and the precipitated crystals were filtered off and dried at room temperature. The yield of amphetamine sulphate was 82 g (0.494 mol, 18%).

2 SYNTHESIS OF IMPURITIES FOUND IN AMPHETAMINE

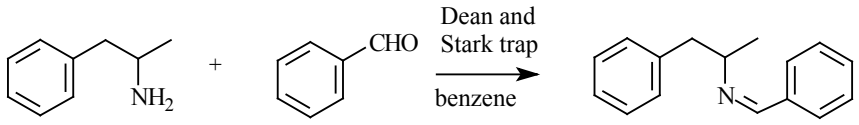
A number of impurities commonly found in amphetamine manufactured in clandestine laboratories have been synthesised. Exemplar syntheses are described below.

2.1 Synthesis of nitrostyrene



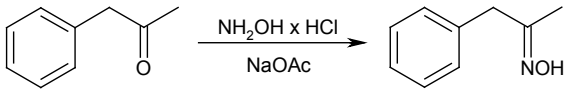
Nitrostyrene was synthesised as described above (1.1).

2.2 Synthesis of N-(β-phenylisopropyl)benzaldimine



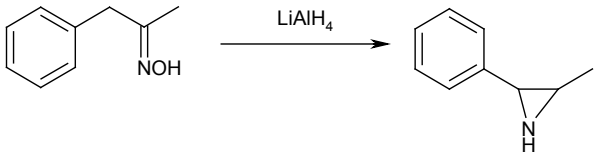
Amphetamine base (2.0 g, 0.015 mol), benzaldehyde (1.0 g, 0.008 mol) and 200 mL of benzene were placed in a flask. The mixture was refluxed at 82°C overnight. A Dean and Stark trap was used to remove the resulting water. The reaction was finished and benzene evaporated. N-(β-phenylisopropyl)benzaldimine was purified by vacuum distillation. The product contained 95% of aldimine and 5% amphetamine.

2.3 Synthesis of benzyl methyl ketoxime



Hydroxylamine hydrochloride (11.98 g, 0.176 mol), benzyl methyl ketone (12.0g, 0.090 mol) and sodium acetate (1.0 g, 0.012 mol) were suspended in distilled water (80 mL) and ethanol (50 mL) added to dissolve all the reagents. The reaction mixture was refluxed at 79°C for 3 hours, at which point the reaction appeared to be complete as monitored by GC – MSD. The reaction mixture was cooled, and the solvent reduced to 1/3 volume *in vacuo*. The sodium acetate was removed by filtration. The mixture was transferred to a separating funnel and diethyl ether (100 mL) added. The ether fraction was collected and the ether removed by drying under nitrogen, yielding finally a pink oil.

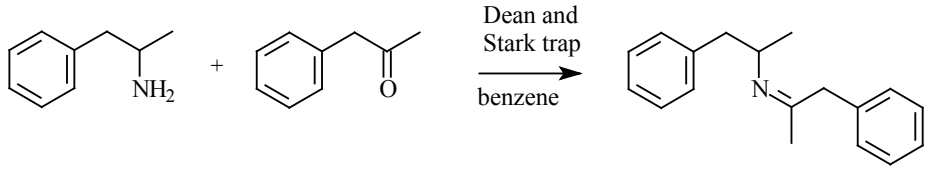
2.4 Synthesis of 2-methyl-3-phenyl aziridine



Benzyl methyl ketoxime (0.74 g, 0.005 mol) was dissolved in distilled THF (20 mL), the vessel evacuated and the solution stored under argon. $LiAlH_4$ (0.5 g, 0.013 mol) was placed in a Schlenk tube which had previously been evacuated and filled with argon. $LiAlH_4$ was suspended in THF and cooled to -78°C. The ketoxime solution was slowly added to the suspension slowly via a syringe while argon was bubbled through the suspension. The reaction mixture was allowed to warm to room temperature with stirring. The reaction mixture was subsequently refluxed for 4 hours with stirring, cooled to room temperature and quenched using 5% NaOH until no more gas evolved and a white precipitate formed.

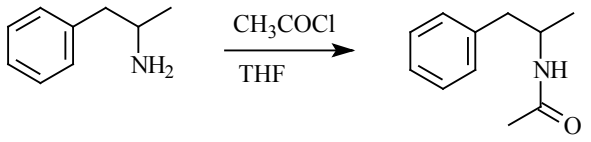
The organic phase was extracted with sodium dried diethyl ether (3 × 20 mL). The ether was removed *in vacuo* leaving an oil, which was removed and dissolved in ethanol for GC-MSD analysis. The aziridine isomers were separated by column chromatography using a 20 cm x 1 cm i.d. column packed with silica gel (230 – 400 mesh), eluting with hexane : ethyl acetate (3:2 v:v). The column fractions were analysed by GC-MSD and those containing aziridine combined and the solvent removed, yielding finally 2-methyl-3-phenyl aziridine (0.40 g, 0.003 mol, 60%).

2.5 Synthesis of N-(β-phenyl-isopropyl)benzyl methyl ketiimine



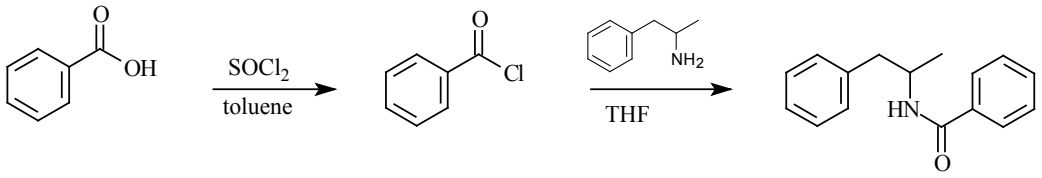
Amphetamine (10 g, 0.075 mol), benzyl methyl ketone (5 g, 0.037 mol) and 200 mL of benzene were placed into a flask and the mixture was refluxed overnight. A Dean and Stark trap was used to remove the resulting water. The organic solvent was distilled under reduced pressure and the obtaining product was analysed by GC-MS. The purity of ketimine was 75%, and no other purification steps were taken since ketimine/amphetamine mixture was too unstable to be purified.

2.6 Synthesis of N-acetylamphetamine



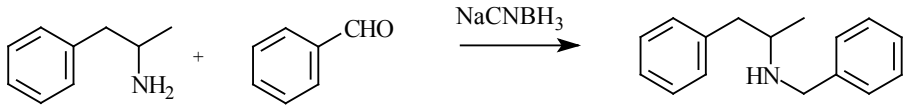
Acetylchloride (3.0 g, 0.037 mol) was dissolved in THF (300 mL) and amphetamine base (5.0 g, 0.037 mol) added. The reaction mixture was stirred at room temperature overnight. The solvent was evaporated under reduced pressure. The resulting oily residue was dissolved in water and acidified with HCl. The water phase was extracted with dichloromethane and the organic solvent was evaporated. The yield of N-acetylamphetamine, which was 95% pure, was 3.0 g (0.017 mol, 46%). The product was recrystallised from petroleum benzene and ethanol. The yield of pure product was 1.5 g (23%).

2.7 Synthesis of benzoylamphetamine



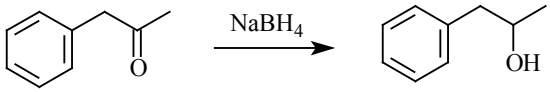
Benzoic acid (5.1 g, 0.042 mol) and thionyl chloride (9.2 mL, 0.126 mol) were refluxed in toluene for 3.5 h. The toluene was evaporated and the residue washed with benzene. The resulting benzoyl chloride (5.4 g, 0.039 mol, 92%) was dissolved in THF, amphetamine (5.8 g, 0.043 mol) added and the mixture stirred at room temperature overnight. The solvent was removed under reduced pressure and the solid residue dissolved in water and HCl added. The mixture was extracted with dichloromethane and organic phase washed with $NaHCO_3$ solution. The solvent was evaporated, yielding a solid product (6.1 g, 0.025 mol, 66%). The product was recrystallised from petroleum benzene and ethanol, which gave benzoyl amphetamine in 40% yield (3.7 g, 0.015 mol).

2.8 Synthesis of benzylamphetamine



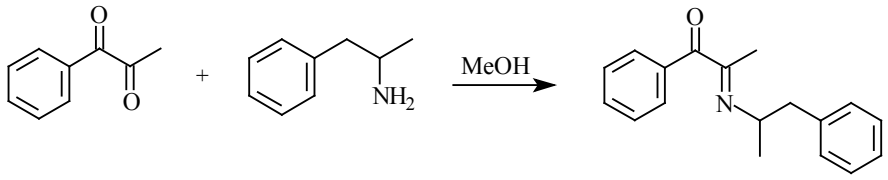
Amphetamine (1 g, 0.0075 mol), benzaldehyde (0.8 g, 0.0075 mol), sodium cyanoborohydride (0.54 g, 0.0087 mol) and MeOH (50 mL) were placed in a flask. The mixture was stirred at room temperature overnight. The pH of the reaction mixture was adjusted to pH 7.0 by adding HCl as necessary. The reaction was quenched by evaporation of the solvent. The residue was dissolved in water and acidified with concentrated HCl and stirred overnight. The mixture was washed with CH_2Cl_2. The water phase was basified with NaOH pellets, extracted with CH_2Cl_2, the organic solvent evaporated and the residue (0.2 g, 0.0008 mol, 12%) analysed by GC - MS. The resulting reaction mixture contained 92.4% benzylamphetamine and 7.4% N-(β-phenylisopropyl)-benzaldimine.

2.9 Synthesis of phenyl-2-propanol



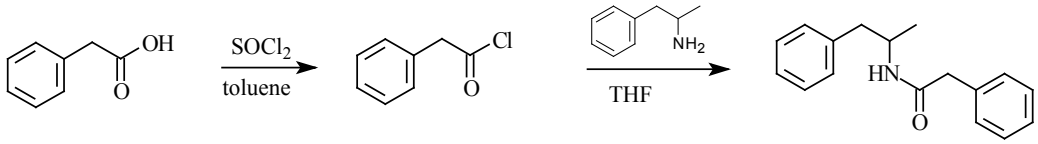
A mixture of benzyl methyl ketone (1.01 g, 0.0075 mol) and sodium borohydride (0.33 g, 0.0087 mol) in 30 mL of propan-2-ol was stirred at room temperature overnight. The reaction was quenched by adding water and concentrated HCl followed by stirring overnight. The solvent was evaporated and the mixture extracted with CH_2Cl_2. The residue was analysed by GC - MS. The resulting reaction mixture contained 99.4% phenyl-2-propanol and 0.6% benzyl alcohol.

2.10 Synthesis of 1-oxo-1-phenyl-2-(β-phenylisopropylimino)propane



Phenyl-1,2-propanedione (1.2 g, 0.0082 mol) and amphetamine (1.2 g, 0.0088 mol) were stirred in methanol (200 mL) at room temperature overnight. The solvent was evaporated, yielding 1-oxo-1-phenyl-2-(β-phenylisopropylimino)propane (1.4 g, 0.0052 mol, 64%). The purity of the product was only 86%, since any subsequent attempt to purify the product was unsuccessful.

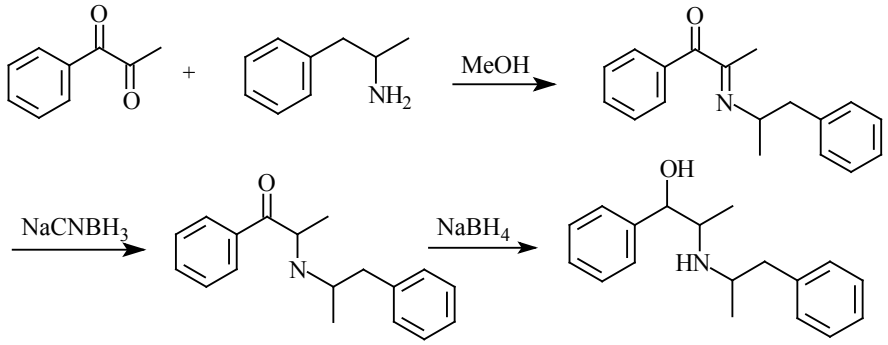
2.11 Synthesis of 2-oxo-1-phenyl-(β-phenylisopropylamine)ethane



Phenylacetic acid (4.4 g, 0.032 mol) was dissolved in toluene and thionylchloride (7 mL, 0.097 mol) added. The mixture was refluxed for 6 hours. The solvent was evaporated under reduced pressure. Benzene was added and evaporated twice to remove the resulting water from the reaction mixture. The reaction mixture was dissolved in THF and amphetamine (4.4 g, 0.033 mol) added. The mixture was stirred overnight at room temperature, and the THF removed *in vacuo*. The oily product was dissolved in water and acidified with HCl. The water phase was extracted with dichloromethane and the regenerated organic phase washed with $NaHCO_3$ to remove phenylacetic acid and other acidic compounds. The dichloromethane phase was removed and solvent evaporated. The yield of solid product was 4.8 g (0.019 mol, 65%).

To purify the product, the 2-oxo-1-phenyl-(β-phenylisopropylamine)propane was recristallised from petroleum benzene and ethanol. The yield of pure product was 2.0 g (0.008 mol, 27%).

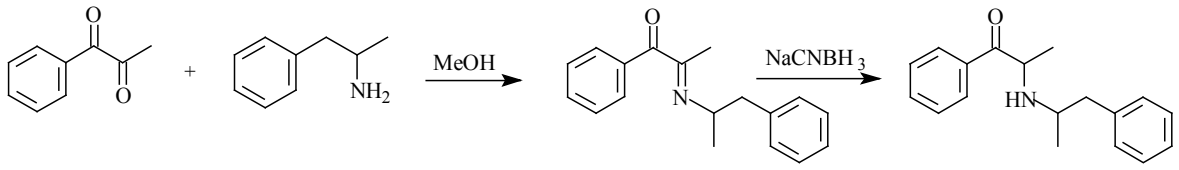
2.12 Synthesis of N,β-hydroxy-N,N-di(β-phenylisopropyl)amine



Amphetamine (1.2g) and phenyl-1,2-propanedione (1.1g) in MeOH (200 mL) were stirred at room temperature overnight. An intermediate product, 1-oxo-1-phenyl-2-(β-phenylisopropylimino)-propane, was reduced with sodium cyanoborohydride (0.7g) to N-(β-phenylisopropyl)cathinone. The carbonyl group of cathinone was reduced to the corresponding alcohol by adding sodium borohydride (0.5 g) and stirring for 2 days. The MeOH was evaporated and the reaction quenched by addition of H_2O and triethylamine (2mL). The reaction mixture was extracted with dichloromethane, the solvent evaporated and the oily product (1.0g) was purified by washing with saturated $NaHCO_3$ solution.

The product which contained two diastereoisomers of N,β-hydroxy-N,N-di(β-phenylisopropyl)-amine was dissolved in ether and white crystals formed. The insoluble crystals were filtered and dried. Recristallisation gave one pure diastereoisomer (0.15g).

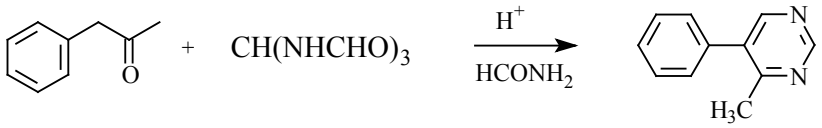
2.13 Synthesis of N-(β-phenylisopropyl)cathinone



Phenyl-1,2-propandione (0.56 g, 0.0038 mol) and amphetamine (0.52 g, 0.0038 mol) was stirred at room temperature for 12 hours. The intermediate product, 1-oxo-1-phenyl-2-(β-phenylisopropyl-imino)propane was reduced to N-(β-phenylisopropyl)cathinone by addition of sodium cyanoborohydride (0.5 g, 0.008 mol). The reaction mixture was stirred for an hour. MeOH was evaporated and the reaction quenched by adding water and HCl. The reaction mixture was stirred for 2 days. The acidic mixture was washed with dichloromethane. The water phase was basified with NaOH and extracted with dichloromethane. Acidic dichloromethane was evaporated and the cathinone product was anlalysed by GC-MS (78% pure).

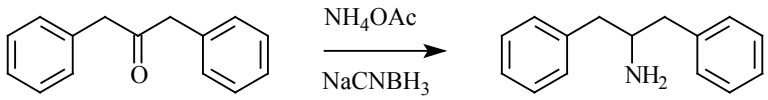
The product was recrystallised from diethyl ether and dichloromethane, which gave pure cathinone 0.3 g (0.0011 mol, 29%).

2.14 Synthesis of 4-methyl-5-phenylpyrimidine



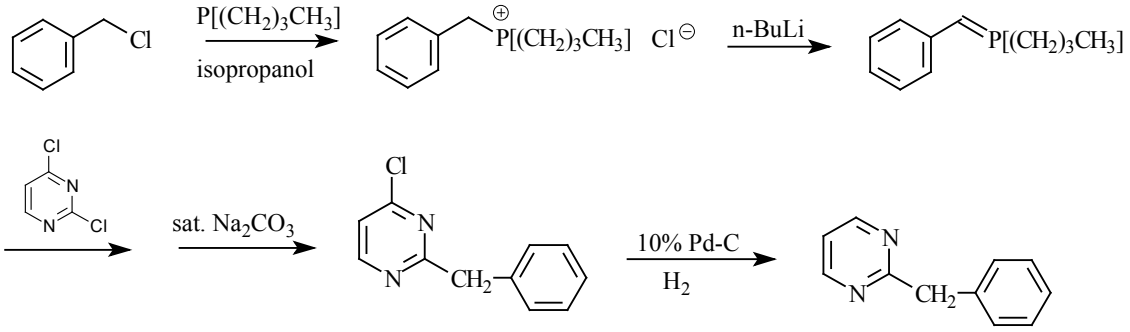
A mixture of benzyl methyl ketone (8.9 g, 0.066 mol), trisformylaminomethane (20 g, 0.138 mol), formamide (14.7 g, 0.326 mol) and p-toluenesulfonic acid (0.7 g, 0.004 mol) was refluxed for 8 hours. The reaction mixture was basified with 2 M NaOH and extracted once with 100 mL toluene:ether (1:1), followed by washing four times with 100 mL distilled water. The organic solvent was dried with $MgSO_4$, filtered and evaporated to dryness to yield a crystalline mass, which was recrystallised from hexane/diethyl ether (2:1 v/v). The crystals (1.5 g) were 90% pure with respect to the target product. These were recrystallised with a subsequent syntheses from hexane / diethylether (1:1) to yield 6.0 g (0.035 mol, 53%) pure product.

2.15 Synthesis of 1,3-diphenyl-2-propylamine



Diphenylacetone (10 g, 0.048 mol), ammonium acetate (30 g, 0.390 mol) and sodium cyanoborohydride (3 g, 0.048 mol) were added to methanol (200 mL) in a 500 mL round bottomed flask. The solution was stirred at ambient temperature for 48 hours. The mixture was basified with sodium hydroxide and refluxed for 2 hrs. The methanol was removed under reduced pressure, distilled water (100 mL) added to the residue and the water phase extract three times with 100 mL chloroform. The chloroform extract was dried over anhydrous magnesium sulphate, filtered and removed under reduced pressure yielding a yellow oil from which the product was crystallised after dissolving in diethylether and bubbling HCl gas through the solution, yielding finally 6.0 g (0.028 mol, 58%) of product.

2.16 Synthesis of 4-benzylpyrimidine



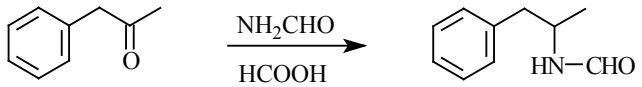
Benzylchloride (18.4 g, 0.145 mol) and tri-butylphosphine (20 g, 0.227 mol) were added to 2-butanone (200 mL) in a three-necked 500-mL round bottomed flask. The reaction mixture was refluxed for five hours under a nitrogen atmosphere. The mixture was cooled overnight and the resulting product removed by vacuum filtration. Recristallisation was performed from toluene and the product was dried for 10 days in a dessicator at 60 °C, yielding finally 15.0 g of tri-n-butylbenzylphosphonium chloride (0.070 mol, 48%).

Tri-n-butylbenzylphosphonium chloride (15 g, 0.070 mol) was added to dry dimethoxyethane (40 mL) in a three-necked 250-mL round bottomed flask. The system was kept inert, under nitrogen. Butyllithium in hexane (29 mL, 1.6 M) was added dropwise to the mixture at an initial reaction temperature that was below –35 ºC. Thereafter, the temperature was allowed to rise slowly for two hours. Dichloropyrimidine (3 g, 0.020 mol) and dry dimethoxyethane (20 mL) was added to the reaction mixture and the temperature was kept below -35 ^{o}C. The reaction was allowed to proceed for 17 hours under nitrogen atmosphere. The temperature of the reaction vessel was gradually increased to ambient temperature.

Saturated aqueous Na_2CO_3 (50 mL) was added into the reaction mixture and refluxed for five hours. It was extracted four times with 100 mL diethylether and the extract was dried, filtered and the solvent removed under reduced pressure. The residue was dissolved in dichloromethane : ethyl acetate (8:1 v/v) and was purified twice on a silica gel (230-400 mesh) using dichloromethane: ethyl acetate (8:1 v/v) as the mobile phase. The solvent fractions containing 4-benzyl-6-chloropyrimidine were combined and the solvent was removed under reduced pressure. The mass of the desired product was 1.64 g (0.008 mol, 40%).

The 4-benzyl-6-chloropyrimidine (1.64 g, 0.008 mol) and magnesium oxide (0.95 g) were added to 95% ethanol (20 mL). This suspension was added to a mixture of distilled water (20 mL), ethanol (20 mL) and 10% Pd-C (0.5 g). The mixture was hydrogenated for 1.5 hrs. The Pd-C was removed by filtration and washed with dichloromethane (30 mL), which was then added to the ethanol/water solution. The organic solvents were removed under reduced pressure and the aqueous residue was extracted with 50 mL dichloromethane. The dichloromethane was dried with $MgSO_4$, filtered and removed under reduced pressure yielding 0.65 g pure 4-benzylpyrimidine (0.0038 mol, 48%).

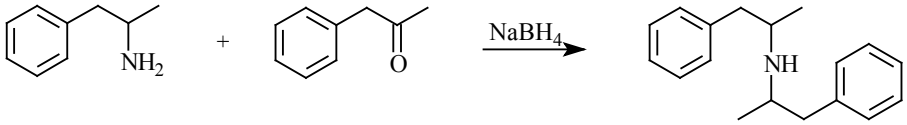
2.17 Synthesis of N-formylamphetamine



Benzyl methyl ketone (50 g, 0.373 mol) was refluxed with formamide (100 mL) and formic acid (50 mL) for 6 hours at 175-180°C. The reaction was monitored by GC-MSD at regular intervals to determine the end of the reaction. After cooling, the reaction mixture was diluted with 400 mL deionized water and extracted with dichloromethane (3 x 100 mL). The organic phase was dried over sodium sulphate and the organic solvent removed under vacuum.

The product was purified by column chromatography using silica gel 60 (70-230 Mesh). The oily product was first eluted with petroleum ether / ethyl acetate (6 : 4) to remove impurities and then with ethyl acetate to obtain the N-formylamphetamine. The extracts containing the N-formylamphetamine were combined, dried over sodium sulphate and evaporated under nitrogen yielding 32.5 g of N-formylamphetamine (0.199 mol, 53%) as an orange oil.

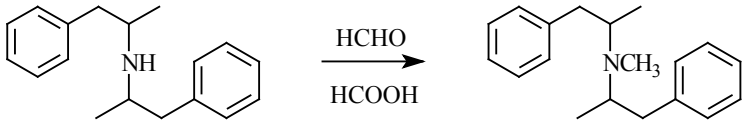
2.18 Synthesis of N,N-di(β-phenylisopropyl)amine



Amphetamine oil (2.8 g, 0.021 mol) was added to a solution of benzyl methyl ketone (1.4 g, 0.010 mol) in methanol (10 mL). The reaction mixture was left at room temperature for 3 hours. Sodium borohydride (0.20 g, 0.005 mol) were added and the solution stirred at room temperature for 30 minutes. Deionized water (200 mL) was added and the aqueous solution extracted with dichloromethane (3 x 50 mL). The combined extracts were evaporated under vacuo and 8 mL of deionized water were added to the remaining oil.

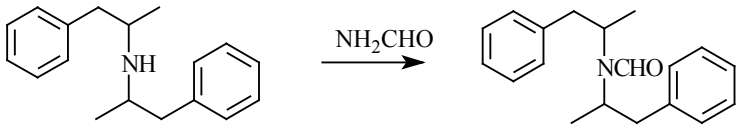
Concentrated hydrochloric acid (0.8 mL) was added dropwise and the resulting precipitate filtered and washed with diethyl ether. The precipitate was recrystallised in isopropanol yielding 1 g of N,N-di(β-phenylisopropyl)amine hydrochloride (0.0034 mol, 34%) as white crystals.

2.19 Synthesis of N,N-di(β-phenylisopropyl)methylamine



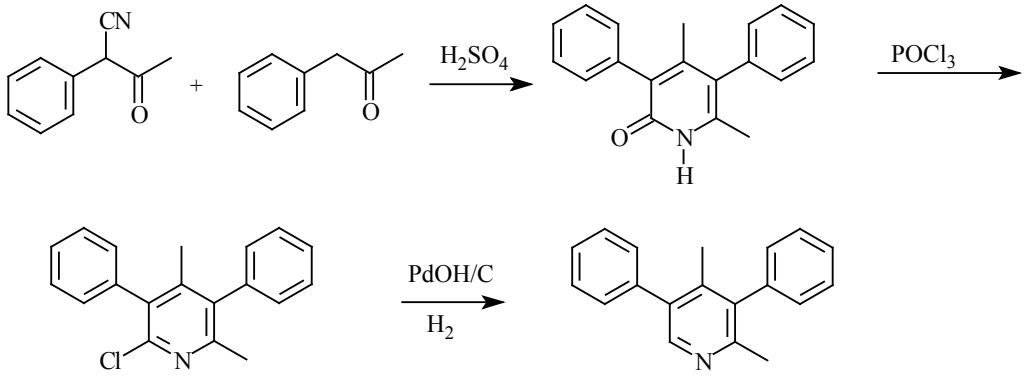
Formic acid (5 mL) and formaldehyde (8 mL) were added to N,N-di(β-phenylisopropyl)amine base (2 g, 0.0079 mol). The reaction mixture was kept overnight at 80°C on an oil bath. After cooling, the mixture was diluted with 150 mL of deionized water and extracted with dichloromethane (3 x 50 mL). The organic solution was dried over sodium sulphate and evaporated in vacuo. N,N-di(β-phenylisopropyl)methylamine (2.0 g, 0.0075 mol, 95%) as a transparent yellow-green oil was obtained.

2.20 Synthesis of N,N-di(β-phenylisopropyl)formamide



N,N-di(β-phenylisopropyl)amine base (2 g, 0.0079 mol) was refluxed 30 minutes with formamide (50 mL) at 210°C. The reaction mixture was diluted with 250 mL of deionized water and extracted with dichloromethane (3 x 100 mL). The dichloromethane was dried over sodium sulphate and evaporated in vacuo yielding an orange-brown oil.The oil was purified by column chromatography using Silica Gel 60 (70-230 Mesh). The oil was eluted with dichloromethane / methanol (9 : 1). The extracts containing the N,N-di(β-phenylisopropyl)formamide were combined, dried over sodium sulphate and evaporated under nitrogen yielding 1.3 g of product (0.0046 mol, 59%) as a yellow-orange oil.

2.21 2,4-dimethyl-3,5-diphenylpyridine



α-Acetylbenzylcyanide (6.36 g, 0.040 mol) was mixed with benzyl methyl ketone (10.72 g, 0.080 mol) and 4 mL of concentrated sulphuric acid. The mixture was slowly heated to 80°C with stirring. After two hours the temperature was raised to 100°C and after another two hours to 120°C. This temperature was maintained for one hour. The reaction mixture was poured into water and neutralized with saturated aqueous sodium carbonate solution. After leaving the solution stand overnight, the solid was filtered off, washed with water and dried.

The obtained 3,5-diphenyl-4,6-dimethyl-2-pyridone (5.5 g, 0.020 mol, 50%) and 20 mL of phosphorous oxychloride ($POCl_3$) were stirred under reflux for 72 hours. After cooling, the mixture was poured into sodium carbonate solution. The mixture was then extracted with 3 x 50 mL of dichloromethane. The combined organic extracts were evaporated yielding brownish crystals. Because the chlorination process was not complete, these were purified by column chromatography using Silica Gel 60 (70-230 Mesh). Elution was performed with petroleum ether / ethyl acetate (80 : 20). The obtained extract was evaporated yielding 1.4 g of light orange crystals of 2,4-dimethyl-3,5-diphenyl-6-chloropyridine (1.4 g, 0.0048 mol, 28%).

