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PROFILING OF DRUGS OF ABUSE: A NEW METHOD FOR AMPHETAMINE

by

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ABBREVIATIONS

AAS	Atomic absorption spectrofotometry
AES	Atomic emission spectrometry
BHT	2,6-Di-tert-butylhydroxytoluene
BMK	Benzyl methyl ketone
BSA	N,*O-bis*-Trimethylsilylacetamide
CAPE	The Central Analysis Program Ecstasy -project
CBC	Cannabichromene
CBD	Cannabidiol
CBN	Cannabinol
CEC	Capillary electrochromatography
CV	Coefficient of variation
DAD	Diode array detector
DEA	Drug Enforcement Administration
DPIA	N,N-Di-(β-phenylisopropyl)amine
DPIF	N,N-Di-(β-phenylisopropyl)formamide
DPIMA	N,N-Di-β-(phenylisopropyl)methamine
ECD	Electron capture detector
EAFS	European Academy of Forensic Science
ENFSI	European Network of Forensic Science Institutes
EU	European Union
FID	Flame ionisation detector
FTIR	Fourier transform infrared spectroscopy
GC	Gas chromatography
ICP	Inductively coupled plasma
IRMS	Isotope ratio mass spectrometry
HCA	Hierarchical clustering analysis
HFB	Heptafluorobutyrate
HFBA	Heptafluorobutyric anhydride
HPLC	High performance liquid chromatography
HS-GC	Head space-gas chromatography

k-NN	*k*-Nearest neighbour
LC	Liquid chromatography
LLE	Liquid-liquid extraction
LSD	Lysergic acid diethylamide
MDA	3,4-Methylenedioxyamphetamine
MDMA	3,4-Methylenedioxymethamphetamine
MS	Mass spectroscopy
MSD	Mass selective detector
MSTFA	N-Methyl-N-(trimethylsilyl)trifluoroacetamide
MW	Molecular weight
NMR	Nuclear magnetic resonance spectroscopy
NPD	Nitrogen and phosphorus selective detector
PC	Principal components
PCA	Principal component analysis
RI	Retention index
RRF	Relative response factor
RSD	Relative standard deviation
RTL	Retention time locking
SI	Similarity index
SIM	Selected ions monitoring
SIMCA	Soft Modelling of Class Analogy
SPE	Solid phase extraction
THC	Tetrahydrocannabinol
THF	Tetrahydrofuran
TLC	Thin layer chromatography
TMS	Tetramethylsilane
UV	Ultraviolet

SYMBOLS

α	Selectivity factor
α_i, β_i, ...	Discriminant coefficients
$\delta^{13}C$	Ratio of $^{13}C/^{12}C$
ξ_{ij}	Discriminant values
d	Euclidean distance
d_f	Film thickness of stationary phase in capillary column
h	Peak height
H	Column plate height
i.s.	Internal standard
i.d.	Internal diameter of a column
k	Retention factor, capacity factor
k_1, k_2, k_3 and w	Coefficients
L	Column length
N	Theoretical plate number
N_{eff}	Effective plate number
N_i	Total number of peaks
q	Quotient
r	Pearson product-moment coefficient of correlation
R^2	Coefficient of determination
R_S	Chromatographic resolution
s	Standard deviation
t_0	Retention time of a non-retaining compound (column dead time)
t_R	Retention time
t'_R	Adjusted retention time
$\bar{u}$	Average linear carrier gas velocity
$w_{½}$	Peak width at half height
w_b	Peak width at the baseline
$\bar{x}$	Average value (the arithmetic mean)
x_1, y_1...	Parameters e.g. peak areas

ABSTRACT

In the literature survey, a summary of chemical impurity profiling methods is presented for the most common drugs. These include cannabis, LSD, heroin, cocaine and amphetamine-type stimulants. The survey also details the statistical techniques commonly used for profiling. The main aim of the experimental work was carried out as part of a European project to develop a harmonised amphetamine profiling method which could be applied at national laboratories utilising an international database. The optimisation of the method was divided into four steps including (i) identification and synthesis of standard impurities, (ii) optimisation of the GC method, (iii) optimisation of the extraction procedure and (iv) evaluation of the suitability of the method between different laboratories.

Ten standard substances were synthesised and the structure of the compounds confirmed through spectrometric data. The optimisation of the GC method was based on the optimisation of sample introduction, chromatography and detection. In the optimisation of the extraction procedure, liquid-liquid extraction (LLE) and solid phase extraction (SPE) techniques were compared. Using the optimised profiling method, several synthesised amphetamine batches and street samples were analysed. Repeatability and reproducibility at the intra- and inter-laboratory level indicated that the method is suitable to use as the harmonised method at national laboratories utilising a common database.

1 GENERAL INTRODUCTION

1.1 Drugs of abuse and drug control

The illicit use of narcotic drugs has become a serious problem in Europe over the years. The statistics provided by national forensic laboratories indicate a significant increase in the use of illicit drugs. The trend of amphetamine, cannabis and heroin seizures in Finland is shown in Figure 1 [1].

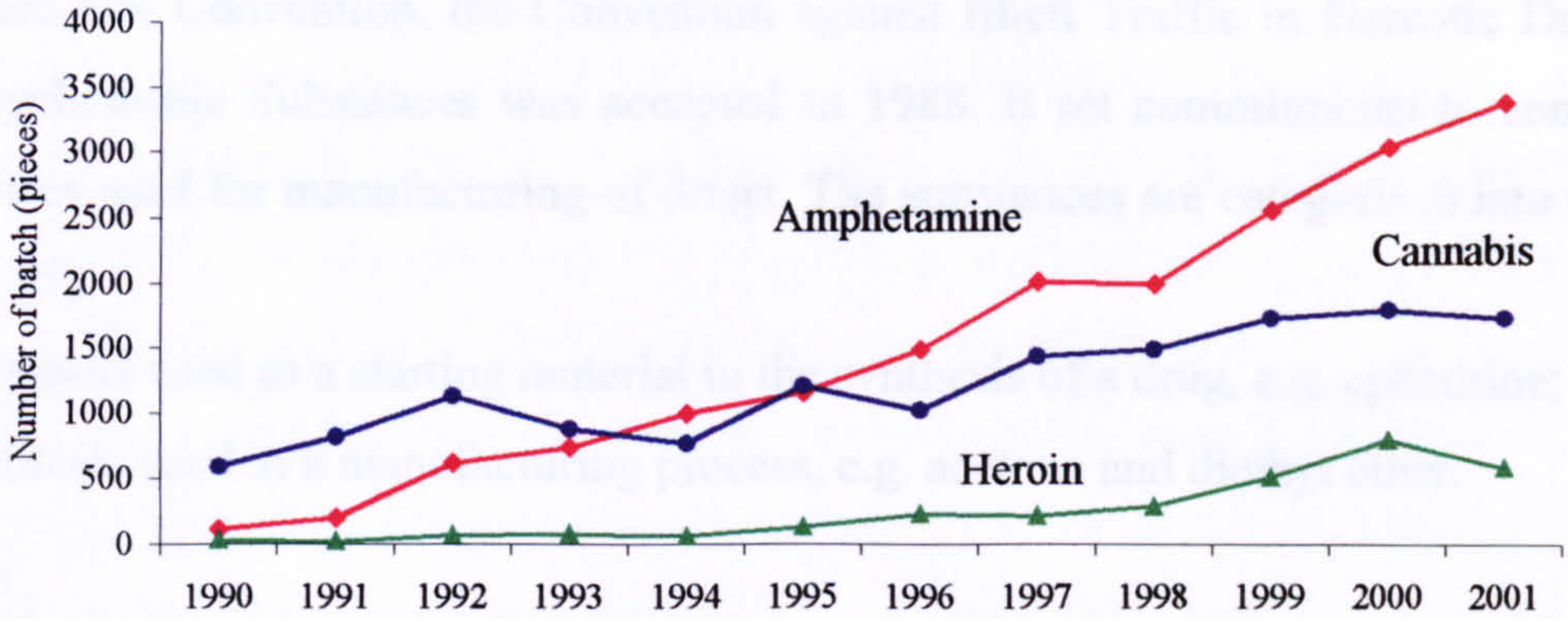


Figure 1: Number of seized batches of amphetamine, cannabis and heroin in Finland during 1990 - 2001.

In general, controlled substances have been listed by the United Nations (UN) [2]. In Finland, all three UN Conventions have been ratified in the Drugs Act, 1993 [3,4]. The conventions form an internationally consistent base for the control of drugs. The first, the Single Convention on Narcotic Drugs, 1961, and its 1972 amendment is aimed to control the abuse, manufacturing, dealing, importation and exportation of drugs. The Single Convention categorised narcotic drugs into four classes:

1. substances that cause addiction and serious drug abuse, e.g. cocaine and heroin;
2. substances that have a medical purpose, e.g. codeine;
3. derivative substances that are based on the substances mentioned in classes 1 and 2;
4. the most hazardous substances from class 1 that have only a limited medical significance.

In 1971, the United Nations Convention on Psychotropic Substances listed psychotropic substances under international control [2,3], again into four classes:

1. hallucinogens that have a significant risk for drug abuse and do not have medical use, e.g. LSD and ecstasy;
2. stimulants that have only an insignificant medical purpose, e.g. amphetamines;
3. barbiturates that have an insignificant or significant medical purpose;
4. central depressants and anaesthetics that have significant medical use; might be abused but are a minor risk to one's health.

The third UN Convention, the Convention against Illicit Traffic in Narcotic Drugs and Psychotropic Substances was accepted in 1988. It set commitments to control substances used for manufacturing of drugs. The substances are categorised into two classes [5]:

1. precursors used as a starting material in the synthesis of a drug, e.g. ephedrine;
2. chemicals used in a manufacturing process, e.g. acetone and diethyl ether.

Controlled substances defined in the United Kingdom as drugs are listed in the "Misuse of Drugs Act", 1971 and its subsequent amendments. Drugs of abuse were grouped into three classes in the 1971 Act, but in 1985 the "Misuse of Drugs Act Regulations" classified drugs of abuse into five Schedules [6]. The first Schedule includes natural drug products, such as cannabis, the majority of which are hallucinogens [7]. Drugs which can be manufactured by synthesis or semi-synthesis from natural starting materials in illegal laboratories, have been classified into Schedule 2. These are collectively known as stimulants and narcotic analgesics, such as amphetamine and heroin (diamorphine), respectively. The drugs in Schedules 3, 4 and 5, which include, for example, prescription drugs are considered less dangerous than the drugs in Schedules 1 and 2.

1.2 Forensic investigation techniques

For the purpose of law enforcement, officers often demand a rapid analysis of seizures of illicit drugs [8]. The main requirements are (i) to identify the material as a controlled substance or otherwise and (ii) sometimes to provide a purity percentage in terms of drug content. Identification and quantification are necessary in order to bring about any criminal charges quickly and correctly. Moreover, further investigation may depend on the nature of the material submitted, and the analysis may indicate chemical features common to other seizures.

In addition to identifying the active compounds of a sample and quantitative analysis, comparative analysis has become a much more significant area of drug analysis in forensic science. The chemical impurity profiling and chemical signature are used as a synonym for the concept of the comparative analysis. The majority of the drugs are produced by batch processes and variations can be observed between batches. When profiling is carried out, it is important to consider possible synthetic impurities, by-products, intermediates and adulterants. The analysis may reveal links between seizures and/or between a dealer and users.

The forensic investigation of drugs is based on the determination of physical and chemical characteristics [9]. Chemical analyses are divided into four stages by the European Network of Forensic Science Institutes (ENFSI) [10]. These stages are (i) identification of controlled substances, (ii) quantification of controlled substances, (iii) chemical characterisation and (iv) chemical impurity profiling, and these stages are detailed below.

Identification

The first item in the sequence of the analysis should be a physical description of the seizure. Data should include, for example, (i) colour, (ii) shape, (iii) weight, (iv) packing and (v) labelling. Seizures that contain crystalline substances should be examined to determine whether the sample is homogenous or composed of a number

of different components. Physical characterisation, such as the determination of a colour is sometimes difficult in terms of objectivity [11].

After outward examination, the first 'presumptive tests' for the identification of a drug are typically colour tests [12]. The use of these tests is an effective first step in the identification of the drug classes. The techniques are based on the development of colour following reaction of the drug with chemical reagents. There are a number of reagents available, although few are specific to one class of drugs. The advantages of colour test include (i) ease of use, (ii) rapidity and (iii) cost effectiveness. However, there are a number of disadvantages, including non-specificity of the colour reaction and relative insensitivity compared to instrumental methods [12].

Thin layer chromatography (TLC) has long been used in drug analysis to identify members of a drug class. The method used depends on the drug class identified. TLC has many advantages, including (i) simplicity, (ii) minimal sample preparation and instrumentation, (iii) low cost and (iv) rapidity of analysis [13]. Its principle purpose is to assist in the correct choice of method for subsequent instrumental analysis.

Fourier transform infrared spectroscopy (FTIR) is another basic identification technique. An infrared spectrum indicates the presence, or absence, of particular functional groups [14]. Secondly, a comparison of infrared spectra shows also structural similarities between two compounds. Even if many frequencies may be similar to closely related compounds, in almost every case there are differences in the range of 1600 cm^{-1} to 600 cm^{-1} [14]. This range is often called the "fingerprint region".

Since the middle of 1970's, mass spectroscopy (MS) has become a routine method of analysis [15]. It is most commonly used in tandem with gas chromatography (GC). GC-MS is one of the most powerful analytical techniques for the separation and identification of drugs. In addition to GC-MS, GC-FTIR and liquid chromatography - mass spectroscopy (LC-MS) are modern techniques used for screening and confirmation of the identification of drug samples.

Quantification

In some drug cases, quantitative analysis is required to classify the drug into a legal or illegal category. For instance, morphine and cocaine have been accorded a minimal concentration by the Single Convention on Narcotic Drugs, 1961 [2], and the Misuse of Drugs Act Regulations, 1985 [16]. Any preparation of morphine containing more than 0.2% of anhydrous morphine base has been controlled. The equal control limit for cocaine is 0.1% of cocaine base. Of the instrumental methods used in quantification studies, high performance liquid chromatography (HPLC) and GC are suitable for the widest application.

Chemical characterisation

In the chemical characterisation of drugs, a chosen number of properties of the sample are described and determined, including identification and quantification of (i) the main compound, (ii) the main impurities, (iii) the adulterants (cutting agents) and diluents and (iv) whether the drugs are present in the free base or salt form [11]. The salt or base forms are not, however, always easily recognised. The presence of some characteristic adulterants may indicate the involvement of certain drug manufacturing or trafficking organisations [17]. The adulterants may be used to link samples from a series of manufacturing batches to a single chemist or laboratory and to identify the source of supply or distribution. The fundamental tools in chemical characterisation are chromatographic techniques.

HPLC has been widely employed for the analysis of drugs of abuse [18-20]. This method can be used to isolate, identify and quantify controlled substances and adulterants. HPLC is more flexible than GC, as the former offers much higher selectivity through the high number of potential mobile phases. The HPLC technique is also non-destructive and it requires only simple sample preparation. However, it requires large amount of high purity solvents leading to high acquisition and disposal costs [21].

GC allows for high resolution and fast run time. It is also very sensitive due to high resolution capillary columns and a variety of sensitive detectors [22]. Even with a flame ionisation detector (FID), the GC analysis can be performed in the nanogram (ng) range. The change from one set of conditions, e.g. for amphetamine to another set (e.g. heroin) takes only a few minutes. The results of GC are reproducible and this facilitates data transfer between laboratories. Despite the apparent advantages of the technique, tailing and co-elution of polar compounds in complex matrices are problems associated with GC. It is known that chemical reactions, such as transacetylation, can occur in the hot GC injector.

Chemical impurity profiling

Chemical profiling of seized synthetic illicit drugs has become one of the most important parts of strategic forensic investigation as the profile of an illicit drug sample provides information at several levels [23]. It can be used to determine links between two or more samples. Comparison of drugs can be divided into several disparate, but complementary steps or methods, including the profiling methods based on residual solvents, isotope ratios and trace level impurities [24,25]. The profiling methods are discussed in detail in Chapter 2.

Purity is one of several parameters used for profiling and to establish relationships between sample groups. Drug purities are considered to give useful information on the economic shifts in the illicit drug markets [26]. A summary of chemical impurity profiling methods is presented for the most common drugs, such as cannabis, LSD, heroin, cocaine, ecstasy and methamphetamine in Chapter 2.8 and for amphetamine in Chapter 3.

2 DRUG PROFILING TECHNIQUES

2.1 Introduction

There are three levels of comparison in the interpretation, which can be recognised as: (i) sample A compared with sample B, (ii) sample A compared with sample B and with other samples or (iii) all samples compared with each other using database and classification [11]. The similarities can be found on a batch or a class level [27]. Analytical data of profiles can be collected and handled with different methods. This includes paper copies of chromatograms, which are often difficult to handle. Different computerised methods have been developed for the comparison of the samples. The data, in the form of a database or spreadsheet, is excellent for classification but cannot easily be presented in court.

2.2 Ballistic profiling

The ballistic or physical profiling of illicit drug preparations is largely limited to tablets and capsules [9]. The profiling based on ballistic features is a method for comparing the appearance of drugs, e.g. shape, colour, diameter, thickness, weight, and logos. The ballistic features can help to link the samples to a distribution network, or to a single production site [17]. Moreover, microscopy has been used to detect punch marks, which serve to identify tablets with a particular set of punches and can be used to link to the actual equipment used for tableting [9,17]. The technique can be used for comparison of ecstasy tablets, printed LSD paper squares and hashish bars.

2.3 Residual solvents

The residual solvents from drug samples can be examined. The solvents can either be bound to the surface of the forming crystals during the synthetic processes, or

alternatively they may be trapped (occlusion) in the interstitial spaces in crystals as they form [28]. Traces of the solvents may still be found even after a long period of time. Links between samples have been studied using this technique, for example, for heroin and cocaine [29,30].

2.4 Inorganic compounds

Another chemical profiling method is to determine various inorganic compounds of the drug [24]. The differences in trace level concentration of metals, e.g. calcium, copper, iron, zinc etc., can be examined by atomic absorption spectrometry (AAS), inductively coupled plasma atomic emission spectrometry (ICP-AES) and ICP-MS. These differences arise due to differences in impurities in starting materials, differences in the metal catalysts that are used for synthetic processes and differences in the vessels in which the drugs are synthesised. The method has been used for methamphetamine [31], heroin [32], cocaine [33] and ecstasy [34,35].

2.5 Isotope ratios

Isotopic ratios of carbon, nitrogen, hydrogen and oxygen in organic matter can also be studied [24]. Environmental conditions, such as humidity, temperature, and differences in starting materials and synthetic processes can all lead to differences in ratios of $^{13}C/^{12}C$, $^{15}N/^{14}N$, $^{2}H/^{1}H$ and $^{18}O/^{16}O$. The discrimination of the source of cannabis [36], MDMA [37], heroin and cocaine [38] samples has been studied. Chemical profiling based on isotopic ratios is possible for semi-synthetic drugs since starting materials, for example, in MDMA and heroin synthesis are natural products [37]. Isotope ratios are analysed by gas chromatography-isotope ratio mass spectrometry (GC-IRMS).

2.6 Chemical impurity profiling

More often profiling of drugs is based on the analysis of manufacturing impurities and by-products. This is carried out using different chromatographic methods that have been examined and published by several authors. Chemical profiling of complex samples has traditionally been based on GC analysis of impurities [39]. Seized drugs usually have a complex composition making automatic profiling difficult. There are two major prerequisites for successful automated comparison: (i) high resolution of the compounds of interest and (ii) automated identification of impurity peaks, typically based on the use of a highly selective MS detector or retention index (RI) monitoring technique [39].

Profiling of drugs includes, in addition to chemical analysis, another interdependent step, the interpretation and handling of the data. After chemical profiling the classification, i.e. grouping is commonly achieved using a statistical data handling method. The different statistical methods are summarised in Chapter 2.7. Profiling results typically fall into three different categories: the sample patterns are either (i) identical, (ii) completely different or (iii) in between (i) and (ii) [11]. For instance, a similar impurity profile indicates a link between the samples. The samples having different profiles clearly do not belong to the same batch due to differences in starting materials or production methods. The illicit laboratory may produce batches having quite different chemical compositions.

2.7 Statistical techniques used for profiling

A number of the commonly used statistical techniques for drug profiling are summarised below. Distance methods, such as similarity index (SI) and Quotient methods, have been commonly used for batch level comparison and hierarchical clustering analysis (HCA) and principal component analysis (PCA) for classification, i.e. grouping of the samples.

2.7.1 Batch level profiling methods

Euclidean distance

Euclidean distance is one of the most commonly known methods for batch level comparison. Binary classification is known as the simplest situation in which a sample may belong to one of two classes [40]. The distance, *d*, between two data points P and Q in n-dimensional co-ordinates is usually called as the Euclidean distance or straight-line distance. The distance can be described, according to the Pythagorean theorem, where co-ordinates are $P = (x_1, x_2, \ldots, x_n)$ and $Q = (y_1, y_2, \ldots, y_n)$ and when parameters are e.g. peak areas [41]. By definition, the distance between these points, $d(P,Q)$, is given by equation 1.

$$d(P,Q) = \sqrt{\sum_{i=1}^{n}(x_i - y_i)^2} \quad (1)$$

Weighting and normalisation of the variables is necessary for each profile when Euclidean distance is employed [42]. The peak areas were weighted by calculating the ratio of the peak area of an impurity to a standard deviation of that impurity (equation 2). Using unweighted variables, a big peak may dominate the distance whereas a small peak may become less significant [27].

$$\text{Weighed } A_i = \frac{A_i}{s} \quad (2)$$

Furthermore, the obtained values were normalised by dividing the values by the sum of the weighed peak areas of all compounds (equation 3). Normalisation is needed to eliminate differences between drug batches caused by different degree of cutting agents. The Euclidean distance method has been used, for example, for batch level comparison of methamphetamine [43,44].

$$\text{Normalised } A_i = \frac{\text{Weighed } A_i}{\sum_{i}^{n} \text{Weighed } A_i} \quad (3)$$

Similarity index (SI) method

Commercial software SC-WorkStation (SuniCom Oy, Finland) has been developed to utilise an exponential comparison algorithm [39]. The program compares two samples by calculating a similarity index (SI) which varies between 1 ... 100 (equation 4). A high value corresponds to strong similarity.

$$SI = \frac{\sum_{i=1}^{n} \frac{k_1}{A} \times 100}{n} \qquad A = \left(\frac{x_i}{y_i} \times w - k_2 \right)^{k_3} - 1{,}0 + k_1 \qquad q_i = \frac{x_i}{y_i} \qquad (4)$$

The SI method was originally utilised to calculate similarities between individual bacterial strains by comparing cellular fatty acids [45]. Analyses of about 4500 strains gave empirically obtained optimal values 50.0, 0.25, 6.0 and 1 for coefficient k_1, k_2, k_3 and w in the equation 4, respectively. Symbols x_i and y_i are peak areas of peak i in sample 1 and sample 2, respectively. Thus, the equation can be further developed to:

$$SI = \frac{\sum_{i=1}^{n} \frac{50}{(q_i - 0.25)^6 - 1 + 50}}{n} \times 100 \qquad (5)$$

Target compounds are identified from GC chromatograms using the retention index (RI) method. The target peaks are normalised as shown in equation 6:

$$A_i = \frac{x_i}{x_t - x_s - \sum x_{RI} - x_a - x_c - x_{is}} \times 100\,\% \qquad (6)$$

where variables x are peak areas of peak i (x_i), sum of all peaks (x_t), solvent peak (x_s), RI standards (x_{RI}), amphetamine (x_a), caffeine (x_c) and internal standard (x_{is}), respectively. In Finland, the similarity index (SI) method is used for amphetamine profiling [39].