2,4-dimethyl-3,5-diphenyl-6-chloropyridine (1.4 g, 0.0048 mol) was dissolved in 180 mL of ethanol – ethyl acetate (1 :1). 1 g of palladium hydroxide on charcoal was added to the solution. This was hydrogenated for two days at atmospheric pressure and room temperature. The catalyst was removed by filtration and the solvent evaporated leaving yellowish-white crystals of 2,4-dimethyl-3,5-diphenylpyridine (0.560 g, 0.0022 mol, 45%)

ANNEX 2 GC METHODS USED IN DIFFERENT TASKS

	TASK 1	**TASK 2**	**TASK 3: Subtask 3.1**	**TASK 3: Subtask 3.2**
Instrument:	HP 6890 gas chromatograph, HP 5973 mass selective detector with HP MS Chemstation rev. B.01.00			
Column:	Methyl silicone column (HP Ultra-1) *)	5% Phenyl methyl silicone column 25 m (L) x 0.20 mm (i.d.), d_f 0.33 µm (HP Ultra-2) *)		HP Ultra-2, HP Ultra-1, HP-50+, HP-1701, HP-25, DB-35MS and DB-17MS *)
Pre-column:	Deactivated retention gap 2 m (L) x 0.32 mm (i.d.) (HP part no. 19091-60600)			
Column connector	Deactivated press-fit connector (HP part no. 5181-3396)	Deactivated press-fit connector (HP part no. 5181-3396) was used to connect the retention gap pre-column onto the analytical column Y-shaped press-fit connector (HP part no. 5181-3398) was used to split the sample between two columns, one for each detector		
Carrier gas:	Helium, 25 cm/s at 60 °C at FID, constant flow			
Sample introduction: Volume: Total flow: Liner*:* **or/and** Volume: Total flow: Liner*:*	Split, 1:148 1 µl 60 mL/min Glass wool packed liner with a volume of 990 µl (19251-60540) **or** Splitless 1 µl 30 mL/min, 1 min splitless time Splitless liner with a volume of 250 µl (HP part no. 18740-80220)	Splitless 1 µl 30 mL/min, 1 min splitless time Splitless liner with a volume of 250 µl. (HP part no. 18740-80220)	Split, 1:20 1 µl 60 mL/min Glass wool packed liner with a volume of 990 µl (19251-60540) **and** Splitless 1 µl 60 mL/min, 1 min splitless time Silanised splitless liner with a volume of 250 µL (HP part no. 18740-80220) **and** Cool on-column (oven track mode)	Split, 1:20 1 µl 60 mL/min Glass wool packed liner with a volume of 990 µl (19251-60540)
Gas saver:	20ml/min after 1.5 min			
Temperatures: Injector: GC-MS interface: FID:	 260°C 305°C 305°C		 220, 240, 260 and 280°C 305°C 305°C	 260°C 305°C 305°C
Oven T-program:	60°C (1 min), 10°C/min, 300°C (10 min)			2, 4, 6, 8, 10 and 12°C/min **)
MS information: Solvent delay: Mass range: Sample rate #: MS quad temp: MS source:	 3.5 min – 6 min depending upon the solvent 30 - 550 a.m.u. 2, A/D samples 4 150 °C 230 °C			

FID sample rate:		20 Hz

	TASK 3: Subtask 3.3	TASK 4	TASK 5	TASK 6
Instrument:	HP 6890 gas chromatograph, HP 5973 mass selective detector with HP MS Chemstation rev. B.01.00			
Column:	35% phenyl methyl silicone column 30 m (L) x 0.25 mm (i.d.), d_f 0.25 µm (DB-35MS, HP part no. 122-3832)	50% diphenyl dimethyl silicone column 25 m (L) x 0.20 mm (i.d.), d_f 0.31 µm (HP-50+, HP part no. 19091L-105)	35% phenyl methyl silicone column 30 m (L) x 0.25 mm (i.d.), d_f 0.25 µm (DB-35MS, HP part no. 122-3832)	
Pre-column:	Deactivated retention gap 2 m (L) x 0.25 mm (i.d.) (HP part no. 160-2255-5)	Deactivated retention gap 2 m (L) x 0.25 mm (i.d.) (HP part no. 19091-60610)	Deactivated retention gap 2 m (L) x 0.25 mm (i.d.) (HP part no. 160-2255-5)	
Column connector:	Non-deactivated press-fit connector (HP part no. 5181-3395) A) Y-shaped press-fit connector (HP part no. 5181-3398) **or** B) divider (HP part no. 5021-7148) **or** C) two-hole ferrule (HP part no. 5062-3580) ***)			
Carrier gas:	Helium, 25 cm/s at 90 °C at FID, constant flow			
Sample introduction: Volume: Total flow: Liner*:*	Splitless 1 µl 60 mL/min, 1 min splitless time Single-tapered (glass-wool packed, deactivated) splitless liner (HP part no. 5062-3587)	Split, 1:20 **or** Splitless 2 µl 1 µL 30 mL/min 60 mL/min Glass wool packed liner with a volume of 990 µl Splitless liner with a volume of 250 µl	Splitless 1 µl, 60 mL/min, 1 min splitless time Single-tapered (deactivated) splitless liner (HP part no. 5181-3316).	Splitless 1 µl, 60 mL/min, 1 min splitless time Single-tapered glass wool packed liner (HP part no. 5062-3587).
Gas saver:	20ml/min after 1.5 min			
Detector cond's: Injector: GC-MS interface: FID: NPD:	 250°C 310°C 310°C, Hydrogen flow : 40 mL/min, Air flow : 450 mL/min, Helium was used as make-up gas at 30 mL/min. Hydrogen flow: 4 mL/min, Oxygen: 55 mL/min, Nitrogen as make-up gas: 10 mL/min NPD bead: TID-4 from Detector Engineering & Technology NPD jet:Agilent part no. G1534-80580 (Subtask 3.3), Agilent part no. 18789-80070 (Task 5) Bead voltage: 2.8 volts Adjust offset: Not used			
Oven T-program:	90°C (1 min), 8°C/min, 300°C (10 min)			
MS information: Solvent delay: Mass range: Sample rate #: MS quad temp: MS source:	 4 min 4 –300 a.m.u. (0-30 min), 30 to 500 amu. 3, A/D samples 8 150 °C 230 °C ****)	 3.5 min – 6 min depending upon the solvent 30 - 550 a.m.u. 2, A/D samples 4 150 °C 230 °C	 4 min 4 -300 a.m.u. (0-30 min), 30 to 500 amu. 3, A/D samples 8 150 °C 230 °C	
FID sample rate:	20 Hz			
RTLocking:	The retention time of nonadecane was set to 16.30 minutes (FID)			

*) 5% Phenyl methyl silicone column 25 m (L) x 0.20 mm (i.d.), d_f 0.33 µm
(HP Ultra-2, HP part no. 19091B-105)

Methyl silicone column 25 m (L) x 0.20 mm (i.d.), d_f 0.33 µm
(HP Ultra-1, HP part no. 19091A-105)

50% diphenyl dimethyl silicone column 25 m (L) x 0.20 mm (i.d.), d_f 0.31 µm
(HP-50+, HP part no. 19091L-105)

17% cyanopropyl phenyl methyl silicone column 25 m (L) x 0.20 mm (i.d.), d_f 0.20 µm
(HP-1701, HP part no. 19091U-102)

50% phenyl methyl silicone column 30 m (L) x 0.25 mm (i.d.), d_f 0.25 µm
(DB-17MS, HP part no. 122-4732)

35% diphenyl dimethyl silicone column 25 m (L) x 0.20 mm (i.d.), d_f 0.33 µm
(HP-35, HP part no. 19091G-105)

35% phenyl methyl silicone column 30 m (L) x 0.25 mm (i.d.), d_f 0.25 µm
(DB-35MS, HP part no. 122-3832)

**) 60°C (1 min), 2°C/min, 300°C (10 min) 60°C (1 min), 4°C/min, 300°C (10 min)
60°C (1 min), 6°C/min, 300°C (10 min) 60°C (1 min), 8°C/min, 300°C (10 min)
60°C (1 min), 10°C/min, 300°C (10 min) 60°C (1 min), 12°C/min, 300°C (10 min)

***)

A

B

C

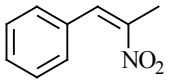
****) SIM ions used for the modified Grob mixture. Tgt, Q_1 and Q_2 are the target ion and the first and the second qualifier ions, respectively.

Group #	Compound	Time (min)	Tgt	Q1	Q2
1	Octanol	4.00	69	84	97
2	Hexanoic acid	4.30	88	101	116
3	Dodecane	4.60	57	170	85
4	2,6-dimethylphenol	5.00	122	107	121
5	Tridecane	5.70	57	184	85
6	2,6-dimethylaniline	6.50	121	106	120
7	Decanoic ester	7.20	74	186	87
8	Undecanoic ester	9.00	74	200	87
9	Dicyclohexylamine	9.40	138	181	152
10	Dodecanoic ester	10.00	74	214	87
11	Heptadecane	11.50	57	240	85
12	Octadecane	13.00	57	254	85
13	Nonadecane	14.00	57	268	85
14	Eicosane	15.30	282	127	113
15	Ketamine	18.00	57	71	85
16	Tetracosane	20.00	57	294	85
17	Trimipramine	21.50	282	127	113

ANNEX 3 SPECTROMETRIC DATA OF SYNTHESISED COMPOUNDS

The chemical structures and analytical data obtained for the synthesised compounds are given in full detail below.

(1) Nitrostyrene [(2-Nitroprop-1-enyl)benzene]



MS m/z = 115 100%, 91, 105, 163, 146, 77, 130.

IR γ_{max} (cm^{-1}) : 766, 943, 1504, 869, 709, 1216, 505, 1650, 592.

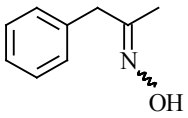
^{1}H NMR δ = 2.46 (3H, s, CH_3), 7.46-8.10 (5H, m, aromatic), 8.10 (1H, s, CH).

^{13}C NMR δ = 136.5 (CH-C), 157 (C-CH_3), 13.1 (CH_3), 126 – 129 (aromatic).

UV λ_{max} (nm) [MeOH] 220; [0.1M NaOH] 212, 220; [0.1M H_2SO_4] 196, 216, 224 (sh).

Purity 98 – 100%

(2) Benzyl methyl ketoxime [1-Phenylpropan-2-one oxime]



MS z-isomer: m/z = 91, 149, 116, 131, 117, 130, 132, 77.
e-isomer: m/z = 91, 116, 131, 130, 117, 132, 149, 77.

IR γ_{max} (cm^{-1}) : 1454, 701, 1494, 1371, 1670, 744, 1031, 1078.

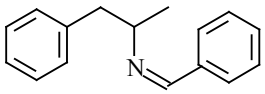
^{1}H NMR z-isomer δ = 1.85 (3H, s, CH_3), 3.77 (2H, s, CH_2), 7.29 (5H, m, aromatic).
e-isomer δ = 1.83 (3H, s, CH_3), 3.53 (2H, s, CH_2), 7.29 (5H, m, aromatic).

^{13}C NMR z-isomer δ = 13.2 (CH_3), 42.0 (CH_2), 126.7 – 136.7 (aromatic), 157.6 (C=NOH).
e-isomer δ = 19.0 (CH_3), 35.0 (CH_2), 126.7 – 1136.7 (aromatic), 157.0 (C=NOH).

UV λ_{max} (nm) [MeOH] 212, 220, 260, 284(sh); [0.1M NaOH] 208, 224, 260; [0.1M H_2SO_4] 196, 216, 224 (sh).

Purity 98 – 100%

(3) *N*-(*β*-Phenylisopropyl)benzaldimine [1-Phenyl-N-(phenylmethylidene)propan-2-amine]



MS m/z = 132 100%, 133, 105, 91, 117, 222.

IR γ_{max} (cm^{-1}) : 3027, 1645, 1122, 1581, 2928, 1381, 2845, 1308, 699, 745.

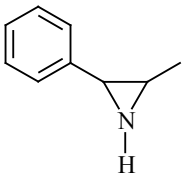
^{1}H NMR δ = 1.30 (3H, d, CH_3, J=6.6Hz), 2.90 (2H, m, CH_2), 3.54 (1H, m, CH), 7.16-7.67 (10H, m, aromatic), 8.01 (1H, s, N=CH).

^{13}C NMR δ = 22.24 (CH_3), 44.59 (CH_2), 68.20 (CH), 125.97-139.35 (aromatic), 159.30 (N=CH).

UV λ_{max} (nm) [MeOH] 247, [0.1 M NaOH] 275 and [0.1 M H_2SO_4] 247.

Purity 98 – 100%

(4) 2-Methyl-3-phenylaziridine



MS m/z = 132 100%, 118. 91, 117, 104, 77.

IR γ_{max} (cm^{-1}) : 808, 1103, 1031, 1263, 2966, 702, 736, 1496, 1452, 3322.

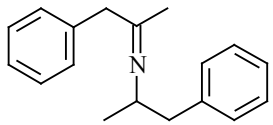
^{1}H NMR δ = 0.87 (3H, CH_3), 2.31 and 3.14 (1H, CH), 7.2 – 7.3 (5H, phenyl)

^{13}C NMR δ = 13.86 (CH_3), 31.96 (CH-CH_3), 36.93 (CH-Ph), 126.40-137.39 (aromatic).

UV λ_{max} (nm) [MeOH] 212, 260 (sh); [0.1M NaOH] 216, 224, 264 (sh); [0.1M H_2SO_4] 212, 220, 256 (sh).

Purity 98 – 100%

(5) *N*-(*β*-Phenylisopropyl)benzyl methyl ketimine [N-(1-Methyl-2-phenylethylidene)-1-phenylpropan-2-amine]



MS m/z = 160 100%, 91, 119.

IR γ_{max} (cm^{-1}) : 699, 1495, 743, 1452, 1658, 3026, 2965, 2926, 1600, 1713.

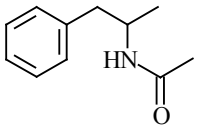
^{1}H NMR Compound is unstable and too impure for NMR data.

^{13}C NMR Compound is unstable and too impure for NMR data.

UV λ_{max} (nm) [MeOH] <220; [0.1M NaOH] 247; [0.1M H_2SO_4] 250.

Purity 86 – 100%

(6) *N*-Acetylamphetamine [N-Benzyl-1-phenylpropan-2-amine]



MS m/z = 44 100%, 86, 118, 91, 117, 134,

IR γ_{max} (cm^{-1}) : 1653, 155, 701, 747, 1372, 507, 3250, 2968, 608, 1298.

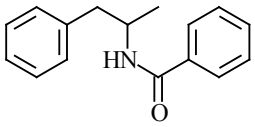
^{1}H NMR δ = 1.10 (3H, d, Me, J=6.6Hz), 1.91 (3H, s, CO-Me), 2.70-2.83 (2H, dq, CH_2, J=7.2 Hz), 4.20 (1H, m, CH), 5.66 (1H, NH, d, J=6Hz), 7.16-7.29 (5H, m, aromatic).

^{13}C NMR δ = 19.93 (CH_3), 23.93 ($\underline{C}H_3$-CO), 42.43 (CH_2), 46.13 (CH), 126.4-138.05 (aromatic), 169.38 (CO).

UV λ_{max} (nm) [MeOH] 259; [0.1M NaOH] 259; [0.1M H_2SO_4] 259.

Purity 98 – 100%

(7) Benzoylamphetamine [N-(1-Methyl-2-phenylethyl)benzamide]



MS m/z = 105 100%, 148, 77, 118, 91, 233.

IR γ_{max} (cm^{-1}) : 1630, 1539, 694, 3320, 1489, 678, 1447, 1351, 1579, 747.

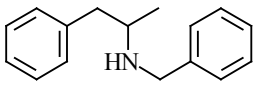
^{1}H NMR δ = 1.22 (3H, d, Me, J=6.9Hz), 2.81-2.98 (2H, dq, CH_2, J=6.9 Hz), 4.47 (1H, m, CH), 6.02 (1H, NH, d, J=7.2 Hz), 7.21-7.71 (10H, m, aromatic).

^{13}C NMR δ = 19.99 (CH_3), 42.39 (CH_2), 46.49 (CH), 126.53-137.86 (aromatic).

UV λ_{max} (nm) [MeOH] < 220; [0.1M NaOH] <220; [0.1M H_2SO_4] <220.

Purity 98 – 100%

(8) Benzylamphetamine [N-Benzyl-1-phenylpropan-2-amine]



MS m/z = 91 100%, 134, 77, 224.

IR γ_{max} (cm^{-1}) : 698, 1452, 3026, 1495, 2963, 1374, 1140, 1028, 1602, 615.

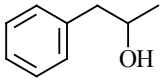
^{1}H NMR δ= 1.09 (3H, d, CH_3, J=6.0Hz), 2.65-2.67 (2H, dq, CH_2, J=6.6Hz), 2.73-2.76 (2H, dq, NH-C$\underline{H}_2$, J=6.6Hz), 2.93-2.95 (1H, m, CH), 3.70-3.87 (1H, q, NH), 7.14-7.30 (10H, m, aromatic).

^{13}C NMR δ= 20.21 (CH_3), 43.60 (CH_2), 51.29 (CH), 53.70 (NH-CH_2), 126.15-140.54 (aromatic).

UV λ_{max} (nm) [MeOH] 259; [0.1M NaOH] 259, [0.1M H_2SO_4] 257.

Purity 98 – 100%

(9) 1-Phenyl-2-propanol [1-Phenylpropan-2-ol]



MS m/z = 92 100%, 91, 136, 71.

IR γ_{max} (cm^{-1}) : 3413, 700, 1455, 742, 2968, 3027, 1078, 1495, 939, 505.

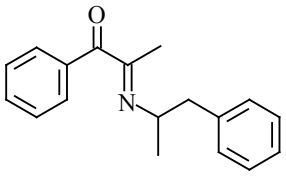
^{1}H NMR δ= 1.21 (3H, d, CH_3, J=6.6Hz), 1.80 (1H, s, OH), 2.60-2.80 (2H, m, CH_2), 4.0 (1H, m, CH), 7.20-7.40 (5H, m, aromatic).

^{13}C NMR δ= 22.68 (CH_3), 45.17 (CH_2), 68.77 (CH), 126.36-138.49 (aromatic).

UV λ_{max} (nm) [MeOH] 259; [0.1M NaOH] 259; [0.1M H_2SO_4] 259.

Purity 98 – 100%

(10) 1-Oxo-1-phenyl-2-(*β*-phenylisopropylimino)propane [2-[(1-Methyl-2-phenylethyl)imino]-1-phenylpropane-1-one]



MS M/z = 91 100%, 119, 160, 105, 77, 174, 265.

IR γ_{max} (cm^{-1}) : 700, 1670, 1448, 747, 1166, 2968, 1597, 1495, 3027, 1371.

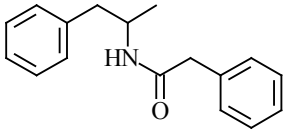
^{1}H NMR δ = 1.25 (3H, d, CH_3, J=6.3.Hz), 1.94 (3H, s, N=CCH_3), 2.92 (2H, m, CH_2), 4.15 (1H, m, CH), 7.18-7.36 (10H, m, aromatic).

^{13}C NMR δ = 14.30 (CH_3), 21.02 (N=C-$\underline{C}H_3$), 44.13 (CH_2), 58.63 (CH), 126.09-139.59 (aromatic), 164.40 (C=N), 194.19 (CO).

UV λ_{max} (nm) [MeOH] 249; [0.1M NaOH] 249; [0.1M H_2SO_4] 249.

Purity 86 – 100%

(11) 2-Oxo-1-phenyl-(β-phenylisopropylamino)ethane [N-(1-Methyl-2-phenylethyl)-2-phenylacetamide]



MS m/z = 162 100%, 91, 119, 44, 252.

IR γ_{max} (cm^{-1}) : 1638, 1539, 696, 3308, 743, 1496, 1453, 2970, 1359, 1204.

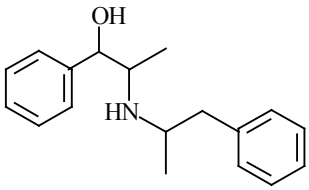
^{1}H NMR δ = 1.04 (3H, d, CH_3, J=6.6Hz), 2.66 (2H, d, CH_2, J=6.6Hz), 3.49 (2H, s, CO-CH_2), 4.25 (1H, m, CH), 5.20 (1H, d, NH, J=6.6Hz), 7.01-7.33 (10H, m, aromatic).

^{13}C NMR δ = 19.98 (CH_3), 42.17 (CH_2), 46.04 (CH), 126.39-137.61 (aromatic)

UV λ_{max} (nm) [MeOH] 259; [0.1M NaOH] 259; [0.1M H_2SO_4] 259.

Purity 98 – 100%

(12) N-β-Hydroxy-N,N-di(β-phenylisopropyl)amine [2-[(1-methyl-2-phenylethyl)amino]-1-phenylpropan-1-ol]



MS m/z = 162 100%, 91, 119, 178, 44, 105, 268.

FTIR γ_{max} (cm^{-1}): 745, 997, 1377, 1437, 1453, 1491, 1602, 1583, 3288.

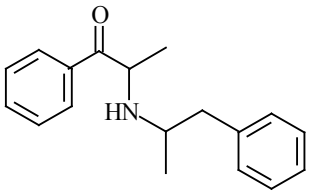
^{1}H NMR δ = 0.67 (3H, d, CH_3, J=6.5Hz), 1.10 (3H, d, CH_3, J=6.5Hz), 2.71 (2H, dd, CH_2, J=6.6Hz), 3.04 (1H, qd, NH-C<u>H</u>-COH, J=6.6Hz), 3.06 (1H, m, NH-C<u>H</u>, J=6.3Hz), 3.90 (1H, s, NH), 4.68 (1H, d, C<u>H</u>-OH, J=4.0Hz), 7.15-7.31 (10H, m, aromatic).

^{13}C NMR δ = 14.9 (CH_3), 21.3 (OHC-<u>C</u>H_3), 43.90 (CH_2), 51.30 (NH-CH), 55.4 (OHC-<u>C</u>H), 73.60 (OH-C), 126.14-141.30 (aromatic).

UV λ_{max} (nm): [MeOH] 256, [0.1 M NaOH] 260 and [0.1 M H_2SO_4] 260.

Purity 98 – 100%

(13) N-(β-Phenylisopropyl)cathinone [2-[(1-Methyl-2-phenylethyl)amino]-1-phenylpropan-1-one]

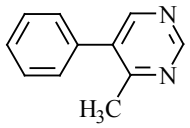


MS m/z = 162 100%, 91, 176, 119, 105, 132, 77, 44, 266.

IR, NMR and UV Compound is too unstable to be analysed by other spectrometric techniques.

Purity 98 – 100%

(14) 4-Methyl-5-phenylpyrimidine



MS m/z = 170 100%, 169, 102, 115, 116.

IR γ_{max} (cm^{-1}) : 700, 768, 570, 559, 1430, 728, 1400, 520, 1544, 1576.

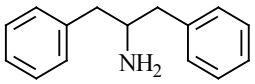
^{1}H NMR δ = 2.52 (3H, s, CH_3), 7.28-7.53 (5H, m, aromatic), 8.54 (1H, s, CH), 9.08 (1H, s, N=CH-N).

^{13}C NMR δ = 22.85 (CH_3), 128.33-128.99-134.86 (aromatic), 135.80 (C-5), 156.33 (C-2) 157.18 (C-6), 164.41 (C-4).

UV λ_{max} (nm) [MeOH] 202, 210(sh), 226(sh), 232, 259; [0.1M NaOH] 219, 233, 263(sh), 281(sh), 283, 297(sh); [0.1M H_2SO_4] 203, 209(sh), 246(sh), 253, 266(sh).

Purity 98 – 100%

(15) 1,3-Diphenyl-2-propylamine



MS m/z = 120 100%, 91, 103, 77.

IR γ_{max} (cm^{-1}) : 698.8, 737.6 750.3, 1495, 1453, 2360, 507, 820, 3026, 1081, 2342.

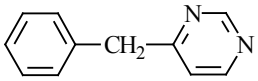
^{1}H NMR δ = 1.42 (2H, broad s, NH_2), 2.51-2.60 (2H, dd, J=13.5Hz; J=8.8Hz, CH_2), 2.81-2.88 (2H, dd, J=13.5Hz; J=4.5Hz, CH_2), 3.22-3.32 (1H, m, CH), 7.19-7.32 (10H, m, aromatic).

^{13}C NMR δ = 44.14 (CH_2); 54.14 (CH), 126.30 (C-4), 128.48-139.39 (aromatic).

UV λ_{max} (nm) [MeOH] 201 (sh), 210, 222 (sh), 253; [0.1M NaOH] 218 (sh), 223, 225 (sh), 252 (sh), 259, 270 (sh); [0.1M H_2SO_4] 200 (sh), 210, 219 (sh), 258.

Purity 98 – 100%

(16) 4-Benzylpyrimidine



MS m/z = 169 100%, 170, 115, 91, 142.

IR γ_{max} (cm^{-1}) : 1578, 697, 616, 1385, 1548, 743, 562, 483, 1472, 1495.

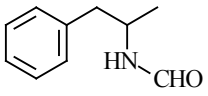
^{1}H NMR δ = 4.12 (2H, s, CH_2), 7.09 (1H, d, J=5.5Hz, H-5), 7.11-7.37 (5H, m, aromatic), 8.57-8.59 (1H, d, J=5.5 Hz, H-6), 9.14 (1H, s, H-2).

^{13}C NMR δ = 44.17 (CH_2), 120.57 (C-5), 127.00 -137.30 (aromatic), 157.00 and 158.71 (C-2 and C-6), 169.38 (C-4).

UV λ_{max} (nm) [MeOH] 203, 214 (sh), 239 (sh), 247, 250 (sh); [0.1M NaOH] 216 (sh), 218, 221 (sh), 239 (sh), 247, 250 (sh); [0.1M H_2SO_4] 203, 212 (sh), 236 (sh), 245, 254 (sh).

Purity 98 – 100%

(17) N-Formylamphetamine [1-Methyl-2-phenylethylformamide]



MS m/z = 118 100%, 72, 91, 44, 117, 119.

IR γ_{max} (cm^{-1}) : 1661, 2379, 3029, 1533, 2971, 1382, 1452, 2854, 736, 701.

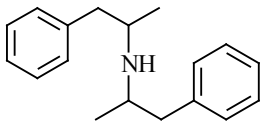
^{1}H NMR δ = 1.15 (3H, d, J = 6.4Hz, CH_3), 2.71 (2H, m, CH_2), 3.7 and 4.3 (1H, m, CH), 5.5 and 5.7 (1H, N-H), 7.1 – 7.3 (5H, m, aromatic) and 8.08 (1H, s, CHO).

^{13}C NMR δ = 20.0 (CH_3), 42.3 (CH_2), 44.6 (C-N), 126 – 129 (aromatic), 137.6, (CH), 160.4 (CHO).

UV λ_{max} (nm) [MeOH] 204, 212, 224 (sh), 252, 260; [0.1M NaOH] 216, 228 (sh), 264 (sh); [0.1M H_2SO_4] 204, 216 (sh), 252, 260.

Purity 98 – 100%

(18) N,N-di-(β-Phenylisopropyl)amine [N-(1-Methyl-2-phenylethyl)-1-phenylpropan-2-amine]



MS m/z = 162 100%, 91, 119, 44.

IR γ_{max} (cm^{-1}) : 2962, 744, 2924, 1451, 3025, 1493, 703, 1373, 1141, 1342.

^{1}H NMR δ = 0.95 – 1.05, (3H, d, J=6.1Hz, CH_3), 2.51-2.68 (2H, dd, J=6.7Hz, 6.9Hz, CH_2), 2.83 (1H, dd, J=6.1Hz, J=6.1Hz, CH), 3.04 (1H, q, CH), 7.2 (10H, m, aromatic).

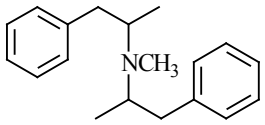
^{13}C NMR δ = 20.2 and 21.3 (CH_3), 43.4 and 44.2 (CH_2), 51.4 and 51.6 (C-NH), 126 – 139.6 (aromatic).

Note! Resolution of some of the signals due to the isomers was possible using high field NMR. The data presented below is subject to confirmation.

UV λ_{max} (nm) [MeOH] 204, 252, 260; [0.1M NaOH] 204, 252, 260; [0.1M H_2SO_4] 208, 256.

Purity 98 – 100%

(19) N,N-di-(β-Phenylisopropyl)methylamine [N-Methyl-N-(1-methyl-2-phenylethyl)-1-phenylpropan-2-amine]



MS m/z = 176 100%, 91, 58, 119, 177.

IR γ_{max} (cm^{-1}) : 2934, 3025, 2788, 1452, 698, 740, 1496, 1601, 1368, 1233.

^{1}H NMR δ = 0.95 and 0.98 (3H, d, J=3.7Hz, CH_3), 2.37 (N-C<u>H</u>$_3$), 2.43 and 2.48 (2H, d, J=4.5Hz, CH_2), 3.03 (1H, m, CH), 7.12-7.29 (aromatic).

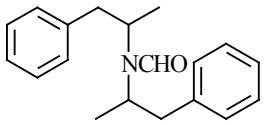
^{13}C NMR δ = 13.8 and 14.1 (CH_3), 31.9 and 32.3 (CH_2), 38.1 and 38.4 (<u>C</u>-NH), 60.4 (N-<u>C</u>H$_3$), 127 – 136.6 (aromatic).

Note! Resolution of some of the signals due to the isomers was possible using high field NMR. The data presented below is subject to confirmation.

UV λ_{max} (nm) [MeOH] 204, 216 (sh) 252, 260; [0.1M NaOH] 216, 228 (sh), 264 (sh); [0.1M H2SO4] 204, 216 (sh), 252, 260.

Purity 98 – 100%

(20) N,N-di-(β-Phenylisopropyl)formamide [bis-(1-methyl-2-phenylethyl)-formamide]



MS m/z = 190 100%, 91, 119, 162.

IR γ_{max} (cm^{-1}) : 1659, 2973, 3056, 2936, 1451, 1494, 745, 1156, 1271, 1316.

^{1}H NMR δ = 0.9 – 1.25 (3H, d, J=6.1Hz, CH_3), 2.5 – 3.1 (2H, dd, J=8.0Hz, J=8.6Hz, CH_2), 3.5 and 4.0 (1H, m, CH), 7.0 – 7.2 (aromatic), 8.1 and 8.2 (C<u>H</u>O).

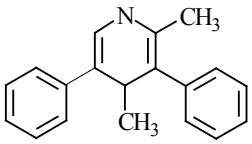
^{13}C NMR δ = 17.7 – 20.8 (CH_3), 43.0 – 43.5 (CH_2), 51.7 – 54.9 (<u>C</u>-N), 126.4 – 139.3 (aromatic), 162.5 (<u>C</u>HO).

Note! Resolution of some of the signals due to the isomers was possible using high field NMR. The data presented below is subject to confirmation.

UV λ_{max} (nm) [MeOH] 204, 252, 260; [0.1M NaOH] 212, 252, 260; [0.1mH_2SO_4] 208, 220.

Purity 98 – 100%

(21) 2,4-Dimethyl-3,5-diphenylpyridine



MS 259 100%, 258, 244, 134, 115.

IR γ_{max} (cm^{-1}) : 703, 768, 1441, 1390, 1575, 3056, 3027, 1602, 1504, 1010.

^{1}H NMR δ = 1.97 (3H, s, CH_3), 2.36 (3H, s, CH_3), 7.20 (2H, d, J ≅ 7 Hz, $2CH_{ar}$), 7.34 (2H, d, J ≅ 7 Hz, $2CH_{ar}$), 7.38-7.44 (2x1H, m, $2xCH_{ar}$), 7.46 (2H, t, J ≅ 7 Hz, $1x2CH_{ar}$), 7.49 (2H, t, J ≅ 7 Hz, $1x2CH_{ar}$), 8.37 (1H, s, $CH_{ar\text{-}pyr}$).

^{13}C NMR δ = 18.4 and 23.0 (q, $\underline{C}H_3$), 127.6 and 127.7 (t, $2x\underline{C}H_{ar}$), 128.5-129.5 (t, $2\underline{C}H_{ar}$), 136.1 (s, $\underline{C}_{ar}$), 137.6 (s, $\underline{C}_{ar}$), 137.9 (s, $\underline{C}_{ar}$), 138.5 (s, $\underline{C}_{ar}$), 144.6 (s, $\underline{C}_{ar}$), 146.5 (t, $\underline{C}H_{ar\text{-}pyr}$), 154.2 (s, $\underline{C}_{ar}$).

UV λ_{max} (nm) [MeOH] 210; [0.1M NaOH] 215; [0.1M H_2SO_4] 215.

Purity 98 – 100%

Conclusions

All together 21 compounds found as impurities in street amphetamines could be successfully synthesised. Full spectroscopic data of these compounds was recorded. This data set is unique and it did not only enable the present research project but also offers a huge potential for future developments in chemical profiling of amphetamine.

ANNEX 4 ANALYSIS OF LINEARITY

The linearity of calibration curves is studied by measuring the Pearson product-moment coefficient of correlation, r, and the coefficient of determination, R^2 [Miller, J.M. and Miller, J.N., Statistic for Analytical Chemistry, 1993]. The individual calibration points have values $y_1, y_2, \ldots, y_n$, and average value $\overline{y}$. The Pearson product-moment coefficient of correlation, r, is calculated using equation 23. The value of r varied $-1 \le r \le 1$. Values of r obtained in instrumental analysis are normally very close to 1.

$$r = \frac{SS_{xy}}{\sqrt{SS_{xx} \times SS_{yy}}} = \frac{\sum (x_i - \bar{x}) \times (y_i - \bar{y})}{\sqrt{(x_i - \bar{x})^2 \times (y_i - \bar{y})^2}} \qquad \textbf{(23)}$$

The deviation of an observation y from the regression line is ($y - \hat{y}$). As the line goes through the point $\overline{x}$ and $\overline{y}$, the regression equation can be written as follows:

$$\overline{y} = a + b\overline{x} \quad \rightarrow \quad a = \overline{y} - b\overline{x}$$

Therefore :

$$\hat{y} = a + bx = (\overline{y} - b\overline{x}) + bx = \overline{y} + b(x - \overline{x})$$
$$\rightarrow \quad \hat{y} - \overline{y} = b(x - \overline{x})$$

where the term ($\hat{y}_i - \overline{y}$) is known as the deviation, estimated y from average of y, and the sum of squares is defined $\Sigma(\hat{y}_i - \overline{y})^2$. The sum of squares $\Sigma(y_i - \hat{y}_i)^2$ is the error of observed y from estimated y. The term $\Sigma(y_i - \overline{y})^2$ is known as the 'sum of squares about $\overline{y}$' (SS_{yy}) and it can be defined by the sum of two terms referred to above. This can be proved as follows:

$$(\hat{y}_i - \overline{y}) + (y_i - \hat{y}_i) = \hat{y}_i - \overline{y} + y_i \quad \hat{y}_i = y_i - \overline{y}$$

The term of sum of square y-residuals (SSE) $\Sigma(y_i - \hat{y}_i)^2$ should be as small as possible if the curve is a good fit to the data points. The coefficient of determination (R^2) is defined using equation 30 and the value of R^2 varied $0 \le R^2 \le 1$.

$$R^2 = \frac{SS_{yy} - SSE}{SS_{yy}} = \frac{\sum (y_i - \bar{y})^2 - \sum (y_i - \hat{y})^2}{\sum (y_i - \bar{y})^2} = \frac{\sum (\hat{y}_i - \bar{y})^2}{\sum (\hat{y}_i - \bar{y})^2 + \sum (y_i - \hat{y}_i)^2}$$

In the straight-line graph, $R^2 = r^2$, where r is the product-moment correlation coefficient.

ANNEX 5 : SUMMARY OF THE LINEARITY STUDY

Summary of the linearity study. "Equation" describes the calibration curve through the slope and intercept. r^2 is the correlation coefficient. Linearity has been calculated for concentrations of 0.1, 1.0, 5.0, 10.0 and 20.0 µg/mL for Partners 2 and 3. Concentrations used by Partners 1 and 4 were 0.1, 1.0, 10, 100 and 500, and 0.1, 1.0, 10, 100 and 250, respectively. All solutions were prepared in iso-octane. Only three significant figures are available for Partner 2 data.