Quotient methods

The basis of the Quotient method is that two profiles are likely to match if the chosen peak areas have approximately the same relative values (quotients) [46]. The quotients between corresponding peak areas are calculated with equation 7 by taking total profile intensity into consideration.

$$r_{i,n} = \left| \frac{q_i - q_n}{q_i + q_n} \right| \times 100 \qquad q_i = \frac{x_i}{y_i} \tag{7}$$

In the equation 7, x_i is peak area of peak i in sample 1 and y_i peak area of peak i in sample 2. The number of quotients with $r_{i,n} < r_{max}$ was calculated. The $r_{max} = 20\%$ has been found to be useful to retrieve the pairs of profiles [46]. In Sweden, the automatic profiling and the classification of impurity profiles is undertaken using the Quotient method [47].

A similar Quotient method has been also introduced in Australia. The retention times of two methamphetamine profiles were compared and a quotient (q) calculated using equation 8 [48].

$$q = \frac{2 \times n}{N_1 + N_2} \tag{8}$$

where n is the number of matched peaks in two profiles and N_i is the total number of peaks in each of two profiles.

2.7.2 Classification methods

Principal component analysis (PCA)

Principal component analysis (PCA) involves a mathematical procedure that transforms a number of correlated variables into a smaller number of uncorrelated variables called principal components (PC) [49]. The principal components are extracted in such a way that the first principal component (PC_1) accounts for as much of the variability in the data as possible. Variables in PCA are x_1, x_2, ..., x_p which

establish the sensible completeness of the problem. The weights c_{11}, c_{12}, ..., c_{1p} of the first principal component (equation 9) have been chosen to maximise the ratio of the variance of PC_1 to the total variation.

$$PC_1 = c_{11}x_1 + c_{12}x_2 + ... + c_{1p}x_p \quad (9)$$

Graphical performance of the first principal component has been illustrated in Figure 2. The second and other principal components (PC_m) are chosen such that weighed linear combination of the observed variables (x_p) are uncorrelated with all of the previously extracted components [49].

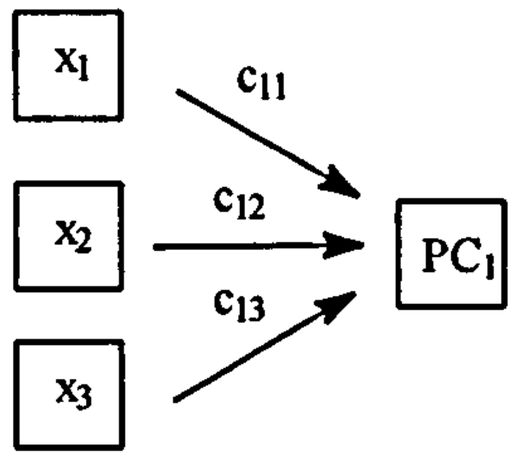


Figure 2: One principal component [50].

The technique is used for amphetamine profiling in many forensic laboratories, for example in Sweden [46] and Poland [51]. It is also applied for batch-to-batch comparison and geographical origin determination of heroin based on concentration of opium alkaloids or inorganic trace elements [52].

Hierarchical cluster analysis (HCA)

Hierarchical cluster analysis (HCA) is a method to find clusters or classes of the observations within a data set [53]. Most commonly, the similarity of clustering technique can be based on the Euclidean distances. The two closest clusters are merged to form a new cluster replacing the two previous clusters. The distance between the clusters can be described with a dendrogram.

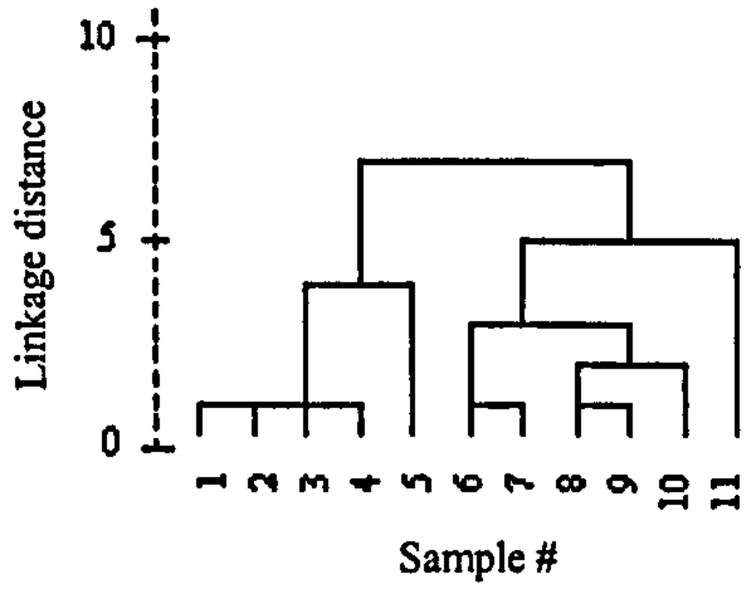


Figure 3: An example of the tree structure of a dendrogram.

Cluster analysis and dendrograms have been used for heroin [54] and amphetamine classification [51,27]. The technique, however, has some disadvantages. The data set, which contains many profiles, is difficult to illustrate. Moreover, the method is very sensitive to errors and thus small inaccuracies in distances can result in significant changes in the dendrogram tree [27].

Discriminant analysis

In order to predict the category to which a particular sample belongs, the data can be handled using Fisher's linear discriminant analysis. This analyse provides models shown below [42]:

$$\begin{aligned} \xi_1 &= \alpha_1 x_1 + \beta_1 y_1 + \ldots \\ \xi_2 &= \alpha_2 x_2 + \beta_2 y_2 + \ldots \end{aligned} \qquad (10)$$

where ξ_i are the calculated discriminant values for a particular sample, α_i, β_i, ... are the discriminant coefficients from the data set and x_i, y_i, ... are the values of variables. The discriminant values can be calculated and defined using a matrix formula (equation 11):

$$\begin{bmatrix} \alpha_1 & \alpha_2 & \ldots & \alpha_n \\ \beta_1 & \beta_2 & \ldots & \beta_n \\ \vdots & \vdots & & \vdots \\ \delta_1 & \delta_2 & \ldots & \delta_n \end{bmatrix} \times \begin{bmatrix} x_1 & y_1 & \ldots & z_1 \\ x_2 & y_2 & \ldots & z_1 \\ \vdots & \vdots & & \\ x_p & y_p & \ldots & z_p \end{bmatrix} = \begin{bmatrix} \xi_{11} & \xi_{12} & \ldots & \xi_{1n} \\ \xi_{21} & \xi_{22} & \ldots & \xi_{2n} \\ \vdots & \vdots & & \vdots \\ \xi_{n1} & \xi_{n2} & \ldots & \xi_{nn} \end{bmatrix} \qquad (11)$$

where discriminant values, ξ_{ij}, has been calculated as:

$$\xi_{11} = \alpha_1 x_1 + \alpha_2 x_2 \ \ldots$$
$$\xi_{12} = \alpha_1 y_1 + \alpha_2 y_2 \ \ldots \qquad (12)$$
$$\xi_{21} = \beta_1 x_1 + \beta_2 x_2 \ \ldots$$

A particular sample is assigned to the group for which the highest discriminant value is calculated. In this model, the correlated variables are utilised such that the most significant variables are used in the calculation whereas the others are discarded or reduced. The method has been used for heroin profiling [55].

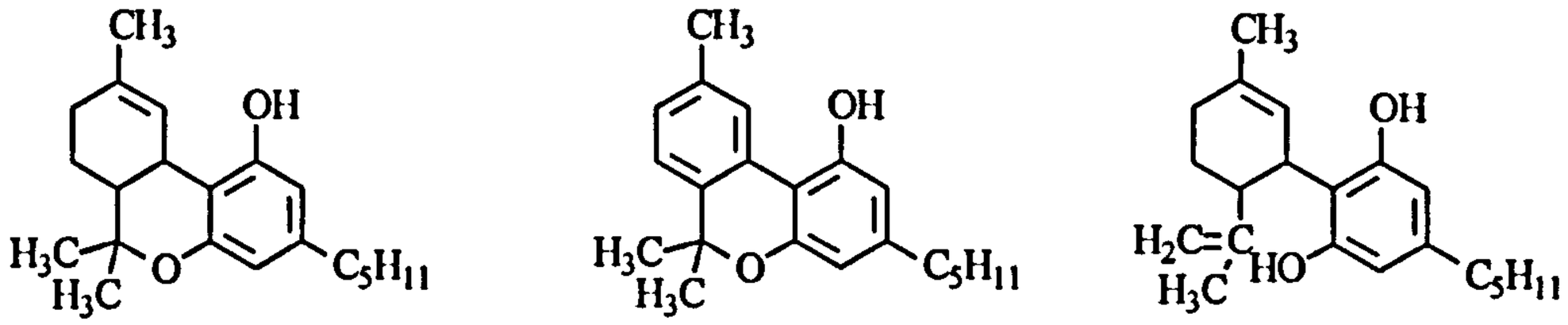
2.8 Applications

2.8.1 Cannabis

Cannabis is the most frequently used and trafficked hallucinogenic drug in the world [56,57]. Cannabis products are prepared directly from *Cannabis sativa L.* Cannabis appears in the illicit market as herbal cannabis (marijuana), cannabis resin (hashish) and liquid cannabis (hashish oil). Morocco dominates the source of cannabis resin for the European Union (EU) [57]. Herbal cannabis is smuggled to the Member States mainly from Colombia, Jamaica, South Africa and Nigeria [57]. Usually cannabis products are smoked in the form of cigarettes (marijuana) or using pipes (hashish). The most common effects of cannabis are talkativeness, cheerfulness and relaxation [58]. However, not all the effects are pleasant. High doses can cause mild hallucinations. On average, at least one in four 15- to 34-years-olds in the European Union has tried cannabis [57].

The *Cannabis* plants have been classified into two categories, fibre-type and drug-type [59]. Cannabis products are prepared directly from the plants and contain several cannabinoids, e.g. Δ^9-tetrahydrocannabinol (THC **1**), cannabinol (CBN **2**) and cannabidiol (CBD **3**) shown in Figure 4. *Cannabis* is characterised by the presence of compounds responsible for the pharmacological effect. THC is believed to be mainly responsible for the psychoactivity of the cannabis products [60].

Division into fibre and drug type cannabis is achieved calculating the ratio of CBD and THC. The CBD/THC ratio is usually < 0.2 for drug-type cannabis and > 5 for the fibre-type [61].



Δ^9-Tetrahydrocannabinol (THC) **1** Cannabinol (CBN) **2** Cannabidiol (CBD) **3**

Figure 4: Structural formulas of common cannabinoids.

Two cannabis products do not necessarily have exactly similar appearances [59]. Cannabis plants grown in different origins contain varying amounts of cannabinoids [60]. Moreover, the carbon isotope ratio, $^{13}C/^{12}C$, also used for characterisation and comparison of cannabis samples, depends on environmental conditions [36]. Thus, marijuana and hashish are produced from a natural product of highly varying characteristics. Cannabis is a difficult material for chromatographic analysis and therefore only few chromatographic methods for chemical profiling of cannabis have been published. Chromatographic separation and mass spectrometric data of cannabinoids have been introduced [62,63]. The methods used in profiling are described below. Of other profiling methods, ballistic profiling and visual comparison can be mentioned. Sometimes logos or other marks of hashish bars can be identified and compared.

A comparative analysis has been made for 100 marijuana and hashish samples using a GC equipped with FID [64,65]. The comparison was based on the relative amounts of the main cannabinoids, THC, CBN, CBD and cannabichromene (CBC). Packed columns, namely slightly polar methyl silicone (3% PC-3210) and polyethylene glycol column (Ultrabond coated of Carbowax 20M), were used. It was impossible to achieve baseline separation for CBC and CBD and, at the same time, for CBC and THC with this column system. This can be seen as the main drawback of the method

due to the poor resolution and peak shape. The percentages for cannabinoids were, however, calculated and the samples were classified into eight individual groups.

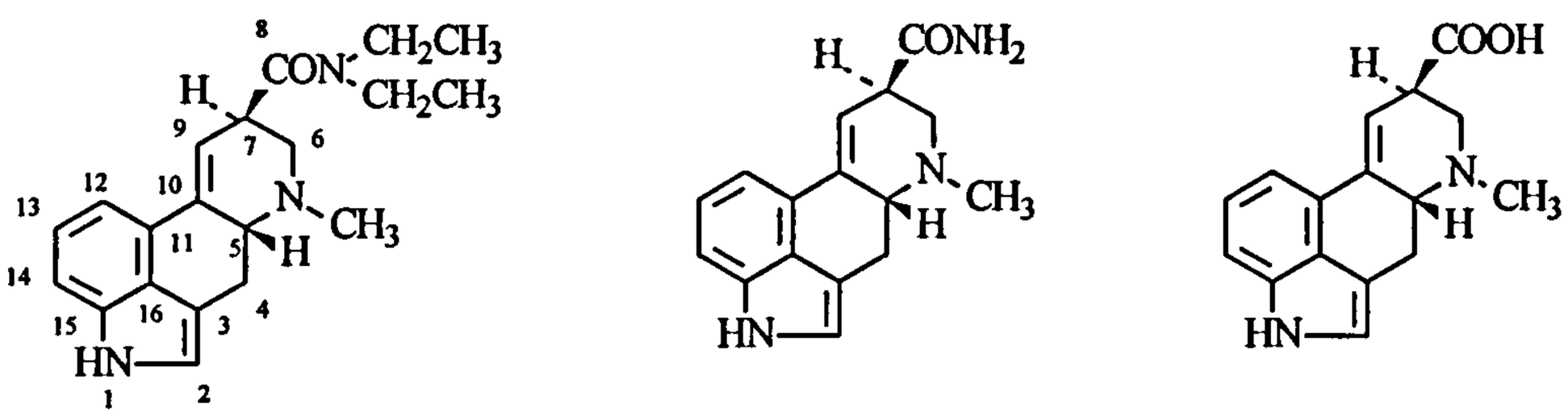
Most of the illicit cannabis seized in the USA is smuggled from Mexico, Colombia, Jamaica and Thailand [66]. A comparison analysis of samples of different origins, including domestic samples was carried out using GC-FID. The profiling method seemed to give a good resolution and repeatability when a DB-1 capillary column was used. The chemical profiling was based on 81 chromatographic peaks. The chemical structure of some of the peaks was identified using mass spectroscopy, but most of the compounds remained unknown. Relative response factors (RRF), i.e. the peak area ratios to internal standard were calculated for each analyte peak. It emerged that the ratios of major cannabinoids, THC, CBD and CBC, were not significant and that they do not correlate with any geographical origin. Some individual compounds were, however, typical to one origin.

HPLC was also tested as a profiling technique for cannabis [66]. The profiling was based on the use of 45 peaks. The use of this technique was limited in comparative analysis due to the lack of resolution. The technique was preferred for screening and it was only suitable for discrimination between different cannabis types, i.e. drug and fibre cannabis.

2.8.2 Lysergic acid diethylamide (LSD)

Lysergic acid diethylamide (LSD **4**) is strongly psychoactive and is the most potent hallucinogenic substance known [67]. It has hallucinogenic effects; anxiety, euphoria, difficulty in thinking and concentration and hallucinations. The content of the LSD dosage varies, typically between 20 - 80 μg [68]. Tolerance develops after a couple of days of continuous use. Frequently, LSD appears in the illicit market in the form of paper squares and small tablets. It has been obtained in the same illicit market as marihuana.

LSD is a semisynthetic drug derived from ergot alkaloids e.g. lysergic acid amide **5** [69]. Alkaloids are present in the seeds of morning glory (*Rivea corymbosa*) and a rhizomorph of *Claviceps purpurea*. The alkaloids are hydrolysed resulting in lysergic acid **6**, which is reacted with diethylamine to produce the final product [70]. LSD was synthesised for the first time in 1938 and two years later it was thought to be a possible treatment for schizophrenia [71]. It has been manufactured illegally since the 1960's and adopted into popular drug culture during the early 1970's.



Lysergic acid diethylamide (LSD) **4** Lysergic acid amide **5** Lysergic acid **6**

Figure 5: Structural formulas of LSD analogues.

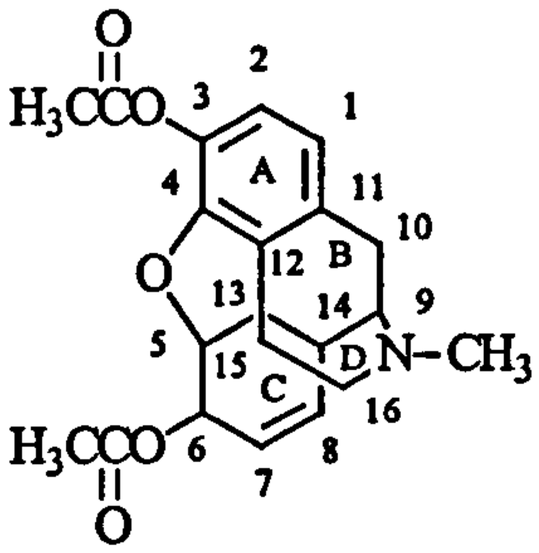
In the GC analysis, LSD produces a poor chromatographic peak due to the poor volatility of the substance. Thus, only the ballistic features have been applied for LSD profiling.

2.8.3 Heroin

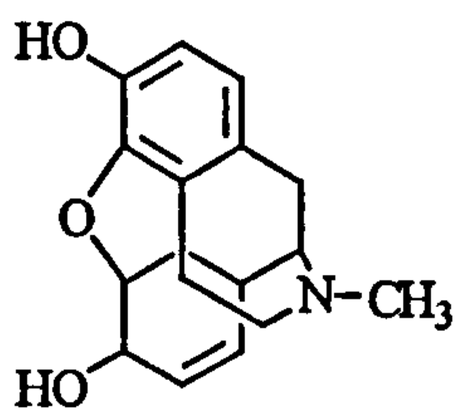
Heroin (diamorphine, diacetylmorphine **7**) originates directly or indirectly from opium. Opium is obtained by splitting and extracting the unripe, dried poppy capsules from *Papaver somniferum L.* [72]. It is established that 3 - 5 million European Union citizens have tried heroin. Turkey is the gateway for trafficking of heroin along the Balkan routes into the European Union [57]. At least 80% of heroin available in Europe is transported across the Balkan routes.

Raw opium is a complex mixture containing sugars, proteins, fatty acids, water and approximately 40 alkaloids. The identified and most common fatty acids are

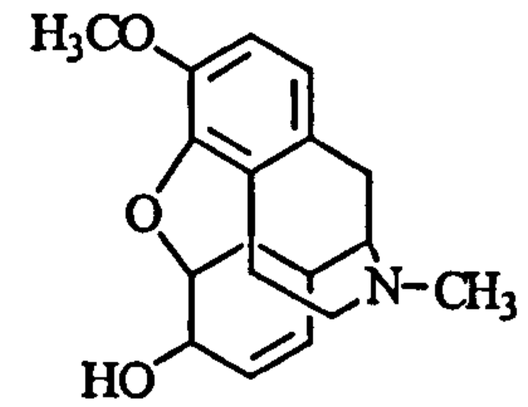
palmitic, linoleic, oleic and strearic acids [73]. Five of the alkaloids can be considered as main alkaloids, namely morphine **8**, which is the principal alkaloid of opium, codeine **9**, thebaine **10**, papaverine **11** and noscapine **12** [74-76]. The structures of alkaloids are shown in Figure 6.



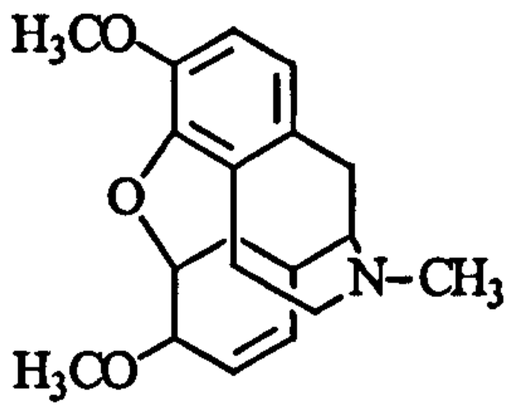
Diamorphine **7**



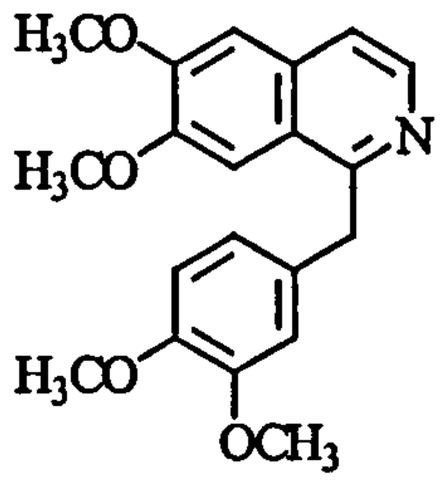
Morphine **8**



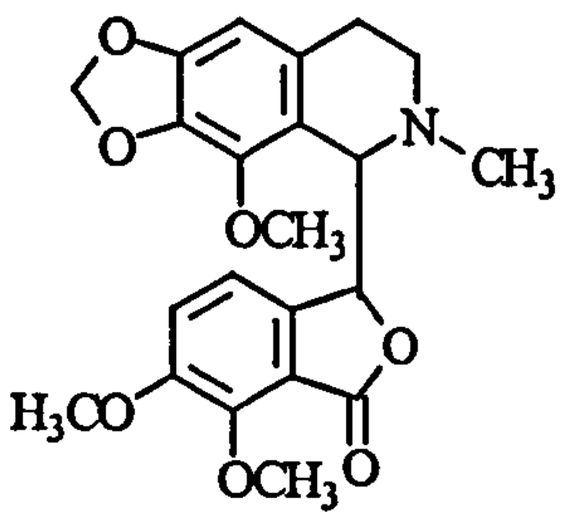
Codeine **9**



Thebaine **10**



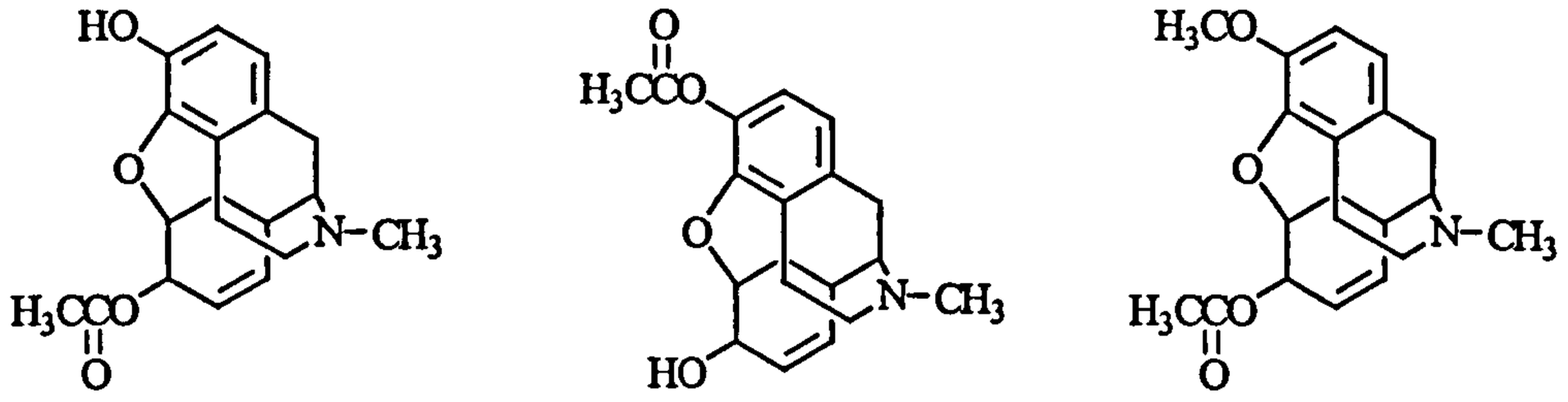
Papaverine **11**



Noscapine **12**

Figure 6: Structural formulas of heroin main alkaloids.