Partner	Compound	Equation	r^2	Range (µg/mL)
1	Benzaldehyde	Y=0.0082x + 0.0073	0.9999	0.1 - 500
1	Ketoxime (1) and (2)	Y=0.0074x - 0.0016	0.9998	0.1 - 500
1	Nitrostyrene	Y=0.0088x + 0.0043	0.9999	0.1 - 500
1	Benzaldimine	Y=0.0104x + 0.0126	0.9999	0.1 - 500
2	Phenyl-2-propanol	Y=0.0680x - 0.0255	0.9994	0.1 - 20
2	N-acetylamphetamine	Y=0.0639x - 0.0374	0.998	0.1 - 20
2	Aldimine	Y=0.07290x - 0.0144	1.00	0.1 - 20
2	Benzylamphetamine	Y=0.0719x - 0.0301	0.998	0.1 - 20
2	1-oxo	Y=0.0442x - 0.001876	0.998	0.1 - 20
2	Benzoylamphetamine	Y=0.0729x - 0.0270	0.998	0.1 - 20
2	2-oxo	Y=0.0722x - 0.0338	0.998	0.1 - 20
2	Cathinol	Y=0.0382x - 0.148	1.00	0.1 - 20
3	4-benzylpyrimidine	Y=0.0938x - 0.0139	0.9991	0.1 - 20
3	4-methyl-5-phenylpyrimidine	Y=0.0732x - 0.0141	0.9987	0.1 - 20
3	DPPA	Y=0.0777x - 0.0111	0.9980	0.1 - 20
3	DPIA	Y=0.0965x - 0.0244	0.9987	0.1 - 20
3	DPIMA	Y=0.0481x - 0.0168	0.9949	0.1 - 20
3	DPIF	Y=0.0621x - 0.0062	0.9998	0.1 - 20
4	N-formylamphetamine	Y=0.0055154x + 0.025	0.9994	0.1 - 250
4	DPIA	Y=0.0079664 + 0.0415	0.9991	0.1 - 250
4	DPIMA	Y=0.0049421x + 0.0124	0.9996	0.1 - 250
4	DPIF	Y=0.0075347x + 0.0359	0.9995	0.1 - 250
4	2,4-dimethyl-3,5-diphenylpyridine	Y=0.0089333x + 0.0427	0.9994	0.1 - 250

ANNEX 6 RESULTS OF THE STABILITY STUDY

Table A Partner 1 synthetic mixture at 25 ºC in different solvents.

Iso-octane	**T=0**	**T=4**	**T=12**	**T=24**	**T=48**	**T=96**	**RSD (%)**
Benzyl methyl ketone	100	101	102	102	100	100	0.8
2-methyl-3-phenyl aziridine	100	101	102	101	101	101	0.6
Benzyl aziridine	100	101	98	100	103	104	2.2
Benzyl methyl ketoxime (1+2)	100	102	103	102	102	105	1.5
Nitrostyrene	100	101	102	101	101	102	0.7
N-(β-phenylisopropyl)benzaldimine	100	101	102	101	101	101	0.7
Average							**1.1**
Toluene	**T=0**	**T=4**	**T=12**	**T=24**	**T=48**	**T=96**	**RSD (%)**
Benzyl methyl ketone	100	100	102	101	102	103	1.1
2-methyl-3-phenyl aziridine	100	100	102	101	102	103	1.0
Benzyl aziridine	100	100	102	102	102	103	1.1
Benzyl methyl ketoxime (1+2)	100	100	98	98	97	95	1.9
Nitrostyrene	100	100	101	101	101	100	0.7
N-(β-phenylisopropyl)benzaldimine	100	100	101	101	101	102	0.7
Average							**1.1**
Dichloromethane	**T=0**	**T=4**	**T=12**	**T=24**	**T=48**	**T=96**	**RSD (%)**
Benzyl methyl ketone	100	99	100	99	99	101	0.8
2-methyl-3-phenyl aziridine	100	99	99	99	100	100	0.6
Benzyl aziridine	100	95	96	96	94	94	2.4
Benzyl methyl ketoxime (1+2)	100	96	98	98	98	93	2.4
Nitrostyrene	100	97	99	98	98	77	9.3
N-(β-phenylisopropyl)benzaldimine	100	96	93	87	79	59	17.6
Average							**5.5**
Diethyl ether	**T=0**	**T=4**	**T=12**	**T=24**	**T=48**	**T=96**	**RSD (%)**
Benzyl methyl ketone	100	102	99	99	100	98	1.3
2-methyl-3-phenyl aziridine	100	102	98	99	101	97	1.6
Benzyl aziridine	100	102	91	100	100	87	6.4
Benzyl methyl ketoxime (1+2)	100	107	104	108	113	106	4.0
Nitrostyrene	100	104	102	103	104	101	1.6
N-(β-phenylisopropyl)benzaldimine	100	101	100	100	101	99	0.8
Average							**2.6**
Ethyl acetate	**T=0**	**T=4**	**T=12**	**T=24**	**T=48**	**T=96**	**RSD (%)**
Benzyl methyl ketone	100	99	100	101	101	102	1.0
2-methyl-3-phenyl aziridine	100	99	100	101	100	100	0.5
Benzyl methyl ketoxime (1+2)	100	102	103	103	102	102	1.2
Nitrostyrene	100	99	100	101	100	99	0.7
N-(β-phenylisopropyl)benzaldimine	100	99	100	101	100	102	0.8
Average							**0.8**
Ethanol	**T=0**	**T=4**	**T=12**	**T=24**	**T=48**	**T=96**	**RSD (%)**
Benzyl methyl ketone	100	99	100	100	100	99	0.5
2-methyl-3-phenyl aziridine	100	101	101	102	101	100	0.8
Benzyl methyl ketoxime (1+2)	100	95	93	94	95	88	4.0
Nitrostyrene	100	99	99	97	93	85	5.9
N-(β-phenylisopropyl)benzaldimine	100	98	98	97	93	89	4.1
Average							**3.1**

Table B Partner 2 synthetic mixture at 25ºC in different solvents.

Iso-octane	**T=0**	**T=4**	**T=12**	**T=24**	**T=48**	**T=96**	**RSD (%)**
N-acetylamphetamine	100	101	101	101	99	95	2.3
Aldimine	100	99	97	98	99	99	1.0
Benzylamphetamine	100	100	103	102	101	102	1.3
Benzoylamphetamine	100	100	102	101	101	100	0.6
2-oxo	100	101	102	102	101	100	0.9
Cathinol	100	122	145	135	132	146	13.3
Average							**3.2**
Toluene	**T=0**	**T=4**	**T=12**	**T=24**	**T=48**	**T=96**	**RSD (%)**
N-acetylamphetamine	100	95	90	93	99	89	4.6
Aldimine	100	94	91	93	97	93	3.5
Benzylamphetamine	100	97	93	95	98	92	3.1
Benzoylamphetamine	100	99	98	99	100	97	1.2
2-oxo	100	100	99	99	101	98	1.0
Cathinol	100	115	112	111	113	107	5.0
Average							**3.1**
Dichloromethane	**T=0**	**T=4**	**T=12**	**T=24**	**T=48**	**T=96**	**RSD (%)**
N-acetylamphetamine	100	99	102	106	104	110	3.8
Aldimine	100	100	102	102	104	101	1.4
Benzylamphetamine	100	101	100	101	99	105	2.0
Benzoylamphetamine	100	100	100	101	101	105	1.8
2-oxo	100	100	100	102	101	106	2.2
Cathinol	100	114	111	131	126	49	28.3
Average							**6.6**
Diethyl ether	**T=0**	**T=4**	**T=12**	**T=24**	**T=48**	**T=96**	**RSD (%)**
N-acetylamphetamine	100	98	101	100	102	103	1.8
Aldimine	100	99	100	96	98	96	1.9
Benzylamphetamine	100	101	104	102	97	109	3.7
Benzoylamphetamine	100	97	99	100	102	104	2.4
2-oxo	100	98	99	101	102	105	2.4
Cathinol	100	123	136	149	0	0	79.8
Average							**15.3**
Ethyl acetate	**T=0**	**T=4**	**T=12**	**T=24**	**T=48**	**T=96**	**RSD (%)**
N-acetylamphetamine	100	98	99	95	95	95	2.4
Aldimine	100	53	52	49	47	40	38.1
Benzylamphetamine	100	97	103	99	103	93	3.9
Benzoylamphetamine	100	100	101	100	101	101	0.5
2-oxo	100	100	101	100	101	101	0.5
Cathinol	100	0	0	0	0	0	40.8
Average							**14.4**
Ethanol	**T=0**	**T=4**	**T=12**	**T=24**	**T=48**	**T=96**	**RSD (%)**
N-acetylamphetamine	100	99	98	99	98	100	0.9
Aldimine	100	89	91	90	86	78	8.1
Benzylamphetamine	100	103	102	102	102	103	1.1
Benzoylamphetamine	100	101	100	101	100	101	0.5
2-oxo	100	103	101	101	101	101	0.9
Cathinol	100	141	141	128	148	148	13.7
Average							**4.2**

Table C Partner 3 synthetic mixture at 25 ºC in different solvents.

Iso-octane	**T=0**	**T=4**	**T=12**	**T=24**	**T=48**	**T=96**	**RSD (%)**
4-methyl-5-phenylpyrimidine	100	98	98	98	99	99	0.9
4-benzylpyrimidine	100	99	99	99	99	100	0.6
DPPA	100	99	101	101	99	97	1.5
DPIA	100	97	99	100	100	100	1.3
DPIMA	100	103	107	107	106	107	2.7
DPIF (1)	100	101	100	100	101	100	0.5
DPIF (2)	100	100	99	99	101	99	1.0
Average							**1.2**
Toluene	**T=0**	**T=4**	**T=12**	**T=24**	**T=48**	**T=96**	**RSD (%)**
4-methyl-5-phenylpyrimidine	100	101	100	100	98	100	1.1
4-benzylpyrimidine	100	101	100	100	98	99	1.3
DPPA	100	101	100	100	99	94	2.6
DPIA	100	102	101	101	100	99	0.9
DPIMA	100	101	99	101	91	92	4.6
DPIF (1)	100	99	98	100	98	99	0.9
DPIF (2)	100	99	98	100	98	99	0.8
Average							**1.7**
Dichloromethane	**T=0**	**T=4**	**T=12**	**T=24**	**T=48**	**T=96**	**RSD (%)**
4-methyl-5-phenylpyrimidine	100	102	98	99	97	97	1.9
4-benzylpyrimidine	100	102	99	99	97	98	1.7
DPPA	100	103	100	94	79	86	10.3
DPIA	100	101	101	100	100	104	1.5
DPIMA	100	102	97	102	98	101	2.0
DPIF (1)	100	101	101	100	101	102	0.8
DPIF (2)	100	101	100	100	100	101	0.7
Average							**2.7**
Diethyl ether	**T=0**	**T=4**	**T=12**	**T=24**	**T=48**	**T=96**	**RSD (%)**
4-methyl-5-phenylpyrimidine	100	96	98	97	93	95	2.6
4-benzylpyrimidine	100	97	98	97	94	95	2.3
DPPA	100	95	87	95	84	82	7.9
DPIA	100	98	100	101	98	101	1.4
DPIMA	100	100	95	97	97	103	2.7
DPIF (1)	100	99	100	100	100	101	0.8
DPIF (2)	100	100	100	100	100	100	0.2
Average							**2.6**
Ethyl acetate	**T=0**	**T=4**	**T=12**	**T=24**	**T=48**	**T=96**	**RSD (%)**
4-methyl-5-phenylpyrimidine	100	99	96	97	99	94	2.3
4-benzylpyrimidine	100	99	96	98	98	95	1.8
DPPA	100	95	88	88	89	83	6.7
DPIA	100	99	98	98	98	97	1.0
DPIMA	100	105	98	103	95	109	5.0
DPIF (1)	100	100	100	101	101	99	0.6
DPIF (2)	100	100	100	101	100	99	0.7
Average							**2.6**
Ethanol	**T=0**	**T=4**	**T=12**	**T=24**	**T=48**	**T=96**	**RSD (%)**
4-methyl-5-phenylpyrimidine	100	95	93	93	97	97	2.8
4-benzylpyrimidine	100	95	93	94	96	97	2.7
DPPA	100	101	101	100	98	97	1.5
DPIA	100	97	96	98	98	100	1.4
DPIMA	100	96	98	100	95	106	4.0
DPIF (1)	100	100	100	101	100	100	0.5
DPIF (2)	100	100	100	101	100	100	0.5
Average							**1.9**

Table D Partner 4 synthetic mixture at 25 ºC in different solvents.

Iso-octane	**T=0**	**T=4**	**T=12**	**T=24**	**T=48**	**T=96**	**RSD (%)**
N-formylamphetamine	100	98	98	103	97	99	2.0
4-benzylpyrimidine	100	99	99	103	98	100	1.8
DPPA	100	100	100	104	99	100	1.8
DPIA	100	98	99	101	99	100	0.9
DPIMA	100	99	98	102	100	103	1.9
2,4-dimethyl-3,5-diphenylpyridine	100	99	99	100	99	99	0.7
DPIF	100	99	100	101	97	101	1.3
Average							**1.5**
Toluene	**T=0**	**T=4**	**T=12**	**T=24**	**T=48**	**T=96**	**RSD (%)**
N-formylamphetamine	100	102	102	102	102	104	1.2
4-benzylpyrimidine	100	101	101	101	101	102	0.6
DPPA	100	101	100	100	98	97	1.3
DPIA	100	100	100	101	101	102	0.7
DPIMA	100	103	104	105	103	105	1.8
2,4-dimethyl-3,5-diphenylpyridine	100	101	101	99	101	103	1.4
DPIF	100	101	101	100	102	104	1.4
Average							**1.2**
Dichloromethane	**T=0**	**T=4**	**T=12**	**T=24**	**T=48**	**T=96**	**RSD (%)**
N-formylamphetamine	100	99	99	100	99	97	1.1
4-benzylpyrimidine	100	99	99	99	98	95	1.8
DPPA	100	101	104	101	111	106	4.1
DPIA	100	99	100	99	102	97	1.7
DPIMA	100	97	97	96	96	94	2.1
2,4-dimethyl-3,5-diphenylpyridine	100	99	100	100	103	99	1.6
DPIF	100	99	100	101	101	107	2.8
Average							**3.2**
Diethyl ether	**T=0**	**T=4**	**T=12**	**T=24**	**T=48**	**T=96**	**RSD (%)**
N-formylamphetamine	100	102	100	103	103	102	1.4
4-benzylpyrimidine	100	103	100	102	102	102	1.2
DPPA	100	101	103	103	109	113	4.9
DPIA	100	102	101	105	105	101	1.9
DPIMA	100	101	100	99	99	99	0.7
2,4-dimethyl-3,5-diphenylpyridine	100	101	103	107	106	104	2.7
DPIF	100	92	103	102	106	111	6.4
Average							**2.7**
Ethyl acetate	**T=0**	**T=4**	**T=12**	**T=24**	**T=48**	**T=96**	**RSD (%)**
N-formylamphetamine	100	100	100	100	99	102	0.9
4-benzylpyrimidine	100	99	100	99	98	101	1.2
DPPA	100	96	96	94	91	90	3.8
DPIA	100	100	101	100	99	101	0.8
DPIMA	100	98	97	95	93	91	3.5
2,4-dimethyl-3,5-diphenylpyridine	100	100	100	100	100	101	0.5
DPIF	100	100	100	99	101	99	0.5
Average							**1.6**
Ethanol	**T=0**	**T=4**	**T=12**	**T=24**	**T=48**	**T=96**	**RSD (%)**
N-formylamphetamine	100	102	99	100	100	97	1.7
4-benzylpyrimidine	100	102	97	99	99	97	2.0
DPPA	100	103	100	101	100	98	1.5
DPIA	100	101	100	101	101	100	0.8
DPIMA	100	101	99	99	98	96	1.6
2,4-dimethyl-3,5-diphenylpyridine	100	101	99	100	100	100	0.6
DPIF	100	101	100	99	100	101	0.6
Average							**1.3**

Table E Partner 2 amphetamine extracts at 25 ºC in different solvents.

Iso-octane	**T=0**	**T=4**	**T=12**	**T=24**	**T=48**	**T=96**	**RSD (%)**
Aldimine	100	90	94	97	79	107	10.0
Benzylamphetamine	100	101	101	102	106	102	1.9
Benzoylamphetamine	100	115	99	99	94	99	7.1
Average							**6.3**
Toluene	**T=0**	**T=4**	**T=12**	**T=24**	**T=48**	**T=96**	**RSD (%)**
N-acetylamphetamine	100	96	92	92	87	103	6.3
Aldimine	100	91	84	88	88	105	8.5
Benzylamphetamine	100	98	92	95	90	99	4.0
Benzoylamphetamine	100	113	106	113	116	106	5.4
Average							**6.1**
Dichloromethane	**T=0**	**T=4**	**T=12**	**T=24**	**T=48**	**T=96**	**RSD (%)**
N-acetylamphetamine	100	102	97	101	100	95	2.8
Benzylamphetamine	100	99	93	94	90	80	8.0
Average							**5.4**
Diethyl ether	**T=0**	**T=4**	**T=12**	**T=24**	**T=48**	**T=96**	**RSD (%)**
N-acetylamphetamine	100	99	101	105	114	137	13.5
Aldimine	100	107	101	94	76	89	11.7
Benzylamphetamine	100	100	101	102	103	123	8.7
Benzoylamphetamine	100	99	99	103	99	-	1.6
Average							**8.9**
Ethyl acetate	**T=0**	**T=4**	**T=12**	**T=24**	**T=48**	**T=96**	**RSD (%)**
N-acetylamphetamine	100	100	99	102	108	117	6.6
Aldimine	100	104	103	104	120	85	10.7
Benzylamphetamine	100	99	99	97	100	96	1.6
Benzoylamphetamine	100	101	101	104	101	100	1.5
Average							**5.1**

Table F Partner 3 amphetamine extracts at 25 ºC in different solvents.

Iso-octane	**T=0**	**T=4**	**T=12**	**T=24**	**T=48**	**T=96**	**RSD (%)**
4-methyl-5-phenylpyrimidine	100	96	99	97	98	97	1.4
4-benzylpyrimidine	100	98	100	98	101	100	1.3
benzylamphetamine	100	100	101	99	102	101	1.2
DPIA	100	99	101	99	100	99	0.8
DPIMA	-	-	-	-	-	-	-
DPIF (1)	100	99	99	99	98	98	0.9
DPIF (2)	100	99	99	98	96	97	1.3
Average							**1.2**
Toluene	**T=0**	**T=4**	**T=12**	**T=24**	**T=48**	**T=96**	**RSD (%)**
4-methyl-5-phenylpyrimidine	100	97	100	99	93	97	2.9
4-benzylpyrimidine	100	98	101	100	94	98	2.6
benzylamphetamine	100	100	103	103	99	100	1.6
DPIA	100	99	102	102	98	101	1.7
DPIMA	-	-	-	-	-	-	-
DPIF (1)	100	99	99	99	98	99	0.7
DPIF (2)	100	99	99	99	97	98	1.3
Average							**1.8**
Dichloromethane	**T=0**	**T=4**	**T=12**	**T=24**	**T=48**	**T=96**	**RSD (%)**
4-methyl-5-phenylpyrimidine	100	101	100	99	98	91	3.6
4-benzylpyrimidine	100	98	100	99	98	87	4.9
benzylamphetamine	100	102	101	101	98	91	4.0
DPIA	100	102	101	101	98	99	1.3
DPIMA	-	-	-	-	-	-	-
DPIF (1)	100	101	101	102	99	101	1.2
DPIF (2)	100	101	101	102	99	99	1.4
Average							**2.7**
Diethyl ether	**T=0**	**T=4**	**T=12**	**T=24**	**T=48**	**T=96**	**RSD (%)**
4-methyl-5-phenylpyrimidine	100	102	99	94	99	-	3.0
4-benzylpyrimidine	100	102	98	97	99	-	1.9
benzylamphetamine	100	102	98	102	97	-	2.3
DPIA	100	86	100	102	102	-	6.9
DPIMA	-	-	-	-	-	-	-
DPIF (1)	100	102	101	105	102	-	1.8
DPIF (2)	100	102	100	105	102	-	1.9
Average							**3.0**
Ethyl acetate	**T=0**	**T=4**	**T=12**	**T=24**	**T=48**	**T=96**	**RSD (%)**
4-methyl-5-phenylpyrimidine	100	101	99	98	100	101	1.3
4-benzylpyrimidine	100	101	99	98	99	100	1.2
benzylamphetamine	100	99	98	98	100	102	1.5
DPIA	100	99	99	99	101	103	1.3
DPIMA	-	-	-	-	-	-	-
DPIF (1)	100	98	101	104	101	99	1.9
DPIF (2)	100	99	101	101	102	99	1.4
Average							**1.4**

Table G Partner 4 amphetamine extracts at 25 ºC in different solvents.

Iso-octane	**T=0**	**T=4**	**T=12**	**T=24**	**T=48**	**T=96**	**RSD (%)**
4-methyl-5-phenylpyrimidine	100	102	103	103	107	103	2.1
DPIA	100	99	100	100	103	100	1.2
DPIMA	100	99	100	98	97	96	1.9
2,4-dimethyl-3,5-diphenylpyridine	100	100	99	103	107	100	2.8
DPIF	100	100	101	100	100	100	0.4
Average							**1.7**
Toluene	**T=0**	**T=4**	**T=12**	**T=24**	**T=48**	**T=96**	**RSD (%)**
4-methyl-5-phenylpyrimidine	100	98	101	99	99	99	1.2
4-benzylpyrimidine	100	99	103	101	100	101	1.6
N-formylamphetamine	100	98	101	99	99	99	1.1
DPIA	100	98	100	99	98	98	0.9
2,4-dimethyl-3,5-diphenylpyridine	100	99	103	97	102	102	2.3
DPIF	100	100	100	100	100	100	0.3
Average							**1.2**
Dichloromethane	**T=0**	**T=4**	**T=12**	**T=24**	**T=48**	**T=96**	**RSD (%)**
4-methyl-5-phenylpyrimidine	100	99	100	103	100	100	1.3
N-formylamphetamine	100	101	103	100	101	104	1.4
DPIA	100	102	101	100	102	103	1.1
DPIMA	100	102	102	100	102	103	1.2
2,4-dimethyl-3,5-diphenylpyridine	100	103	99	111	99	106	4.7
DPIF	100	100	101	100	100	100	0.6
Average							**1.7**
Diethyl ether	**T=0**	**T=4**	**T=12**	**T=24**	**T=48**	**T=96**	**RSD (%)**
4-methyl-5-phenylpyrimidine	100	101	94	105	125	125	12.4
N-formylamphetamine	100	99	95	98	110	111	6.5
DPIA	100	96	97	98	104	103	3.2
DPIMA	100	102	98	99	105	118	7.3
2,4-dimethyl-3,5-diphenylpyridine	100	103	101	102	107	104	2.5
DPIF	100	100	97	100	104	104	2.5
Average							**5.7**
Ethyl acetate	**T=0**	**T=4**	**T=12**	**T=24**	**T=48**	**T=96**	**RSD (%)**
4-methyl-5-phenylpyrimidine	100	101	103	100	104	107	2.8
N-formylamphetamine	100	100	101	98	103	101	1.6
DPIA	100	100	99	97	99	98	1.1
DPIMA	100	99	98	95	97	94	2.3
2,4-dimethyl-3,5-diphenylpyridine	100	92	97	99	104	101	4.2
DPIF	100	100	101	99	101	100	0.8
Average							**2.1**

ANNEX 7 : RESULTS OF LINEARITY STUDY IN SUBTASK 3.4

	Partner 2			Partner 3			Partner 4		
	FID	**SCAN**	**SIM**	**FID**	**SCAN**	**SIM**	**FID**	**SCAN**	**SIM**
Octanol									
r^2	0.99779	0.99999	0.99887	-	-	-	-	-	-
R^2	0.99779	0.99999	0.99887	-	-	-	-	-	-
variable b	-0.1068	0.00355	0.38256	-	-	-	-	-	-
variable a	0.12596	0.60451	0.90707	-	-	-	-	-	-
Dodecane (C12)									
r^2	0.93109	0.98943	0.98376	-	-	-	-	-	-
R^2	0.93109	0.98943	0.98376	-	-	-	0.9999	0.9984	-
variable b	-1.03007	0.48362	1.5712	-	-	-	-	-	-
variable a	0.25511	1.77077	2.73289	-	-	-	-	-	-
2,6-dimethylphenol									
r^2	0.99803	0.99997	0.99897	1.0000	0.9938	0.9959	-	-	-
R^2	0.99803	0.99997	0.99897	1.0000	0.9953	0.9720	0.9999	0.9989	
variable b	-0.12189	0.41087	1.95698	0.1025	0.6663	0.7344	-	-	-
variable a	0.1514	3.09719	4.66908	0.0713	4.0956	3.2534	-	-	-
Tridecane (C13)									
r^2	0.99825	0.99952	0.99774	1.0000	0.9909	0.9987	-	-	-
R^2	0.99825	0.99952	0.99774	1.0000	0.9951	0.9952	0.9999	0.9988	-
variable b	-0.10626	0.88007	2.12257	0.1025	0.5438	0.7997	-	-	-
variable a	0.14952	1.59394	2.45592	0.0713	4.2307	0.3428	-	-	-
2,6-dimethyl aniline									
r^2	0.99807	0.99997	0.99895	0.9999	0.9912	0.9928	-	-	-
R^2	0.99807	0.99997	0.99895	1.0000	0.9925	0.9899	0.9999	0.9983	-
variable b	-0.10379	0.48357	2.38515	0.0896	0.7896	0.8677	-	-	-
variable a	0.13334	3.37029	5.05218	0.4747	6.6523	5.5648	-	-	-
Decanoic acid methyl ester									
r^2	0.99815	0.99976	0.99802	0.9999	0.9897	0.9894	-	-	-
R^2	0.99815	0.99976	0.99802	0.9999	0.9909	0.9852	0.9999	0.9977	-
variable b	-0.06729	0.89822	2.78145	outlier	0.6927	0.7777	-	-	-
variable a	0.10476	2.54436	3.8578	outlier	6.2908	5.8583	-	-	-
Undecanoic acid methyl ester									
r^2	0.99826	0.99953	0.99761	-	-	-	-	-	-
R^2	0.99826	0.99953	0.99761	-	-	-	-	-	-
variable b	-0.17031	1.22746	3.27682	-	-	-	-	-	-
variable a	0.24325	2.42514	3.67272	-	-	-	-	-	-
Dicyclohexylamine									
r^2	0.99785	0.99998	0.99869	1.0000	0.9872	0.9789	-	-	-
R^2	0.99785	0.99998	0.99869	1.0000	0.8753	0.9773	0.9999	0.9937	-
variable b	-0.13723	2.71827	-0.35236	0.0949	0.1120	0.1001	-	-	-
variable a	0.13505	10.3525	0.12945	0.4241	1.1522	1.2155	-	-	-
Dodecanoic acid methyl ester									
r^2	0.99668	0.99882	0.99556	-	-	-	-	-	-
R^2	0.99668	0.99882	0.99556	-	-	-	-	-	-
variable b	-0.42095	1.60993	3.9259	-	-	-	-	-	-
variable a	0.24382	2.30159	3.48151	-	-	-	-	-	-
	Partner 2			**Partner 3**			**Partner 4**		
	FID	**SCAN**	**SIM**	**FID**	**SCAN**	**SIM**	**FID**	**SCAN**	**SIM**
Heptadecane (C17)									
r^2	0.99825	0.99931	0.99651	0.9999	0.9912	0.9924	-	-	-
R^2	0.99825	0.99931	0.99651	1.0000	0.9945	0.9945	0.9999	0.9986	-
variable b	-0.11131	0.97261	2.70882	0.1013	0.5559	0.5937	-	-	-
variable a	0.15332	1.72628	2.63626	-0.2443	3.8793	3.7978	-	-	-

Octadecane (C18)									
r^2	0.99814	0.99814	0.99676	0.9999	0.9885	0.9875	-	-	-
R^2	0.99814	0.99814	0.99676	0.9999	0.9947	0.9945	0.9999	0.9988	-
variable b	-0.09716	-0.09716	2.11271	0.0961	0.5524	0.5966	-	-	-
variable a	0.12928	0.12928	2.26256	0.1316	3.8193	3.5914	-	-	-
Nonadecane (C19)									
r^2	0.99798	0.99954	0.99727	0.9999	0.9747	0.9869	-	-	-
R^2	0.99798	0.99954	0.99727	0.9999	0.9887	0.9942	0.9999	0.9988	-
variable b	-0.09154	0.55792	1.72402	0.0960	0.5217	0.5974	-	-	-
variable a	0.11744	1.35732	2.08564	0.1337	4.5324	3.6546	-	-	-
Tetracosane (C24)									
r^2	0.99742	0.99955	0.99702	-	-	-	-	-	-
R^2	0.99742	0.99955	0.99702	-	-	-	1.0000	0.9991	-
variable b	-0.12068	0.45172	1.86171	-	-	-	-	-	-
variable a	0.12589	1.46322	2.23888	-	-	-	-	-	-

Partner 3 only

	FID	**NPD**	**SCAN**	**SIM**
Ketamine				
r^2	0.9996	0.9917	0.9989	0.9975
R^2	0.9998	0.9918	0.9915	0.9994
variable b	0.0704	0.1310	0.0754	0.0700
variable a	-0.1225	-1.2460	-0.0550	-0.0397
Diphenethylamine				
r^2	0.9998	0.9999	0.9598	0.9687
R^2	0.9999	0.9999	0.9434	**0.9773**
variable b	0.1005	0.0968	0.0784	0.1520
variable a	-0.0599	-0.0029	3.2599	2.1374
4-Methyl-5phenylpyrimidine				
r^2	1.0000	0.9976	0.9951	0.9990
R^2	1.0000	0.9982	0.9469	0.9955
variable b	0.0837	0.1505	0.0890	0.1100
variable a	0.3279	5.6250	0.4975	0.0400
Dicyclohexylamine				
r^2	1.0000	0.9991	0.9872	0.9789
R^2	1.0000	0.9994	0.8753	0.9773
variable b	0.0949	0.0947	0.1120	0.1001
variable a	0.4241	2.5085	1.1522	1.2155
Trimipramine				
r^2	0.9995	0.9997	0.9910	0.9876
R^2	0.9999	0.9998	outlier	0.9912
variable b	0.0710	0.0979	0.0444	0.0350
variable a	-0.0592	-0.0304	0.2603	0.3329

ANNEX 8 SYNTHESIS OF AMPHETAMINE IN TASK 6

Synthesis of the benzyl methyl ketone

First synthesis of benzyl methyl ketone

A first synthesis of benzy methyl ketone was made according to Bobranski [Bobranski and Drabik, 1941]. α-acetylbenzyl cyanide (32 g) was added to 60 mL of concentrated sulfuric acid (cooled in freezer and standing in ice). Water (20 mL) was also added. The solution was stirred during this process. The mixture was then heated under reflux for 21 hours (melting point of acetylbenzylcyanide: 92-94 °C). 50 % of benzyl methyl ketone was obtained (the other 50 % being unreacted acetylbenzylcyanide). This is probably due to the fact that a layer is formed above the acid hampering the reaction (not homogeneous mixture).

The mixture was allowed to cool down and 200 mL water added. The aqueous solution was extracted with 3 x 50 mL of dichloromethane. The combined extracts were left to evaporate at room temperature. Upon evaporation, crystals of acetylbenzylcyanide were formed. The remaining oil was then recovered and placed in the fridge. After a few days in the fridge, more crystals of acetylbenzylcyanide formed. The remaining oil was again recovered. A total amount of 8 g of benzyl methyl ketone was obtained (purity 95 %). The yield was 25 %.

Second synthesis of benzyl methyl ketone

The second synthesis of benzyl methyl ketone was made according to Shulgin and Shulgin [Shulgin and Shulgin, 1991]. Phenyl-2-nitropropene (25 g, Sigma-Aldrich®) was dissolved in190 mL acetic acid and slowly dripped into a 2 L Erlenmeyer containing 61 g of iron in 270 mL acetic acid. The mixture was shaken manually and then slowly heated to 60 °C. The mixture was left on low heat for 2h while manual shaking took place every 10 min. Then, 1.5 L of water was added and the solution was filtered to remove the iron particles.

The aqueous solution was extracted thrice with 300 mL dichloromethane. The combined extracts were then washed with 500 mL water and dried over magnesium sulphate. Finally, the solvent was evaporated under vacuum yielding a pale yellow oil. The weight of the obtained benzyl methyl ketone was 15 g (yield 60 %). The two batches of benzyl methyl ketone oils obtained during these syntheses were combined and used in the synthesis of batch 3 (IPSC 3).

Leuckart recipe used in the repeated synthesis

Benzyl methyl ketone (15 mL), formamide (30 mL) and formic acid (15 mL) were mixed and refluxed for 2.5 hours at approximately 160 ºC (molar ratio: 1:6.7:3.5). The reaction was stopped and the mixture was allowed to cool at room temperature for 1 hour (<100 ºC). Water (60 mL) and the mixture from the first step was added to a 250 mL separation funnel and the mixture were shaken for 1 minute. The two-phase system was allowed to equilibrate for 20 minutes after which approximately 15 mL formylamphetamine was recovered. HCl 32 % (same volume as formylamphetamine) was added and the mixture was refluxed at approximately 110 ºC for 1.5 hours. The mixture was then allowed to cool over night. NaOH (60 mL 5M) was added dropwise to the mixture. The mixture was allowed to cool for 1 hour after which about 12-14 g of amphetamine oil were obtained. Methanol (60 mL) was added followed by sulphuric acid (32 % in water) until pH 7. The obtained amphetamine sulphate crystals were filtered off (filter paper without vacuum). The crystals were then washed thrice with acetone (100 mL + 50 mL + 50 mL). The amphetamine sulphate was then allowed to dry at room temperature.

ANNEX 9

SWISS SEIZED AMPHETAMINE SAMPLES USED IN TASK 6

Database name	IPS name	Police number	Provenance	Year
I00G19I	00ge19	20916.1	Genève	2000
I00N68AI	00ne68	12221.5	Neuchâtel	2000
I00N68BI	00ne68	12221.7	Neuchâtel	2000
I00N68CI	00ne68	12221.9	Neuchâtel	2000
I00N68DI	00ne68	12221.11	Neuchâtel	2000
I01G250I	01ge250-07.01	414973.5	Genève	2001
I1104BI	-	1104-00 B	WD Zurich	2000
I136I	-	136-99	WD Zurich	1999
I176I	-	176-99	WD Zurich	1999
I18AI	-	18A -01	WD Zurich	2001
I18BI	-	18B -01	WD Zurich	2001
I195I	-	195-99	WD Zurich	1999
I2006AI	-	2006-02 A	WD Zurich	2002
I2006BI	-	2006-02 B	WD Zurich	2002
I2009I	-	2009-99	WD Zurich	1999
I2058I	-	2058-99	WD Zurich	1999
I2061I	-	2061-02	WD Zurich	2002
I2062I	-	2062-02	WD Zurich	2002
I2097I	-	2097-00	WD Zurich	2000
I2317I	-	2317-01	WD Zurich	2001
I2349I	-	2349-00	WD Zurich	2000
I2418I	-	2418-00	WD Zurich	2000
I2436I	-	2436-02	WD Zurich	2002
I2514AI	-	2514A-01	WD Zurich	2001
I2516I	-	2516-02	WD Zurich	2002
I2866I	-	2866-99	WD Zurich	1999
I2937I	-	2937-99	WD Zurich	1999
I3027I	-	3027-99	WD Zurich	1999
I3069I	-	3069-99	WD Zurich	1999
I3126I	-	3126-00	WD Zurich	2000
I3261I	-	3261-00	WD Zurich	2000
I3264I	-	3264-00	WD Zurich	2000
I3343I	-	3343-00	WD Zurich	2000
I3346I	-	3346-00	WD Zurich	2000
I3348I	-	3348-00	WD Zurich	2000
I3497AI	-	3497-00 A	WD Zurich	2000
I3499I	-	3499-00	WD Zurich	2000
I3500I	-	3500-00	WD Zurich	2000
I3631I	-	3631-01	WD Zurich	2001
I3740I	-	3740-99	WD Zurich	1999
I4331I	-	4331-01	WD Zurich	2001
I4469AI	-	4469-00 A	WD Zurich	2000
I4469BI	-	4469-00 B	WD Zurich	2000
I446I	-	446-01	WD Zurich	2001
I4576I	-	4576-99	WD Zurich	1999
I4578I	-	4578-99	WD Zurich	1999
I4695I	-	4695-99	WD Zurich	1999
I4705I	-	4705-01	WD Zurich	2001
I4723I	-	4723-99	WD Zurich	1999
I4837I	-	4837-01	WD Zurich	2001
I4960I	-	4960-00	WD Zurich	2000
I5010I	-	5010-01	WD Zurich	2001
I5012AI	-	5012A-01	WD Zurich	2001
I5012BI	-	5012B-01	WD Zurich	2001
I5150I	-	5150-00	WD Zurich	2000

Database name	IPS name	Police number	Provenance	Year
I5220I	-	5220-00	WD Zurich	2000
I5269AI	-	5269A-01	WD Zurich	2001
I5269BI	-	5269B-01	WD Zurich	2001
I5269CI	-	5269C-01	WD Zurich	2001
I5269DI	-	5269D-01	WD Zurich	2001
I5269FI	-	5269F-01	WD Zurich	2001
I5774I	-	5774-01	WD Zurich	2001
I5832	-	5832-99	WD Zurich	1999
I5921I	-	5921-99	WD Zurich	1999
I6116I	-	6116-01	WD Zurich	2001
I61I	-	61-99	WD Zurich	1999
I6373I	-	6373-01	WD Zurich	2001
I6430I	-	6430-99	WD Zurich	1999
I6501I	-	6501-00	WD Zurich	2000
I6686BI	-	6686-00 B	WD Zurich	2000
I6978I	-	6978-00	WD Zurich	2000
I7001AI	-	7001A-01	WD Zurich	2001
I7001BI	-	7001B-01	WD Zurich	2001
I7044I	-	7044-01	WD Zurich	2001
I7051I	-	7051-00	WD Zurich	2000
I7091I	-	7091-00	WD Zurich	2000
I7449I	-	7449-01	WD Zurich	2001
I7532BI	-	7532B-01	WD Zurich	2001
I7536I	-	7536-01	WD Zurich	2001
I7557I	-	7557-01	WD Zurich	2001
I7723I	-	7723-01	WD Zurich	2001
I7889I	-	7889-01	WD Zurich	2001
I7922I	-	7922-01	WD Zurich	2001
I98N166I	98ne166	9391.4	Neuchâtel	1998
I98N196I	98ne196	9470.3	Neuchâtel	1998
I98N19I	98ne19	8162.1	Neuchâtel	1998
I98N22I	98ne22	8195.2	Neuchâtel	1998
I98N51I	98ne51	8514	Neuchâtel	1998
I98N74AI	98ne74	8732.1	Neuchâtel	1998
I98N74BI	98ne74	8732.2	Neuchâtel	1998
I99G46AI	99ge46	135705.1	Genève	1999
I99G46BI	99ge46	135705.3	Genève	1999
I99N10I	99ne10	10029	Neuchâtel	1999
I99N141I	99ne141	10686.4	Neuchâtel	1999
IB0106I	-	B-0106-99	Kanton ZH	1999
IB1104I	-	B-1104-99	Kanton ZH	1999
IB1221BI	-	B-1221b-99	Kanton ZH	1999
IB1221CI	-	B-1221c-99	Kanton ZH	1999
IB1433I	-	B-1433-00	Kanton ZH	2000
IB1612AI	-	B-1612a-01	Kanton ZH	2001
IB1612BI	-	B-1612b-01	Kanton ZH	2001
IB1613I	-	B-1613-01	Kanton ZH	2001
IB1643I	-	B-1643-01	Kanton ZH	2001
IB1967I	-	B-1967-01	Kanton ZH	2001
IB2338AI	-	B-2338a-01	Kanton ZH	2001
IB2338BI	-	B-2338b-01	Kanton ZH	2001
IB2353I	-	B-2353-01	Kanton ZH	2001
IB2483I	-	B-2483-99	Kanton ZH	1999
IB3001I	-	B-3001-01	Kanton ZH	2001
IGEJUI	-	Jura	Jura	
IGEVSI	-	Valais	Valais	
IT532I	532	6631-97	WD Zurich	1997

ANNEX 10 LIST OF TASK 6 COMPOUNDS

#	Compound
1	2-methyl-3-phenylaziridine [a]
2	Benzylmethylketoxime 1 [a]
3	Benzylmethylketoxime 2 [a]
4	4-methyl-5-phenylpyrimidine [a]
5	Possibly N-propylbenzamide [e]
6	4-Benzylpyrimidine [a]
7	N-Acetylamphetamine [a]
8	N-Formylamphetamine [a]
9	1,2-Diphenylethylamine [e]
10	N,N-Dibenzylamine [d]
11	1,2-Diphenylethanone [d]
12	Benzylamphetamine [a]
13	1,3-diphenyl-2-propylamine [a]
14	N,N-di(β-phenylisopropyl)amine 1 [a]
15	N,N-di(β-phenylisopropyl)amine 2 [a]
16	α-methyldiphenethylamine [e]
17	N,N-di(β-phenylisopropyl)methylamine 1 [a]
18	N,N-di(β-phenylisopropyl)methylamine 2 [a]
19	Unknown A2 [e]
20	1-benzyl-3-methylnaphthalene [b]
21	Unknown A3 [e]
22	1-hydroxy-N,N-di(β-phenylisopropyl)amine 1 [a] (Cathinol 1)
23	1,3-dimethyl-2-phenylnaphthalene [b]
24	Unknown A4 [e]
25	Benzoylamphetamine [a]
26	Unknown B2 [e]
27	2-oxo-1-phenyl-(β-phenylisopropylamino)ethane [a]
28	2,6-dimethyl-3,5-diphenylpyridine [c]
29	2,4-dimethyl-3,5-diphenylpyridine [a]
30	Pyridines 7 and 14 [f]
31	Pyridine 272 [g]
32	2,6-diphenyl-3,5-dimethylpyridine [c]
33	N,N-di(β-phenylisopropyl)formamide 1 [a]
34	N,N-di(β-phenylisopropyl)formamide 2 [a]
35	Benzaldehyde oxime [e]
36	1-phenyl-1,2-propanedione [d]
37	Phenylacetonitrile [e]
38	Ethylamphetamine [d]
39	Isopropylamphetamine [e]
40	Phenyl-2-nitropropane [e]
41	Phenyl-2-nitropropene [a]
42	1,3-diphenyl-2-propanone [e]
43	α-dimethyldiphenethylamine [e]
44	1,5-diphenyl-4-methyl-4-penten-2-one [i]
45	1-benzyl-2,3-dimethylaziridine [e]
46	2-benzyl-2-methyl-5-phenyl-2,3-dihydropyrid-4-one [c]
47	Pyridine X [h]
48	Unknown C [e]
49	Unknown A1 [e]
50	Unknown B1 [e]
51	1-hydroxy-N,N-di(β-phenylisopropyl)amine 2 [a] (Cathinol 2)

a) Target compounds synthesised within the SMT project.

b) Target compounds of which standards were provided by the United Nations Office for Drugs and Crime (UNODC).

c) Target compounds of which standards were provided by the Netherlands Forensic Institute (NFI).

d) Other target compounds for which standards were available.

e) Other target compounds for which standards were not available. Identification based only upon comparison with Wiley and / or NIST MS library.

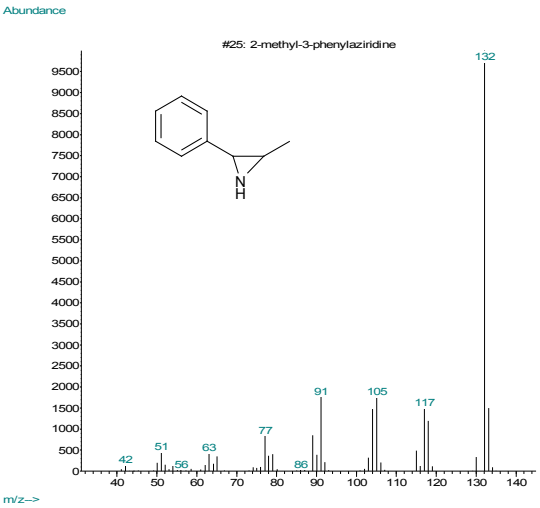
f) Two compounds not separated with the GC method. Their mass spectra indicate that they are pyridine derivatives. The names pyridine 7 and pyridine 14 are those used by partner 3 in their laboratory (SKL, Sweden).

g) Pyridine derivative tentatively identified as 2,4-dimethyl-3-phenyl-6-(phenylmethyl)-pyridine by van den Ark [van den Ark et al., 1978c].

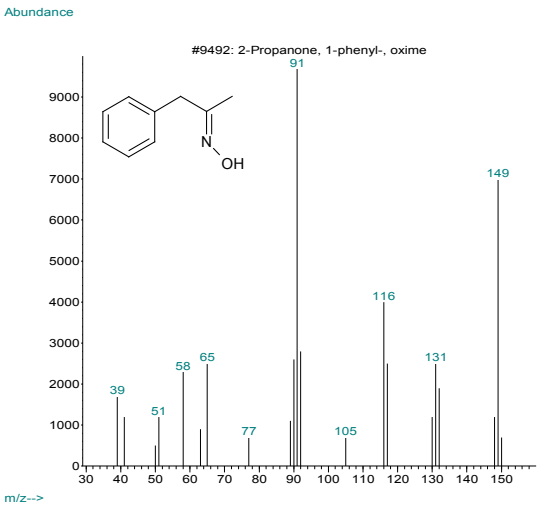
h) Compound with the same mass spectrum as identified pyridines. The name "pyridine x" was given as the exact chemical structure could not be determined.

i) Target compound for which a standard was not available. Identification based only upon comparison with Wiley and / or NIST MS library and with compound tentatively identified by Huizer [Huizer et al., 1981]

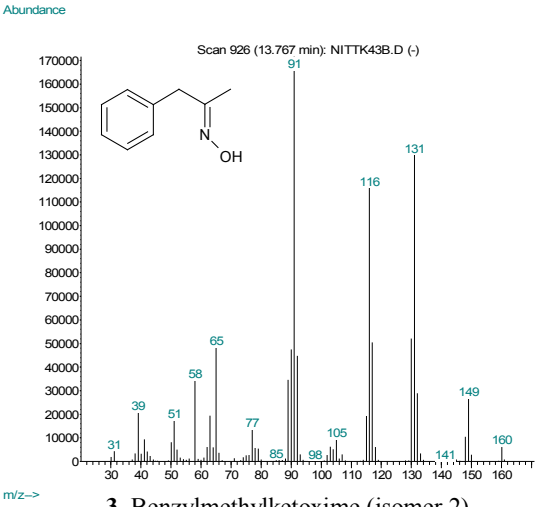
MASS SPECTRA OF THE 51 TARGET COMPOUNDS



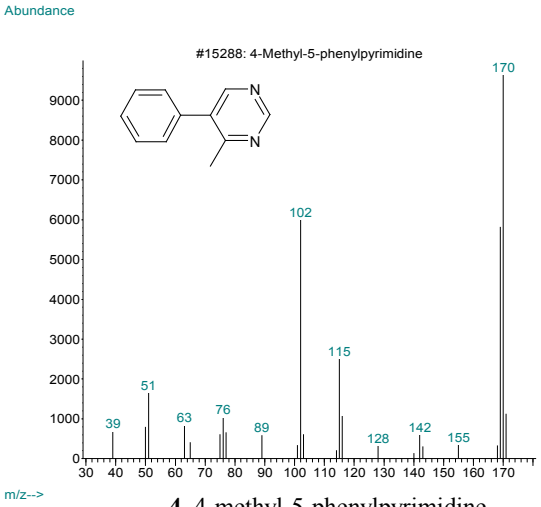
1. 2-methyl-3-phenylaziridine



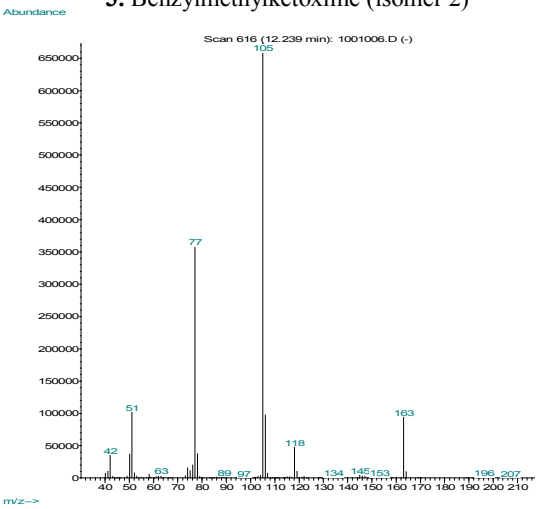
2. Benzylmethylketoxime (isomer 1)



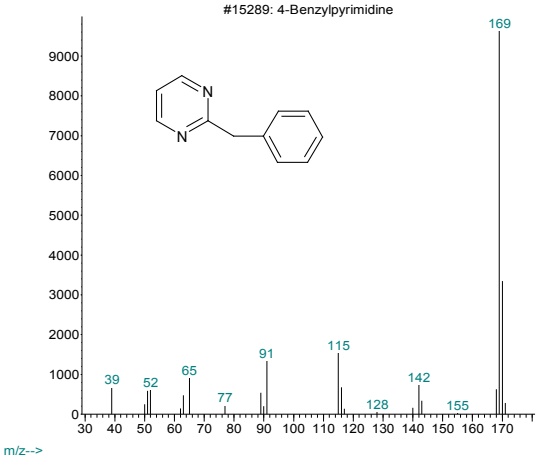
3. Benzylmethylketoxime (isomer 2)



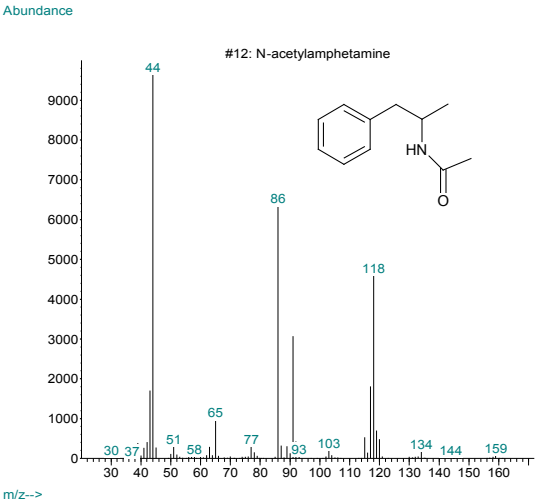
4. 4-methyl-5-phenylpyrimidine



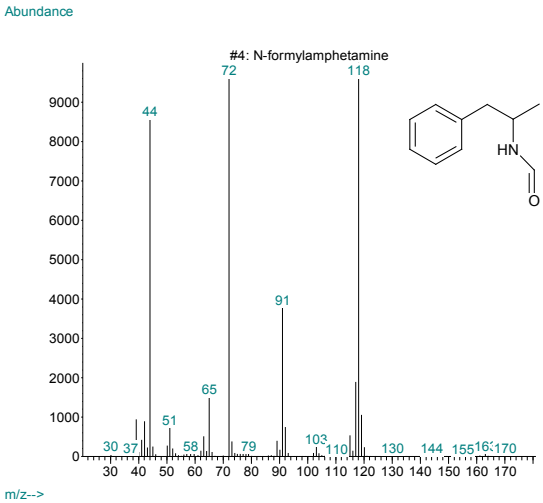
5. Compound which could be N-propylbenzamide



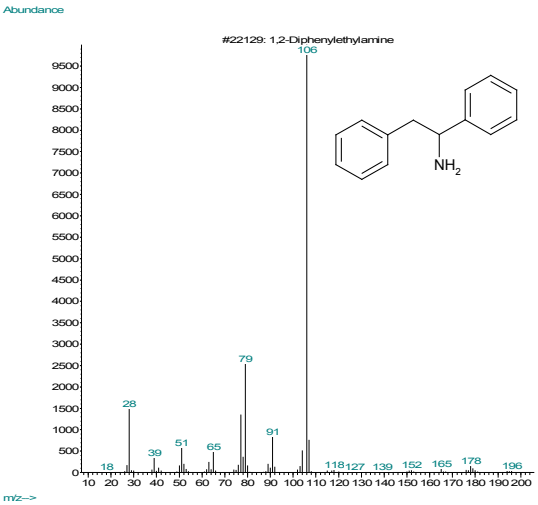
6. 4-benzylpyrimidine



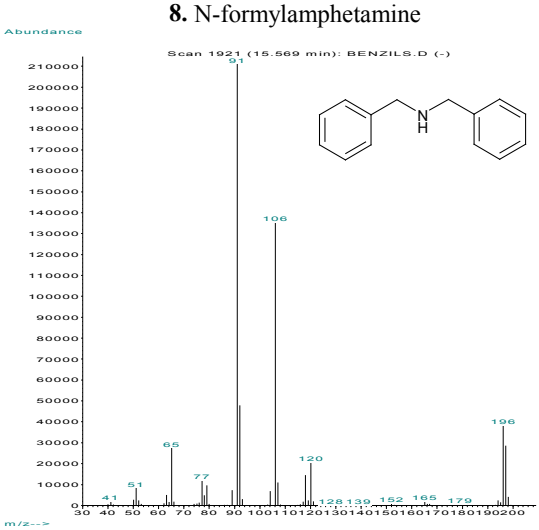
7. N-acetylamphetamine



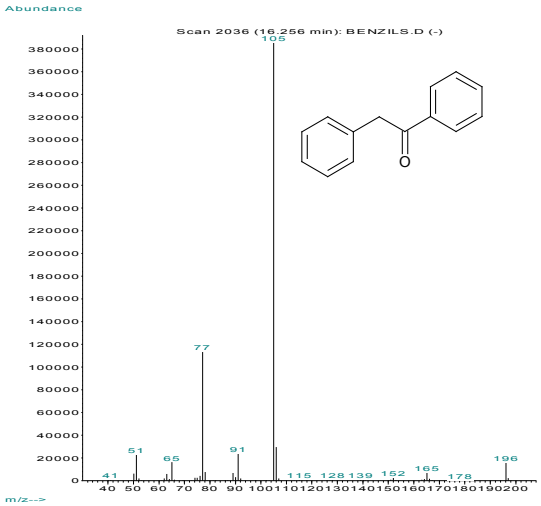
8. N-formylamphetamine



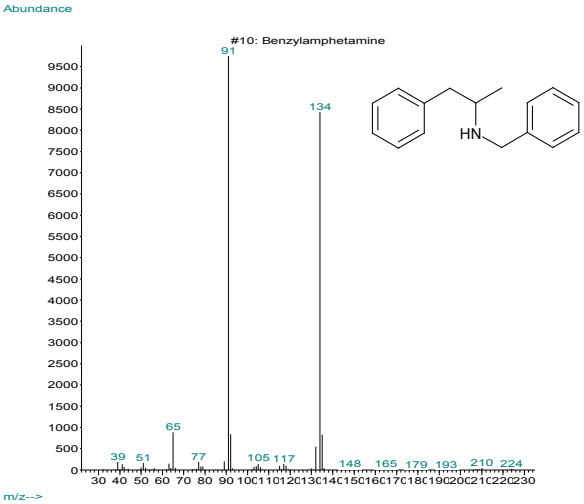
9. 1,2-Diphenylethylamine



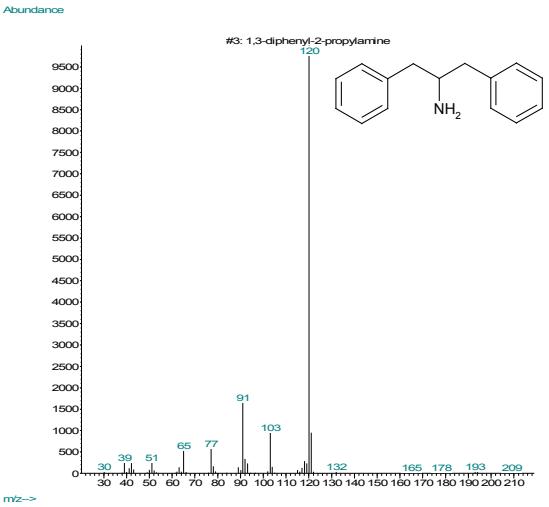
10. N, N-dibenzylamine



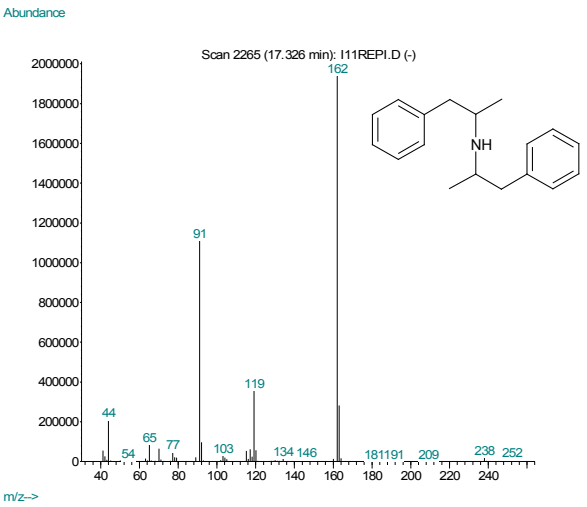
11. 1,2-Diphenyl ethanone



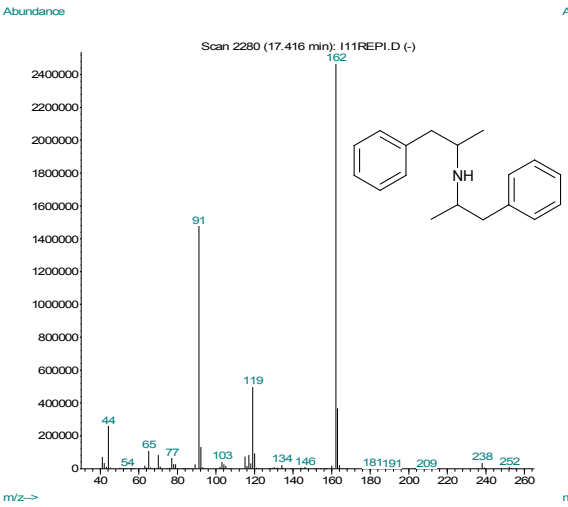
12. Benzylamphetamine



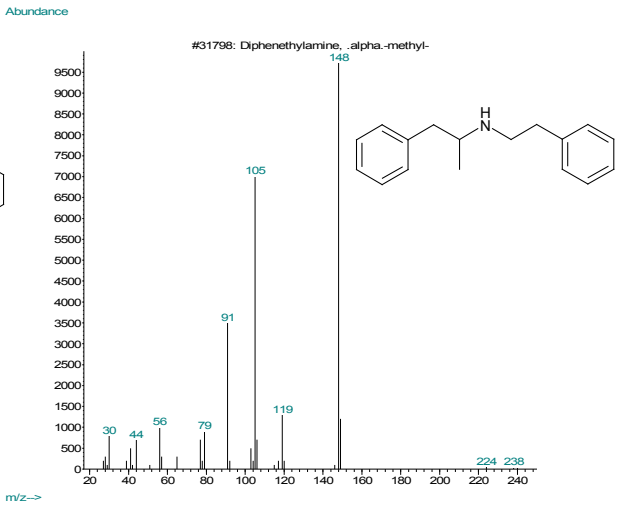
13. 1,3-diphenyl-2-propylamine (DPPA)



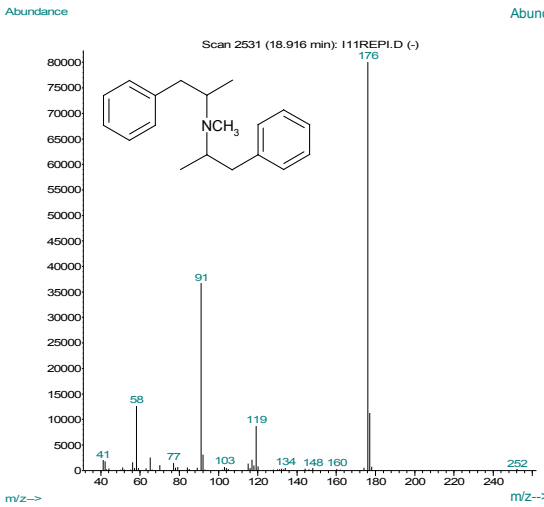
14. N,N-di(β-phenylisopropyl)amine (isomer 1)



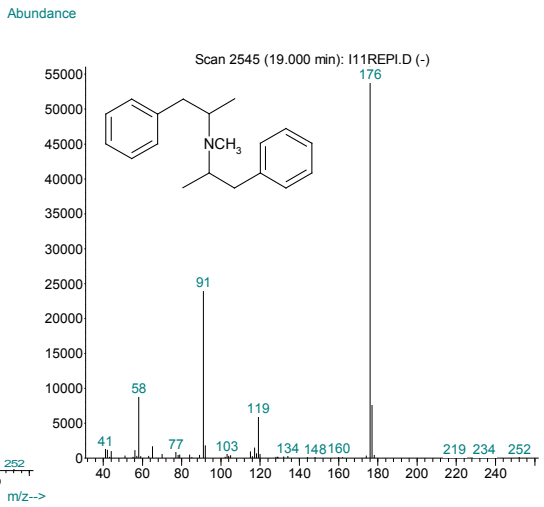
15. N,N-di(β-phenylisopropyl)amine (isomer 2)



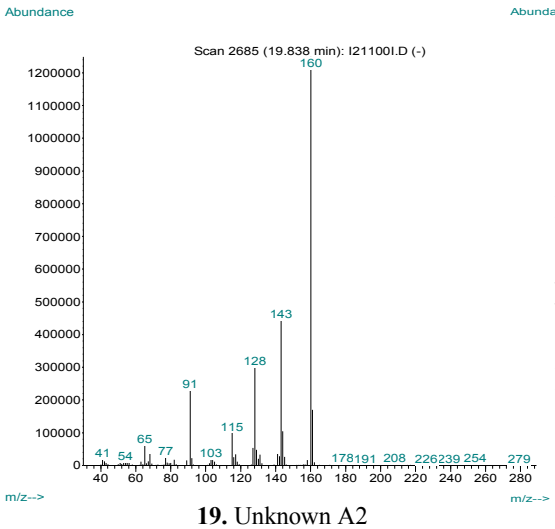
16. α-methyldiphenethylamine



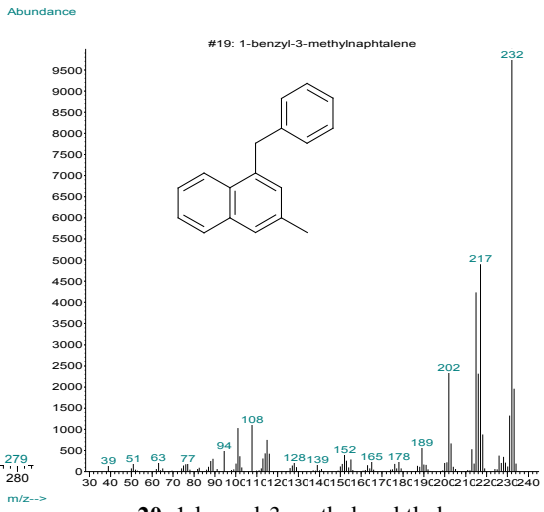
17. N,N-di(β-phenylisopropyl)methylamine (isomer 1)



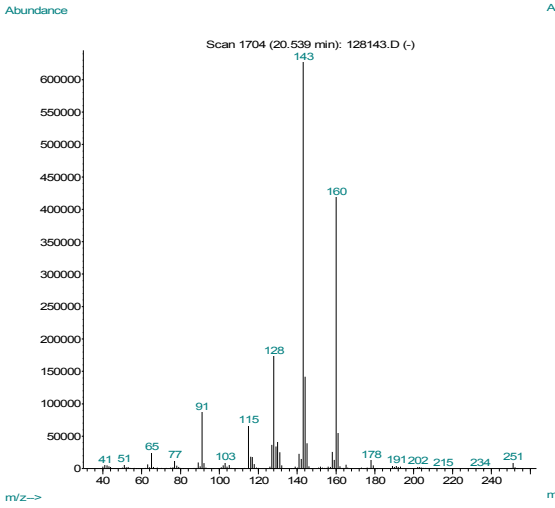
18. N,N-di(β-phenylisopropyl)methylamine (isomer 2)