The synthesis of heroin dates back to the end of the 19th century, i.e. diamorphine was prepared for the first time in 1874 [77]. Usually, diamorphine is prepared by acetylation of morphine [73,78], but a direct acetylation of opium has also been reported [79]. Unlike amphetamine and cocaine, an illicit heroin is a complex mixture and contains both acetylated and unacetylated opium alkaloids and after purification, added adulterants. Several acetylated impurities are identified, such as O^6- and O^3-monoacetylmorphines **13 - 14** and acetylcodeine **15**. The structures of the impurities are given in Figure 7.



O^6-Monoacetylmorphine **13**

O^3-Monoacetylmorphine **14**

Acetylcodeine **15**

Figure 7: Structural formulas of impurities found in heroin samples.

The most common adulterants of heroin samples are caffeine and sugars (lactose and glucose) [80-83]. The term 'heroin purity' always refers to the diamorphine content. It is usually the major component and the most active pharmacologically. Morphine and codeine are effective 'painkillers' and are used in many cough medicines and anti-diarrhoea treatments [77]. Opiates and heroin depress the activity of the nervous system, including such reflexes as coughing, breathing and heart rate [84]. Heroin, like other narcotics, gives a feeling of warmth by causing widening of the blood vessels [77,85]. It also relieves stress and discomfort by creating a relaxed detachment from pain, desires and activity. Physical dependence and tolerance develop rapidly.

Two heroin samples do not have exactly similar chemical characteristics. The routine profiling of illicit heroin is used infrequently but several studies have been done, e.g. in the Netherlands [86] and in Germany over the past 20 years [87,88]. Heroin comparisons are made on the basis of the main compounds, the synthetic impurities and adulterants. The methods used in heroin profiling are summarised in Table 1, and they are discussed below.

Table 1: Chemical profiling methods for heroin.

#	Sample preparation	Analysis method and conditions		Ref.
1	Liquid-liquid extraction (LLE): sulphuric acid/toluene	Method:	GC-FID	89
		Column:	25 m x 0.2 mm, d_f 0.11 µm of 5% Ph Me silicone (Ultra-2)	
		T-program:	50°C (0 min), 2°C/min to 325°C (6 min)	
		Injection:	300°C, split 1:25	
		Carrier gas:	H_2	
		Flow rate:	27 cm/s* (0.6 ml/min)	
2	LLE: chloroform (acidic)/ toluene	Method:	GC-FID	25
		Column:	30 m x 0.25 mm, d_f 0.25 µm of Me silicone (DB-1)	
		T-program:	200°C (1 min), 4°C/min to 300°C (30 min)	
		Injection:	280°C, split 1:25	
		Carrier gas:	He	
		Flow rate:	31 cm/s* (1 ml/min)	
3	Dissolution: chloroform	Method:	GC-MS, Scan mode	54
		Column:	30 m x 0.25 mm, d_f 0.25µm of Me silicone (HP-1)	
		T-program:	180°C (1min), 10°C/min to 240°C (4.5min), 17°C/min to 290°C (4 min)	
		Injection:	280°C, split 1:20	
		Carrier gas:	He	
		Flow rate:	34 cm/s* (1.2 ml/min)	
4	Dissolution: methanol Silylation: BSA heated at 60°C (1 hour)	Method:	GC-FID	55
		Column:	12 m x 0.22 mm, d_f 0.25 µm of Me silicone (BP-1)	
		T-program:	150°C (2 min), 9°C/min to 300°C (5 min)	
		Injection:	280°C, split 1:50	
		Carrier gas:	N_2	
		Flow rate:	Not available	
5	Dissolution: chloroform/pyridine (5:1) Silylation: MSTFA heated at 80°C (30 min)	Method:	GC-FID	90
		Column:	30 m x 0.25 mm, d_f 0.25 µm of Me silicone (DB-1)	
		T-program:	150°C (1 min), 8°C/min to 250°C (1 min), 6°C/min to 320°C (1 min)	
		Injection:	290°C, split 1:17	
		Carrier gas:	He	
		Flow rate:	37 cm/s* (1.43 ml/min)	
6	LLE: sulphuric acid/toluene Silylation: MSTFA or BSA heated at 70°C (3 min)	Method:	Dual oven GC-FID	91
		Column:	20 m x 0.25 mm, d_f 0.15 µm of Me silicone (OV-1) or 5% Ph Me silicone (SE-54) and 5 m or 20 m x 0.32 mm, d_f 0.25 µm of 50% Ph Me silicone (OV-17)	
		T-program:	(one example) 160°C, 12°C/min to 240°C, 5°C/min to 280°C, 20°C/min to 330°C	
		Injection:	300°C, split	
		Carrier gas:	He	
		Flow rate:	Not available	
7	LLE: sulphuric acid/toluene Silylation: MSTFA or BSA heated at 70°C (3 min)	Method:	GC-FID	87 88
		Column:	25 m x 0.2 mm, d_f not available of 5% Ph Me silicone (SE-54)	
		T-program:	150°C (0 min), 6°C/min to 280°C (1 min), 15°C/min to 300°C (5 min)	
		Injection:	270°C, split	
		Carrier gas:	H_2	
		Flow rate:	Not available	

Table 1: Cont'd / ...

#	Sample preparation	Analysis method and conditions		Ref.
8	LLE: sulphuric acid/toluene Silylation: MSTFA heated at 70°C (5 min)	Method: Column: T-program: Injection: Carrier gas: Flow rate:	GC-FID 25 m x 0.27 mm, d_f 0.15 µm of Me silicone (OV-1) or 5% Ph Me silicone (SE-54) 150°C, 6°C/min to 280°C (1 min), 15°C/min to 300°C (20 min) 270°C, split H_2 110 cm/s (OV-1) or 65 cm/s (SE-54)	73

* Flow rate was transformed to correspond to cm/s by FlowCalc. (Version A.02.07, Agilent Technology, 1996).

An inter-laboratory development of a heroin profiling method was carried out by Sweden, Germany and the Netherlands (Method 1) [89]. The aim of the study was to develop a harmonised profiling method and improve the interpretation for the resulting database of the chemical profiles. The profiling was based on 16 variables, i.e. 12 identified and 4 unknown impurity peaks. The stability of impurity profile under different storage conditions and influence of common adulterants were also examined. Only phenobarbital as an adulterant caused interference to the profile. The GC-FID system seemed to be stable and the intra-laboratory repeatability and reproducibility were acceptable. Unfortunately, poor reproducibility at an inter-laboratory level was obtained. Principal component analysis (PCA) and the Quotient method were used for classification and statistical interpretation of the profiles. In conclusion, it was pointed out that a database for heroin comparison should be collected in a central laboratory instead of profiling in many laboratories.

The chemical profiling method based on 18 impurity peaks was used to compare nine illicit heroin samples in another study (Method 2) [24]. Variation between profiles was obtained visually. The visual comparison of profiles was not straightforward. It was pointed out that for court testimony, quantitation of selected impurities might have been necessary. To finalise the chemical profiling method, the quantity and ratio of major opiates, namely diamorphine **7**, papaverine **11**, noscapine **12**, monoacetylmorphines **13 - 14** and acetylcodeine **15** were determined. The PCA based on three alkaloid ratios was used for classification. The samples were classified into four different classes.

PCA with two other chemometric techniques, namely hierarchical cluster analysis (HCA) and *k*-nearest neighbour (*k*-NN) have been compared as classification techniques (Method 3) [54]. The chemical impurity profiles were obtained for 24 heroin samples, which were known to originate from three different batches. The analyses were carried out by GC-MS using a non-polar capillary column. Data was evaluated utilising normalised peak areas, i.e. the absolute peak areas of the meconine, acetylcodeine, acetylthebaine, papaverine and noscapine were divided by the sum of peak areas of O^6-monoacetylmorphine and diamorphine. The PCA grouping was recorded in two- and three-dimensional measurement space. The three-dimensional method was found to be better than two-dimensional without any misclassification. The dendrogram and *k*-NN classification techniques also gave nearly correct classifications. The use of the *k*-NN method was, however, more complicated compared to HCA and PCA techniques. Few or no misclassified samples indicated that there were no significant differences between classification techniques. However, it was pointed out that number of comparable samples was not sufficient to obtain reliable results.

Fisher's linear discriminant analysis can also be used to determine the batches to which the samples are related (Method 4) [55]. The model is suitable as the parameters used for model development are dependent, i.e. correlated with each other. Thus, it has been used for the classification of heroin samples since most opiates are significantly correlated. The Fisher's discriminant values were obtained for 31 derivatised seized street samples with SPSS software. The peak areas of main opiates were determined. The highest Fisher's discriminant value assigned the samples to the same origin. 92% of profiles were completely classified. Utilising the classification technique and the visual comparison the samples could be divided into eight groups.

To measure a link between different impurity profiles at batch level, different distance methods have also been compared (Method 5) [90]. Pearson correlation coefficient method and Cosine function were found to be useful techniques for large

database. The standard deviation within one heroin source was not significant, and the number of false negatives and positives was lower than 4%. Using, for example, the Euclidean distance, the number of false negatives was over 15%. The Cosine function method was the final choice for the comparison of heroin samples.

A single-column GC system may cause problems in the analysis of complex illicit drug samples, such as heroin. The main problems are poor separation of certain parts of impurity profiles, decomposition of substances and overlapping of important compounds. The problem has been investigated and one solution found from a column-switching (Method 6) [91]. The system was based on the use of different stationary phases for overlapping peak groups. The system is not, however, useful for a routine profiling due to its complicated technology.

Problems associated with profiling methods may be also caused by incorrect sample preparation methods. A large solvent peak and components in the beginning of the chromatogram, which are not baseline separated, result in a negative influence on the determination of the peaks [92]. This problem may arise from adsorption of the polar solvent, e.g. ethanol in the injection system or the column [93]. Using silylation, the situation can be improved. Silylation helps also to avoid other possible problems including transacetylation, adsorption and different responses for salt and base [86].

In another experiment, toluene was used as an extraction solvent (Method 7) [87]. The chromatograms were compared visually and highly specific profiles of heroin from different origins, namely Turkey, Malaysia, Lebanon and Near/Middle East were obtained. Different profiles from different geographical regions were also compared based on the main alkaloids and adulterants by Johnston and King [94]. Chemical analysis conditions were not available. In this study, 505 illicit heroin samples from Turkey, Pakistan, India and South East Asia were classified based on discriminant analysis using the SPSS software. The analysis results of classification were correct in over 83% cases. The greatest individual misclassification was obtained from the Indian samples, 59%. It was pointed out that chemical composition of seized heroin changes over time, which might have caused missclassification.

The modified Method 2 has been also used for the profiling of heroin precursors, opium and crude morphine (Method 8) [73]. The comparison of impurities from opium, morphine and heroin origins revealed more significant similarities between opium and crude morphine than the correspondence between opium and heroin.

The comparison based on determination of isotope ratio has been also used in heroin profiling. The ratio of $^{13}C/^{12}C$ ($\delta^{13}C$) has been determined for diamorphine and acetylcodeine [24]. The ^{13}C enrichment of these acetylated opium alkaloids gives information on geographical origin and synthesis of the sample. The profiling was carried out by calculating $\delta^{13}C$ with equation 13.

$$d^{13}C = \frac{(^{13}C/^{12}C)_{sample} - (^{13}C/^{12}C)_{standard}}{(^{13}C/^{12}C)_{standard}} \times 1000‰ \qquad (13)$$

The same nine samples analysed in Method 2 were analysed by GC-IRMS. The samples were clustered correspondingly into the same four groups when using the chemical impurity profiling. Due to the relative ease of using of normal impurity profiling, it is preferred to isotope ratio analysis.

In addition to GC methods, HPLC has been used in heroin profiling [86]. Chromatograms of 24 illicit samples were compared visually. Moreover, percentages of diamorphine, acetylcodeine, noscapine, papaverine and few impurities were determined. Similarities between 33 street samples have been also determined without chemical impurity profiling [95]. Head space-gas chromatography (HS-GC) was used to determine solvent residuals. Heroin content and adulterants were obtained by GC and more information from diluents by HPLC. Atomic absorption (AA) was used for the analysis of traces of metal, namely iron and zinc. The results from each analysis were combined and similarities between the samples were found.

2.8.4 Cocaine

Cocaine (benzoyl methyl ecgonine **16**) is a powerfully active drug of abuse. It has similar effects to amphetamines; a feeling of well-being and euphoria are established by central nervous system stimulation. Cocaine has been an abused drug for more than a century [96]. Illicit cocaine is generally sold on the street as cocaine hydrochloride powder for oral and intravenous use. "Crack" is a street name given to cocaine that has been processed from cocaine hydrochloride to a free base for smoking. Bolivia, Columbia and Peru are the major producers of cocaine [57]. 1 - 5% of young adults has tried cocaine in European Union region [57].

In addition to cocaine, at least 21 different alkaloids have been identified as natural congeners in extracts of coca leaf. Tropane alkaloids **16 - 23** are extracted from the leaves of the genus *Erythroxylon* grown in South America. The structures of alkaloids are shown in Figure 8. Two of 200 different species of *Erythroxylon*, namely *Erythroxylon coca* and *Erythroxylon novogranatense* contain significant amounts of cocaine [97]. The presence and concentration of the alkaloids may vary greatly, depending on the species and growing area.

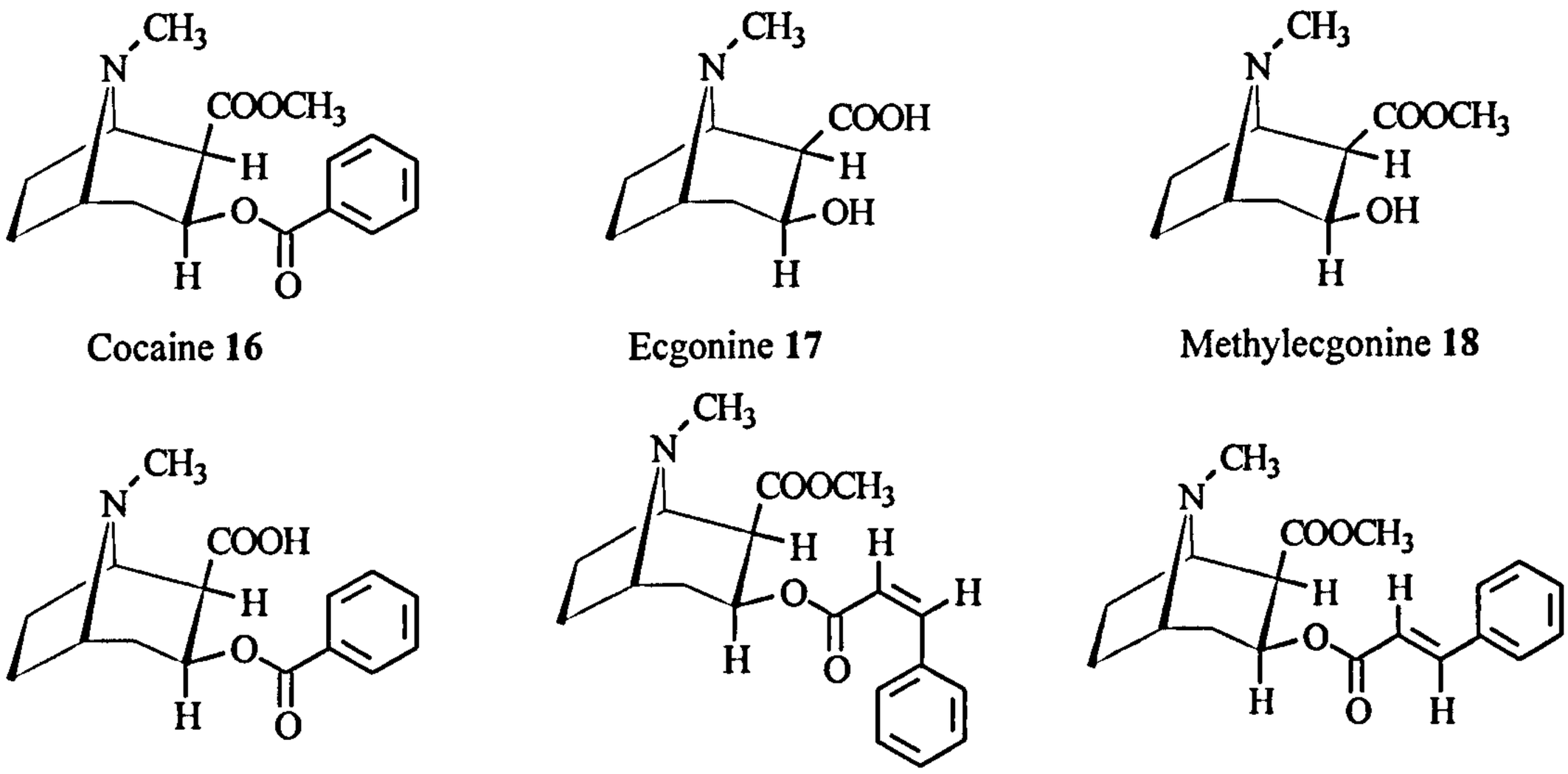
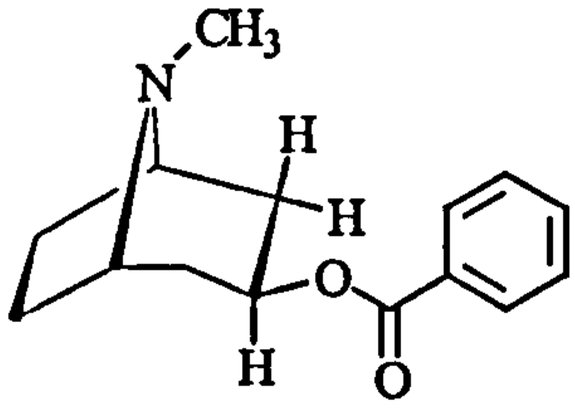
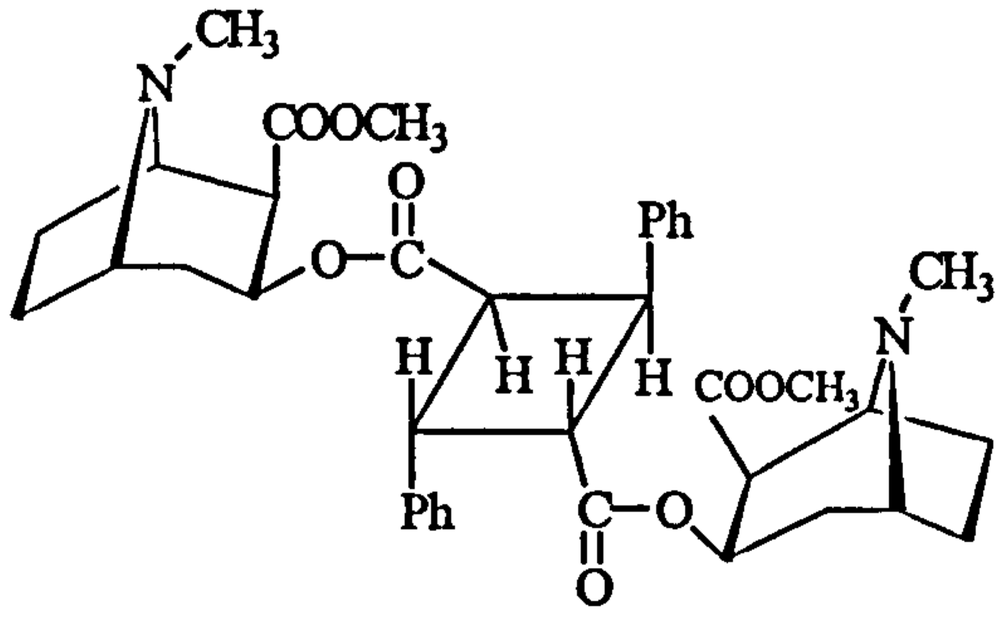


Figure 8: Structural formulas of cocaine alkaloids.



Tropacocaine **22**

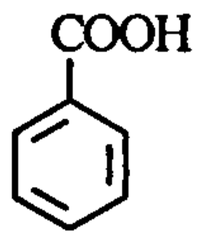


α-Truxilline **23**

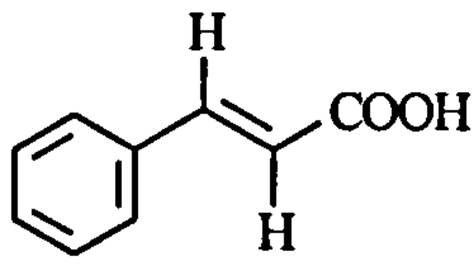
Figure 8: Cont'd / ...

Cocaine was manufactured for the first time in 1855 [98]. The general approach to the production of illicit natural cocaine involves three steps: (i) extraction of crude coca paste from the coca leaf, (ii) purification of coca paste to coca base and (iii) conversion of coca base to cocaine hydrochloride [97]. The cocaine purity level of coca paste varies from 30% to 80%, depending of extraction technique and variety of coca. The coca paste contains several co-extracted alkaloids resulting in a poor quality. Therefore the purification step is rarely skipped in illicit laboratories. Illicit, unadulterated cocaine hydrochloride generally varies from 80% to 97% purity, from an off-white powder to white. In addition to illicit natural cocaine, synthetic cocaine has been obtained [97]. The synthetic route is difficult and the preparation of (-)-cocaine via total synthesis process results in an overall yield of less than 10%, whereupon synthetic cocaine is very unusual.

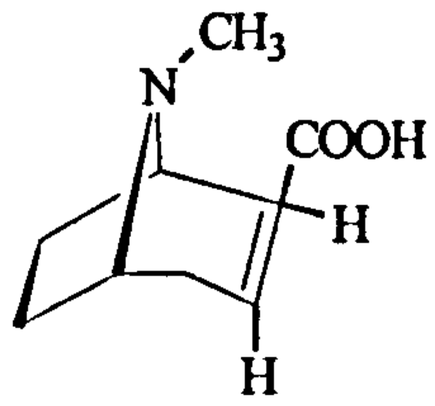
Illegal cocaine samples usually contain varying amounts of several alkaloids and synthetic impurities. Numerous impurities have been identified [99-101] and the structures of some common compounds are shown in Figure 9.