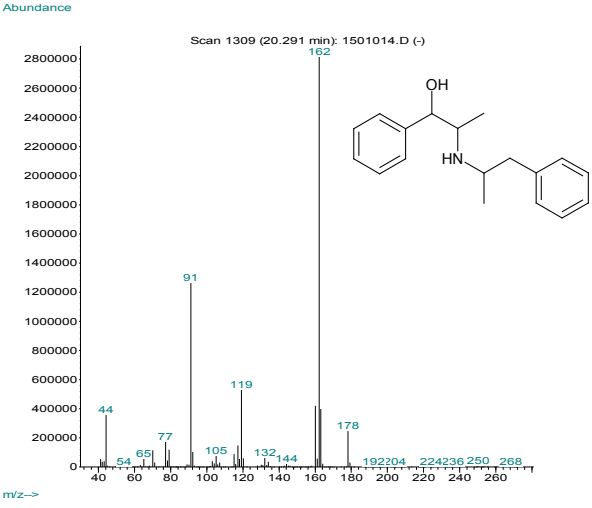
19. Unknown A2



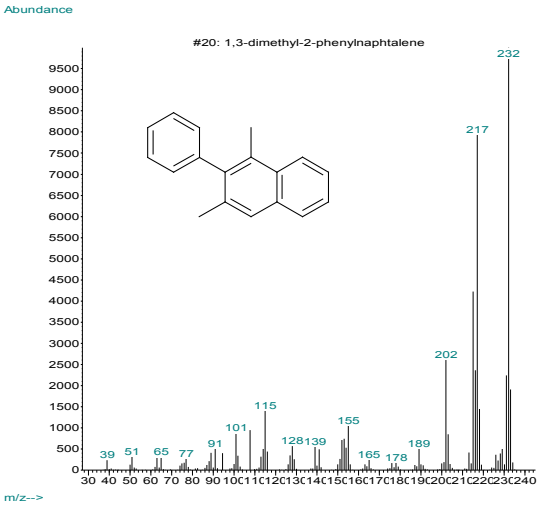
20. 1-benzyl-3-methylnaphthalene



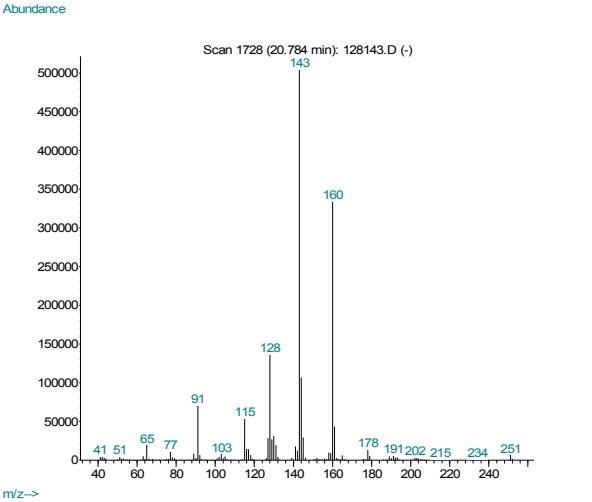
21. Unknown A3



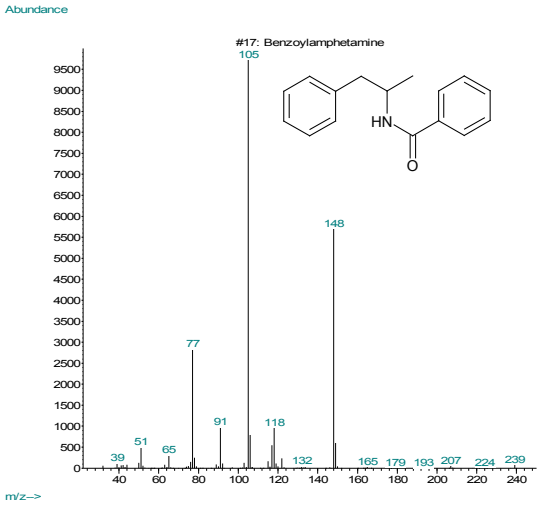
22. Cathinol (isomer 1)



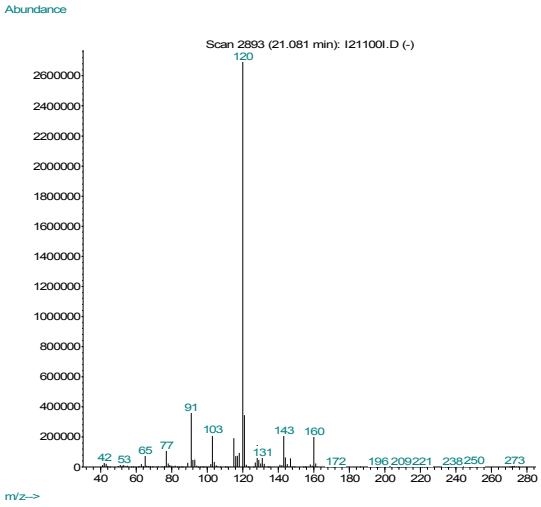
23. 1,3-dimethyl-2-phenylnaphthalene



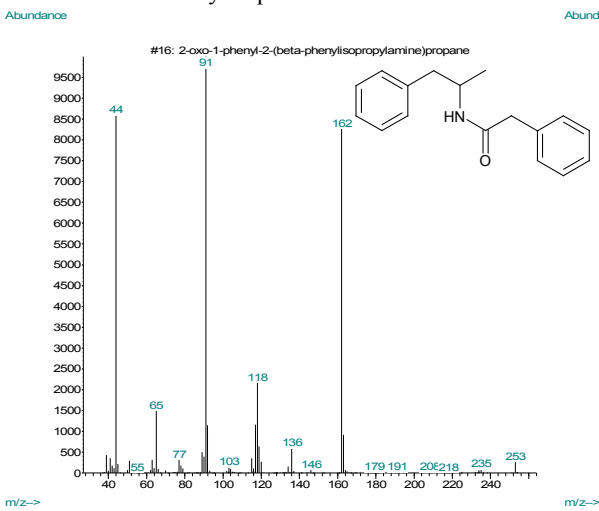
24. Unknown A4



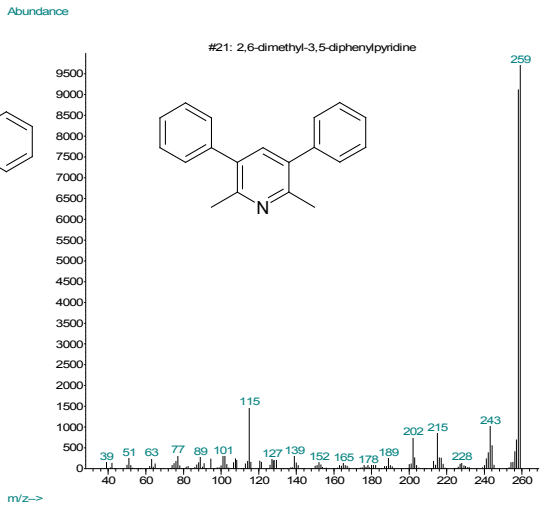
25. Benzoylamphetamine



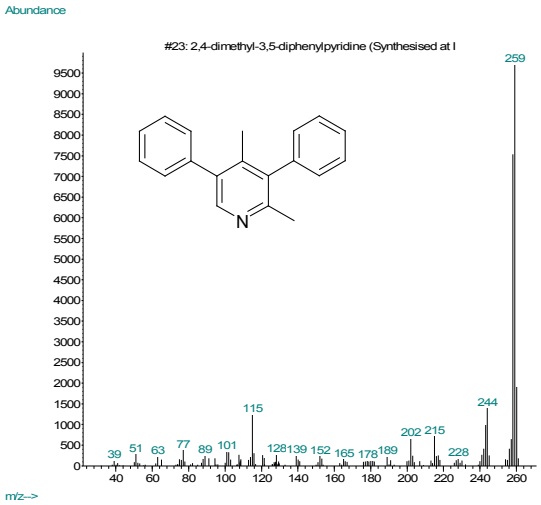
26. Unknown B2



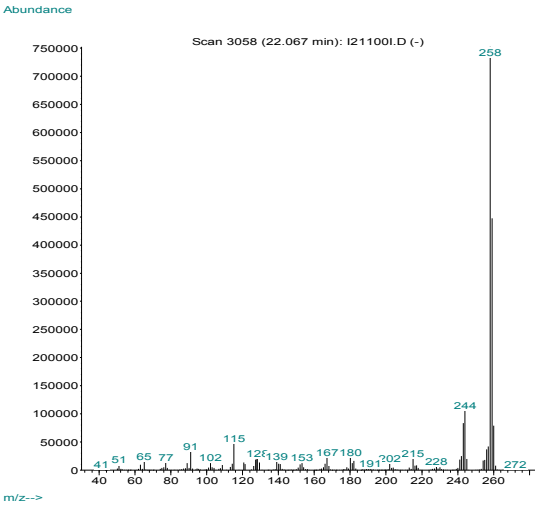
27. 2-oxo-1-phenyl-(β-phenylisopropylamino)ethane



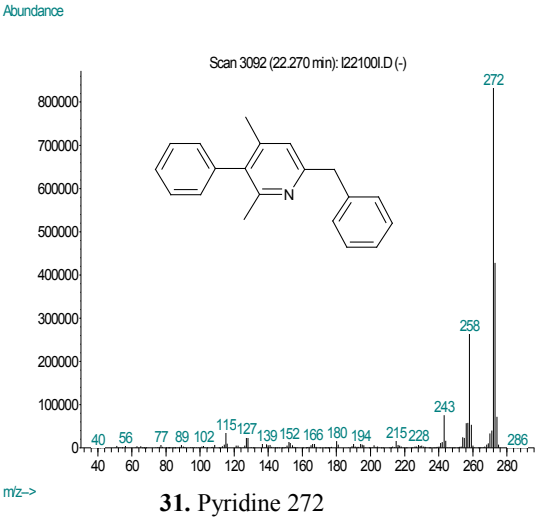
28. 2,6-dimethyl-3,5-diphenylpyridine



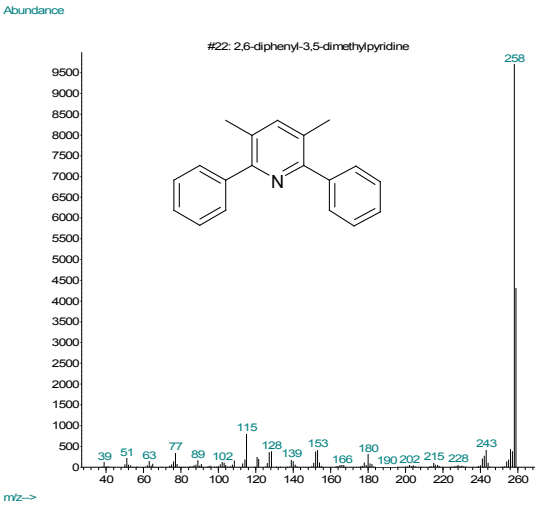
29. 2,4-dimethyl-3,5-diphenylpyridine



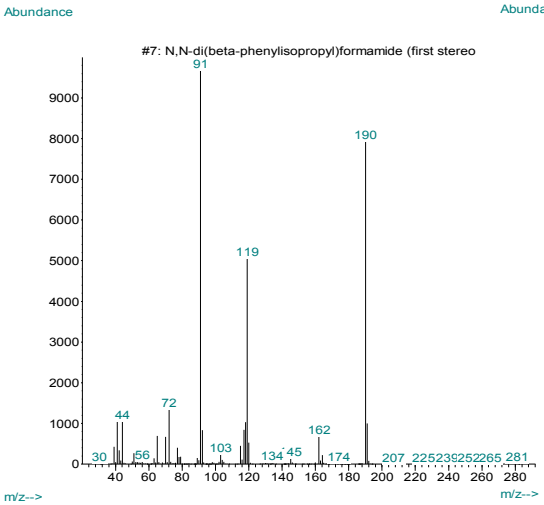
30. Pyridines 7 and 14 (one peak)



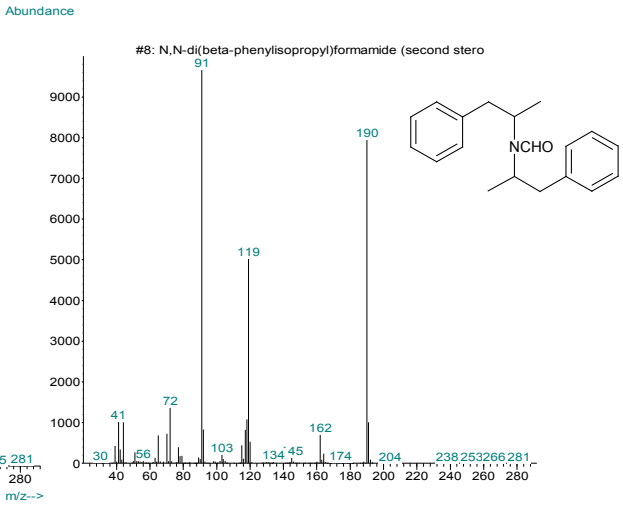
31. Pyridine 272



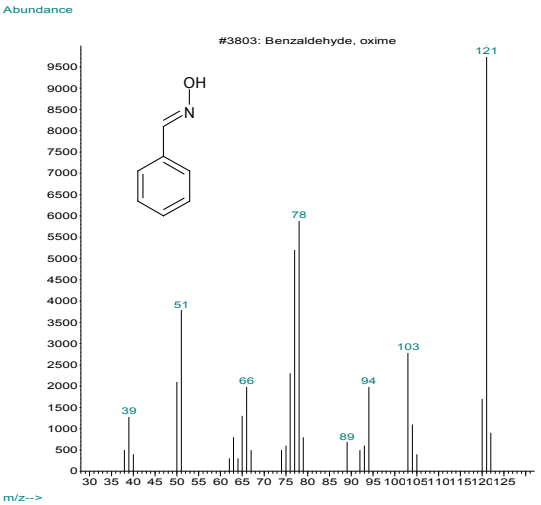
32. 2,6-diphenyl-3,5-dimethylpyridine



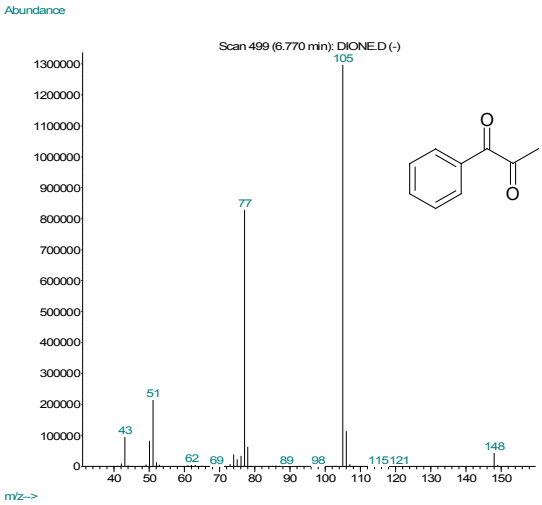
33. N,N-di(β-phenylisopropyl)formamide (isomer 1)



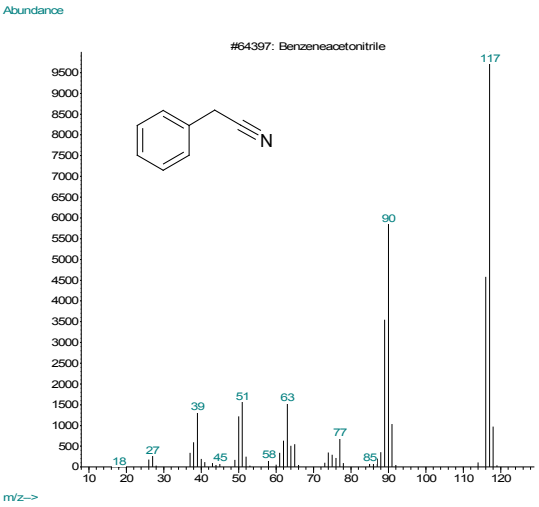
34. N,N-di(β-phenylisopropyl)formamide (isomer 2)



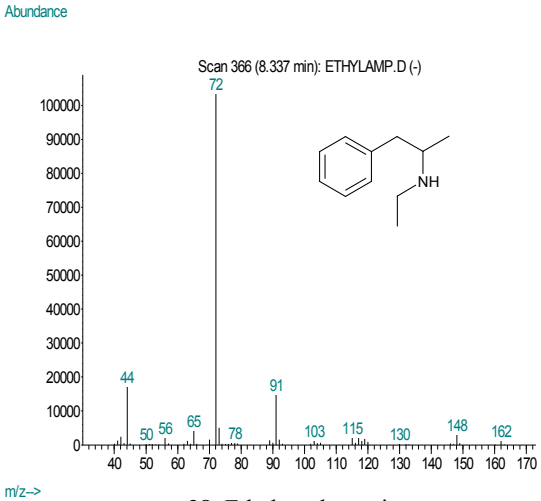
35. Benzaldehyde oxime



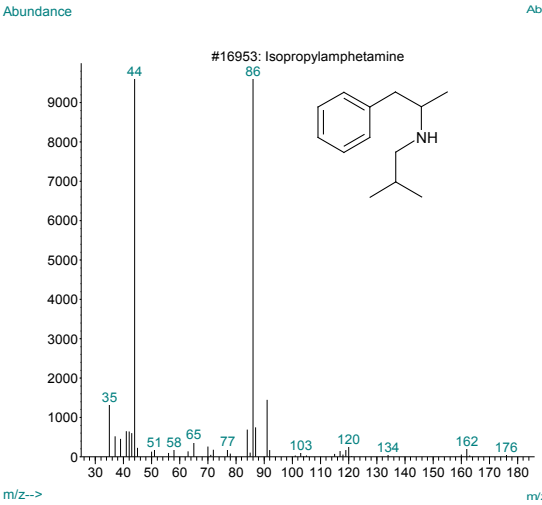
36. 1-phenyl-1,2-propanedione



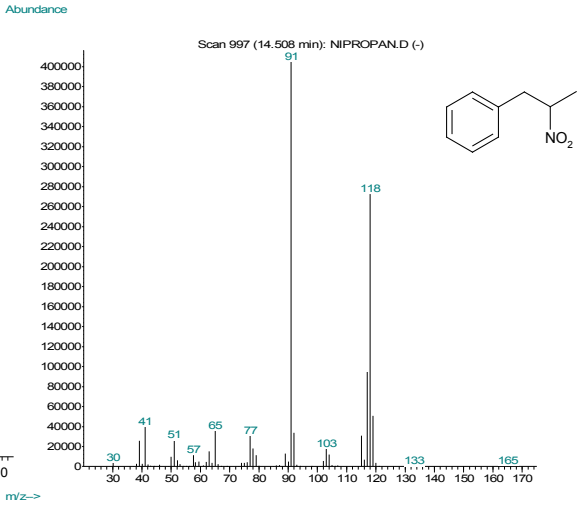
37. Phenylacetonitrile



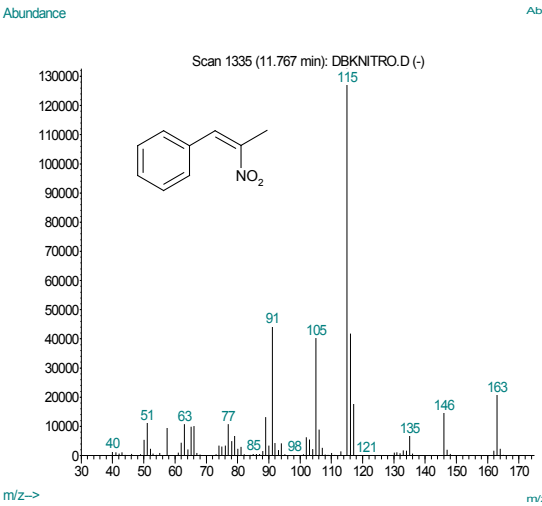
38. Ethylamphetamine



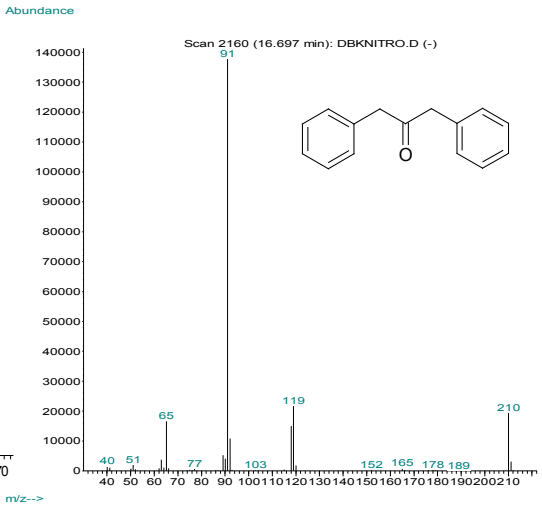
39. Isopropylamphetamine



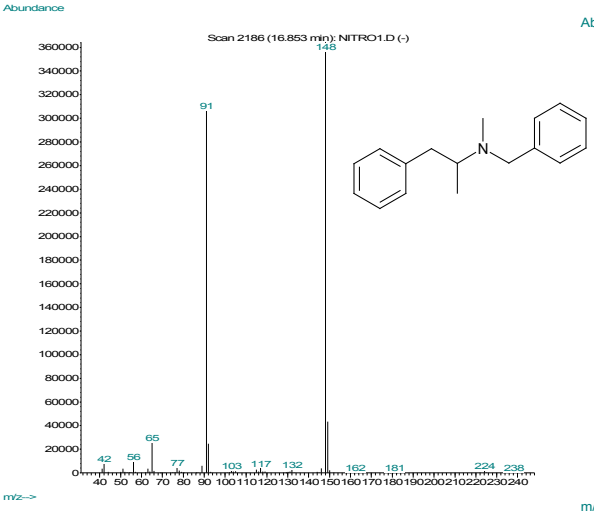
40. Phenyl-2-nitropropane



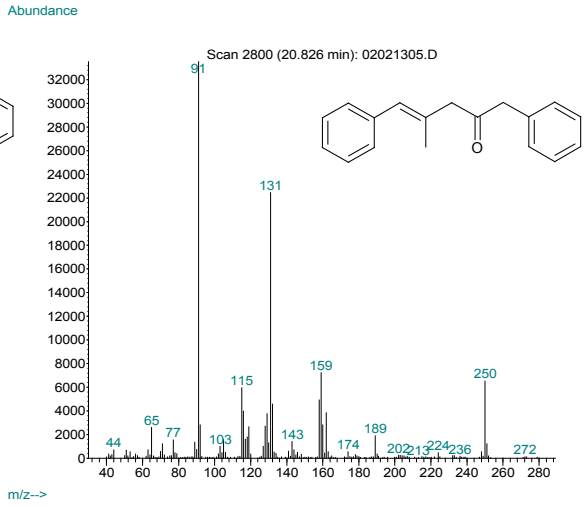
41. Phenyl-2-nitropropene



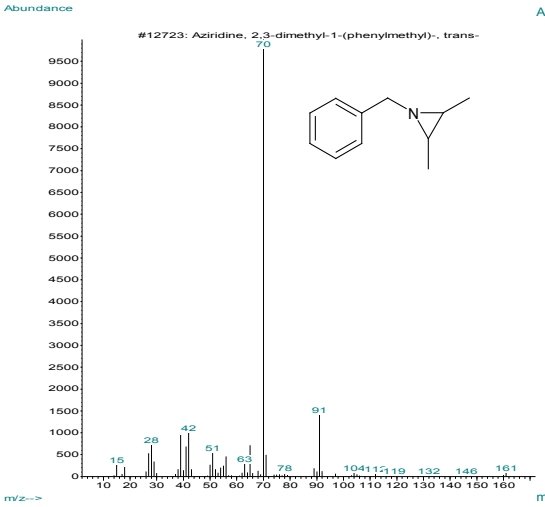
42. 1,3-diphenyl-2-propanone



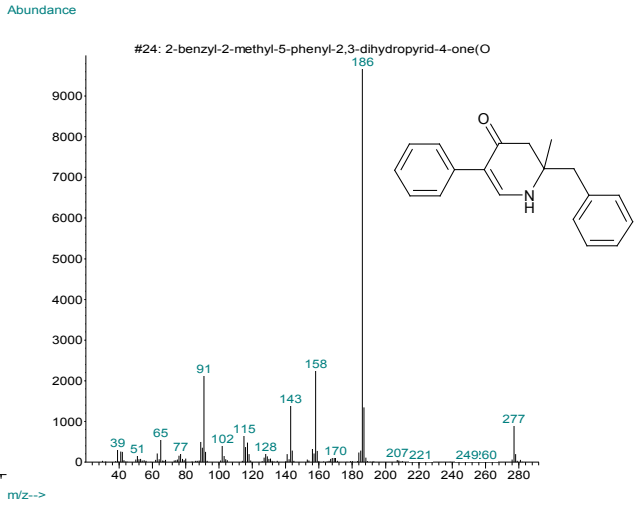
43. α-Dimethyldiphenethylamine



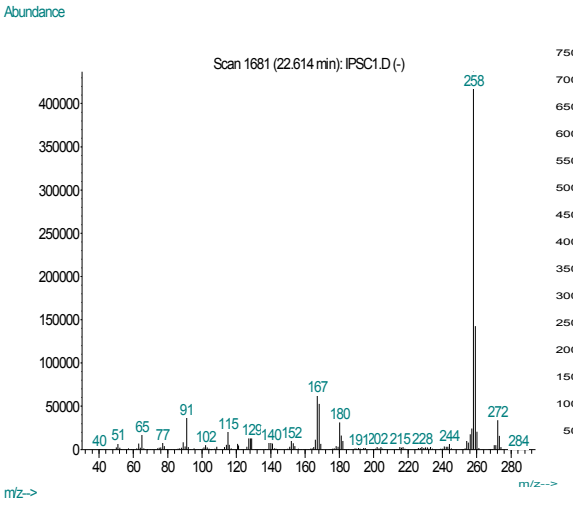
44. 1,5-Diphenyl-4-methyl-4-penten-2-one



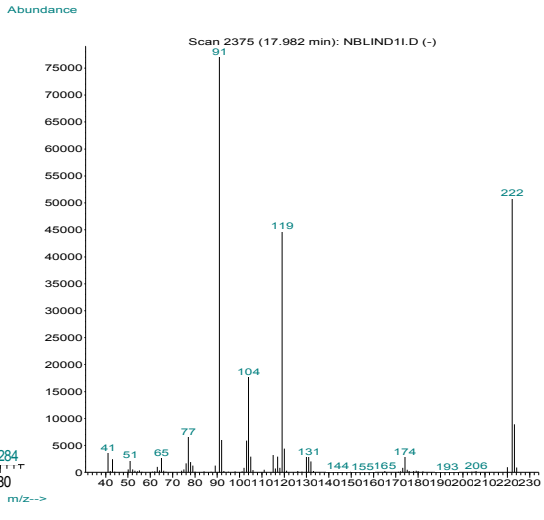
45. 1-Benzyl-2,3-dimethyl-aziridine



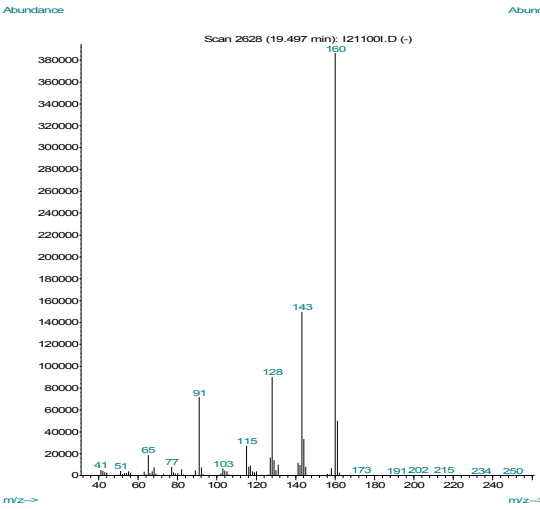
46. 2-Benzyl-2-methyl-5-phenyl-2,3-dihydropyrid-4-one



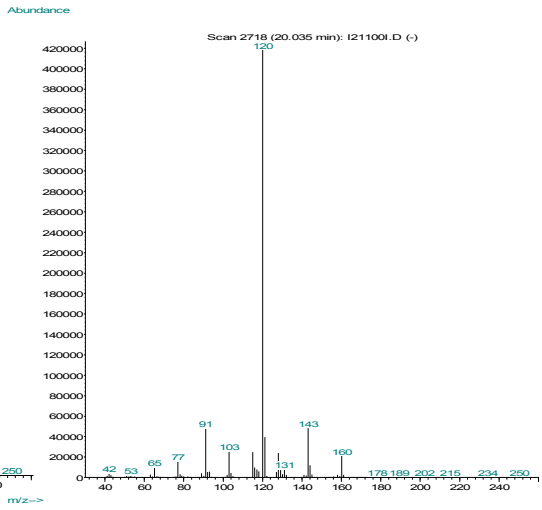
47. Pyridine X



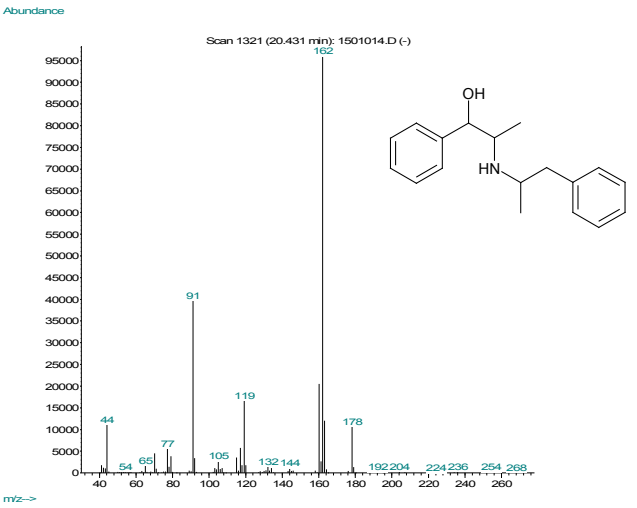
48. Unknown C



49. Unknown A1



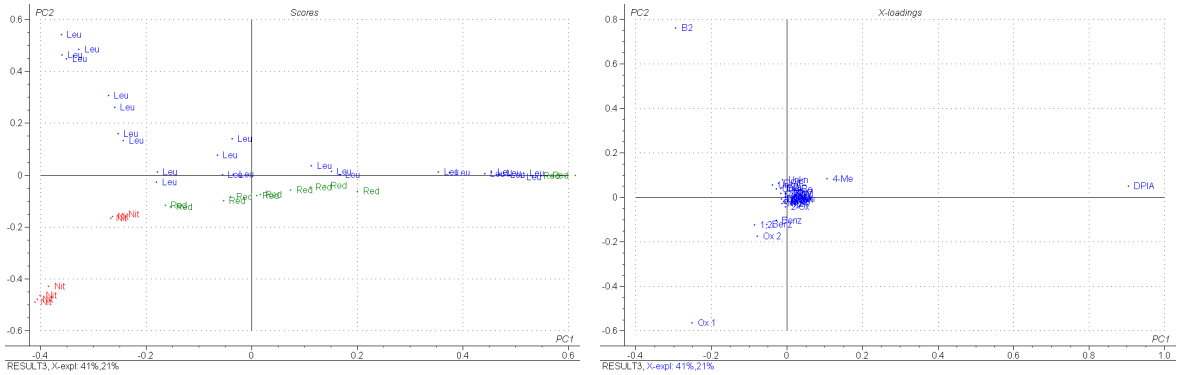
50. Unknown B1



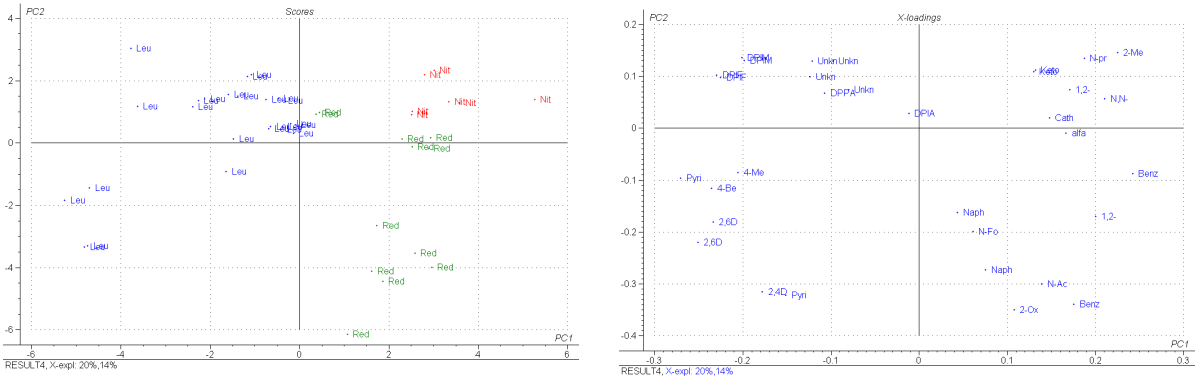
51. Cathinol 2

ANNEX 11

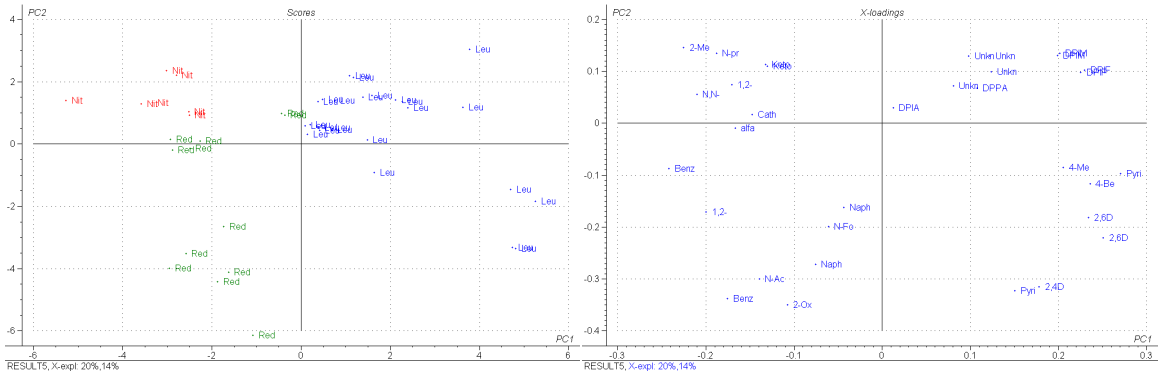
PCA SCORES AND LOADINGS PLOTS OF THE DATA SET OF 44 SAMPLES AND 33 VARIABLES. SECTION 6.6.5



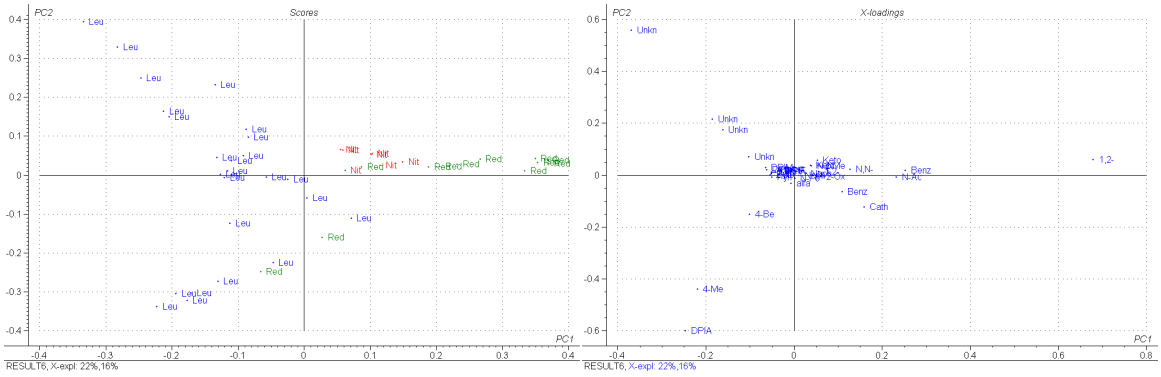
Normalisation to the sum. Alternative 2. Left : scores plot. Right : loadings plot.



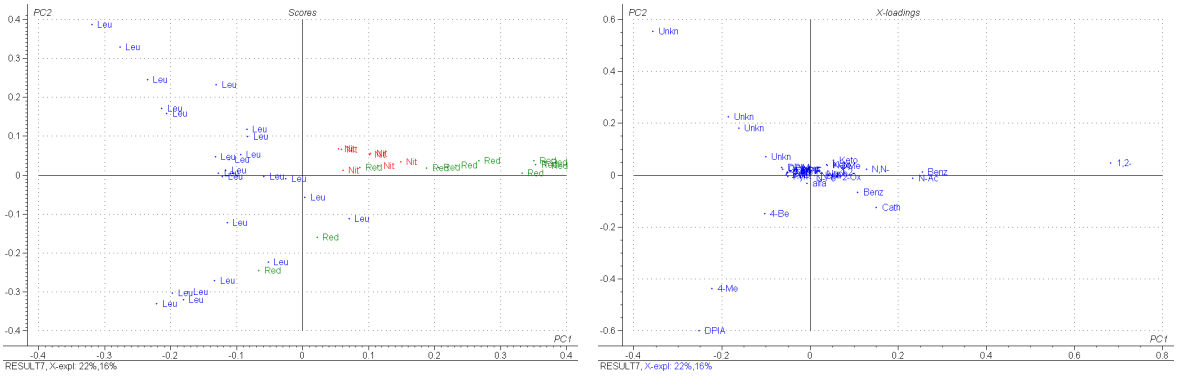
Normalisation to the sum + Weighing. Alternative 1. Left : scores plot. Right : loadings plot.



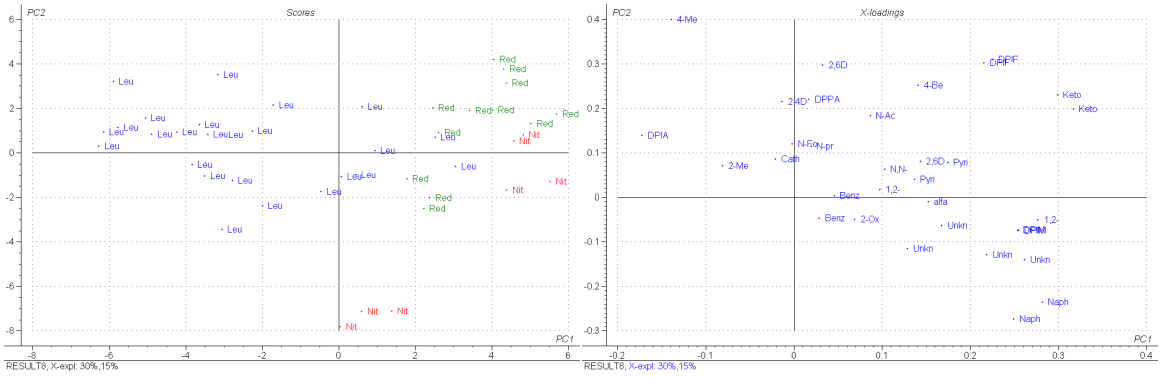
Normalisation to the sum + Weighing. Alternative 2. Left : scores plot. Right : loadings plot.



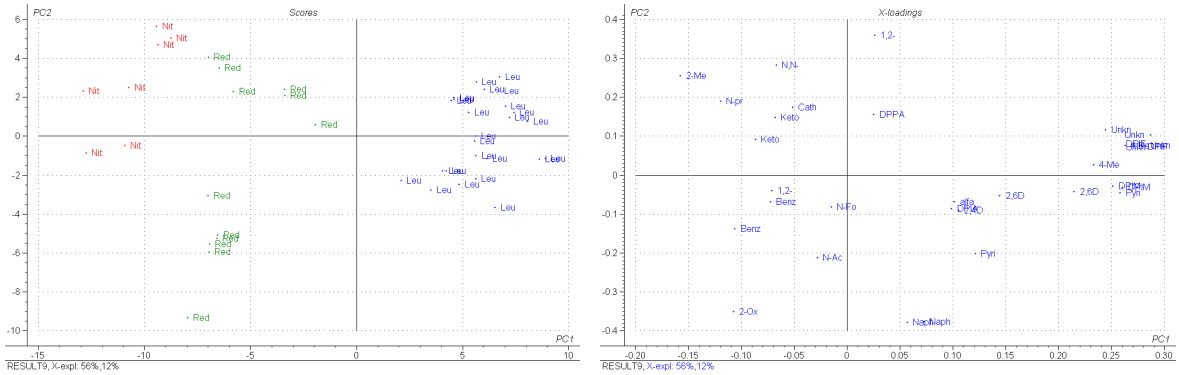
Weighing + Normalisation to the sum. Alternative 1. Left : scores plot. Right : loadings plot.



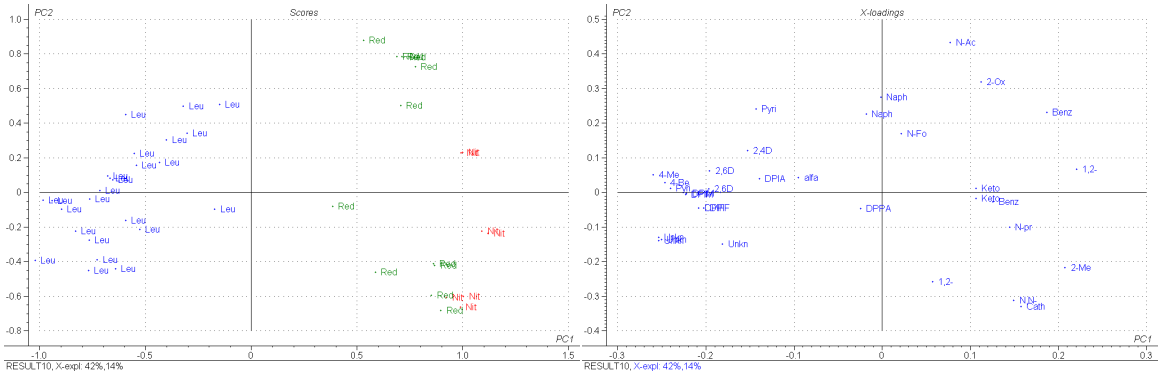
Weighing + Normalisation to the sum. Alternative 2. Left : scores plot. Right : loadings plot.



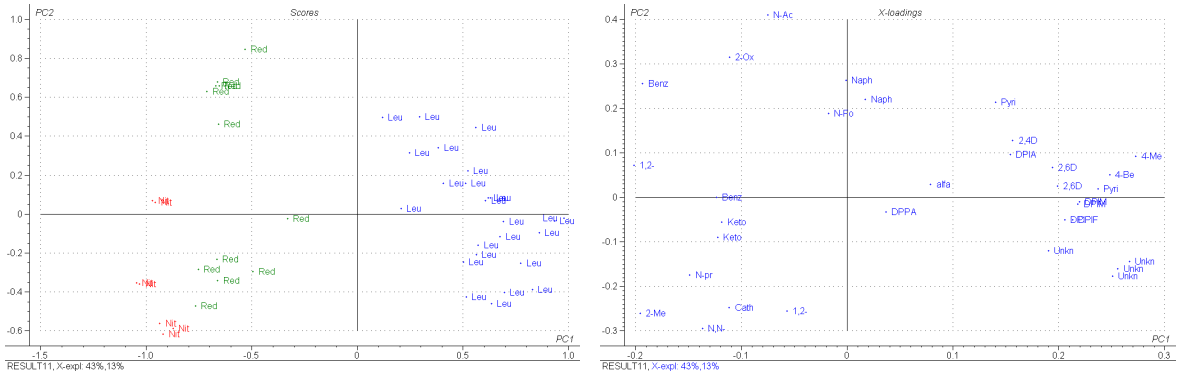
Weighing + Normalisation + Logarithm. Alternative 1. Left : scores plot. Right : loadings plot.



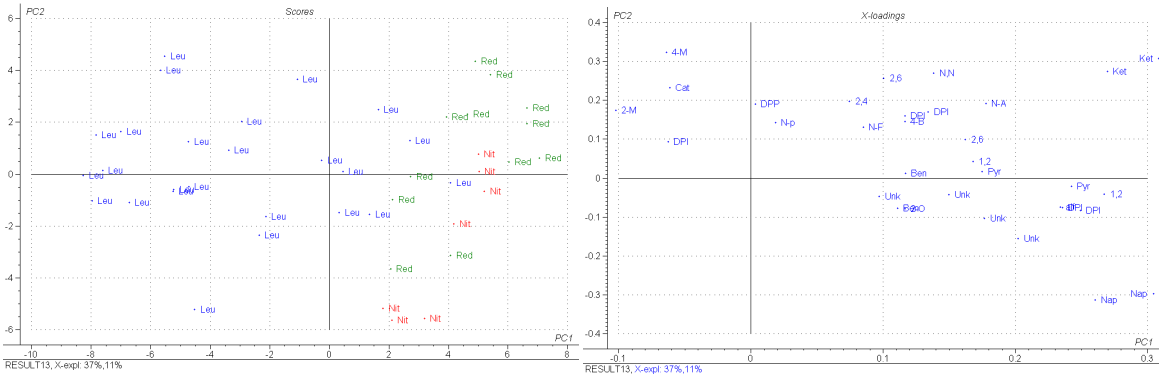
Weighing + Normalisation + Logarithm. Alternative 2. Left : scores plot. Right : loadings plot.



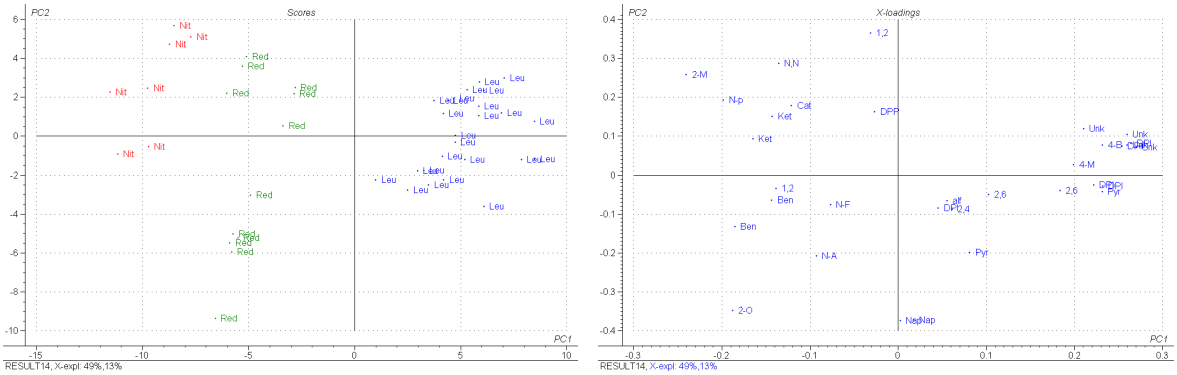
Weighing + Normalisation + 4th square root. Alternative 1. Left : scores plot. Right : loadings plot.



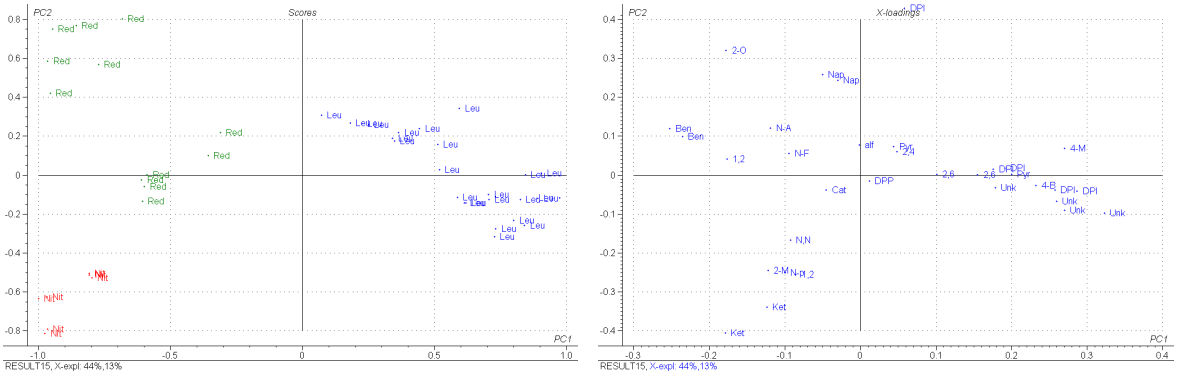
Weighing + Normalisation + 4th square root. Alternative 2. Left : scores plot. Right : loadings plot.



Normalisation + Logarithm. Alternative 1. Left : scores plot. Right : loadings plot.



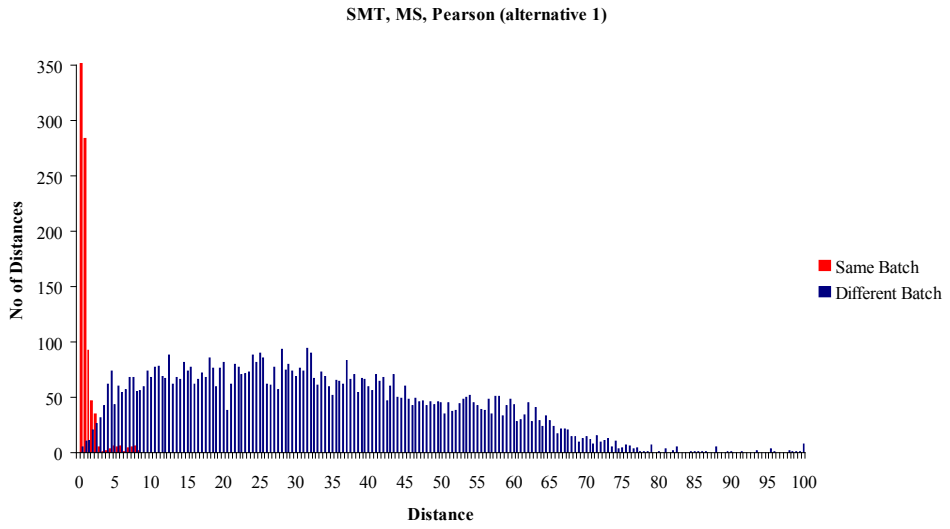
Normalisation + Logarithm. Alternative 2. Left : scores plot. Right : loadings plot.

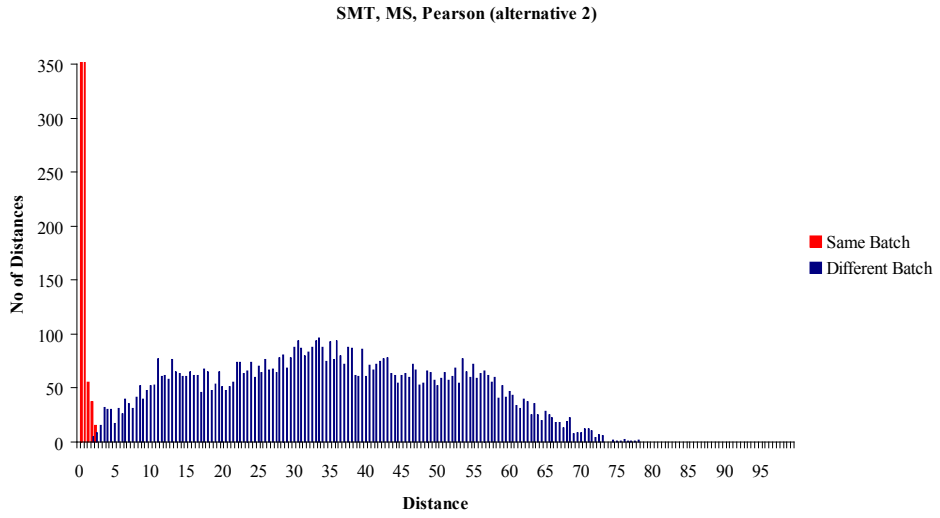


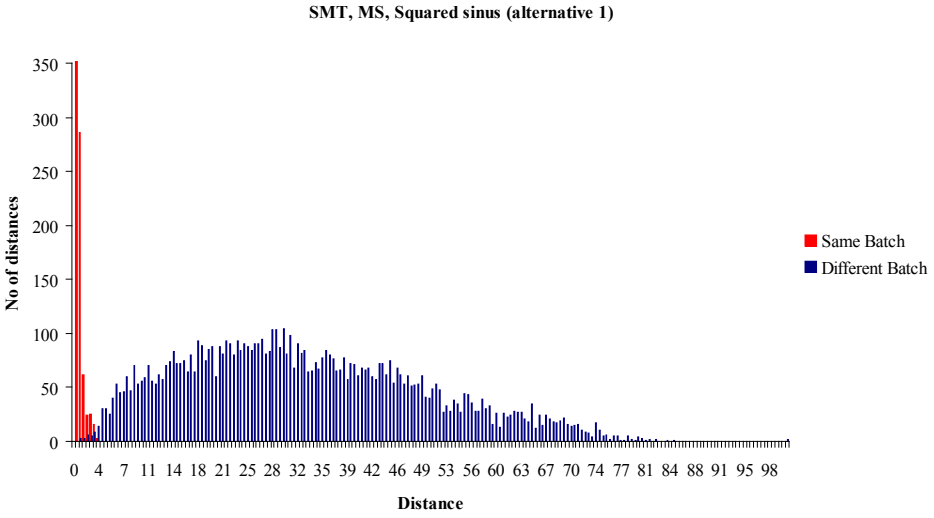
Normalisation + 4th square root. Alternative 1. Left : scores plot. Right : loadings plot.

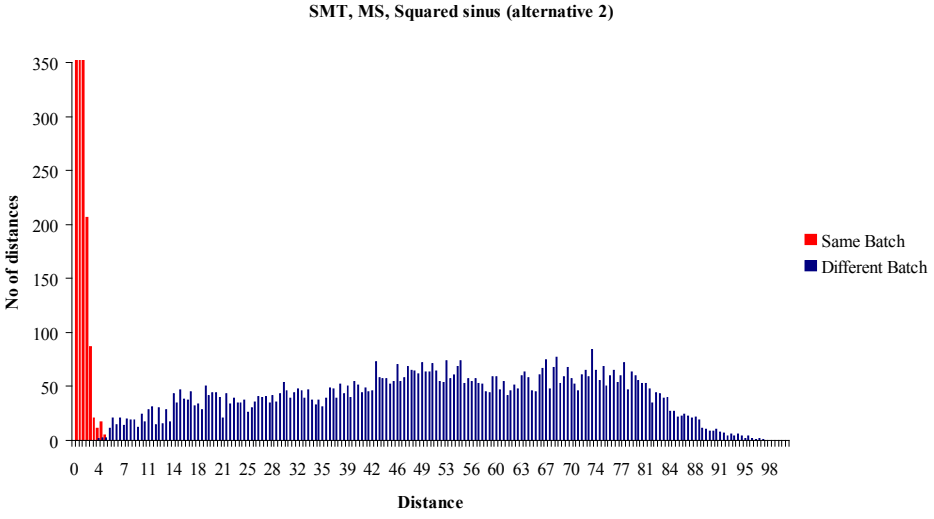
ANNEX 12

HISTOGRAM PLOTS : OVERLAPPING BETWEEN SYNTHESISED SAMPLES AND STREET SAMPLES WITH DIFFERENT DISTANCE METHODS. SECTION 6.8.2

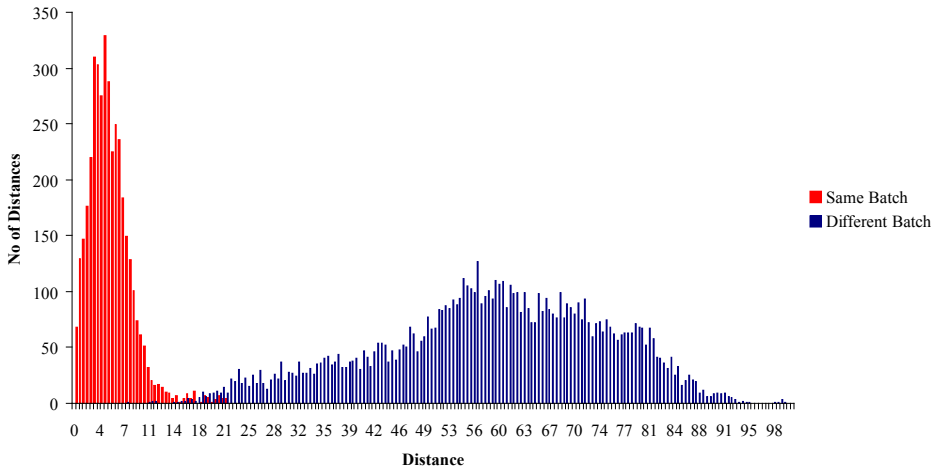




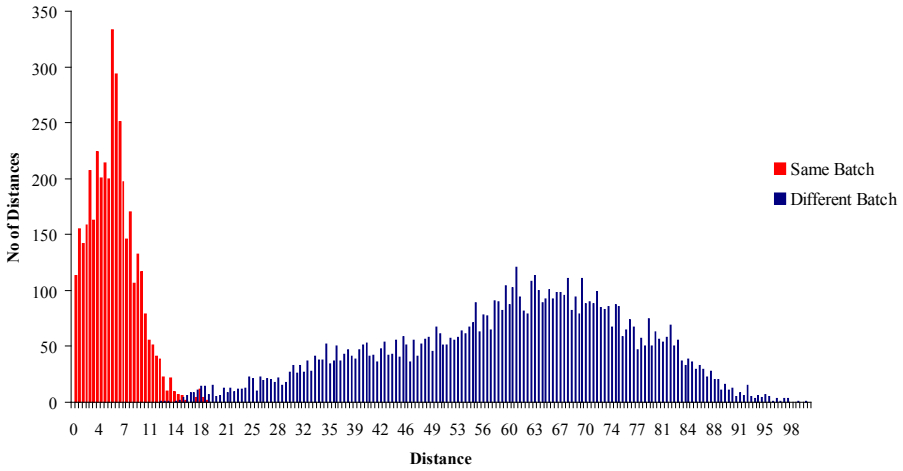

SMT, MS, Squared sinus (alternative 1)
No of distances
350
300
250
200
150
100
50
0
0 4 7 11 14 18 21 25 28 32 35 39 42 46 49 53 56 60 63 67 70 74 77 81 84 88 91 95 98
Distance
Same Batch
Different Batch


SMT, MS, Squared sinus (alternative 2)
No of distances
350
300
250
200
150
100
50
0
0 4 7 11 14 18 21 25 28 32 35 39 42 46 49 53 56 60 63 67 70 74 77 81 84 88 91 95 98
Distance
Same Batch
Different Batch

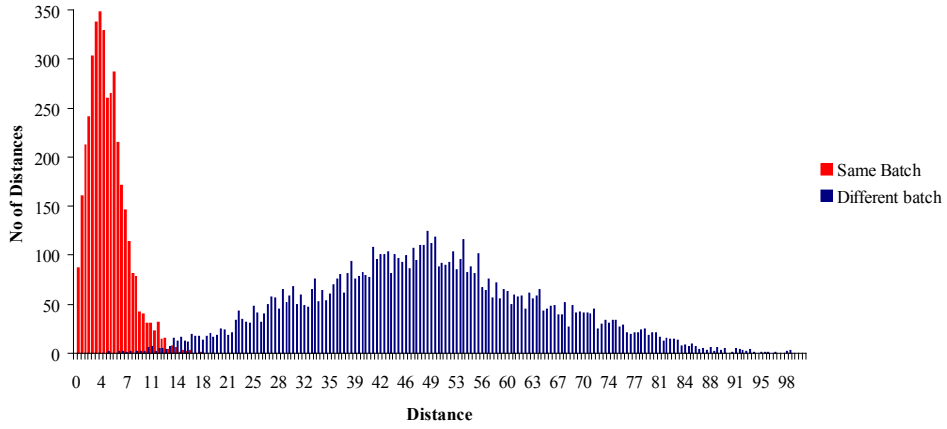
SMT, MS, Euclidian (alternative 1)



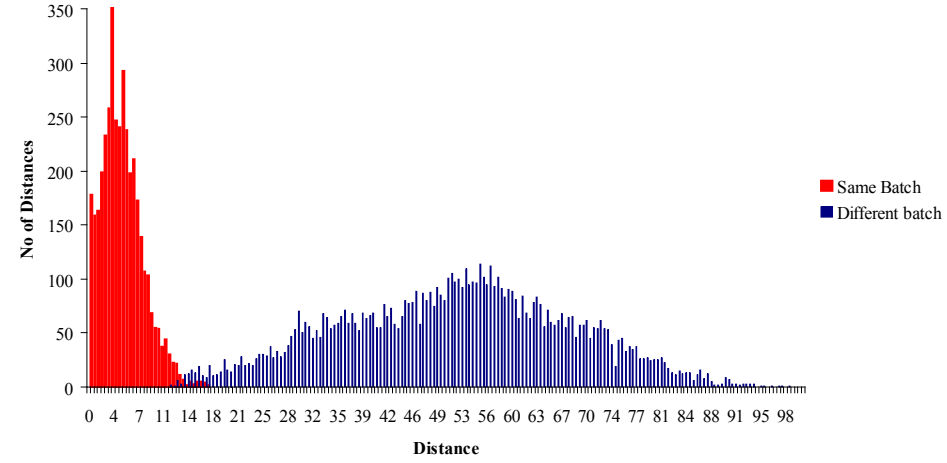
SMT, MS, Euclidian (alternative 2)

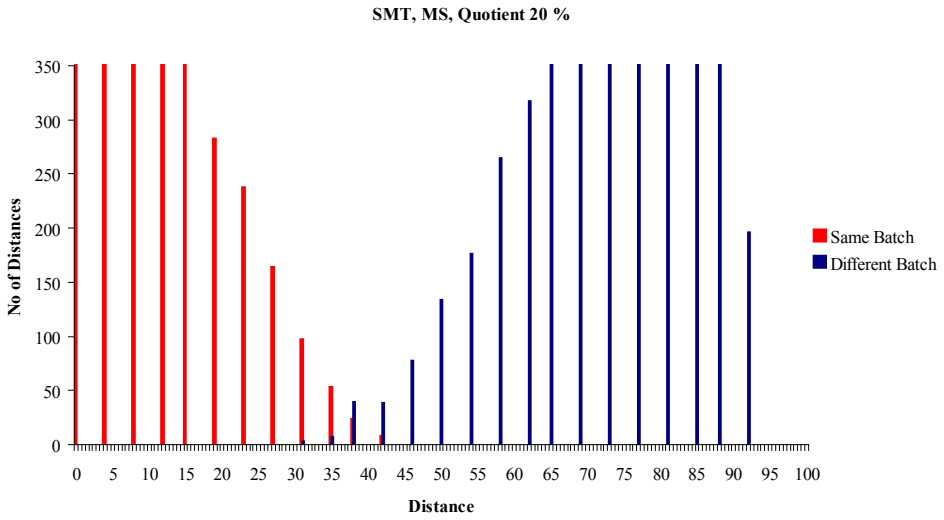


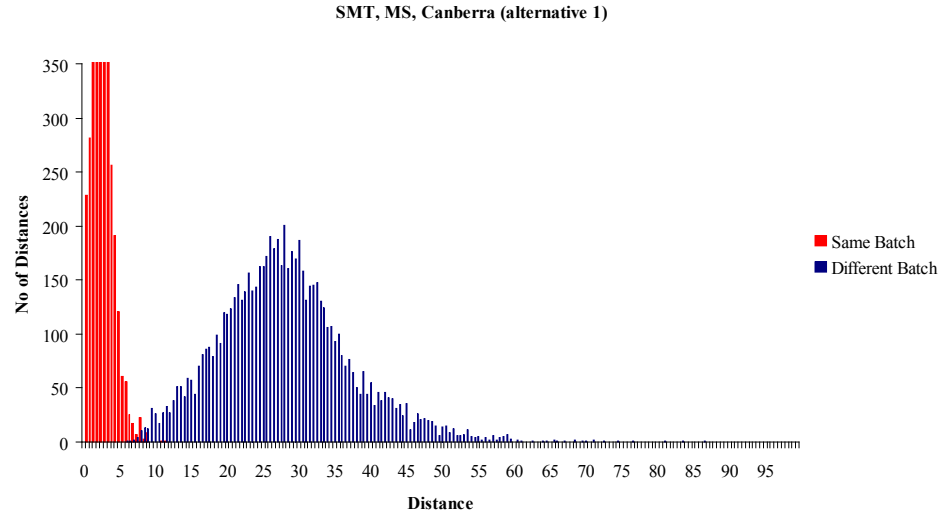
SMT, MS, Manhattan (alternative 1)

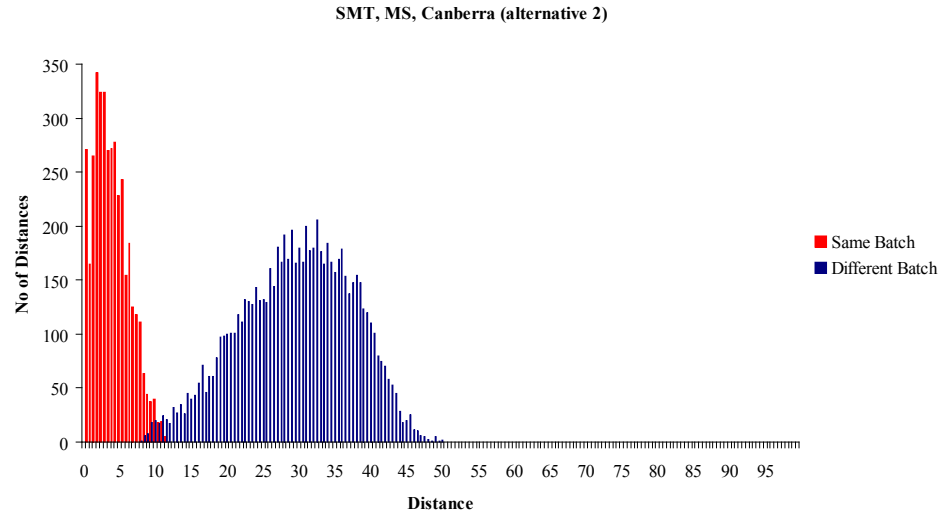


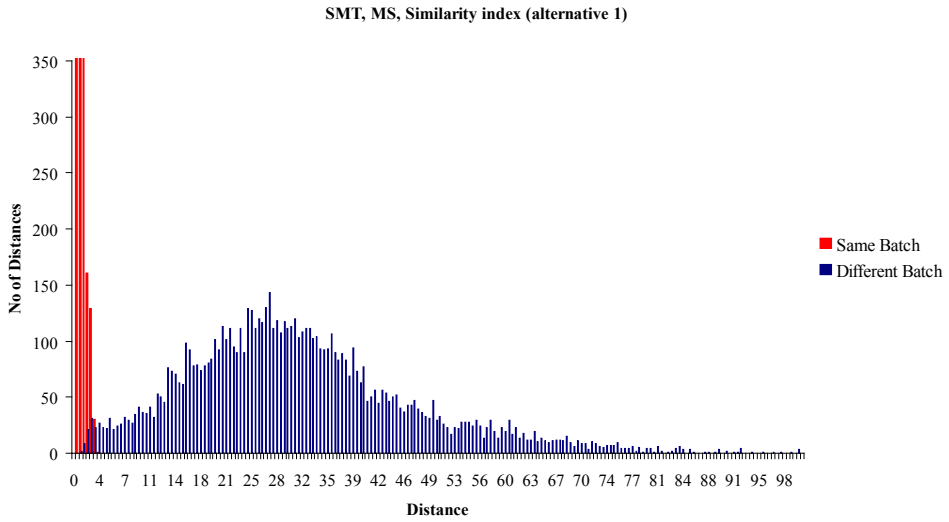
SMT, MS, Manhattan (alternative 2)

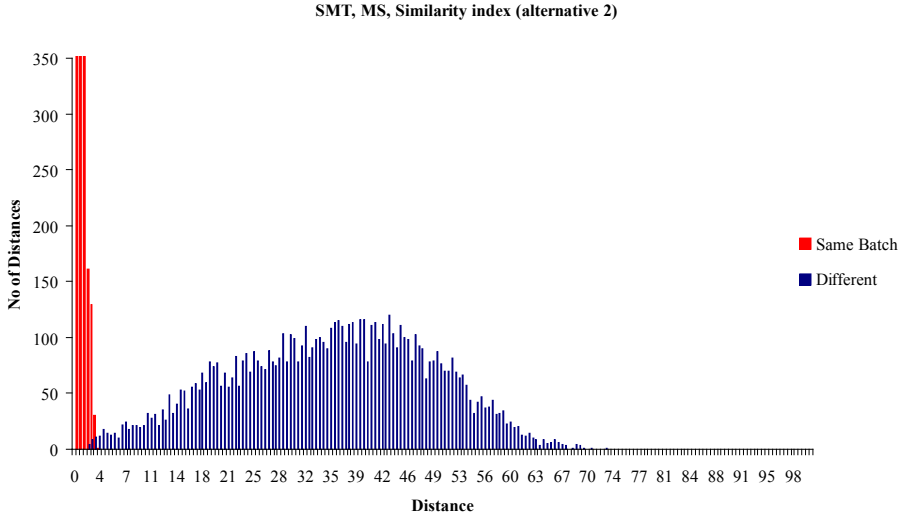



SMT, MS, Quotient 20 %
No of Distances
Distance
Same Batch
Different Batch


SMT, MS, Canberra (alternative 1)
No of Distances
Distance
Same Batch
Different Batch


SMT, MS, Canberra (alternative 2)
No of Distances
Distance
Same Batch
Different Batch


SMT, MS, Similarity index (alternative 1)
No of Distances
350
300
250
200
150
100
50
0
0 4 7 11 14 18 21 25 28 32 35 39 42 46 49 53 56 60 63 67 70 74 77 81 84 88 91 95 98
Distance
Same Batch
Different Batch


SMT, MS, Similarity index (alternative 2)
No of Distances
350
300
250
200
150
100
50
0
0 4 7 11 14 18 21 25 28 32 35 39 42 46 49 53 56 60 63 67 70 74 77 81 84 88 91 95 98
Distance
Same Batch
Different

ANNEX 13 DISTANCES BETWEEN AMPHETAMINE SAMPLES OF DIFFERENT DEGREES OF SIMILARITY.