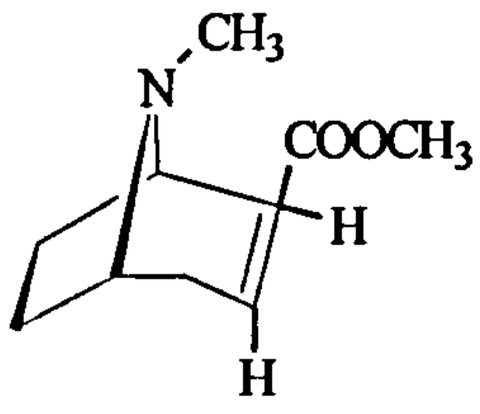
Benzoic acid **24**



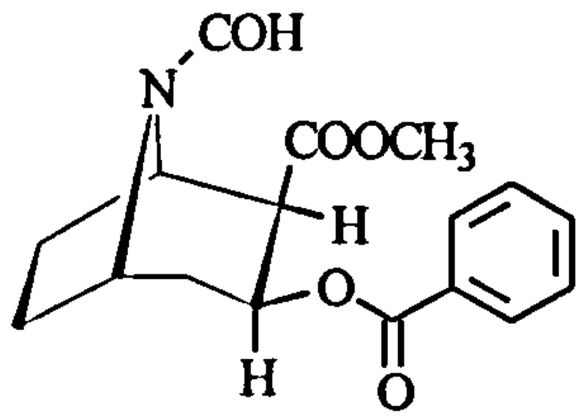
trans-Cinnamic acid **25**



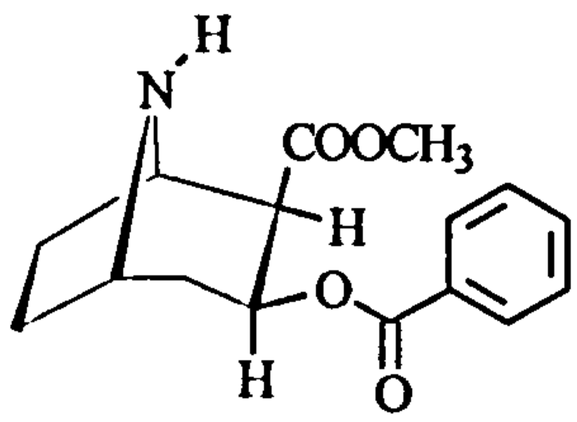
Anhydroecgonine **26**



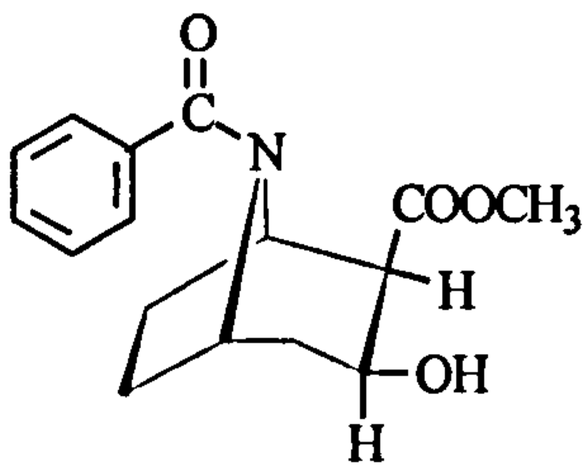
Anhydromethylecgonine **27**



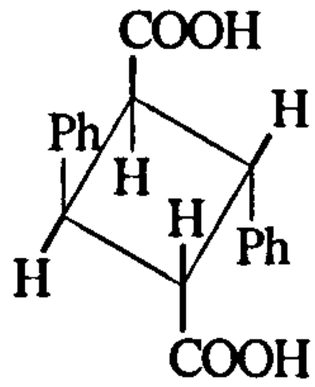
N-Formyl cocaine **28**



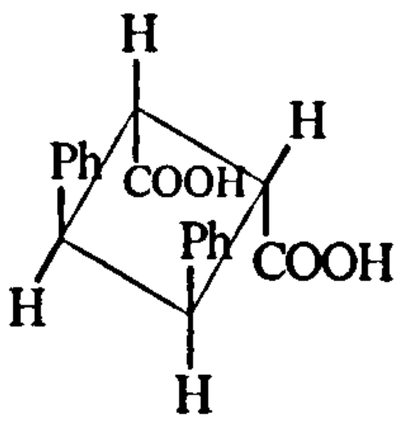
Norcocaine **29**



N-Benzoylnormethylecgonine **30**



Truxillic acid **31**



Truxinic acid **32**

Figure 9: Structural formulas of impurities found in cocaine samples.

The substances originate from extraction process of the coca leaf, from the manufacturing progress or from adulteration. Methylecgonine **18** and benzoylecgonine **19** are present in coca leaves, but are also products from hydrolysis of cocaine [102]. Most common adulterants in illicit cocaine are ephedrine, norephedrine, procaine, lidocaine, tetracaine and benzocaine [103]. The methods used for cocaine comparison are summarised in Table 2, and discussed below.

Table 2: Chemical profiling methods for cocaine.

#	Sample preparation	Analysis method and conditions		Ref.
1	Dissolution: ethanol	Method:	GC-NPD	104
		Column:	12.5 m x 0.2 mm, d_f 0.5 µm of Me silicone (HP-1)	105
		T-program:	i) isotherm 230°C and ii) 120°C (2 min), 6°C/min to 320°C (5 min)	
		Injection:	215°C, split 1:50	
		Carrier gas:	He	
		Flow rate:	45 cm/s (120°C) or 37 cm/s (230°C)* (1.5 ml/min)	
2	Dissolution: chloroform Silylation: BSA heated at 80°C (15min)	Method:	GC-FID	102
		Column:	30 m x 0.25 mm, d_f 0.25 µm of 7% cyanopropyl Ph Me silicone (DB-1701)	
		T-program:	180°C (1 min), 4°C/min to 200°C and 180°C (1 min), 6°C/min to 275°C	
		Injection:	230°C, split 1:50	
		Carrier gas:	He	
		Flow rate	30 cm/s	
3	Dissolution: chloroform	Method:	GC- dual FID	106
		Column:	1.52 m x 2 mm, packed with 3% OV-17	
		T-program:	100°C (2 min), 12°C/min to 320°C (5 min) and 200°C (2 min), 12°C/min to 320°C (5 min)	
		Injection:	250°C, not available	
		Carrier gas:	N_2	
		Flow rate:	30 ml/min**	
4	Dissolution: acetonitrile Silylation: HFBA heated at 75°C (15min). Back LLE: isooctane and sodium bicarbonate/acid phthalate buffer (pH 4)	Method:	GC-ECD	107
		Column:	30 m x 0.25 mm, d_f 0.25 µm of 7% cyanopropyl Ph Me silicone (DB-1701)	
		T-program:	90°C (5 min), 25°C/min to 160°C (1 min) and 90°C (5 min), 4°C/min to 275°C (15 min)	
		Injection:	225-250°C, splitless	
		Carrier gas:	H_2	
		Flow rate:	40-50 cm/s (at 90°C)	
5	LLE: water and sodium bicarbonate/chloroform SPE: alumina column	Method:	GC-MS, SIM mode	108
		Column:	30 m x 0.25 mm, d_f 0.25 µm of 7% cyanopropyl Ph Me silicone (DB-1701)	
		T-program:	100°C (5 min), 3°C/min to (not available) (20 min)	
		Injection:	250°C, split 1:24	
		Carrier gas:	He	
		Flow rate:	33.7 cm/s	

* Flow rate was transformed to correspond to cm/s by FlowCalc.

** The cm/s value cannot be calculated for a packed column.

To confirm the suitability of the chromatographic method for profiling, the stability of the peak area ratios of *cis*- and *trans*-cinnamoylcocaine **20 - 21**, tropacocaine **22**, norcocaine **29** to cocaine **16** were determined (Method 1) [104,105]. Further, the effect of adding of adulterants was examined. The results indicated that the peak area ratios were stable and unaffected by adulterants. The Euclidean distance based on four alkaloid/cocaine ratios was used to measure the similarity between the samples by BASIC program. Final confirmation was obtained by visual comparison. In

conclusion, it was found that this approach to comparison analysis of illicit cocaine is rapid and simple enough for routine use.

Seventeen alkaloids and impurities were identified from 368 seized cocaine samples (Method 2) [102]. The possibility of artefacts produced by GC was taken into consideration when natural products were identified. Fourteen impurity compounds were chosen for statistical analysis and three were discarded due to decomposition. For example, anhydromethylecgonine **27** was found to be formed in the injection port at 250°C [102,109,110]. Moreover, the thermal decomposition of truxillines **23** can yield truxillic **31** and truxinic acids **32** and anhydromethylecgonine [102]. In this experiment, correlation coefficients were determined to show the linear relationship between the variables [102]. The analysis indicated a marginally high correlation between *cis*- and *trans*-cinnamoylcocaine and between benzoic acid **24** and ecgonine **17.** The correlation between substances is logical since *cis*- and *trans*-cinnamoylcocaines are isomers, and benzoic acid and ecgonine are the principal products from acid hydrolysis of cocaine. Chromatograms were compared visually.

Non-polar columns allow elution of most drugs and thus are preferred as columns for screening purposes. In a comparison study, better separation was achieved with a more polar 50% diphenyl dimethyl silicone column (OV-17) (Method 3) [106] or cyanopropyl silicone column (DB-1701) (Method 4) [107]. All compounds were not, however, chromatographed properly due to lack of derivatisation in the previous case. In the latter experiment, good resolution and good peak shapes occurred after silylation. In addition to the comparison of different stationary phases, different type of detectors were compared to evaluate the profiling method. The more selective electron-capture detector (ECD) was found to give advantages compared to FID, especially for halogenated compounds [107]. Thus, in this experiment the cocaine samples were derivatised with heptafluorobutyric anhydride (HFBA), and impurities such as hydroxycocaines were easily detected by GC-ECD (Method 4). Visual comparison based on these hydroxycocaines and other substances was used in the profiling of over 100 seized cocaine samples [107].

In addition to visual comparison, "pictograms" have been utilised in the comparison of illicit cocaine profiles [106]. The peak heights of six compounds **20 - 22** and **28 - 30** were measured from 71 profiles analysed by Method 3. At first, the peak heights of the target compounds were measured and the ratio calculated by equation 14.

$$x_i = \frac{h_i}{H} \times 10 \qquad (14)$$

The highest peak (H) is given a reference value 10 and all other peaks (h) are expressed as ratios of H and multiplied by 10. Pictograms appear to be a powerful tool for sample comparison when the number of samples is limited.

Utilising previously described profiling methods, a positive correlation between samples has been established and comparative cocaine analyses accepted successfully by the court in a smuggling case in the USA [111]. At first, impurities were detected as heptafluorobutyrate (HFB) derivatives by GC-ECD due to excellent reproducibility of the comparison method (Method 4) [107]. More impurities and also adulterants were detected with Method 3, e.g. two isomers of cinnamoylcocaine and citric acid [102]. The third method was provided for the relative determination for 10 of the 11 isomeric truxillines [111]. The chromatograms were compared visually and identical profiles indicated that two samples came from the same source.

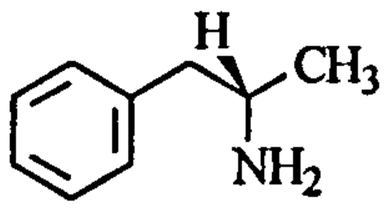
Recently, new cocaine profiling methodology has been introduced by the Drug Enforcement Administration (DEA) in the USA [108]. Previously, six independent profiling methods were used based on the determination of main alkaloids, impurities, 10 isomers of truxilline alkaloids and residual solvent composition. In the new methodology alumina column chromatography was used for isolation of impurities from the cocaine matrix (Method 5) [108]. The residue from sample preparation was eluted through the alumina column using four different eluent systems. The collected fractions were further treated and derivatised with N-methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA). The sensitivity of the profiling method was improved significantly by using GC-MSD in the selected ions

monitoring (SIM) mode. The alkaloids found from the first extraction were not structurally identified. The most important compound group identified from the second fraction is 11 truxilline isomers. The third fraction indicated the most peak rich chromatograms. More interesting alkaloids were identified from the fourth fraction. To prove the suitability of the profiling method these four fractions were collected from 51 cocaine samples.

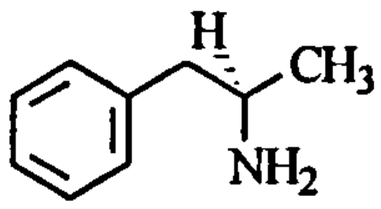
2.8.5 Amphetamine-type stimulants

Amphetamine **33** and the amphetamine-type stimulants are, after cannabis, the second most frequently used illicit drug in the European Union [112,57]. Compared to the plant-based drugs, heroin and cocaine, clandestine synthetic drugs have rapidly become a part of mass youth culture. Trafficking of amphetamine-type stimulants is significantly intra-regional, i.e. drugs are often produced close to the consumer markets [56,57]. Illicit laboratories have already been found in 10 Member States [57]. Most amphetamine is produced in Belgium, the Netherlands and the United Kingdom. The illicit amphetamine product is sold frequently as salt, namely sulphate and hydrochloride, and diluted with different adulterants. The most common adulterants are caffeine and different sugars. In the 1980's, the majority of seized clandestine laboratories in the USA were associated with the production of methamphetamine **34** [113].

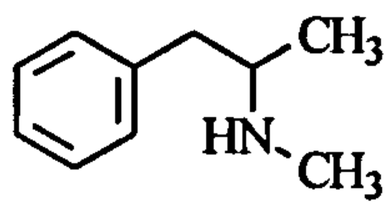
The European Union has become one of the most important production regions for ecstasy, i.e. 3,4-methylenedioxymethamphetamine (MDMA **35**). MDMA is the most popular of the 3,4-methylenedioxyamphetamine (MDA **36**) series and at present is commonly found in street seizures. Young people discovered it in the early part of the 1980's and it became a frequently used drug at clubs and rave parties. Ecstasy became illegal in 1985 in the USA and few years later in the European countries. It occurs in the illicit market usually as tablets. Recently, other "designer drug" analogs of MDA, namely N-ethyl-(3,4-methylenedioxyamphetamine) **37** and N-hydroxy-(3,4-methylenedioxyamphetamine) **38** have also been encountered in illicit ecstasy samples. The structures of amphetamine-type stimulants are shown in Figure 10.



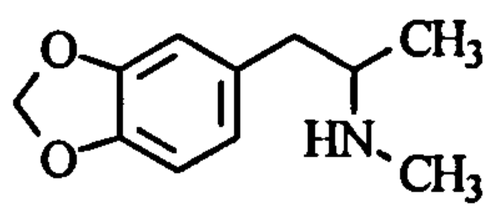
d-Amphetamine **33 a**



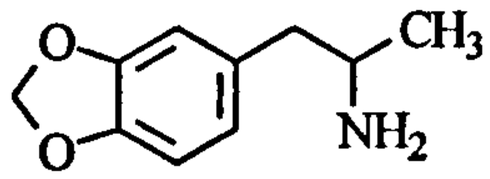
l-Amphetamine **33 b**



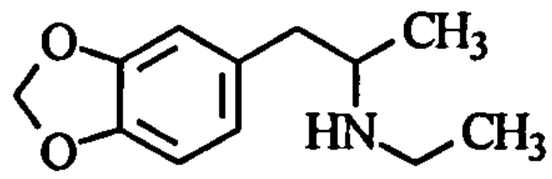
Methamphetamine **34**



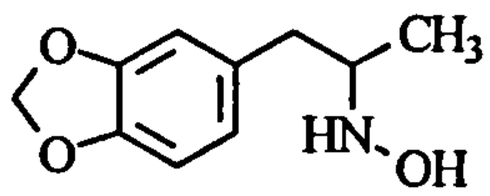
3,4-Methylenedioxymeth-amphetamine (MDMA) **35**



3,4-Methylenedioxy-amphetamine (MDA) **36**



N-Ethyl-(3,4-methylenedioxy-amphetamine) **37**



N-Hydroxy-(3,4-methylenedioxy-amphetamine) **38**

Figure 10: Structural formulas of amphetamine-type stimulants.

The effects of amphetamine-type stimulants are similar to those of cocaine, but their duration is longer. The effect in the central nervous system is similar to adrenaline by causing auditory and visual hallucinations. Over time, because of tolerance which develops to the stimulant effects, chronic users will increase their dose to achieve the same effect. Different enantiomers of optically active amphetamine have very different properties [114]. *d*-Amphetamine **33 a** has a much higher affinity for target sites in the central nervous system and produces significantly greater stimulant effects than the corresponding *l*-enantiomer **33 b**. All frequently used synthetic procedures for the synthesis of amphetamine produce a racemic mixture, i.e. equimolar amounts of *d*- and *l*-enantiomers. The stimulant activity of methamphetamine is greater than the effect of amphetamine. Ecstasy may also be hallucinogenic in large doses [115].

It is known that when analysing impurities originating from the synthetic process, illegal synthetic drugs can be compared and characterised. The methods used for methamphetamine comparison are summarised in Table 3, and discussed below.

Table 3: Chemical profiling methods for methamphetamine.

#	Sample preparation	Analysis method and conditions		Ref.
1	LLE: water/n-heptane	Method: Column: T-program: Injection: Carrier gas: Flow rate:	GC-FID-NPD (1:2) 11 m x 0.2 mm, d_f 0.25 µm of 5% Ph Me silicone (SE-54) 110°C, 6°C/min to 212°C, 10°C/min to 272°C (10 min) 250°C, split 1:40 He 40 cm/s	116
2	LLE: phosphate buffer (pH 7)/ethyl acetate	Method: Column: T-program: Injection: Carrier gas: Flow rate:	GC-FID 15 m x 0.53 mm, d_f 1.5 µm of Me silicone (DB-1) 100°C (1 min), 15°C/min to 200°C, 2°C/min to 208°C, 10°C/min to 300°C (18 min) 270°C, splitless He 59 cm/s* (7 ml/min)	43 44
3	LLE: phosphate buffer (pH 10.5)/ethyl acetate	Method: Column: T-program: Injection: Carrier gas: Flow rate:	GC-FID 25 m x 0.2 mm, d_f 0.33 µm of 5% Me silicone (Ultra-2) 50°C (1 min), 10°C/min to 300°C (4 min) 250°C, splitless N_2 41 cm/s* (1.4 ml/min)	17
4	LLE: phosphate buffer (pH 6)/n-heptane	Method: Column: T-program: Injection: Carrier gas: Flow rate:	GC-FID 25 m x 0.2 mm, d_f 0.33 µm of 5% Ph Me silicone (Ultra-2) 50°C (1 min), 15°C/min to 200°C, 1°C/min to 205°C, 10°C/min to 300°C (3 min) 250°C, splitless He 32 cm/s* (1 ml/s)	48

* Flow rate was transformed to correspond to cm/s by FlowCalc.

Firstly, methamphetamine hydrochloride was dissolved in water and extracted with organic solvent (Method 1) [116]. Buffers are frequently used at pH 7. Capillary columns and a combination of different detectors offer many advantages in the profiling method. FID together with a nitrogen and phosphorus selective detector (NPD) has a high stability and a large linear range [116]. Inter- and intra-laboratory variations in methamphetamine batches were examined by comparing the chromatograms visually. Variation between synthesised methamphetamine batches (inter-variation) depends on how strongly the synthesis conditions influence the chemical profile. The influence of reaction time and temperature, the amount of the starting material and the effect of distillation and crystallisation in the impurity profiles were determined. Significant differences between batches were obtained.

Variation within the batches (intra-variation) can be caused by heterogeneity and chemical instability of impurities.

Tanaka *et al.* [43] studied different extraction solvents and found ethyl acetate to offer the best extraction efficiency (Method 2). Moreover, the stability of impurities was acceptable during an eight-week period and similarity between chromatograms indicates small intra-laboratory variation. More than 130 seized samples were analysed and inter-variation was determined [43,44]. The similarity or dissimilarity of each sample was evaluated by calculation of the Euclidean distances (equation 1). Three individual cases were presented using the described method, and the link between samples could be illustrated.

The United Nations International Drug Control Programme (UNDCP) has been involved in the development of the standard methods for the profiling analysis of methamphetamine (Method 3) [17]. The chemical impurity profiling method was presented and the chromatograms compared visually.

In one experiment, similarities between batch origin have been proved on the basis of three definitions - (i) determination of quotient values (q), (ii) the deviation of matched peaks (r), and (iii) the Euclidean distance (*d*) (Method 4). The target compounds of two profiles were compared and quotient calculated using equation 8. A mean area deviation (r) was calculated from the values of r_i where x_i and y_i are peak areas of the i^{th} pair of matched peaks from the sample profiles 1 and 2 (equation 15). The Euclidean distance was calculated using the equation 1. The following criteria for the definitions were determined and used by Perkal *et al.*: $q = 0.85$, $r < 0.06$ and $d = 0.05$ [48]. If any two of the above criteria are satisfied, two samples are likely to be from the same batch.

$$r = \frac{\sum_i r_i}{n} \qquad r_i = \frac{|x_i - y_i|}{x_i + y_i} \tag{15}$$

In addition to GC, HPLC and capillary electrochromatography (CEC) have been introduced as a methamphetamine profiling techniques [117]. CEC allows the use of smaller particle sizes and longer columns than are possible in HPLC. This results in increased resolution and much higher peak efficiency. The chromatograms were compared visually, but a particular comparison study was not done.

Ecstasy

The chemical impurity profiling of ecstasy tablets is not as simple as comparison of other synthetic drugs. One batch of ecstasy tablets is not as homogenous as one batch of, for example, amphetamine. The concentration of active substance varies and usually the concentration is only few tens of milligrams per tablet. Moreover, even if the tablets have the same ballistic features, it cannot be assumed they are from the same batch. It appears the development of chemical impurity profiling methods for ecstasy will be one of the most interesting subjects for future investigations.

Germany has recently introduced a profiling project for ecstasy tablets [118]. The Central Analysis Program Ecstasy (CAPE) project is based on the determination of the external and internal parameters of tablets. In ballistic profiling, the external parameters include the logos and diameters of tablets. The comparison based on the internal parameters includes the identification of impurities and chemicals used in the synthetic routes. However, analytical methods or individual impurities used in chemical impurity profiling have not been presented until now.

Another ecstasy profiling project is running in Denmark [119,120]. During a two-year period the physical description of ecstasy tablets has been collected into a national database. The database contains information of several thousand ecstasy tablets from around the country. The profiling of tables is based on only the ballistic features. For example, the diameter, weight, logos and identity of active substances have been collected and stored in a database.

Different sample preparation methods in chemical drug profiling have been studied by Rashed *et al.* [121]. Solid phase extraction (SPE) method using C18 columns was compared to the traditional liquid-liquid extractions (LLE)- phosphate buffer/isooctane and ethyl acetate. The comparison was based on three target impurities. The extracts were analysed by GC-FID using 5% phenyl methyl silicone capillary column. The results showed that the SPE method was more efficient than LLE. However, due to limited number of target compounds chosen for the experiment, the results might be unreliable.

Profiling based on the isotope ratios of $^{13}C/^{12}C$ and $^{15}N/^{14}N$ has been carried out [37]. The samples were analysed by GC-IRMS and classification carried out by calculating $\delta^{13}C$ by equation 13 and correspondingly $\delta^{15}N$ values. Several samples were classified into four groups. It was pointed out that even if $\delta^{13}C$ values were comparable, the samples might still be from different batches.

Raman spectroscopy is a new technique for ecstasy profiling [122,123]. It is a rapid and non-destructive technique, i.e. the sample can be analysed without preparation. It is believed to be a potential method for profiling and even for quantitation. The compound is identified according to bands with specific Raman shift values. The method may have many advantages, but it has only been studied for a relatively short period. Problems include the fact that strong colour in the drug sample might complicate the identification [124].

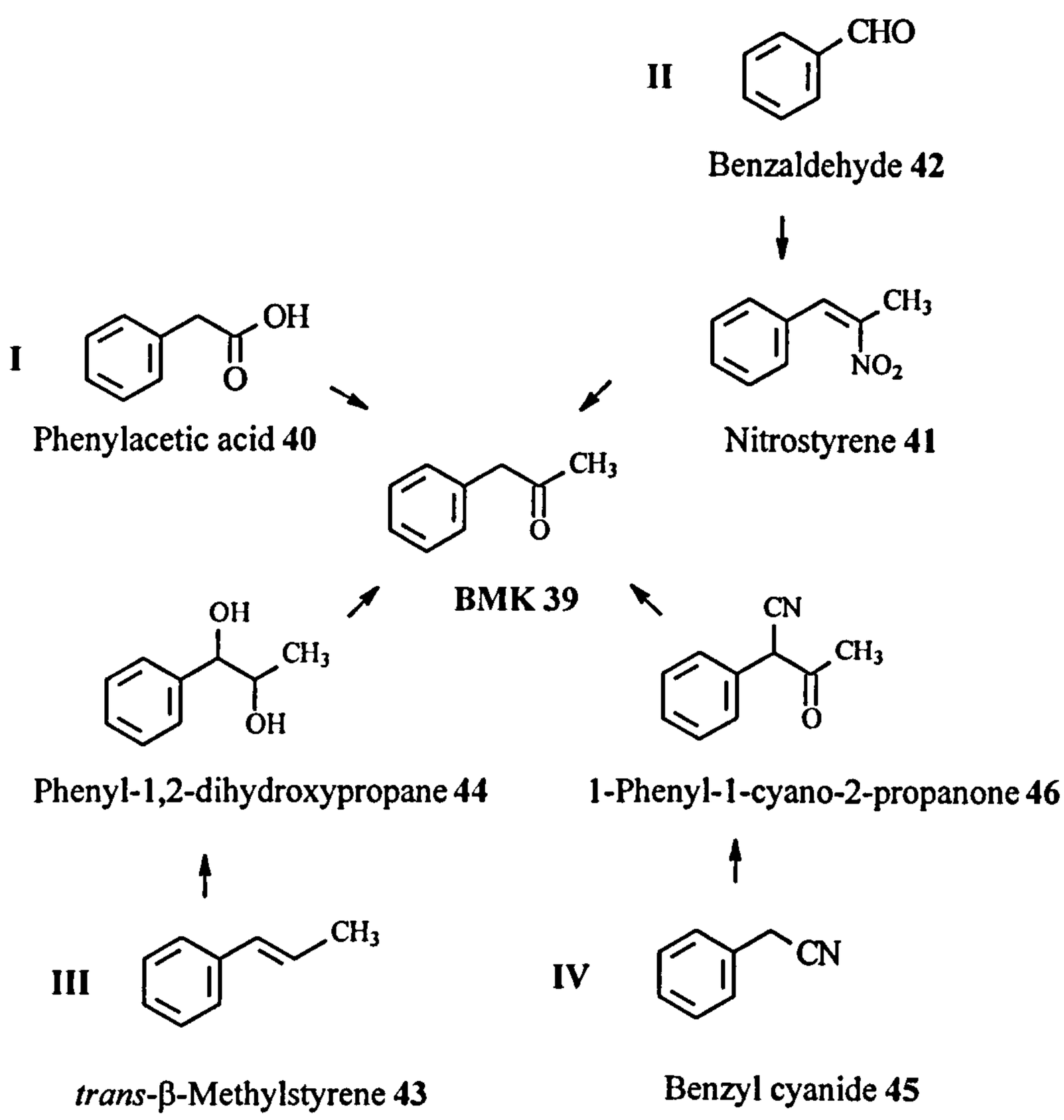
3 AMPHETAMINE

3.1 Introduction

A variety of methods are available for the illicit production of synthetic drugs. Detailed information on the manufacturing process of synthetic drugs is available through the internet and other open sources [57]. The Leuckart method is one of the oldest procedures for the illicit manufacture of amphetamine and amphetamine analogues. Until the 1980's, most amphetamines in Europe had been synthesised by the Leuckart method. Alternative methods for the preparation of amphetamine have been introduced, including reductive amination and nitrostyrene routes. Due to the tight control on frequently needed starting materials, benzyl methyl ketone (BMK **39**) is manufactured in illicit laboratories. Commonly used synthetic methods for BMK and amphetamine, for each synthetic route, are summarised in this chapter. Moreover, typical impurities for each synthetic route are detailed. Analogous reaction routes are also utilised in the synthesis of other amphetamine-type stimulants, including methamphetamine [152] and ecstasy [125,126], resulting in analogous impurities.

3.2 Synthesis of benzyl methyl ketone

One of the most common approaches in the synthesis of amphetamine used by clandestine laboratory operators involves the amination of benzyl methyl ketone (phenylacetone, 1-phenyl-2-propanone **39**). However, BMK is a controlled substance. For example, it is listed in Schedule II of the United States Controlled Substance Act, 1980 [127], and controlled in most European countries. Since the introduction of controls on BMK, clandestine laboratories have developed various methods for the synthesis of this important precursor. The synthesis reactions I - IV are shown in Scheme 1 and discussed below.



Scheme 1: Synthetic routes (I –IV) used for synthesising of benzyl methyl ketone.

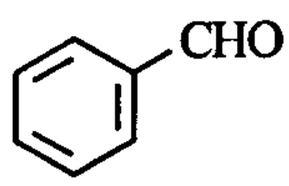
One of the most commonly employed methods for synthesis of BMK is condensation of phenylacetic acid **40** with acetic anhydride [128] or with lead (II) acetate [129] (Scheme 1, Reaction I). BMK is also often prepared by the reduction of 1-phenyl-2-nitropropene (nitrostyrene **41**). Nitrostyrene is synthesised from benzaldehyde **42** and nitroethane in a two step process. BMK is formed by the reaction of the nitrostyrene with iron and HCl in the presence of a catalytic amount of ferric chloride (Scheme 1, Reaction II). Another approach in the synthesis of BMK from nitrostyrene is to use lithium aluminium hydride ($LiAlH_4$) [130].

A commercially available, unregulated precursor for the synthesis of BMK is *trans*-β-methylstyrene **43**. A two step reaction with hydrogen peroxide, formic acid and *trans*-β-methylstyrene results in an intermediate 1-phenyl-1,2-dihydroxypropane **44** (Scheme 1, Reaction III). The product is treated with sulphuric acid resulting in the

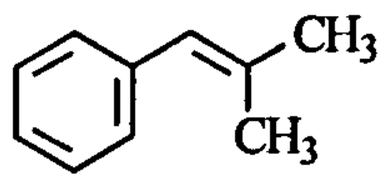
final product [114]. The reaction of benzyl cyanide **45** and ethyl acetate with sodium ethoxide results in the intermediate, 1-phenyl-1-cyano-2-propanone **46**, which is hydrolysed to BMK (Scheme 1, Reaction IV) [131].

The synthesis from benzyl cyanide as well as from other precursors results in BMK, which is mix of various contaminants and impurities. Steam distillation is employed for purification of clandestine BMK. Typical by-products and impurities from these described synthesis methods are summarised and shown in Figure 11.

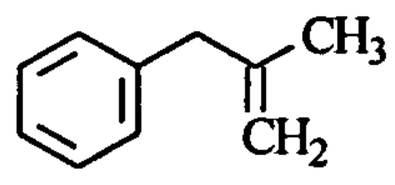
Reaction I: Phenylacetic acid and acetic anhydride



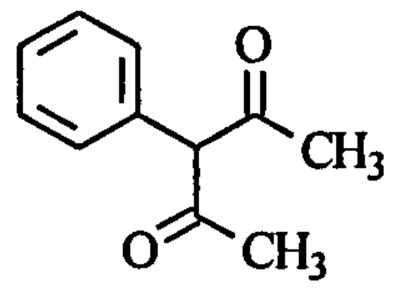
Benzaldehyde **42**



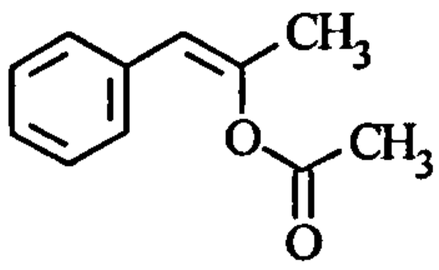
1-Phenyl-2-methyl-1-propene **47**



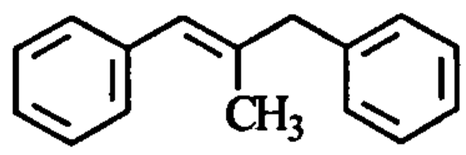
1-Phenyl-2-methyl-2-propene **48**



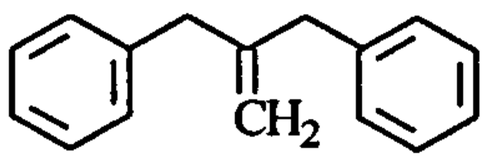
3-Phenylpentan-2,4-dione **49**



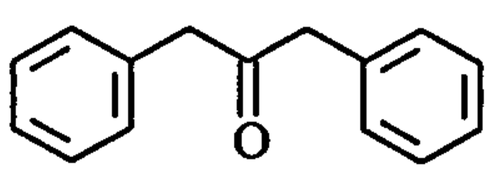
Benzylmethylketone enolacetate **50**



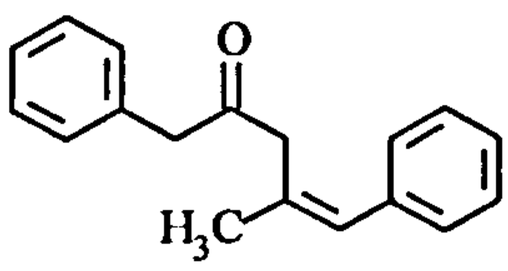
1-Phenyl-2-benzyl-1-propene **51**



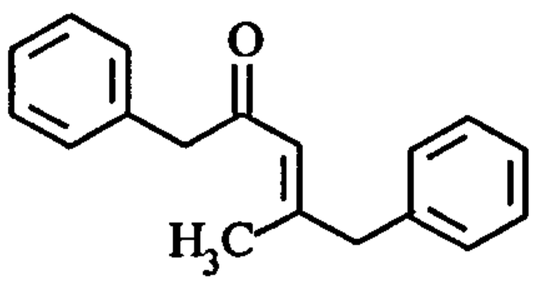
1-Phenyl-2-benzyl-2-propene **52**



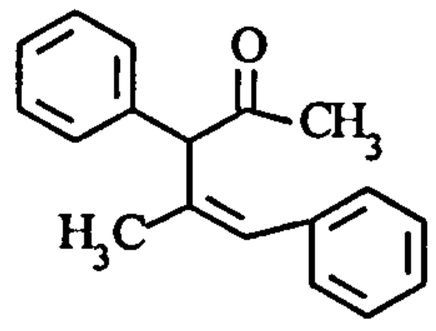
Dibenzylketone **53**



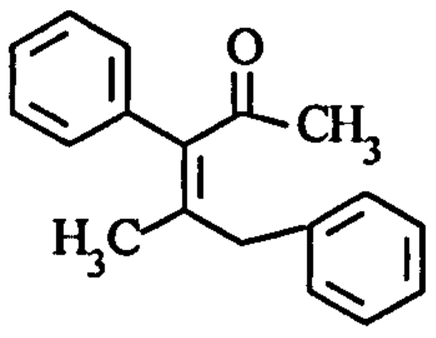
1,5-Diphenyl-2-methyl-1-penten-4-one **54**



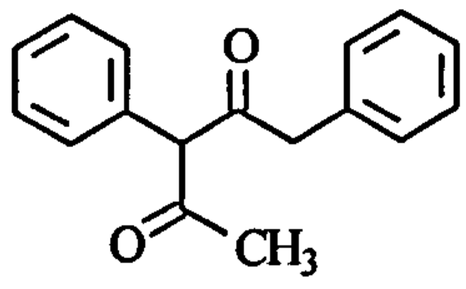
1,5-Diphenyl-2-methyl-2-penten-4-one **55**



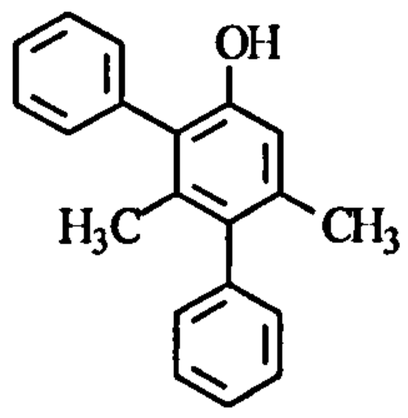
1,3-Diphenyl-2-methyl-1-penten-4-one **56**



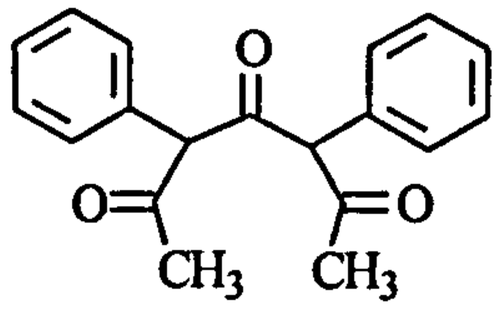
1,3-Diphenyl-2-methyl-2-penten-4-one **57**



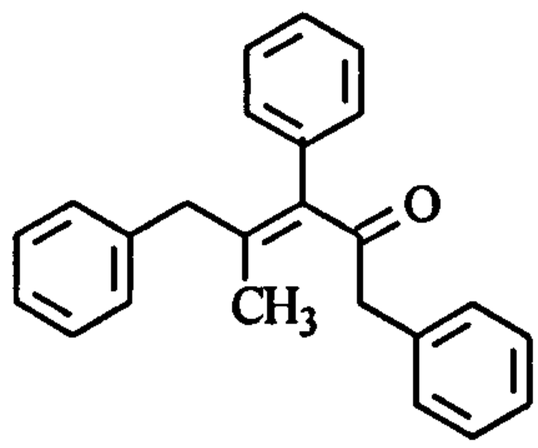
1-Acetyl-1,3-diphenylacetone **58**



2,4-Diphenyl-3,5-dimethylphenol **59**



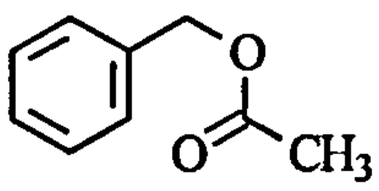
1,3-Diacetyl-1,3-diphenylacetone **60**



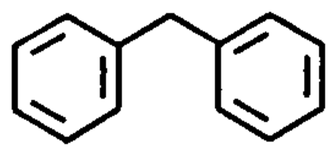
1,3,5-Triphenyl-2-methyl-2-penten-4-one **61**

Figure 11: Structural formulas of impurities found in BMK.

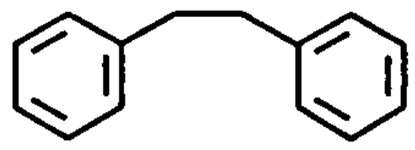
Reaction I: Phenylacetic acid and lead (II) acetate



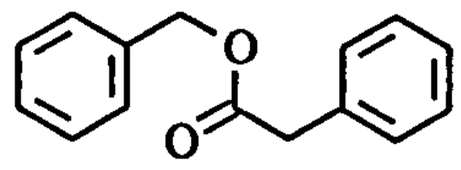
Benzylacetate **62**



Diphenylmethane **63**

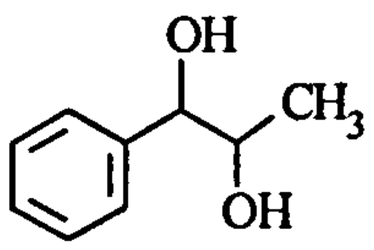


Bibenzyl **64**

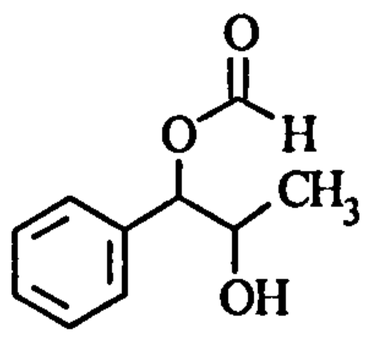


Benzylphenylacetate **65**

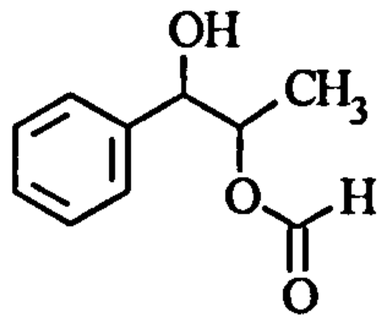
Reaction III: *trans*-β-Methylstyrene



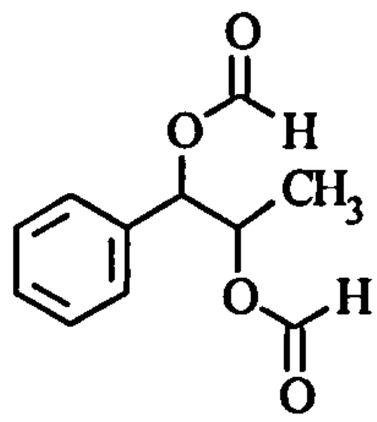
1-Phenyl-1,2-dihydroxypropane **44**



1-Phenyl-1-formyl-2-hydroxypropane **66**

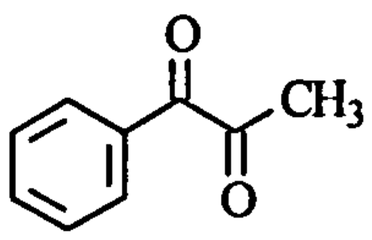


1-Phenyl-1-hydroxy-2-formylpropane **67**

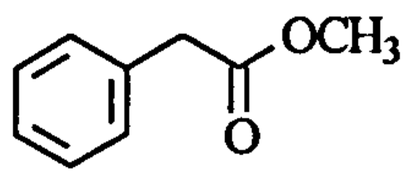


1-Phenyl-1,2-diformylpropane **68**

Reaction IV: Benzyl cyanide



1-Phenyl-1,2-propandione **69**



Methyl ester of phenylacetic acid **70**

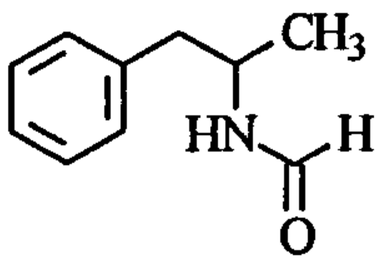
Figure 11: Cont'd /...

3.3 Synthesis of amphetamine

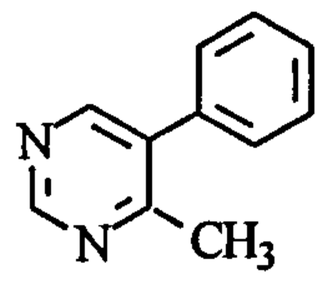
The use of BMK allows many routes to be used to synthesise amphetamine, but other starting materials are also available. The synthesis reactions (I - VI) are summarised in Scheme 2 and discussed below.

frequently with sulphuric acid resulting in amphetamine sulphate. Amphetamine salt may be further purified with recrystallisation.

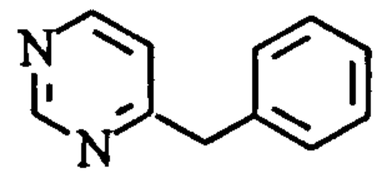
The influence of different reaction conditions on impurity profiles and the yield of amphetamine has been studied by Johansson [137] and Kronstrand [138]. The yield was improved using higher reaction temperatures and replacing ammonium formate with formamide. The results indicated that the use of formic acid reduced the amount of impurities produced. In the Leuckart method, after one hour of reaction a considerable amount of pyrimidines, namely 4-methyl-5-phenylpyrimidine **72** and 4-benzylpyrimidine **73** together with N,N-di(β-phenylisopropyl)amine (DPIA **74**) and N,N-di(β-phenylisopropyl)formamide (DPIF **76**) had been formed [138]. N,N-di(β-phenylisopropyl)methamine (DPIMA **75**) is formed when the refluxing is continued, and subsequently a considerable amount of DPIF is formed. In 1977, it was found that the most common impurity in seized amphetamines was 4-methyl-5-phenylpyrimidine [139,140]. This is a route specific impurity together with another pyrimidine, 4-benzylpyrimidine. Route specificity means that these substances are unique to a particular synthetic route; in this case, the Leuckart route [141]. In the mid 1980's it was discovered that in most amphetamine samples, the main impurity was DPIA [142] – illustrating that the main impurities may differ from one laboratory to another. An important and rich group of impurities includes the different pyridines **80 - 85.** Pyridines have the same molecular weight (MW = 259) and similar mass spectra [143-145]. Moreover, the concentration of these substances is frequently very low, which makes the identification of different isomers difficult. Typical impurities obtained from the Leuckart reaction are shown in Figure 12.