1 MSD DATA

1.1 Pearson (alternative 2)

MS data, 0=200	Pearson, N+4root						
samples	Type of estimate	analysing lab	n	mean	SD	max	min
Control samples	Within day/sequence	Partner 2	5	0.011	0.010	0.025	0.004
Control samples	Within day/sequence	Partner 3	6	0.005	0.004	0.012	0.002
Control samples	Within day/sequence	Partner 4	6	0.008	0.006	0.015	0.001
Control samples	Within day/sequence	All	17	0.007	0.007	0.025	0.001
Control samples	Between day/sequence	Partner 2	10	0.010	0.005	0.017	0.002
Control samples	Between day/sequence	Partner 3	15	0.006	0.004	0.014	0.001
Control samples	Between day/sequence	Partner 4	15	0.004	0.003	0.009	0.001
Control samples	Between day/sequence	All	40	0.006	0.005	0.017	0.001
Control samples	Between lab	Partners 2 vs 3	30	0.053	0.012	0.070	0.031
Control samples	Between lab	Partners 2 vs 4	30	0.13	0.011	0.16	0.11
Control samples	Between lab	Partner 3 vs 4	36	0.073	0.013	0.10	0.049
Control samples	Between lab	All	96	0.086	0.036	0.16	0.031
100 % samples	Within lab, same conc	Partner 2	33	0.024	0.055	0.27	0.001
100 % samples	Within lab, same conc	Partner 3	33	0.010	0.020	0.077	0.001
100 % samples	Within lab, same conc	Partner 4	33	0.005	0.008	0.043	0.0001
100 % samples	Within lab, same conc	All	99	0.013	0.035	0.27	0.0001
40 % samples	Within lab, same conc	Partner 2	27	0.024	0.064	0.25	0.001
40 % samples	Within lab, same conc	Partner 3	24	0.008	0.011	0.038	0.001
40 % samples	Within lab, same conc	Partner 4	24	0.031	0.059	0.20	0.0004
40 % samples	Within lab, same conc	All	75	0.021	0.051	0.25	0.0004
5 % samples	Within lab, same conc	Partner 2	33	0.18	0.42	1.7	0.001
5 % samples	Within lab, same conc	Partner 3	33	0.033	0.037	0.13	0.001
5 % samples	Within lab, same conc	IPSC	33	0.021	0.031	0.12	0.001
5 % samples	Within lab, same conc	All	99	0.077	0.25	1.7	0.001
5, 40 and 100% samples	Within lab, same conc	All	273	0.038	0.16	1.7	0.0001
100 % samples	Between lab, same conc	All	33	0.26	0.20	0.84	0.016
40 % samples	Between lab, same conc	All	23	0.25	0.25	0.82	0.015
5 % samples	Between lab, same conc	All	33	0.34	0.37	1.6	0.027
5, 40 and 100% samples	Between lab, same conc	All	89	0.28	0.28	1.6	0.015
100% vs 40%	Within lab, different conc.	Partner 2	9	0.13	0.11	0.36	0.018
100% vs 40%	Within lab, different conc.	Partner 3	8	0.093	0.11	0.33	0.034
100% vs 40%	Within lab, different conc.	Partner 4	8	0.12	0.12	0.33	0.013
100% vs 40%	Within lab, different conc.	All	25	0.12	0.11	0.36	0.013
100% vs 5%	Within lab, different conc.	Partner 2	11	0.54	0.38	1.3	0.072
100% vs 5%	Within lab, different conc.	Partner 3	11	0.41	0.26	0.97	0.063
100% vs 5%	Within lab, different conc.	Partner 4	11	0.38	0.22	0.63	0.048
100% vs 5%	Within lab, different conc.	All	33	0.45	0.29	1.3	0.048
40% vs 5%	Within lab, different conc.	Partner 2	9	0.42	0.29	0.86	0.041
40% vs 5%	Within lab, different conc.	Partner 3	8	0.25	0.13	0.45	0.033
40% vs 5%	Within lab, different conc.	Partner 4	8	0.35	0.22	0.60	0.038
40% vs 5%	Within lab, different conc.	All	25	0.34	0.23	0.86	0.033
100% vs 40%	Between lab, different conc.	All	50	0.27	0.24	1.1	0.009
100% vs 5%	Between lab, different conc.	All	66	0.58	0.40	2.1	0.051
40% vs 5%	Between lab, different conc.	All	50	0.45	0.42	2.1	0.034
Repeated synthesis, replicates	Within lab	Partner 3 and 4	36	0.041	0.14	0.61	0.001
Repeated synthesis	Within lab	Partner 3	15	1.3	1.1	3.5	0.22
Repeated synthesis	Within lab	Partner 4	15	0.94	1.1	4.0	0.032
Repeated synthesis	Within lab	Partner 3 and 4	30	1.1	1.1	4.0	0.032
Repeated synthesis	Between lab	Partner 3 and 4	36	3.1	1.7	7.5	0.70
Same oil precipitated at diff pH	Within lab/operator	Partner 3	36	1.2	1.4	3.8	0.004
100 % samples	Diff. recipe within synth route	Partner 3	19	15.4	9.1	37.6	3.5
100 % samples	Between synthetic route	Partner 3	36	47.5	13.5	69.4	14.0

1.2 Squared sinus (alternative 2)

MS data, 0=200	Squared sinus, N+4root						
samples	**Type of estimate**	**analysing lab**	**n**	**mean**	**SD**	**max**	**min**
Control samples	Within day/sequence	Partner 2	5	0.023	0.020	0.055	0.009
Control samples	Within day/sequence	Partner 3	6	0.011	0.013	0.036	0.002
Control samples	Within day/sequence	Partner 4	6	0.011	0.008	0.021	0.003
Control samples	Within day/sequence	All	17	0.015	0.014	0.055	0.002
Control samples	Between day/sequence	Partner 2	10	0.017	0.009	0.030	0.002
Control samples	Between day/sequence	Partner 3	15	0.012	0.010	0.028	0.001
Control samples	Between day/sequence	Partner 4	15	0.005	0.004	0.013	0.001
Control samples	Between day/sequence	All	40	0.011	0.009	0.030	0.001
Control samples	Between lab	Partner 2 vs Partner 3	30	0.12	0.032	0.18	0.070
Control samples	Between lab	Partner 2 vs Partner 4	30	0.39	0.031	0.44	0.32
Control samples	Between lab	Partner 3 vs Partner 4	36	0.16	0.036	0.22	0.096
Control samples	Between lab	All	96	0.22	0.12	0.44	0.070
100 % samples	Within lab, same conc	Partner 2	33	0.041	0.090	0.44	0.001
100 % samples	Within lab, same conc	Partner 3	33	0.022	0.045	0.18	0.001
100 % samples	Within lab, same conc	Partner 4	33	0.009	0.015	0.079	0.00005
100 % samples	Within lab, same conc	All	99	0.024	0.060	0.44	0.00005
40 % samples	Within lab, same conc	Partner 2	27	0.031	0.070	0.28	0.001
40 % samples	Within lab, same conc	Partner 3	24	0.016	0.020	0.063	0.001
40 % samples	Within lab, same conc	Partner 4	24	0.049	0.089	0.30	0.001
40 % samples	Within lab, same conc	All	75	0.032	0.067	0.30	0.001
5 % samples	Within lab, same conc	Partner 2	33	0.26	0.55	2.2	0.002
5 % samples	Within lab, same conc	Partner 3	33	0.065	0.088	0.36	0.001
5 % samples	Within lab, same conc	IPSC	33	0.041	0.060	0.21	0.001
5 % samples	Within lab, same conc	All	99	0.12	0.34	2.2	0.001
5, 40 and 100% samples	Within lab, same conc	All	273	0.062	0.21	2.2	0.00005
100 % samples	Between lab, same conc	All	33	0.49	0.39	1.6	0.040
40 % samples	Between lab, same conc	All	23	0.39	0.36	1.3	0.041
5 % samples	Between lab, same conc	All	33	0.63	0.63	2.1	0.056
5, 40 and 100% samples	Between lab, same conc	All	89	0.52	0.49	2.1	0.040
100% vs 40%	Within lab, different conc.	Partner 2	9	0.24	0.18	0.56	0.055
100% vs 40%	Within lab, different conc.	Partner 3	8	0.20	0.20	0.59	0.054
100% vs 40%	Within lab, different conc.	Partner 4	8	0.24	0.23	0.73	0.079
100% vs 40%	Within lab, different conc.	All	25	0.22	0.20	0.73	0.054
100% vs 5%	Within lab, different conc.	Partner 2	11	1.1	0.67	2.4	0.084
100% vs 5%	Within lab, different conc.	Partner 3	11	0.97	0.57	1.9	0.16
100% vs 5%	Within lab, different conc.	Partner 4	11	0.88	0.51	1.7	0.086
100% vs 5%	Within lab, different conc.	All	33	0.99	0.58	2.4	0.084
40% vs 5%	Within lab, different conc.	Partner 2	9	0.90	0.44	1.6	0.18
40% vs 5%	Within lab, different conc.	Partner 3	8	0.66	0.32	1.2	0.25
40% vs 5%	Within lab, different conc.	Partner 4	8	0.76	0.29	1.1	0.18
40% vs 5%	Within lab, different conc.	All	25	0.78	0.36	1.6	0.18
100% vs 40%	Between lab, different conc.	All	50	0.47	0.35	1.7	0.035
100% vs 5%	Between lab, different conc.	All	66	1.3	0.75	4.0	0.33
40% vs 5%	Between lab, different conc.	All	50	0.95	0.67	3.7	0.20
Repeated synthesis, replicates	Within lab	Partner 3 and 4	36	0.056	0.18	0.81	0.002
Repeated synthesis	Within lab	Partner 3	15	2.1	1.8	5.7	0.32
Repeated synthesis	Within lab	Partner 4	15	1.7	1.9	7.2	0.051
Repeated synthesis	Within lab	Partner 3 and 4	30	1.9	1.8	7.2	0.051
Repeated synthesis	Between lab	Partner 3 and 4	36	4.9	2.9	12.8	1.1
Same oil precipitated at diff pH	Within lab/operator	Partner 3	36	2.4	2.6	7.3	0.008
100 % samples	Diff. recipe within synth route	Partner 3	19	25.6	12.3	52.2	7.8
100 % samples	Between synthetic route	Partner 3	36	71.3	15.1	93.4	31.7

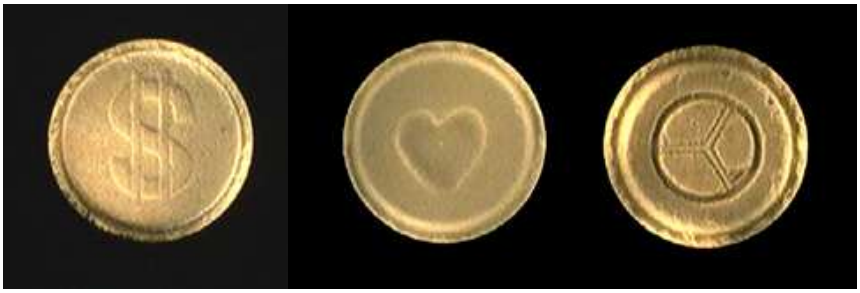
ANNEX 14

LINKS DETERMINED BY ORGANIC PROFILING BETWEEN AMPHETAMINE TABLETS WITH DIFFERENT EXTERNAL CHARACTERISTICS

CASE 1

Colour :	light yellow	white	white
Diameter :	9 mm	9.1 mm	9.1 mm
Width :	3.4 mm	3.3 mm	3.2 mm
Weight :	275 mg	250 mg	252 mg
Amount of amphetamine :	16 mg	12 mg	n.a.
Adulterant(s) :	caffeine	caffeine	caffeine
Diluent(s) :	-	-	-
Shape code :	RBB	RBB	RBB
Breakline :	YES	YES	YES
Location and year of seizure:	ZH, 1999	ZH, 1999	VD, 1998
Internal number .	990a	990b	744

CASE 2

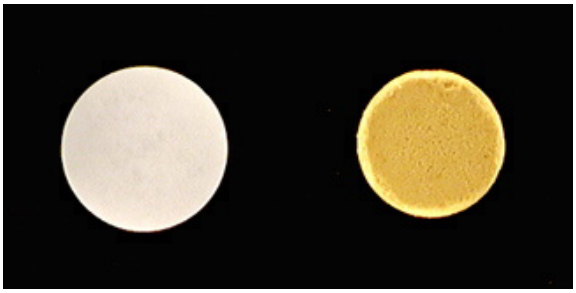


Colour :	beige	beige	beige
Diameter :	11.3 mm	11.3 mm	11.2 mm
Width :	3.6 mm	3.5 mm	3.7 mm
Weight :	455 mg	452 mg	456 mg
Amount of amphetamine :	51 mg	44 mg	51 mg
Adulterant(s) :	-	-	-
Diluent(s) :	glucose, sucrose	glucose, sucrose	glucose, sucrose
Shape code :	RCC	RCC	RCC
Breakline :	NO	NO	NO
Location and year of seizure:	ZH, 1999	ZH, 1999	ZH, 1999
Internal number .	894i	894g	910a

CASE 3

Colour :	light green	light violet	light violet
Diameter :	8.6 mm	8.6 mm	8.7 mm
Width :	5.3 mm	5.4 mm	5.3 mm
Weight :	398 mg	398 mg	392 mg
Amount of amphetamine :	15 mg	18 mg	13 mg
Adulterant(s) :	caffeine	caffeine	caffeine
Diluent(s) :	lactose	lactose	lactose
Shape code :	RCC	RCC	RCC
Breakline :	YES	YES	YES
Location and year of seizure:	ZH, 1997	ZH, 1997	ZH, 1997
Internal number .	540	543	561

CASE 4



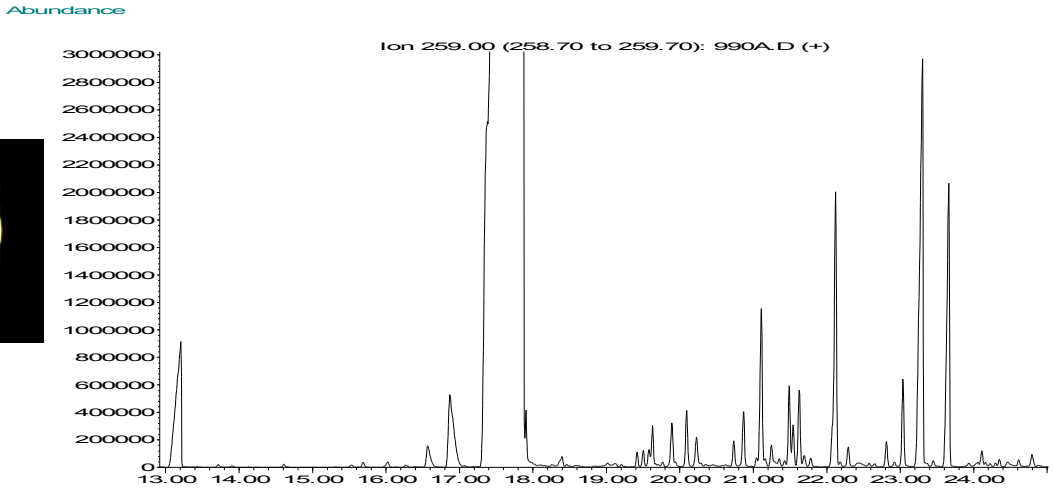
Colour :	white	yellow
Diameter :	9.1 mm	8.6 mm
Width :	3.5 mm	5.4 mm
Weight :	224 mg	203 mg
Amount of amphetamine :	22 mg	18 mg
Adulterant(s) :	caffeine, 1-PEA*	caffeine, 1-PEA*
Diluent(s) :	-	-
Shape code :	R ? ?	R ? ?
Breakline :	NO	YES
Location and year of seizure:	ZH, 1995	ZH, 1995
Internal number .	084	093

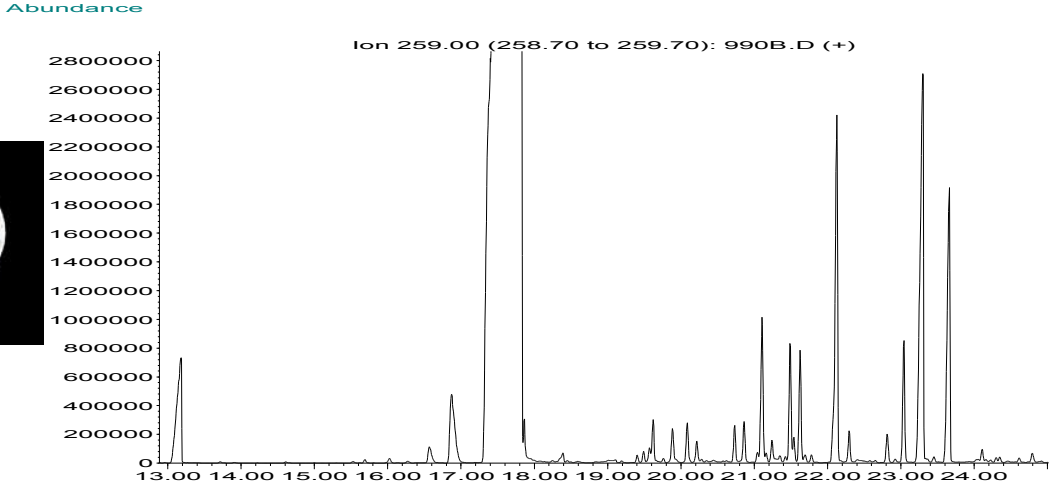
Note : see Annex 19 for explanation of the shape code.

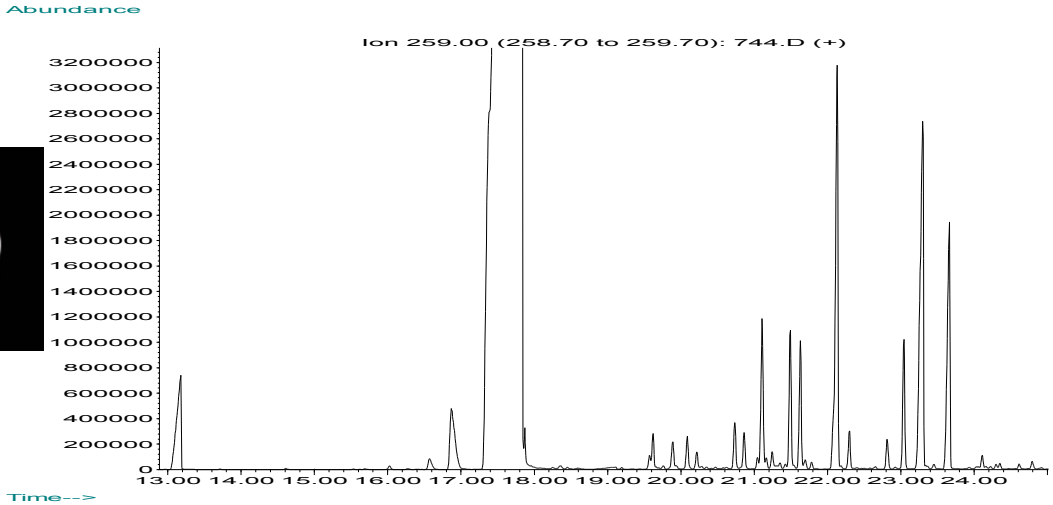
* 1-PEA is the short name for 1-phenethylamine. This compound is sometimes found in illicit amphetamine [Blachut et al., 2001, King, 1996]

ANNEX 14A

EXTRACTED ION CHROMATOGRAMS OF AMPHETAMINE TABLETS : CASE 1 from annex 14

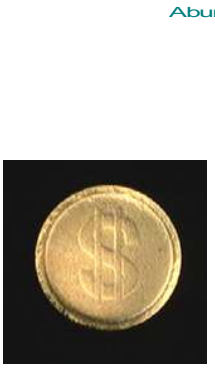


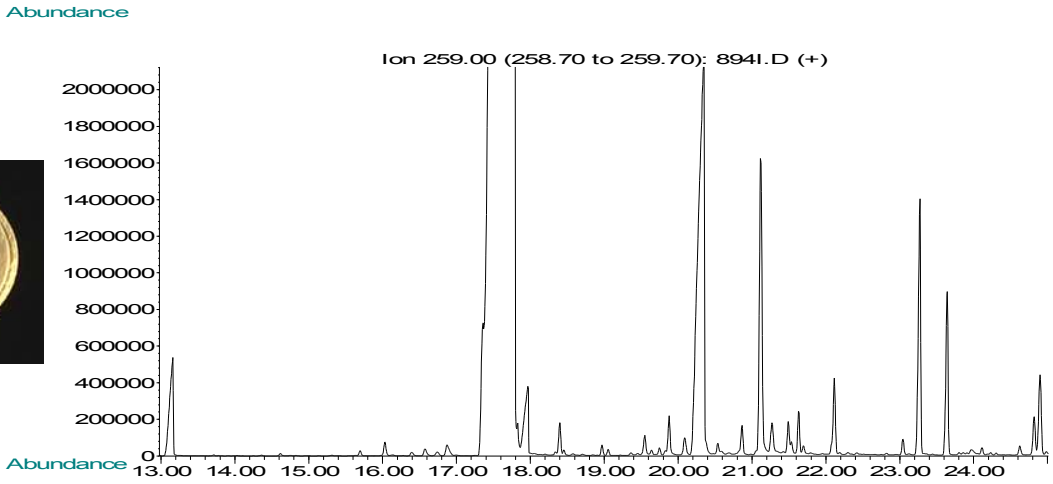


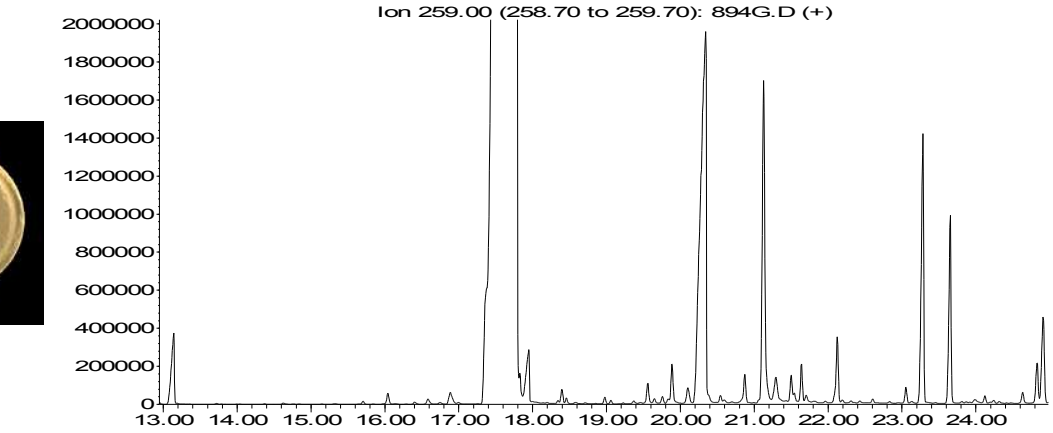


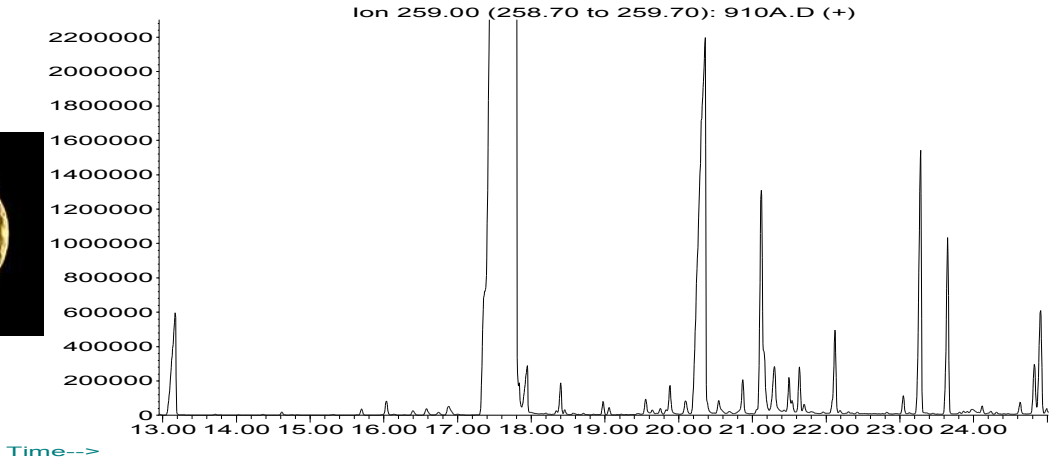
Note : these three profiles are considered to be a match (Pearson correlations of 0.10, 0.18 and 0.49).

EXTRACTED ION CHROMATOGRAMS OF AMPHETAMINE TABLETS : CASE 2 from annex 14



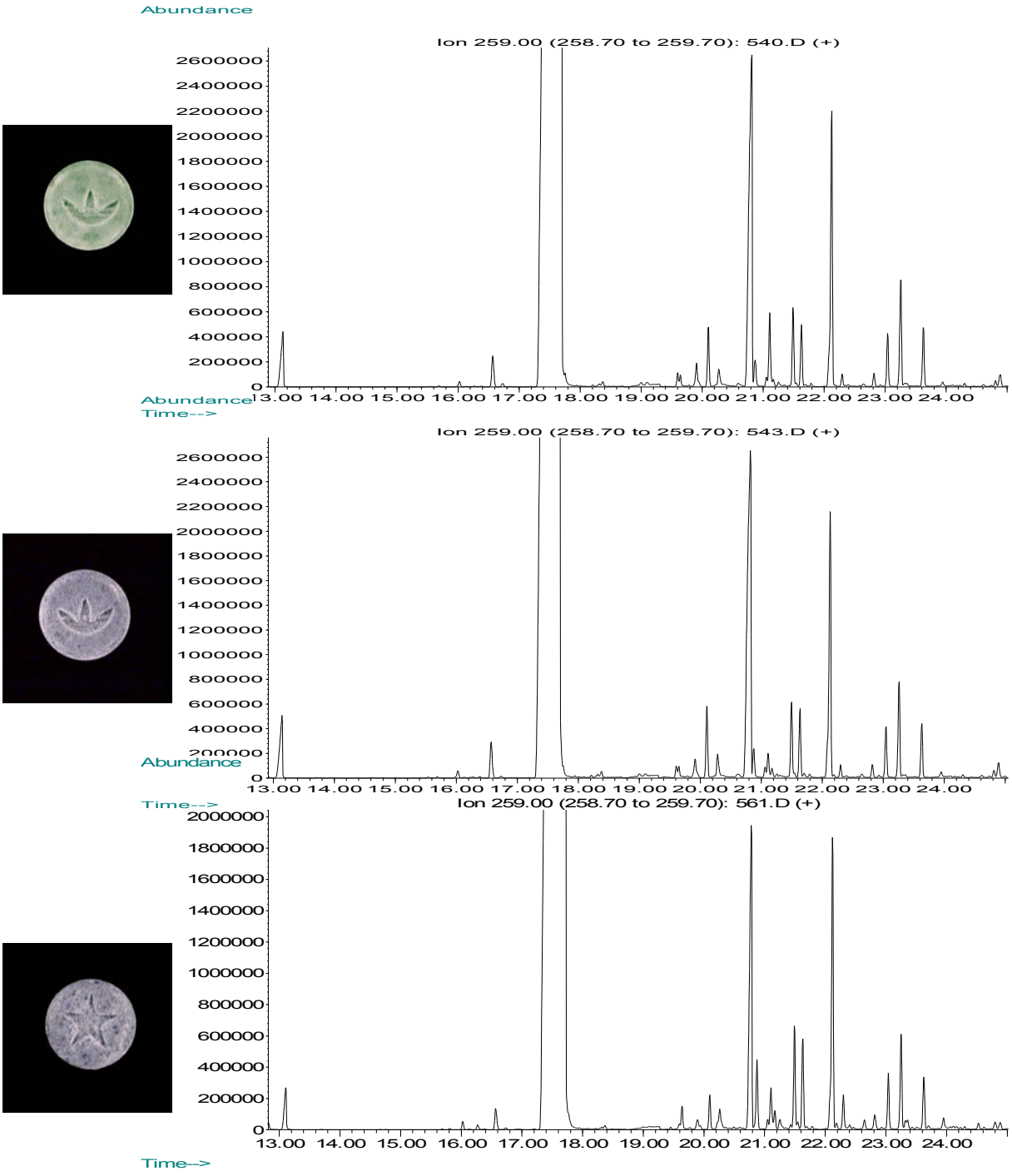






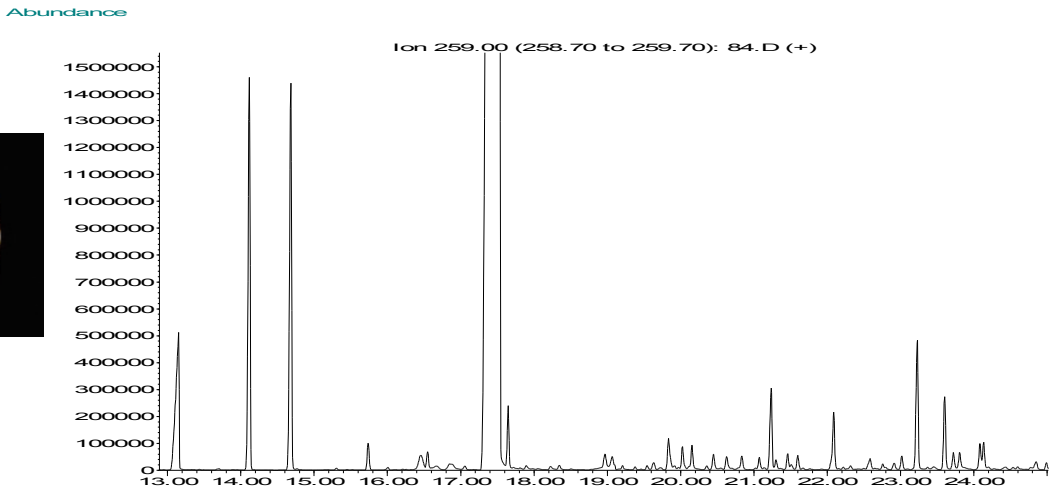
Note : these three profiles are considered to be a match (Pearson correlations of 0.05, 0.09 and 0.10).