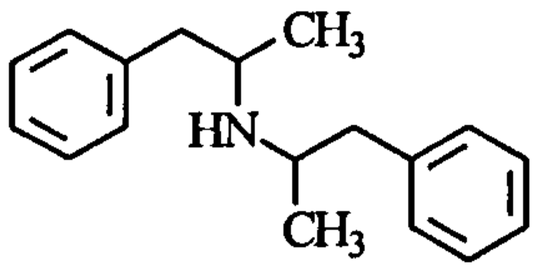
N-Formylamphetamine **71**



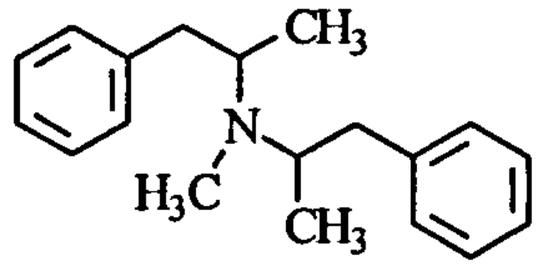
4-Methyl-
5-phenylpyrimidine **72**



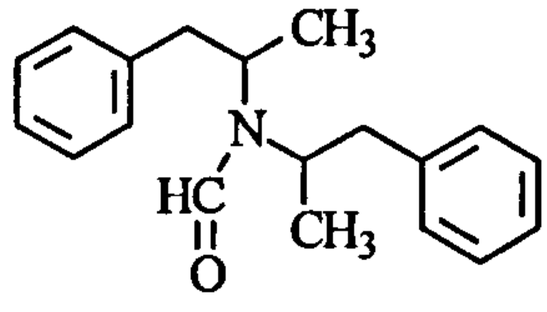
4-Benzylpyrimidine **73**



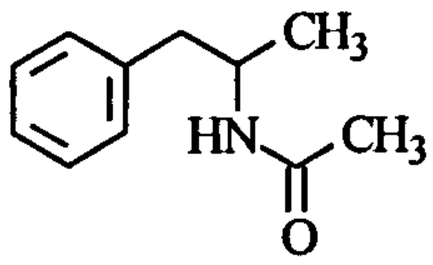
N,N-Di(β-phenylisopropyl)-
amine (DPIA) **74**



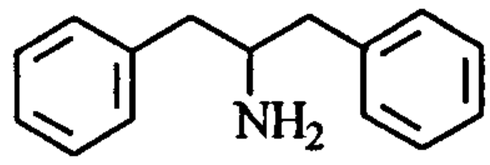
N,N-Di(β-phenylisopropyl)-
methylamine (DPIMA) **75**



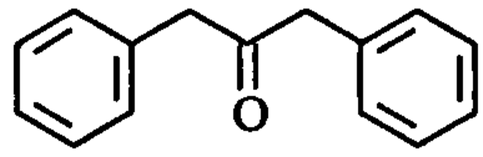
N,N-Di(β-phenylisopropyl)-
formamide (DPIF) **76**



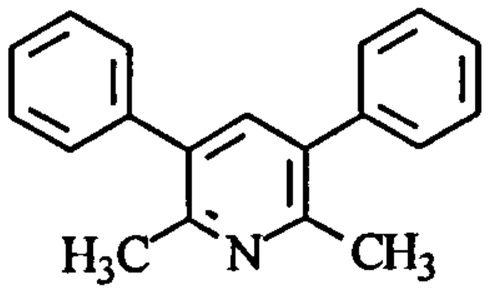
N-Acetylamphetamine **77**



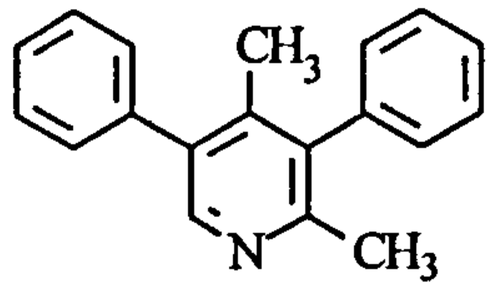
Dibenzylmethylamine **78**



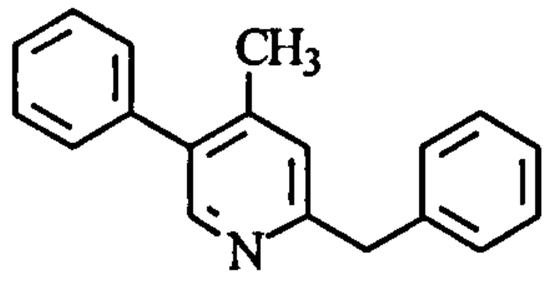
Bibenzylketone **79**



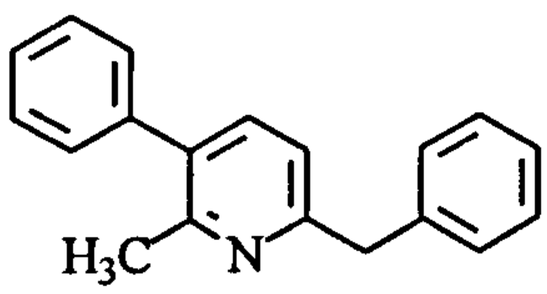
2,6-Dimethyl-3,5-
diphenylpyridine **80**



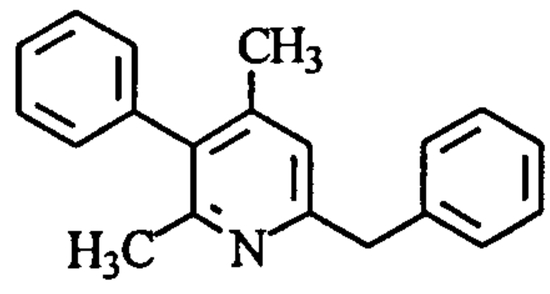
2,4-Dimethyl-
3,5-diphenylpyridine **81**



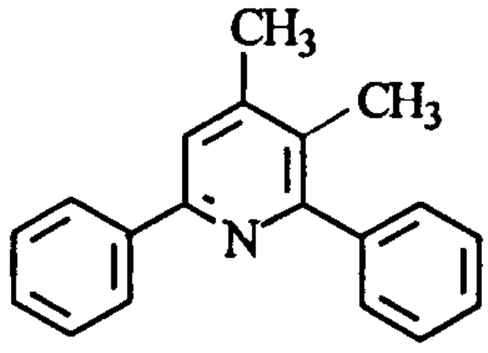
4-Methyl-5-phenyl-2-
(phenylmethyl)pyridine **82**



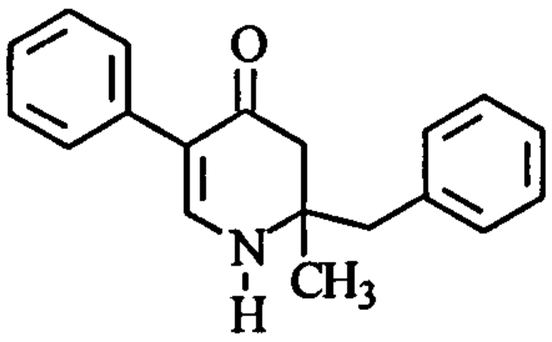
2-Methyl-3-phenyl-6-
(phenylmethyl)pyridine **83**



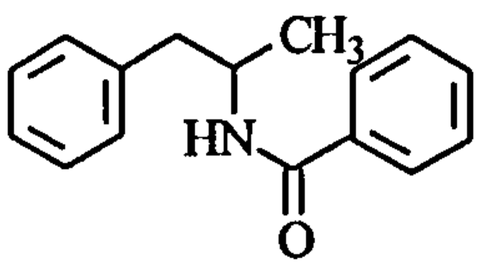
2,4-Dimethyl-3-phenyl-6-
(phenylmethyl)pyridine **84**



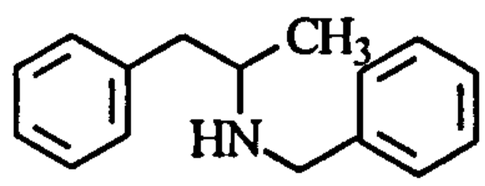
2,6-Diphenyl-3,4-
dimethylpyridine **85**



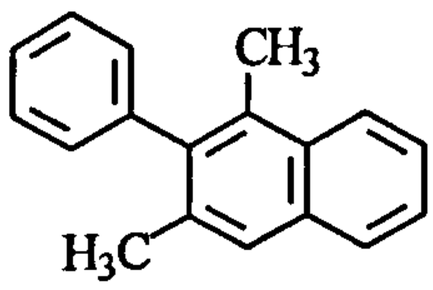
2-Benzyl-2-methyl-5-phenyl-2,3-
dihydropyrid-4-one **86**



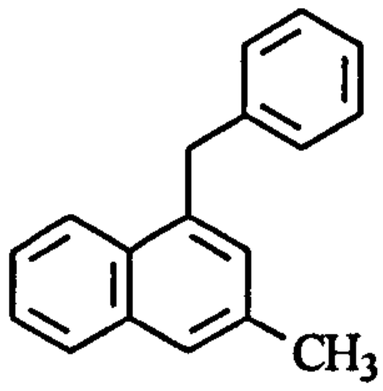
Benzoylamphetamine **87**



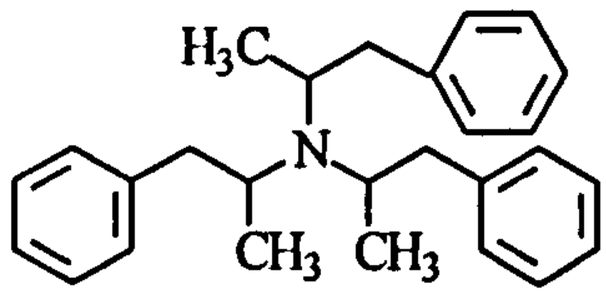
Benzylamphetamine **88**



1,3-Dimethyl-
2-phenylnaphthalene **89**



1-Benzyl-
3-methylnaphthalene **90**

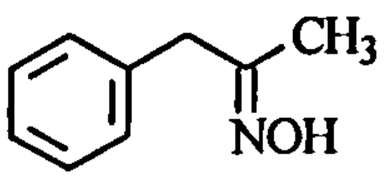


Tri-(phenylisopropyl)-
amine **91**

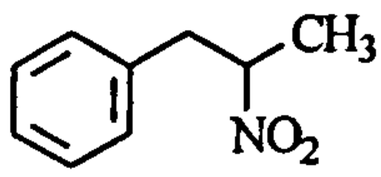
Figure 12: Structural formulas of impurities found in Leuckart amphetamine.

Nitrostyrene and oxime reactions

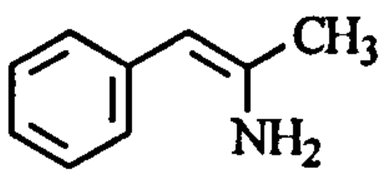
Nitrostyrene **41** may be converted directly to amphetamine by catalytic or metal hydride reduction (Scheme 2, Reaction II) [146]. One approach is hydrogenation using a palladium catalyst or Raney nickel [147]. In the metal hydride reduction $LiAlH_4$ is frequently used. The effect of the amount of reducing agent in the nitrostyrene route has been studied. The major component with a 5-fold excess of $LiAlH_4$ was 1-phenyl-2-propanoxime **92**, indicating incomplete reduction [148]. Several additional components are also suggestive of incomplete reduction. These compounds are the starting material nitrostyrene, 1-phenyl-2-nitropropane **93**, 1-phenyl-2-aminopropene **94** and BMK. BMK is present as a minor compound that forms from hydrolysis of 1-phenyl-2-propanoxime or 1-phenyl-2-aminopropene. In the presence of a large excess of $LiAlH_4$ (20-fold), amphetamine is obtained as the major component. In the corresponding oxime reaction, 1-phenyl-2-propanoxime is synthesised from BMK and hydroxylamine. The intermediate is hydrogenated to amphetamine (Scheme 2, Reaction III). Equal impurities from nitrostyrene and oxime reactions have been found and the structures of the substances formed are shown in Figure 13.



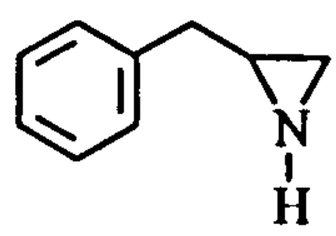
1-Phenyl-2-propanoxime **92**



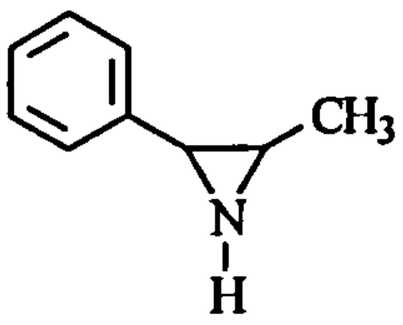
1-Phenyl-2-nitropropane **93**



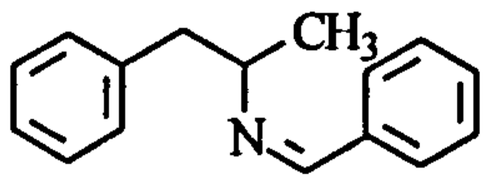
1-Phenyl-2-aminopropene **94**



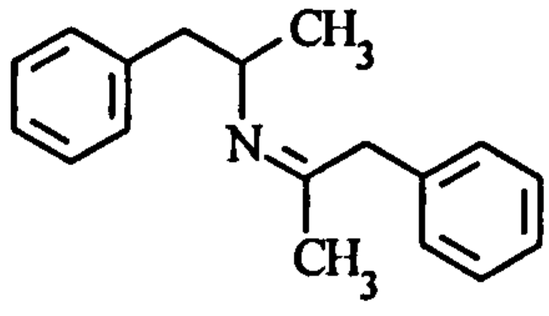
2-Phenylmethyl aziridine **95**



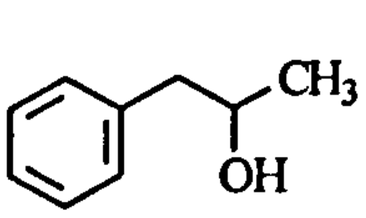
2-Methyl-3-phenylaziridine **96**



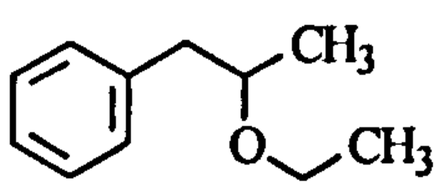
N-(β-Phenylisopropyl)-benzaldimine **97**



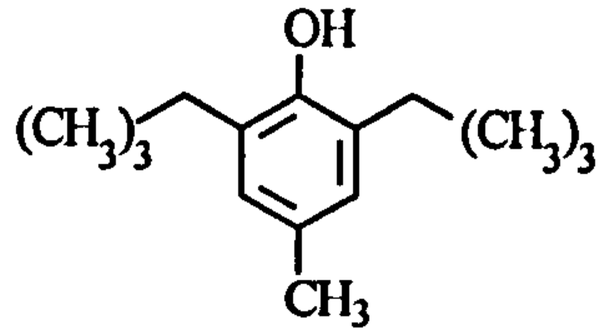
N-(β-Phenylisopropyl)-benzylmethylketimine **98**



1-Phenyl-2-propanol **99**



1-Phenyl-2-ethoxypropane **100**



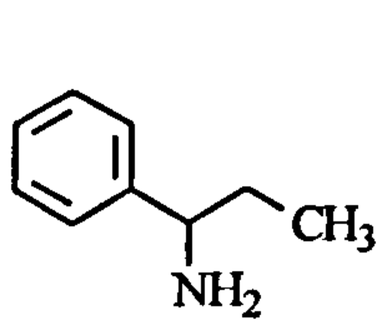
2,6-Di-tert-butylhydroxytoluene (BHT) **101**

Figure 13: Structural formulas of impurities found in nitrostyrene and oxime amphetamine.

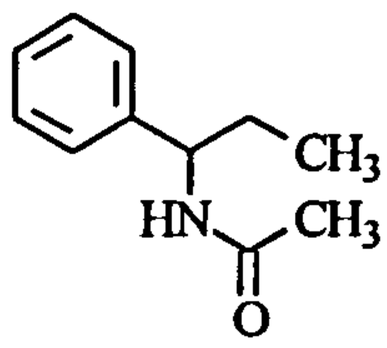
Allylbenzene reactions

Commercially available allylbenzene **102** is also used as a starting material in the amphetamine synthesis. In allylbenzene, the unconjugated double bond can be functionalised at the 2-position by treatment with acetonitrile in sulphuric acid (Scheme 2, Reaction IV). The resulting intermediate, N-acetylamphetamine **77** is hydrolysed to yield amphetamine [149]. Allylbenzene and *trans*-β-methylstyrene **43**, used in the synthesis of BMK, are isomeric differing only in the position of the double bond in the propene side chain. However, the synthesis of *trans*-β-methylstyrene with acetonitrile does not result in amphetamine.

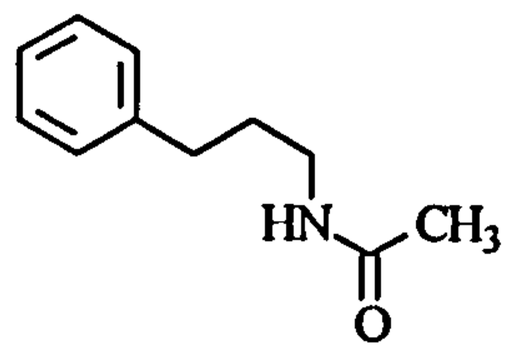
In another approach, the double bond in the allyl substituted aromatic system can be functionalised at the 2-position by the addition of HBr (Scheme 2, Reaction V) [150]. The displacement of bromine from the intermediate, 1-phenyl-2-bromopropane **103** with the appropriate amine results in amphetamine as the major product. For allylbenzene reactions typical impurities are, in addition to N-acetylamphetamine (acetamide of 1-phenyl-2-propaneamine), shown below in Figure 14.



1-Phenyl-1-propanamine **104**



Acetamide of 1-phenyl-1-propanamine **105**



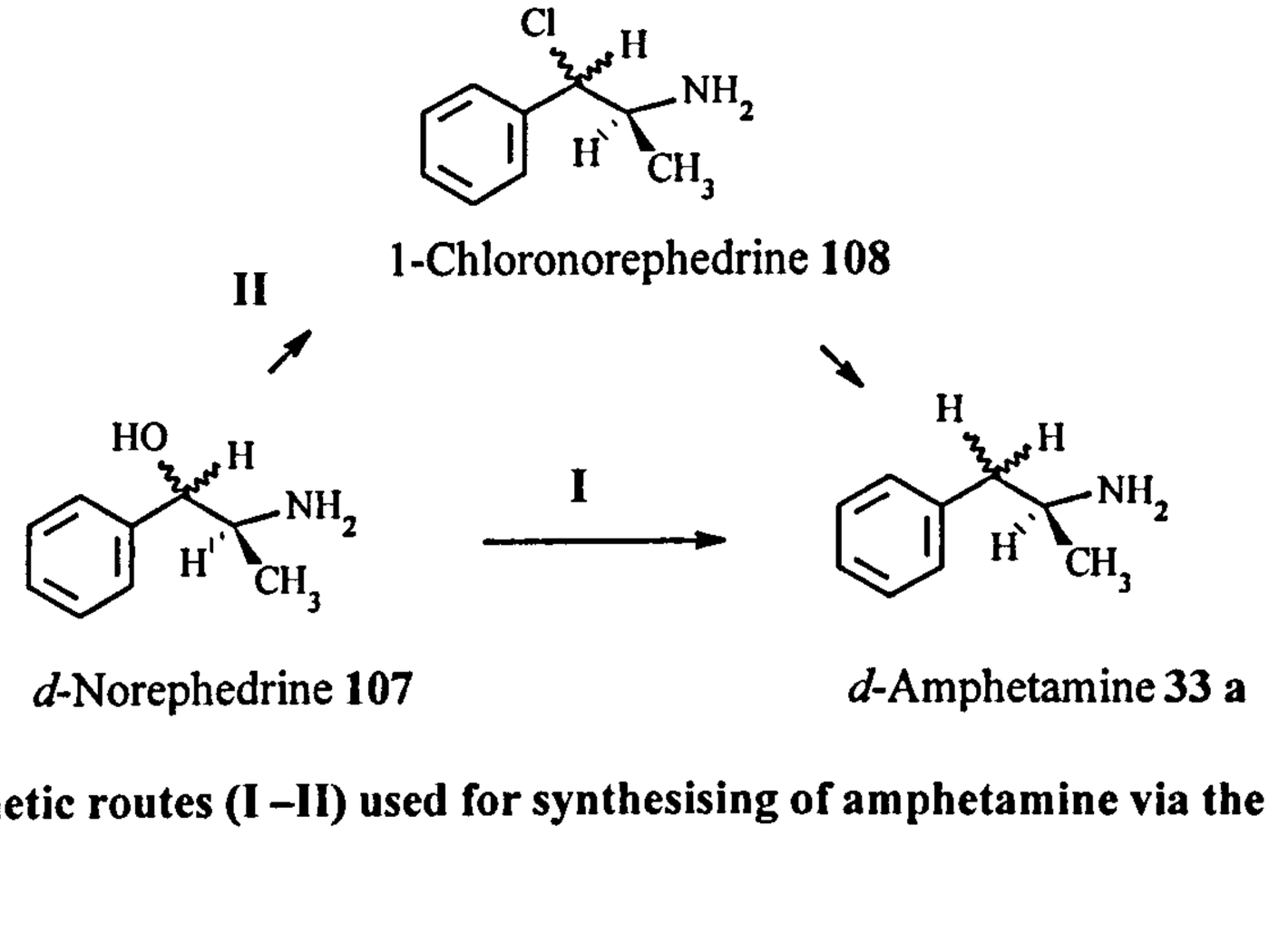
Acetamide of 1-phenyl-3-propanamine **106**

Figure 14: Structural formulas of impurities found in allylbenzene amphetamine.

Hydrogenolysis reactions

While all of the synthetic methods outlined above yield amphetamine as racemic mixtures, hydrogenolysis reactions may be employed to prepare optically pure amphetamine from chiral starting materials [114]. This method is highly desirable since the stimulant activity of amphetamines is due primarily to the *d*-enantiomer. The enantiomers of norephedrine **107** or norpseudoephedrine contain the structural

elements of amphetamine in chiral form. The most commonly employed method is the reduction of the precursor with hydriodic acid and red phosphorous (Scheme 3, Reaction I). Another general approach involves converting norephedrine to the corresponding 1-chloronorephedrine **108** by treatment with thionyl chloride ($SOCl_2$) (Scheme 3, Reaction II). Further, the intermediate is subjected to hydrogenolysis using palladium as the catalyst. Typical impurities for hydrogenolysis reactions are naphthalenes **89 - 90**, aziridines **95 - 96** and 1-phenyl-2-propanol **99**.



Scheme 3: Synthetic routes (I –II) used for synthesising of amphetamine via the hydrogenolysis.

Reductive amination reaction

In reductive amination reactions, BMK reacts with an amine (ammonia or ammonium acetate) to form a Schiff's base **109** (Scheme 2, Reaction VI). The reduction of the double bond of the Schiff's base is based on different reducing agents. Reductive amination reactions can be carried out using (i) heterogeneous catalysis, (ii) metal reductions and (iii) metal hydride reductions.

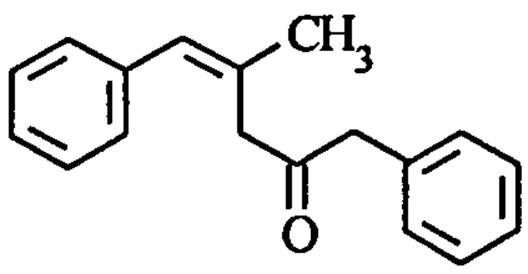
The heterogeneous catalysis reduction is achieved using palladium, platinum and Raney nickel as catalysts for the reducing agents. When the heterogeneous catalysis is used, the reduction of BMK to 1-phenyl-2-propanol limits the yield of amphetamine. In the clandestine laboratories, one of the most popular metal reductions is via aluminium-mercury amalgam reduction [151]. Since the 1980's, metal hydride reductions have become commonly used methods in clandestine laboratories [152,153]. The use of sodium borohydride ($NaBH_4$) and $LiAlH_4$ has

been reported for synthesis of methamphetamine. Unfortunately, $NaBH_4$ is reactive enough to reduce the ketone group of BMK to a corresponding alcohol, 1-phenyl-2-propanol. The reduction can be avoided by using a more selective reducing agent, such as sodium cyanoborohydride ($NaBH_3CN$) whose activity is dependent on the pH of the reaction [154].

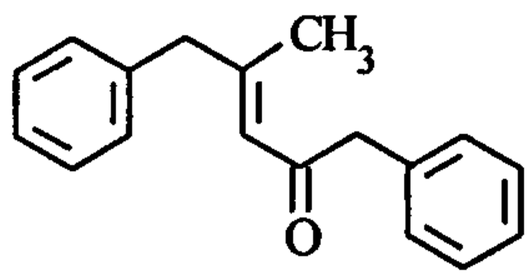
Different ketone compounds **110 - 112** are typical impurity substances for reductive amination reactions utilising heterogeneous catalysis. Using metal or metal hydride reductions, typical impurities are imines **97** and **98.** These and other impurities, N-acetylamphetamine **77** and DPIA **74,** are also identified from other synthetic routes. Therefore, these compounds cannot be accounted to be route specific compounds. Two substances that are not found from other reaction type amphetamines are identified, 1-oxo-1-phenyl-2-(β-phenylisopropylimino)propane **113** and 2,4-dihydroxy-1,5-diphenyl-4-methylpentene-1 **114.** The structures are shown in Figure 15.

In our laboratory, amphetamine has been synthesised using metal hydride reduction. Typical reductive amination reaction impurities were also studied in detail.

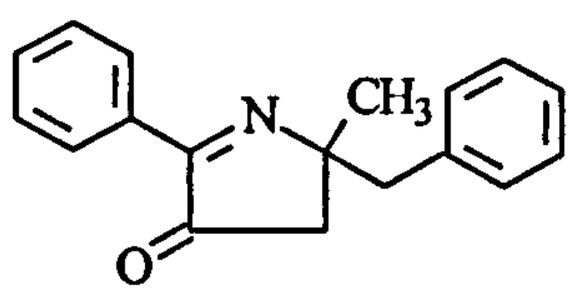
Heterogeneous catalysis reductions:



1,5-Diphenyl-2-methyl-1-penten-4-one **110**

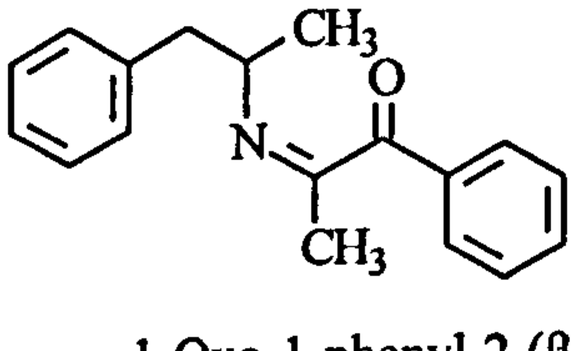


1,5-Diphenyl-2-methyl-2-penten-4-one **111**

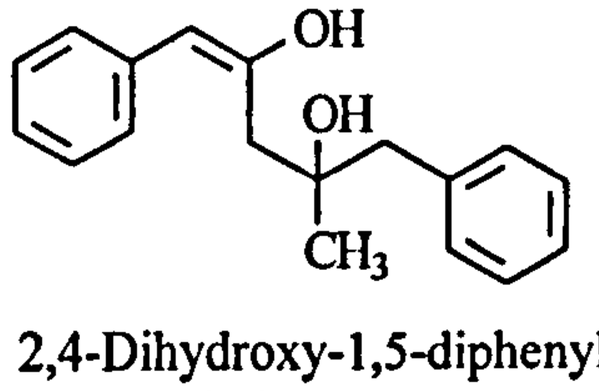


3,4-Dihydro-2-benzyl-2-methyl-4-oxo-5-phenyl-2H-pyrrole **112**

Metal and metal hydride reductions:



1-Oxo-1-phenyl-2-(β-phenylisopropylimino)propane **113**



2,4-Dihydroxy-1,5-diphenyl-4-methylpentene-1 **114**

Figure 15: Structural formulas of impurities found in reductive amination amphetamine.

3.4 Amphetamine profiling

A chemical amphetamine profiling method was mainly developed in Sweden in the early 1970's [155]. During the last 30 years, techniques have advanced and several different sample preparation methods, analysis conditions and means of collecting data have been published. Frequently, in the first stage of comparison, GC chromatograms are compared visually. The method is also laborious and slow, but essential. In several countries a computer aided comparison method has been developed based on amphetamine impurities [27]. The impurities chosen for profiling are identified from Leuckart type amphetamines.

The interest in amphetamine profiling is evident both at the national and international level. Until now, the comparison at an international level has been carried out by sending samples to the central laboratory. The sample exchange process is laborious and slow as each sample has to be analysed in the central laboratory. The central laboratories for amphetamine profiling are located in Sweden and Poland where two projects are currently running; Amphetamine Projects in the Nordic countries [156] and Amphetamine Projects in the Eastern Baltic Sea Region [157]. The methods used for comparing amphetamines are summarised in Table 4, and discussed below.

Table 4: Chemical profiling methods for amphetamine.

#	Sample preparation	Analysis method and conditions		Ref.
1	LLE: water/benzene	Method: Column: T-program: Injection: Carrier gas: Flow rate:	GC-FID-ECD 1.9 m x 2 mm, packed with 3% OV-17 130°C (0 min), 6°C/min to 250°C 250°C, not available N_2 35 ml/min**	155
2	LLE: phosphate buffer (pH 7.0)/n-octane or isooctane	Method: Column: T-program: Injection: Carrier gas: Flow rate:	GC-FID 25 m x 0.2 mm, d_f 0.33 µm of 5% Ph Me silicone (SE-54 or Ultra-2) 120°C or 100°C (0 min), 12°C/min to 240°C (5.5 min), 15°C/min to 300°C (10 min) 250°C, splitless He 30 cm/s	158 159
3	LLE: phosphate buffer (pH 7.0)/n-heptane	Method: Column: T-program: Injection: Carrier gas: Flow rate:	GC-FID 25 m x 0.32 mm, d_f 0.52 µm of 5% Ph Me silicone (HP-5) 100°C (1 min), 12°C/min to 240°C (5.5 min), 15°C/min to 300°C 250°C, splitless not available not available	51
4	LLE: phosphate buffer (pH 7.0)/isooctane	Method: Column: T-program: Injection: Carrier gas: Flow rate:	GC-FID 50 m x 0.2 mm, d_f 0.33 µm of 5% Ph Me silicone (Ultra-2) 80°C (0 min), 15°C/min to 195°C (22 min), 8°C/min to 270°C, 30°C/min to 320°C (15 min) 250°C, splitless H_2 35 cm/s	39
5	LLE: phosphate buffer (pH 7.4)/octane	Method: Column: T-program: Injection: Carrier gas: Flow rate:	GC-FID 25 m x 0.2 mm, d_f 0.25 µm of 5% Ph Me silicone (BP-5) 110°C (0 min), 15°C/min to 200°C, 2°C/min to 208°C 10°C/min to 300°C (5.8 min) not available, splitless He 52 cm/s* (2 ml/min)	160

* Flow rate was transformed to correspond to cm/s by FlowCalc.

** The cm/s value cannot be calculated for a packed column.

To avoid overloading of the column, the components were extracted in such a way that most of the amphetamine was left in the aqueous layer. In earlier work, amphetamine sulphate was dissolved in water (Method 1) [155]. Nowadays, buffers are used and the pH adjusted to improve extraction conditions (Method 2). A frequently used buffer is a phosphate at pH 7 [158,159]. In Method 1, the FID-ECD

detector system was utilised. In the 1970's, the double-detector system was not, however, considered user-friendly in practical work.

Fundamentally, the sample preparation and GC methods used in amphetamine profiling studies are based on the conditions described in Method 2. The experiments focused mainly on developing a practical and effective method for data interpretation. In one experiment, the automatic profiling and the classification of impurity profiles was achieved utilising the Quotient method (equation 7) and the SIMCA (Soft Modelling of Class Analogy) software package [46]. The basis for the computer program is that two profiles are likely to match if the peak areas have approximately the same relative values (quotients). When first used, quotients were determined for nine target compounds identified from Leuckart type amphetamine. Currently, comparison is based on over 20 peaks. In addition to 11 identified compounds **71 - 76, 78 - 79, 85, 88 - 90,** 13 unknown peaks have been chosen to improve the profiling method [27]. The structures of identified substances are shown in Figure 12.

In an another experiment, 15 Leuckart impurity peaks were selected for a comparison experiment (Method 3) [51]. For profiling of amphetamine samples, the special statistical and chemometric methods developed in Poland were applied. The first step of data analysis includes preliminary grouping of the profiles by calculating a correlation coefficient, r, and Euclidean distances between profiles. The application of the cluster analysis was used for verification of the classification of the profiles by STATISTICA StatSoft software. Dendrograms have been also used for illustrating the data and similarity between samples.

Using the column and the temperature program described in Method 2, the resolution was not good enough for obtaining accurate peak abundance data. Some optimisation experiments have been carried out in Finland [39]. A 25 m long column was replaced with a 50 m long column using the same stationary phase (Method 4). The temperature program was optimised by the Drylab computer simulation. The

program is based on the retention times obtained from two initial runs with different temperature program rates [161].

One of the most difficult problems in chemical profiling is the peak identification used for comparison purposes. This is due to the inaccuracy in retention times, especially if the data have been obtained from different laboratories. The problem can be overcome by using a commercially available peak identification system [44]. In this system, the retention time of each peak was corrected, based on the retention time of an internal standard. Some disagreements were observed only for very small peaks. In this experiment (Method 4), 11 indicator peaks were identified from GC chromatograms utilising the retention index (RI) standards method. The same compounds were utilised as identified in Method 2. In addition to these substances chosen for automatic profiling, a few extra compounds were utilised in visual comparison. The similarity index (SI) method has been chosen for the automatic comparison (equation 5).

A few years ago, seized samples could be classified into ten major groups [160]. Nowadays, profiles vary from one other more often and they have been classified into a single group. This indicates that amphetamine is more often synthesised by non-Leuckart reactions. Alternative reactions include, for example, the oxime and allylbenzene routes. The impurity profiles of these reactions have been obtained with Method 5.

4 OBJECTIVE OF THE STUDY

The overall objectives of this study include (i) an extensive literature study for profiling of common drugs of abuse and (ii) the development of chemical profiling method suitable also for non-Leuckart type amphetamines. The main objective is to develop a harmonised method that will enable an exchange of similar impurity profiles produced in different laboratories. The practical work has been carried out as a part of the project "Development of a Harmonised Method for the Profiling of Amphetamines" (SMT4-CT98-2277) funded by the European Commission [162]. The project includes four partners - Finland (NBI), Scotland (University of Strathclyde), Sweden (National Laboratory of Forensic Science, SKL) and Switzerland (University of Lausanne, IPSC). Three different amphetamine synthesis routes, namely Leuckart, reductive amination and nitrostyrene reactions were studied during the project. In this thesis, reductive amination synthesis is examined. The results from other partners are taken into consideration, when relevant.

The experimental work is broken down into a number of phases. Amphetamine is synthesised through the reductive amination route. A number of standard impurities are identified and chosen for the profiling method. The suitability of these impurities is studied in different storage conditions. A GC method is developed and optimised for the chosen standard impurities. The GC method is optimised by selecting the most appropriate sample introduction technique, column, column temperature program and detector. The developed GC method should also be suitable for amphetamine samples at low concentration.

A second part in the method development is the sample preparation technique. The aim is to develop an extraction method which possesses a good extraction power and which holds its nominal pH value steadily without extra pH adjustment, after the addition of the amphetamine. Liquid-liquid extraction (LLE) and solid phase extraction (SPE) techniques are studied utilising different buffer and solvent combinations.

Finally, the new harmonised profiling method will be tested to establish its potential for real world applications. A number of street samples are analysed and a profile database made. Reproducibility of the profiling method will be evaluated at intra- and inter-laboratory level. Different statistical data handling methods are also evaluated for the interpretation.

5 REDUCTIVE AMINATION AMPHETAMINE

5.1 Introduction

As described in Chapter 3.4, most amphetamine profiling methods are, unfortunately, suitable only for some seized amphetamine batches since the impurities chosen for profiling are identified from Leuckart type amphetamines. There is now considerable interest in the possibility of harmonised methods for the comparative analysis of different type of amphetamines. The ideal situation would be to analyse the samples in each national laboratory and collect data for an international database.

The major topic of this thesis is the development of a chemical profiling method suitable for Leuckart, reductive amination and nitrostyrene type amphetamines. In this chapter, the reductive amination type amphetamine itself and synthetic impurities are discussed in detail. Amphetamine batches were synthesised by metal hydride reduction utilising $NaBH_4$ and $NaBH_3CN$ as reducing agents. Typical impurities were collected from synthesised amphetamines and non-Leuckart street samples. The structure of each chosen impurity was confirmed by synthesising and obtaining spectroscopic data for each compound. At the same time, other partners collected typical substances for Leuckart and nitrostyrene type amphetamines [162]. Eventually, all chosen impurities will be used for the profiling method based on Leuckart, reductive amination and nitrostyre impurities.

At the time of writing, all laboratories are using automatic injectors in the chromatographic instruments. This has caused some problems. For example, several amphetamine extracts are prepared beforehand and thus, the time delays between the first and last sample might be several hours, or even days. It is essential to study if storage has an influence on the impurities used for amphetamine profiling and on the impurity profiles. The stability of Leuckart amphetamine extracts has been previously studied in different conditions [163]. The extract was stored at 4°C, at an ambient temperature in the dark and in daylight. The first two storage conditions did result in changes in profiles after 6 days. Many of the peak areas, however, changed

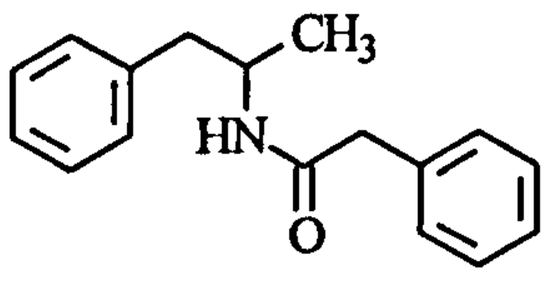
within two days in daylight. In this chapter, stability of synthesised reductive amination impurities was studied at the ambient temperature (25°C) and at 8°C, in daylight. The time delay was up to 96 hours (4 days). The impurities were studied individually, as a synthetic mixture and in a synthesised reductive amination amphetamine matrix.

5.1.1 Identification of impurities

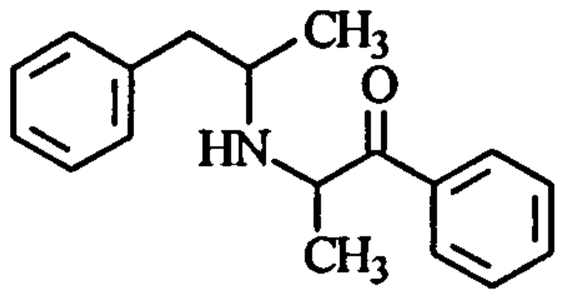
The reductive amination compounds could be split into three categories, including (i) new reductive amination compounds, (ii) previously published reductive amination substances, (iii) substances found also from other types of amphetamines. The compounds are listed below. The compounds were identified comparing the mass spectra of compounds with the available mass spectrum library or literature. The structures of new reductive amination impurities were determined by mass fragmentation patterns.

Table 5: Impurities found in reductive amination amphetamines.

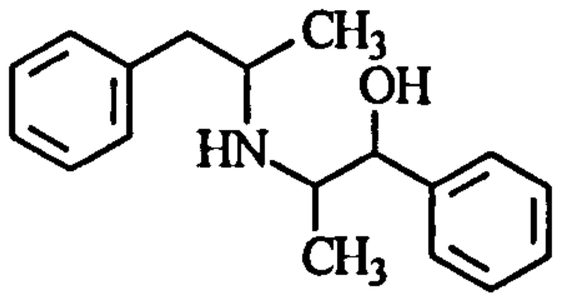
Category	Compound	Abbreviation
1	2-Oxo-1-phenyl-(β-phenylisopropylamine)propane **115**	2-Oxo
	N-(β-Phenylisopropyl)cathinone **116**	Cathinone
	N, β-Hydroxy-N,N-di(β-phenylisopropyl)amine **117**	Cathinol
2	N-Acetylamphetamine **77**	-
	N,N-Di(β-phenylisopropyl)amine **74**	DPIA
	N-(β-Phenylisopropyl)benzaldimine **97**	Aldimine
	N-(β-Phenylisopropyl)benzyl methyl ketimine **98**	Ketimine
	1-Oxo-1-phenyl-2-(β-phenylisopropylimino)propane **113**	1-Oxo
3	Benzoylamphetamine **87**	-
	Benzylamphetamine **88**	-
	1-Phenyl-2-propanol **99**	-



2-Oxo-1-phenyl-(β-phenylisopropylamine)propane **115**



N-(β-Phenylisopropyl)-cathinone **116**



N,β-Hydroxy-N,N-di(β-phenylisopropyl)amine **117**

Figure 16: Structural formulas of new impurities found in reductive amination amphetamine.

The IUPAC names of the compounds are typically long and unpractical for every day use; therefore the abbreviation of the names in Table 5 will be utilised in further chapters. The mass spectra of reductive amination impurities are shown in Figure 17.

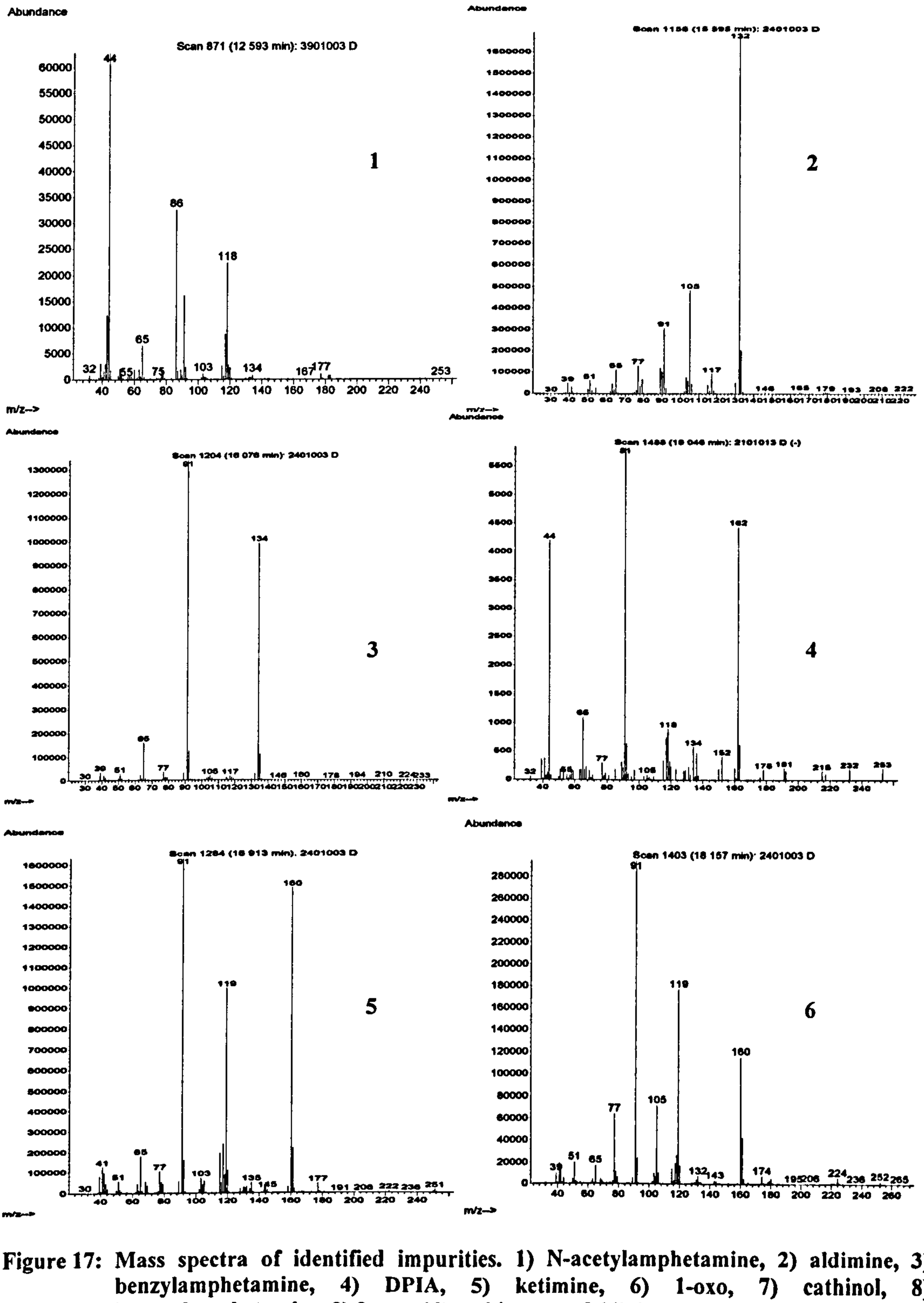
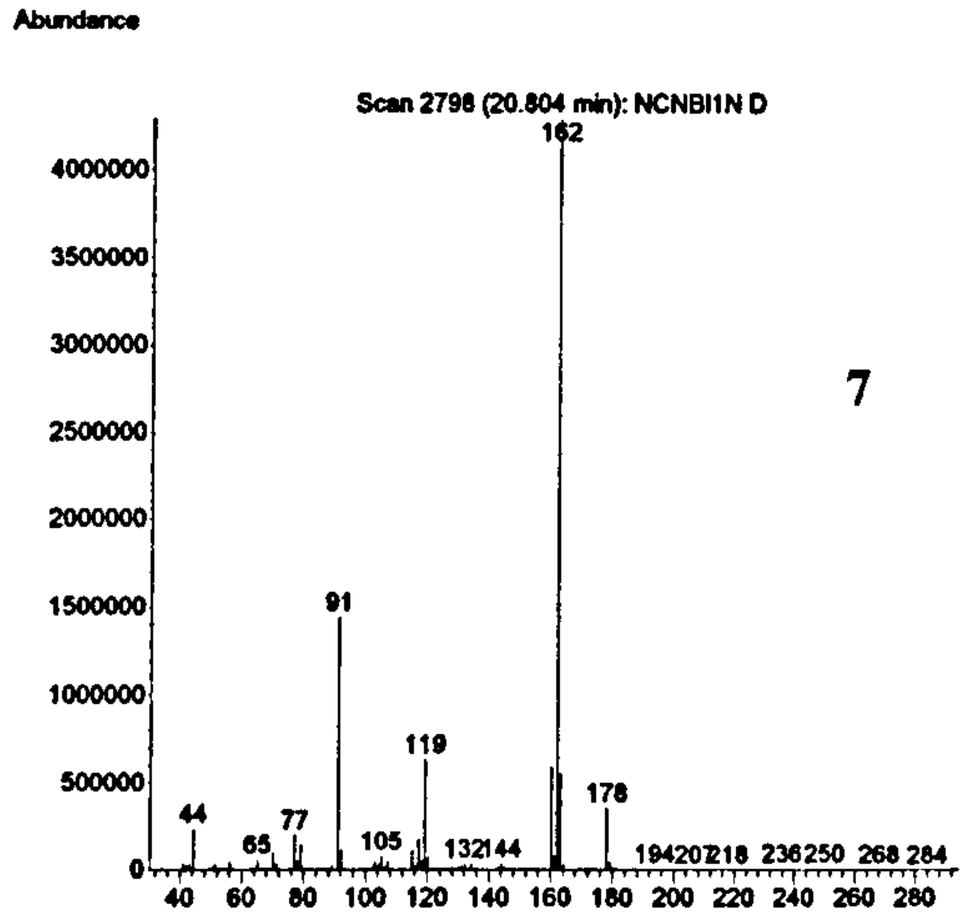


Figure 17: Mass spectra of identified impurities. 1) N-acetylamphetamine, 2) aldimine, 3) benzylamphetamine, 4) DPIA, 5) ketimine, 6) 1-oxo, 7) cathinol, 8) benzoylamphetamine, 9) 2-oxo, 10) cathinone and 11) 1-phenyl-2-propanol.