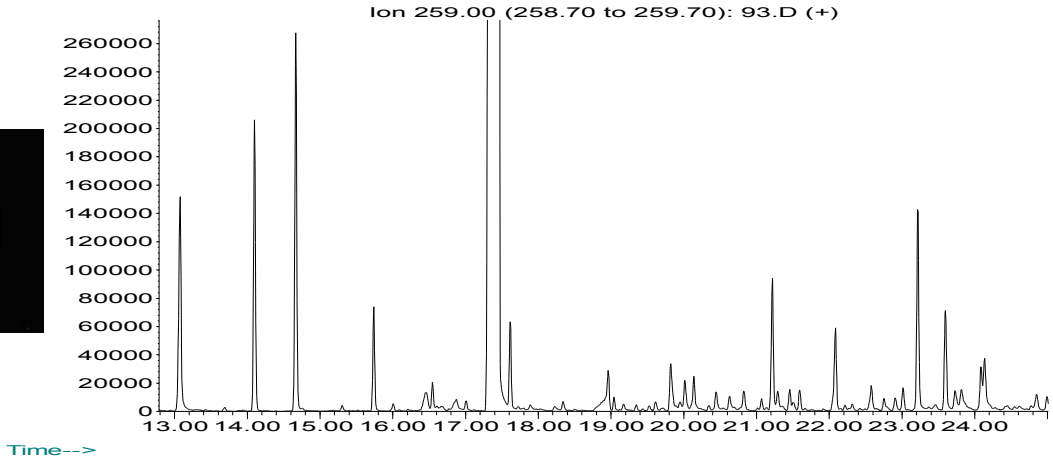
EXTRACTED ION CHROMATOGRAMS OF AMPHETAMINE TABLETS : CASE 3 from annex 14



Note : these three profiles are considered to be a match (Pearson correlations of 0.18, 0.84 and 0.84).

EXTRACTED ION CHROMATOGRAMS OF AMPHETAMINE TABLETS : CASE 4 from annex 14





Note : these two profiles are considered to be a match (Pearson correlation of 0.21).

ANNEX 15

AMPHETAMINE TABLETS SHOWING THE SAME VISUAL, PHYSICAL AND CHEMICAL CHARACTERISTICS BUT WHERE ORGANIC PROFILES DIFFER SIGNIFICANTLY

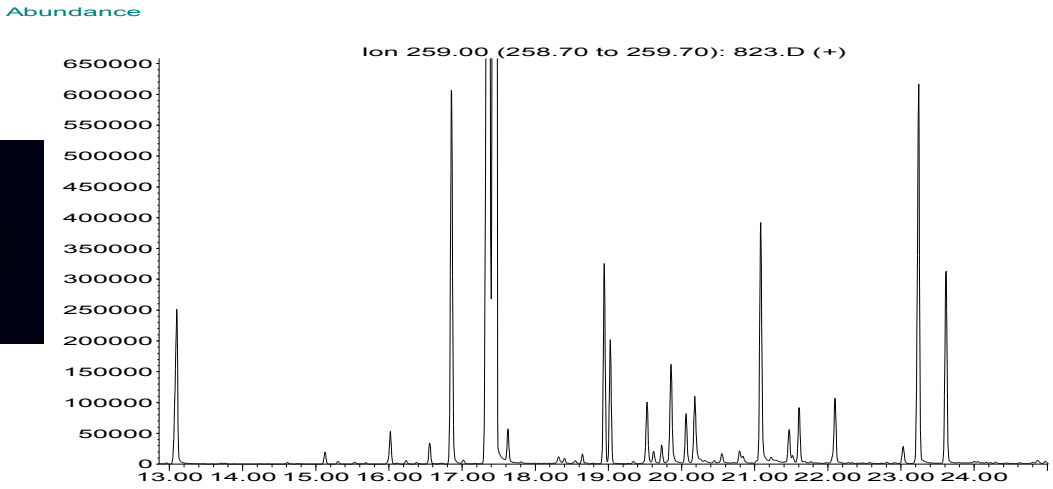
CASE 5

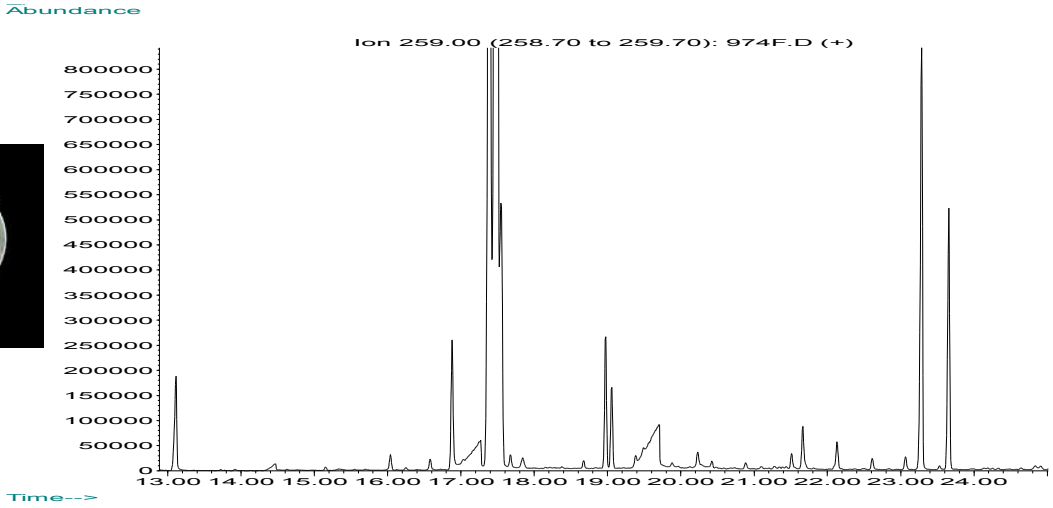
Colour :	light blue	light blue
Diameter :	9.1 mm	9.1 mm
Width :	3.9 mm	3.9 mm
Weight :	292 mg	288 mg
Amount of amphetamine :	21 mg	22 mg
Adulterant(s) :	-	-
Diluent(s) :	lactose, glucose (traces)	lactose, glucose (traces)
Shape code :	RAA	RAA
Breakline :	YES	YES
Location and year of seizure:	NE, Oct. 1998	NE, Jan. 2000
Internal number .	823	974f

Note : see Annex 19 for explanation of the shape code.

ANNEX 15A

EXTRACTED ION CHROMATOGRAMS OF AMPHETAMINE TABLETS : CASE 5 from annex 15





Note : these two profiles show strong dissimilarities (Pearson correlation 5.85).

ANNEX 16

AMPHETAMINE TABLETS WITH SIMILAR VISUAL AND PHYSICAL CHARACTERISTICS BUT WITH SLIGHT DIFFERENCES IN CHEMICAL COMPOSITION INCLUDING THE ORGANIC PROFILES

CASE 6

Colour :	light green	light green	light green
Diameter :	9.1 mm	9.1 mm	9.2 mm
Width :	3.4 mm	3.3 mm	3.5 mm
Weight :	323 mg	315 mg	320 mg
Amount of amphetamine :	18 mg	18 mg	26 mg
Adulterant(s) :	caffeine	caffeine	caffeine
Diluent(s) :	-	-	**lactose**
Shape code :	RAA	RAA	RAA
Breakline :	YES	YES	YES
Location and year of seizure:	ZH, 1998	ZH, 1999	ZH, 2001
Internal number .	Z2B	885a	1461

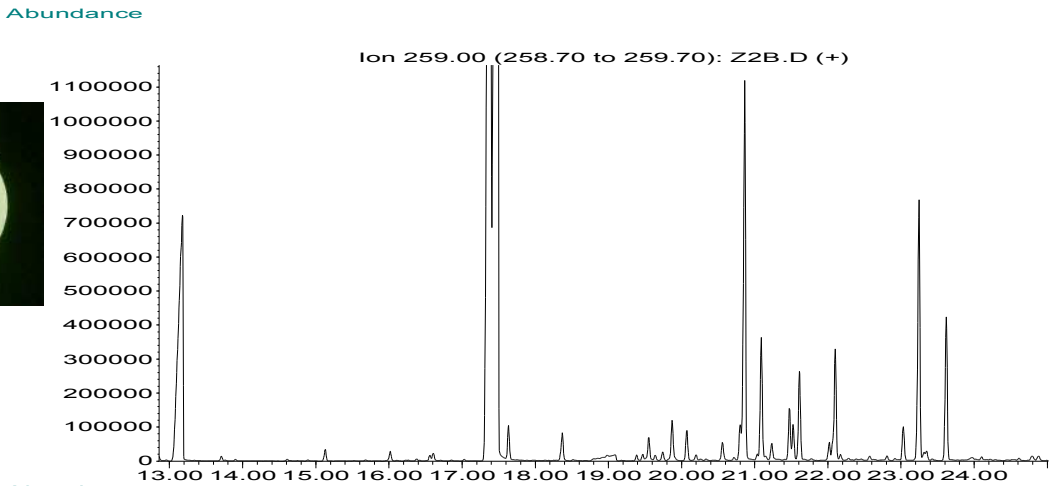
In this case, the first two tablets have almost identical visual, physical and chemical characteristics. Their respective organic profiles are also almost identical. However, the third tablet, which was seized two, respectively three years later, contains also lactose. Its organic profile (see Annex 16A) is very similar compared to the two other tablets although there are some very small differences.

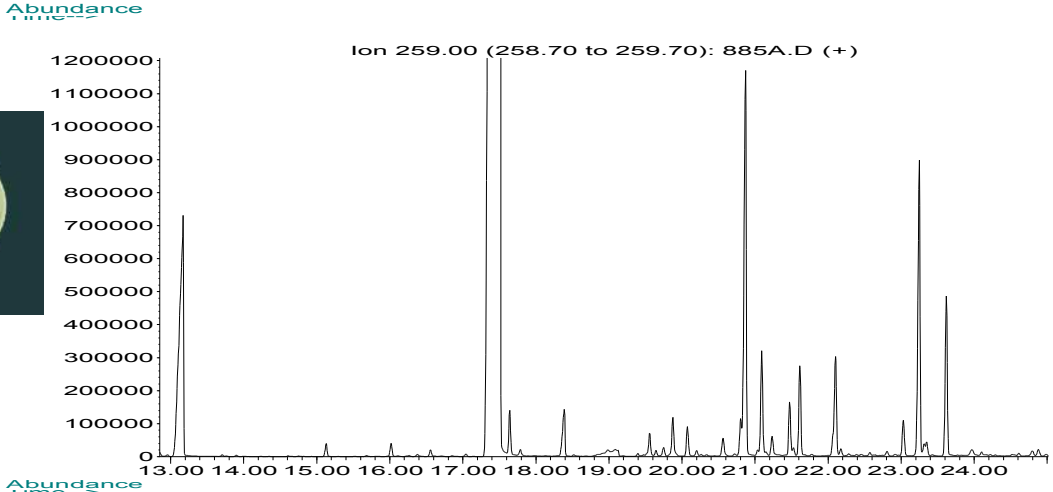
Note 1 : as can be seen above, the pictures are unfortunately not all on the same scale although they should be.

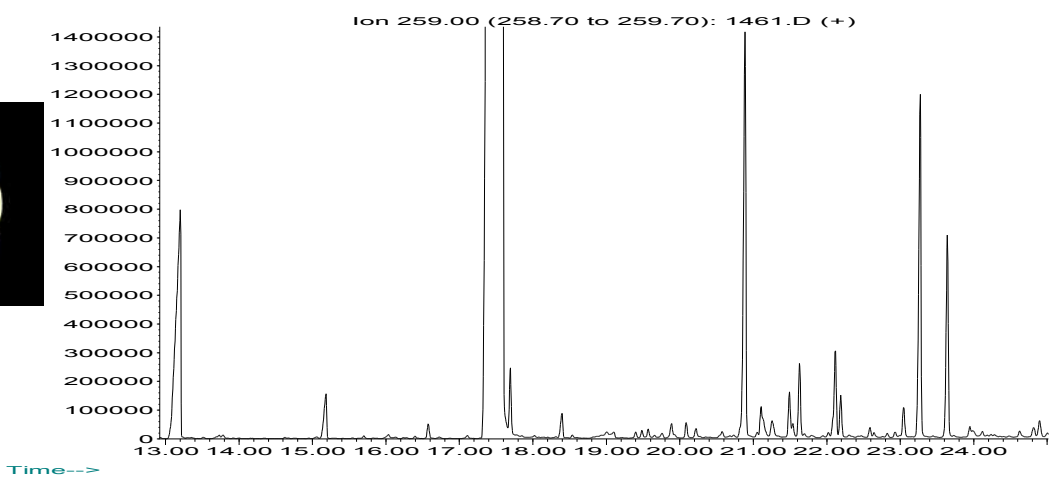
Note 2 : see Annex 19 for explanation of the shape code.

ANNEX 16A

EXTRACTED ION CHROMATOGRAMS OF AMPHETAMINE TABLETS : CASE 6 from annex 16







Note : top and middle profiles are considered to be a match (Pearson correlation : 0.19). Bottom chromatogram shows very slight differences (Pearson correlations : 0.84 and 0.85).

ANNEX 17

AMPHETAMINE TABLETS WITH SIMILAR VISUAL AND PHYSICAL CHARACTERISTICS BUT WITH SLIGHT DIFFERENCES IN CHEMICAL COMPOSITION INCLUDING THE ORGANIC PROFILES

CASE 7

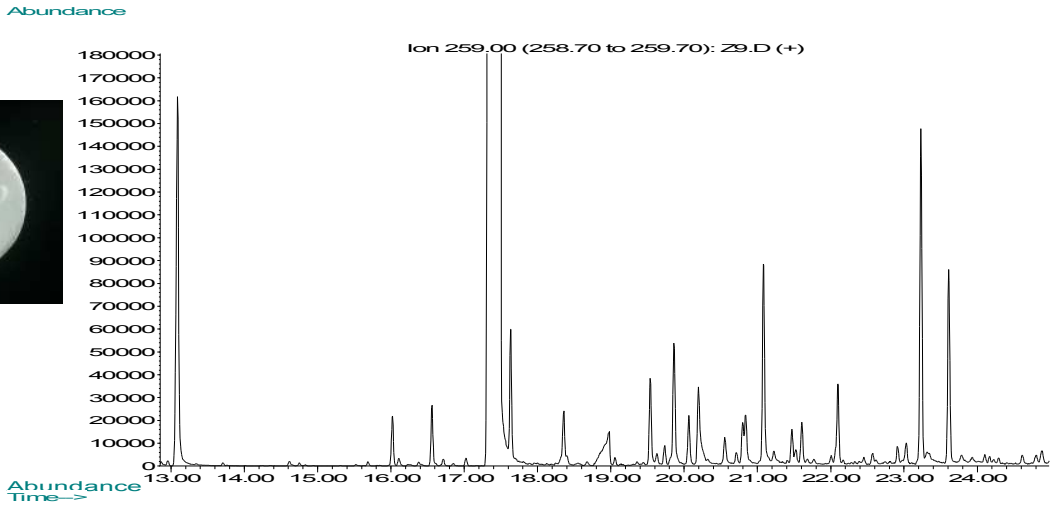
Colour :	white	white	white	white	white
Diameter :	9 mm	9.1 mm	9 mm	9.1 mm	9 mm
Width :	3.4 mm	3.6 mm	3.6 mm	3.5 mm	3.6 mm
Weight :	290 mg	286 mg	291 mg	286 mg	280 mg
Amount of amphetamine :	28 mg	15 mg	13 mg	19 mg	44 mg
Adulterant(s) :	-	caffeine	caffeine	caffeine	caffeine
Diluent(s) :	lactose	lactose	lactose	lactose	-
Shape code :	RBB	RBB	RBB	RBB	RBB
Breakline :	YES	YES	YES	YES	YES
Location and year of seizure:	ZH, 1995	ZH, 1997	ZH, 1997	ZH, 1998	GE, 1999
Internal number .	127	557a	560a	Z9	857

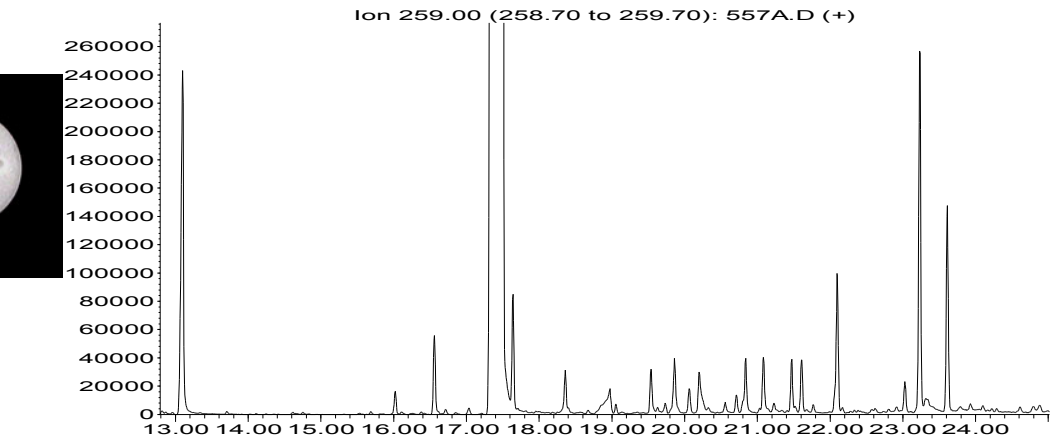
Note 1 : again, the pictures are unfortunately not all on the same scale although they should be. Also, the differences in colour are due to non-standardization (at the time) of the digital recording of the images.

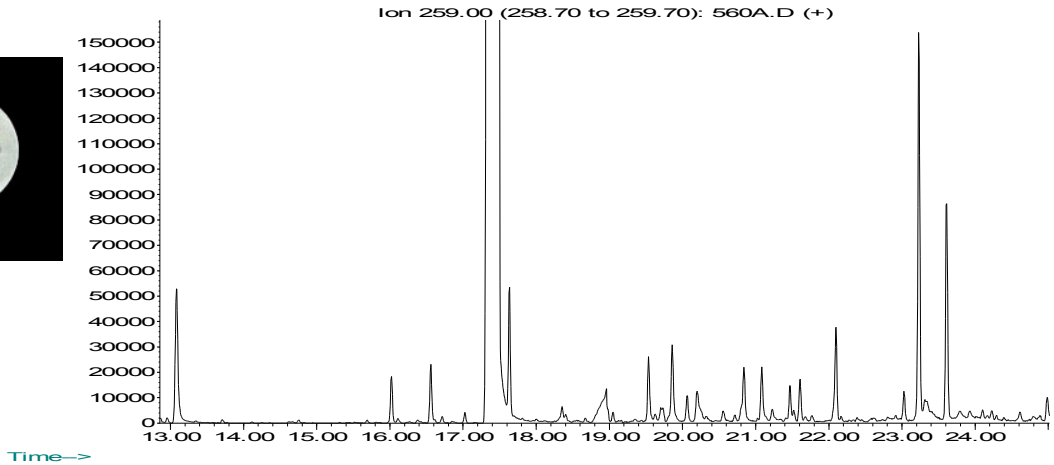
Note 2 : see Annex 19 for explanation of the shape code.

ANNEX 17A

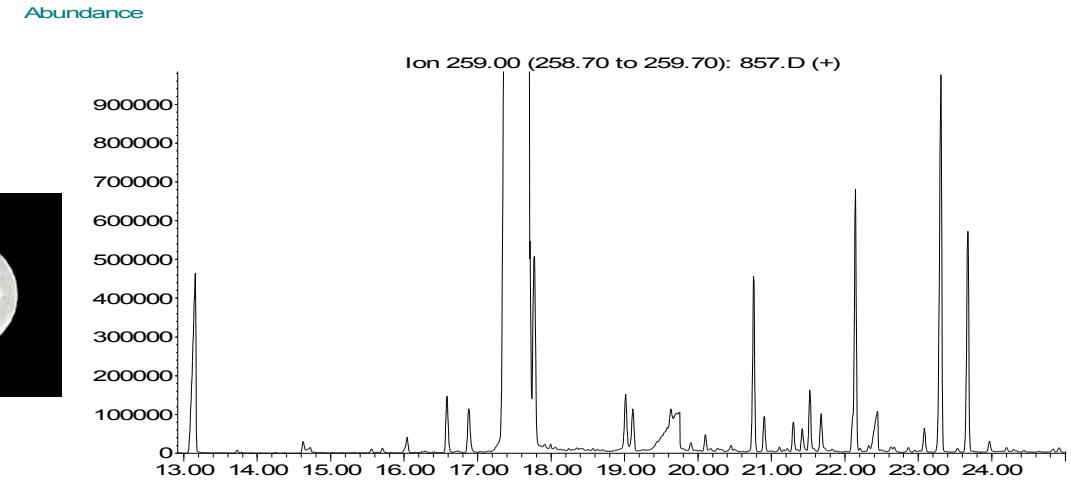
EXTRACTED ION CHROMATOGRAMS OF AMPHETAMINE TABLETS : CASE 7 from annex 17

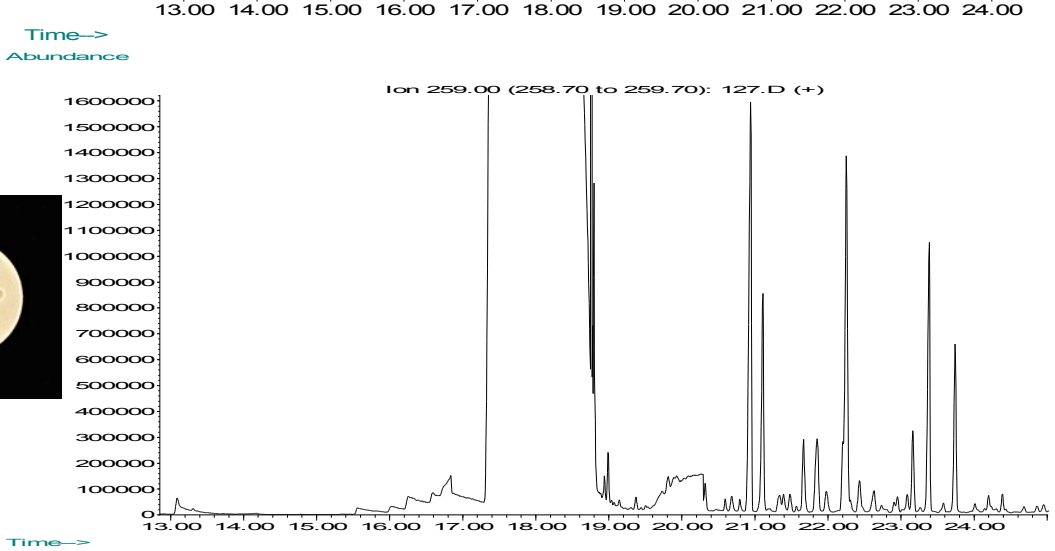






Note : these three samples (Z9, 557a and 560a) are considered as a match (Pearson correlations : 0.32, 0.36 and 0.39 respectively).



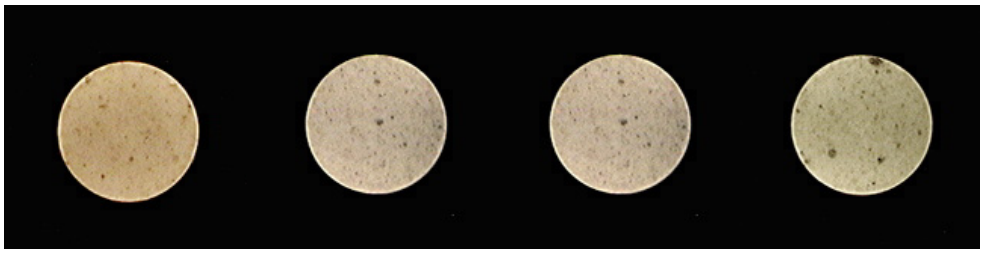


Note : these two samples (127 and 857) are considered different from each other and also different from the three other samples.

ANNEX 18

AMPHETAMINE TABLETS WHERE LINKS WERE ESTABLISHED BY MEANS OF VISUAL, PHYSICAL AND CHEMICAL CHARACTERISTICS AND CONFIRMED BY THE ORGANIC PROFILES

CASE 8

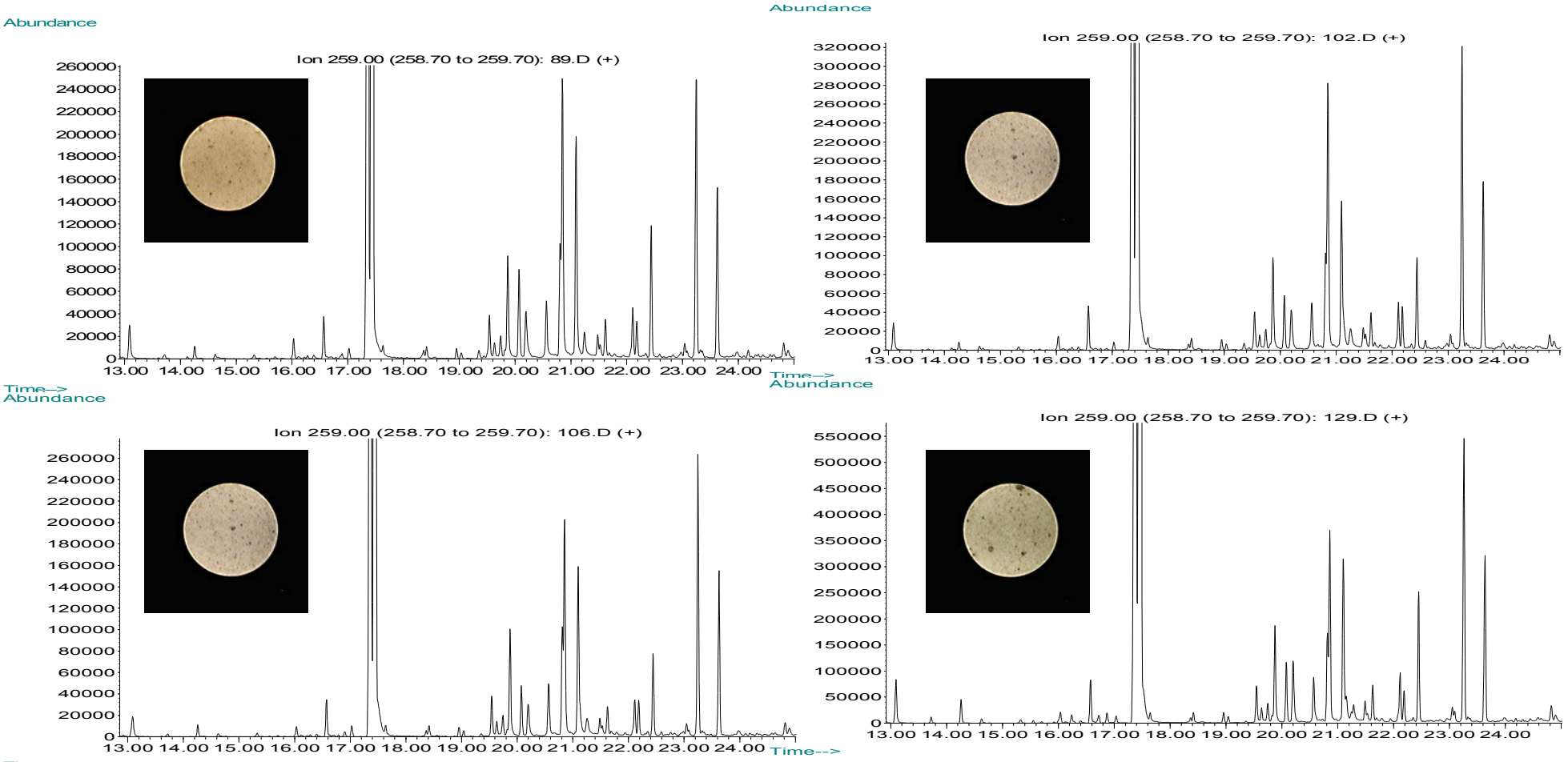


Colour :	beige	beige	beige	beige
Diameter :	9 mm	9 mm	9 mm	9 mm
Width :	4.4 mm	4.4 mm	4.4 mm	4.4 mm
Weight :	321 mg	320 mg	320 mg	320 mg
Amount of amphetamine :	63 mg	59 mg	64 mg	64 mg
Adulterant(s) :	-	-	-	-
Diluent(s) :	lactose	lactose	lactose	lactose
Shape code :	RAA	RAA	RAA	RAA
Breakline :	NO	NO	NO	NO
Location and year of seizure:	ZH, 1995	ZH, 1995	ZH, 1995	ZH, 1995
Internal number .	089	102	106	129

Note 1 : the differences in colour are due to non-standardization (at the time) of the digital recording of the images.

Note 2 : see Annex 19 for explanation of the shape code.

ANNEX 18A EXTRACTED ION CHROMATOGRAMS OF AMPHETAMINE TABLETS : CASE 8 FROM ANNEX 18



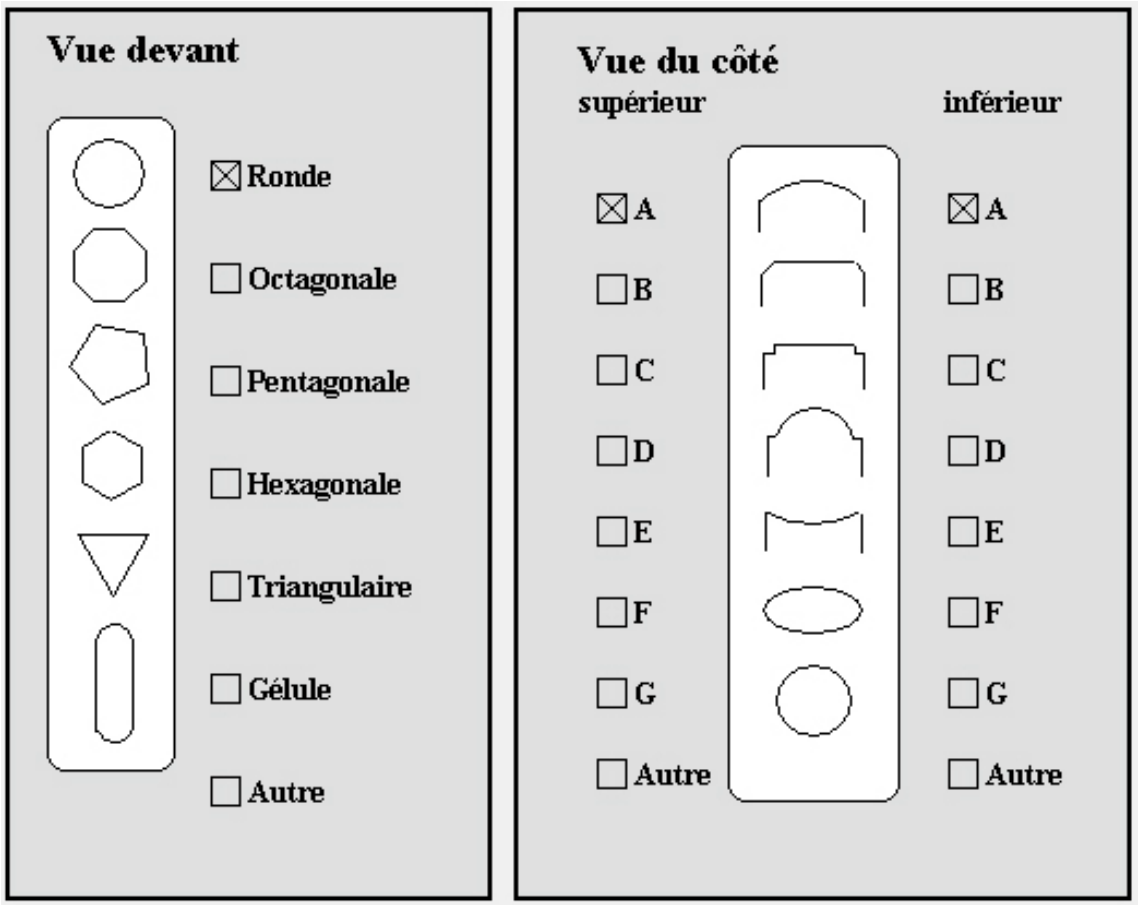
Note : all samples are considered as matching, Pearson correlations from 0.08 to 0.23.

ANNEX 19 CODE SHAPE FOR TABLETS *

This is the three letter code shape used in the IPS tablet database (Institut de Police Scientifique, University of Lausanne, Switzerland).

The first letter represents the shape of the tablet from a top view. If it is round (as in most cases), then the first letter will be R, if it is a capsule, then it will be G, etc.

The second and third letters represents the shape of the tablet from the side views. The second letter is given to the side view which bears an imprint whereas the third letter is given for the side view which generally bears the break line. If no imprint or break lines are present, then the alphabetical rule is followed.



* [Zingg C., 2004]

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