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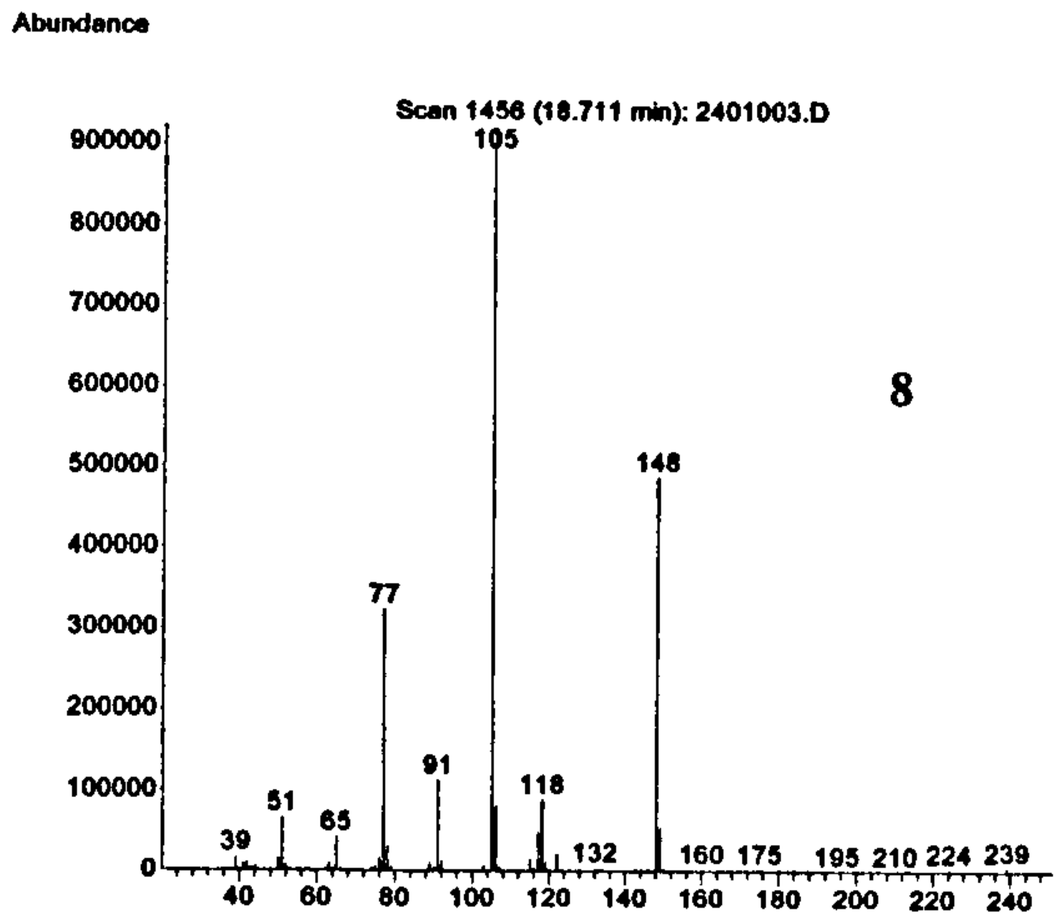

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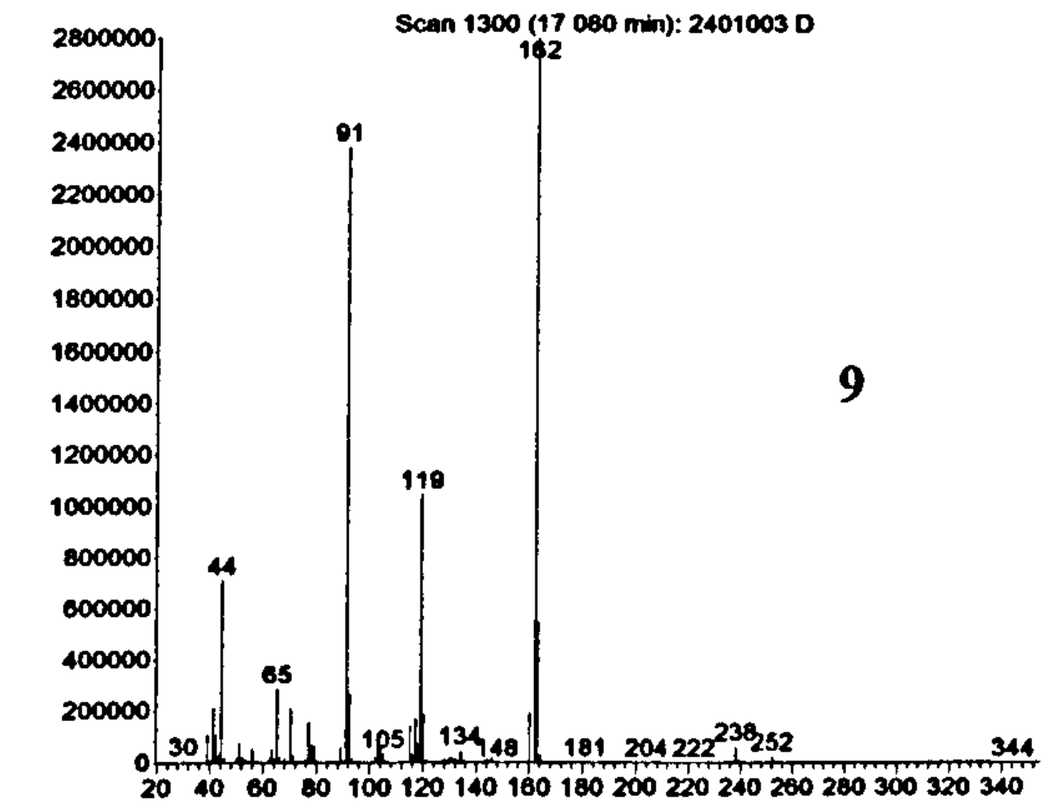

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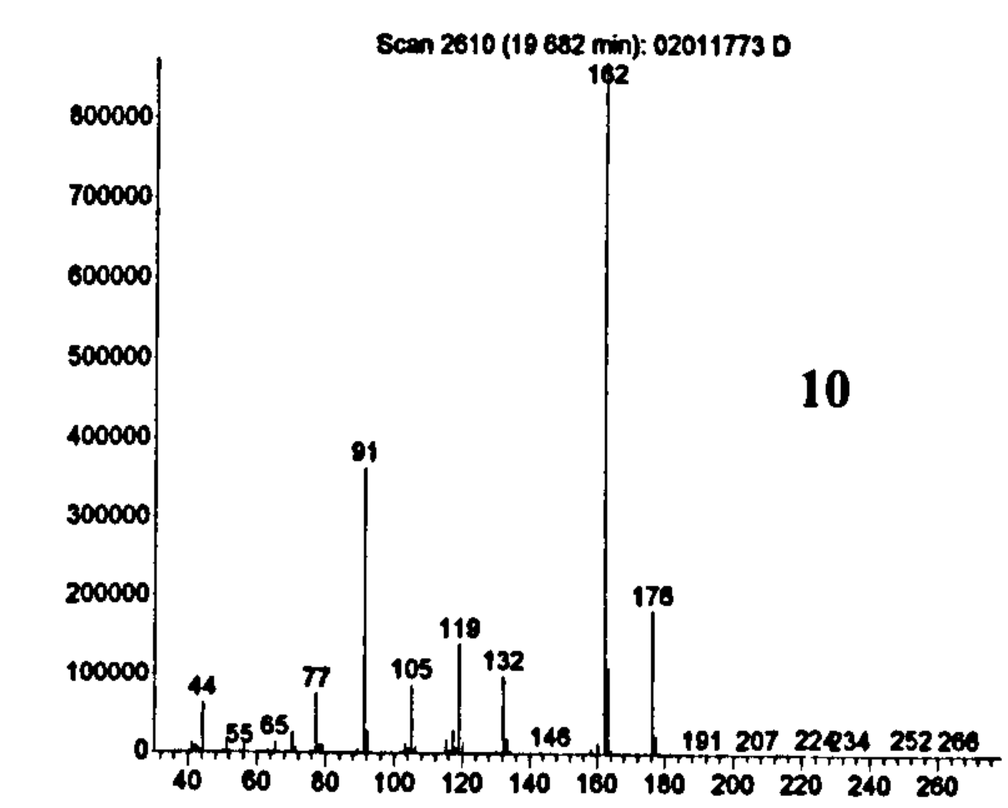

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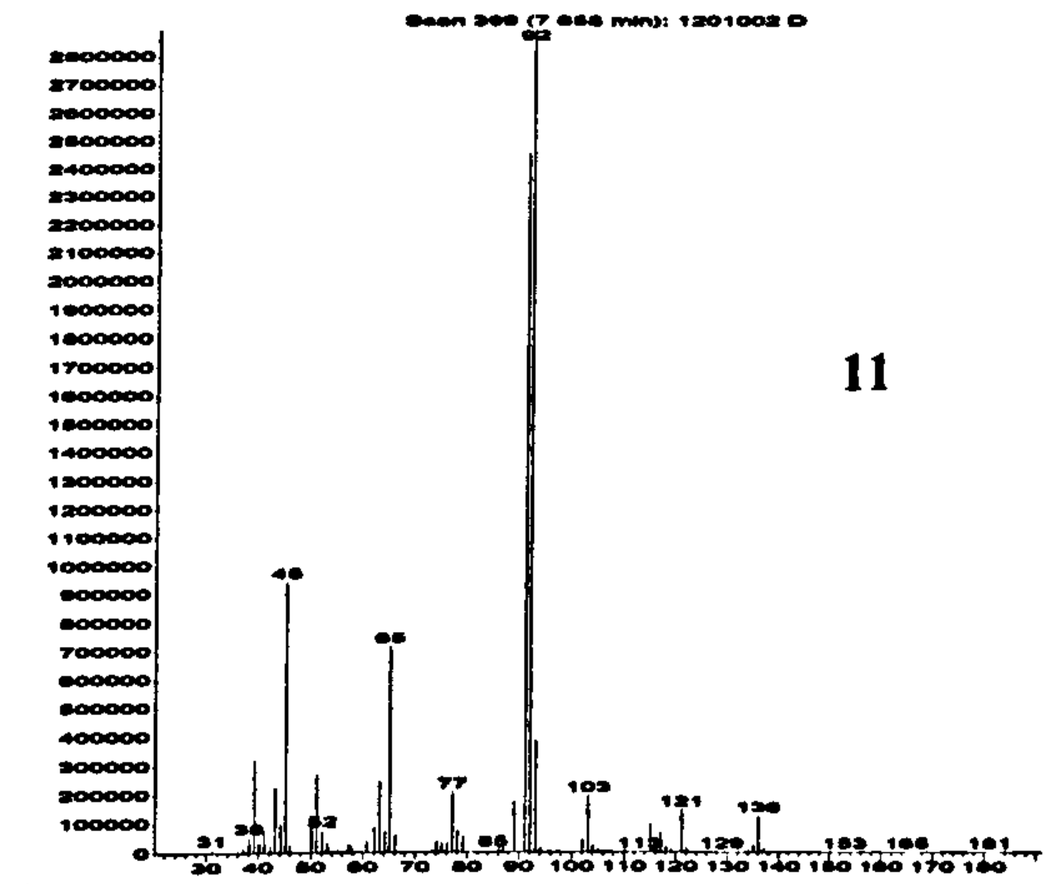

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Figure 17: Cont'd / ...

5.2 Experimental

5.2.1 Materials and methods

Materials

Acetylchloride, 1-phenyl-1,2-propandione, $NaBH_3CN$ and $NaBH_4$ were obtained from Aldrich (Steinheim, Germany) and thionylchloride ($SOCl_2$) from Merck (Darmstadt, Germany). Benzoic acid and ammonium acetate were obtained from Riedel-de Haën (Seelze, Germany) and benzaldehyde from University Pharmacy (Helsinki, Finland). Triethyl amine, phenylacetic acid and BMK were obtained from Fluka (Buchs, Switzerland). Seized BMK available at National Bureau of Investigation (NBI, Finland) was also utilised. The BMK was purified by vacuum distillation before use. Other chemicals, such as sodium hydroxide (NaOH) and sodium hydrogen carbonate ($NaHCO_3$) were obtained from Merck and Riedel-de Haën, respectively.

The solvents, namely diethyl ether, methanol (MeOH), dichloromethane (CH_2Cl_2), acetone, benzene and toluene were obtained from Riedel-de Haën. The purity of all solvents falls into a pro-analyses category. Moreover, ca. 25% ammonia solution (NH_3), 95 - 97% sulphuric acid (H_2SO_4) and 37% hydrochloric acid (HCl) were obtained from Riedel-de Haën. Isooctane was obtained from Merck, tetrahydrofuran (THF), 2-propanol (isopropanol) and ethyl acetate from Rathburn (Walkerburn, Scotland) and ethanol from Primalco (Rajamäki, Finland).

GC method

Gas chromatographic-mass spectrometric (GC-MS) analyses were performed with an HP 6890 gas chromatograph equipped with split/splitless inlet, HP 7683 automatic injector and HP 5973 mass selective detector (MSD) (Agilent Technology, Little Falls, DE, the USA). A 25 m (L) x 0.20 mm (i.d.), d_f 0.33 μm of 5% phenyl methyl silicone capillary column (HP Ultra-2, Agilent Technology) was chosen for the identification analyses. Carrier gas (helium) was adjusted at 60°C to give average velocity to the optimum, 25 cm/s. Inlet pressure was converted according to the

constant flow mode and the total flow was 60 ml/min. The injection was in the split mode (1:62) with an injector temperature at 260°C. ChemStation software Rev. B.01.00 was used for data acquisition and processing.

For stability analysis, the GC-MS was also equipped with flame ionisation detector (FID) (Agilent Technology). Signals from the MSD and FID were detected simultaneously using a GC-MS-FID system. Two similar Ultra-2 columns were installed into the FID and MSD with a 2 m (L) x 0.32 mm (i.d.) deactivated and uncoated pre-column and an Y-shape splitter. The Y-shape splitter could be also replaced with a divider obtained from Gerstel (Mülheim an der Ruhr, Germany) or a two-hole ferrule (Figure 18).

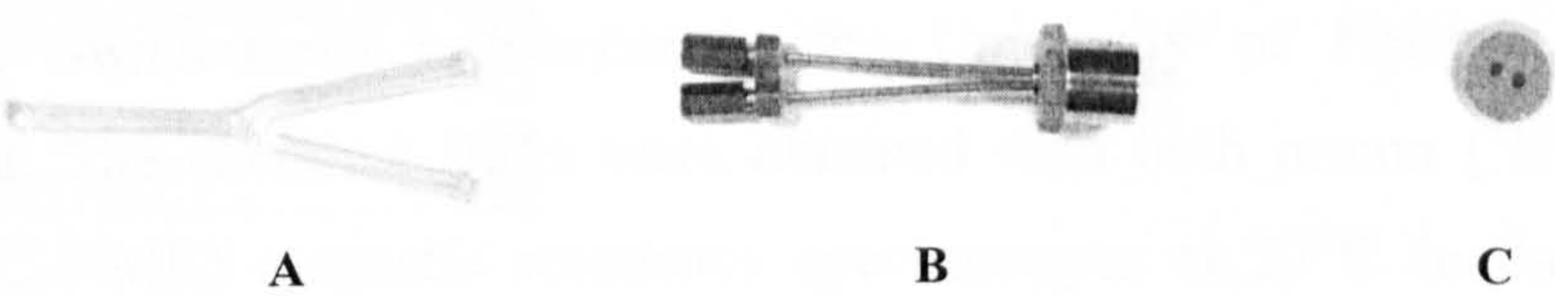


Figure 18: Different connectors used to connect two columns into the same injector. A) Y-shape splitter, B) divider and C) two-hole ferrule.

In the stability study, injection was carried out using splitless mode where the split purge valve opened after 1 min. The detector temperatures were 305°C and 250°C for MSD and FID, respectively. The injection volume was 1 µl per column. The temperature program started with 1 min isothermal hold at 60°C, followed by a linear ramp (10°C/min) to the final temperature of 300°C for 10 min. The same temperature program was used for both split and splitless injections to obtain fully comparable retention times.

Spectrometric methods

Synthesised impurities were identified with spectrometric methods. Fourier transformed infrared spectroscopy (FTIR) runs were performed with Perkin-Elmer

1600 Series. The spectrum v2.00 program was used for the data analysis. The samples were analysed between two KBr tablets.

Ultraviolet (UV) spectra were obtained using high performance liquid chromatography (HPLC) HP 1090 (Agilent Technology, Waldbronn, Germany) coupled with diode array detector (DAD). The samples were injected directly into the detector in three different solutions, (i) MeOH, (ii) 0.1 M sodium hydroxide in MeOH and (iii) 0.1 M sulphuric acid in MeOH. The UV absorption spectra were recorded in the 220 - 400 nm range. ChemStation program Rev. A.08.03 was used for data acquisition.

Nuclear magnetic resonance spectroscopy (NMR) analyses were performed by Varian Inova 300 (Palo Alto, CA, the USA) and Bruker Avance DRX 500 (Fällanden, Switzerland) instrument in the University of Helsinki, Chemistry Department. The chemical shifts were obtained with both proton (^{1}H NMR) and carbon (^{13}C NMR) magnetic resonance spectroscopes at 27°C in the deuterated chloroform ($CDCl_3$) with tetramethylsilane (TMS) as the internal standard.

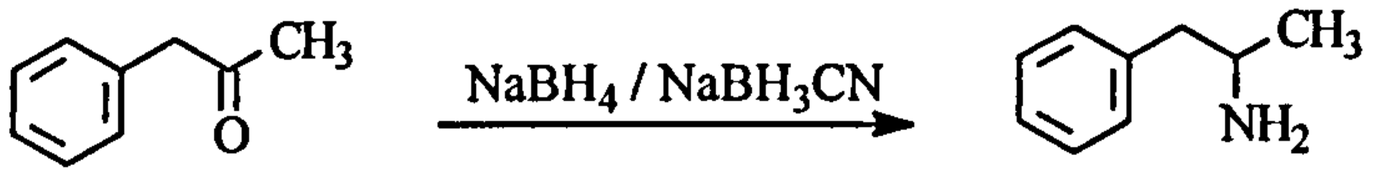
5.2.2 Synthesis of amphetamine

<u>$NaBH_3CN$ reduction</u>

5.0 g (0.037 mol) of BMK **39** and 28.2 g (0.37 mol) of ammonium acetate were stirred in MeOH at room temperature for 3 hours. 2.4 g (0.037 mol) of $NaBH_3CN$ was added and stirred for 4 hours (Scheme 4). 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 CH_2Cl_2. The water phase was alkalised with NaOH and extracted with CH_2Cl_2. The organic solvent was evaporated and amphetamine oil was obtained. The yield of amphetamine oil was 2.5 g (50%). The oil was crystallised to amphetamine sulphate by addition of 37% sulphuric acid and MeOH. The reaction was also carried out using ammonia as a base but the yield of amphetamine was only 11%.

<u>$NaBH_4$ reduction</u>

The mixture of 2.02 g (0.015 mol) of BMK, 12.51 g (0.162 mol) of ammonium acetate in 100 ml of 2-propanol (isopropanol) was stirred at room temperature for 3 hours. 0.07 g (0.0018 mol) of $NaBH_4$ was added and stirred for another 3 hours (Scheme 4). The reaction was quenched and amphetamine oil prepared as described above. The yield of amphetamine oil was 0.64 g (32%). The reaction was carried out also using ammonia but BMK reduced only to corresponding alcohol, 1-phenyl-2-propanol **99**.



BMK **39** Amphetamine **33**

Scheme 4: Synthesis of amphetamine.

5.2.3 Synthesis of impurities

Amphetamine base was used as a starting material in the synthesis of standard impurities. The base was obtained from seized amphetamine sulphate batches. The amphetamine sulphate was purified by re-crystallisation. The sulphate was refluxed in ethanol and more ethanol was added drop by drop until all the sulphate was dissolved. The solution was cooled down and diethyl ether added. A white precipitate started to form and crystallisation completed by keeping the mixture in a fridge/freezer. The amphetamine sulphate crystals were filtered and dried. Amphetamine oil was prepared by dissolving the sulphate in water, alkalising with NaOH (pH 10) and extracting oil product with CH_2Cl_2. The solvent was evaporated and amphetamine base was obtained.

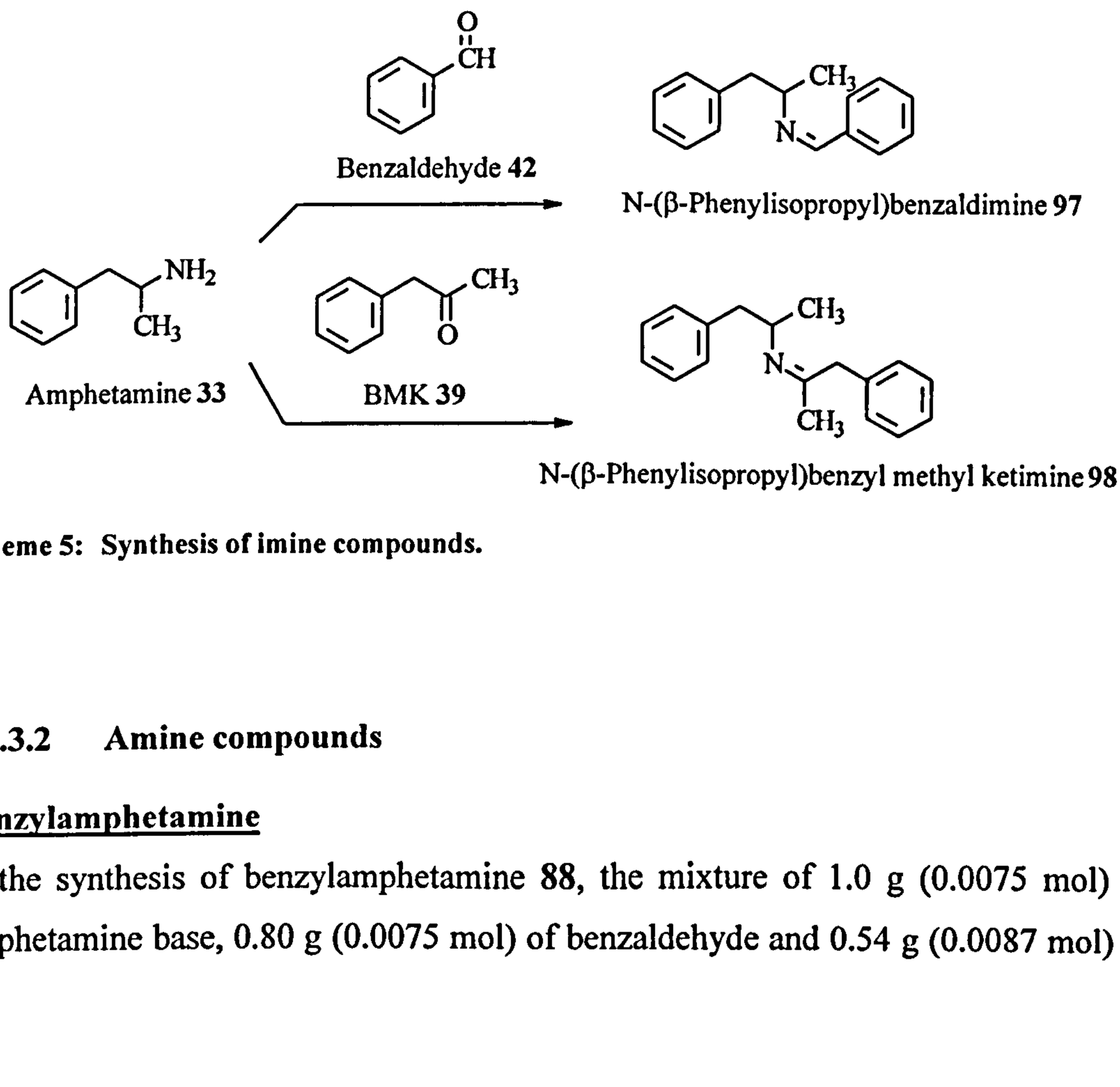
5.2.3.1 Imine compounds

N-(β-Phenylisopropyl)benzaldimine

2.0 g (0.015 mol) of amphetamine base, 1.0 g (0.008 mol) of benzaldehyde **42** and 200 ml of benzene were placed in a flask. The mixture was refluxed at 82°C overnight (Scheme 5, Reaction I). A Dean Stark trap was used to remove the resulting water. The reaction was finished and benzene evaporated. N-(β-phenylisopropyl)benzaldimine (aldimine **97**) was purified by vacuum distillation. The product contained 95% of aldimine and 5% amphetamine.

N-(β-Phenylisopropyl)benzyl methyl ketimine

Correspondingly, in the synthesis of N-(β-phenylisopropyl)benzyl methyl ketimine (ketimine **98**) 10 g (0.075 mol) of amphetamine base, 5 g (0.037 mol) of BMK were refluxed in benzene overnight (Scheme 5, Reaction II). The purity of ketimine was 75%, but ketimine/amphetamine mixture was too unstable to purify.



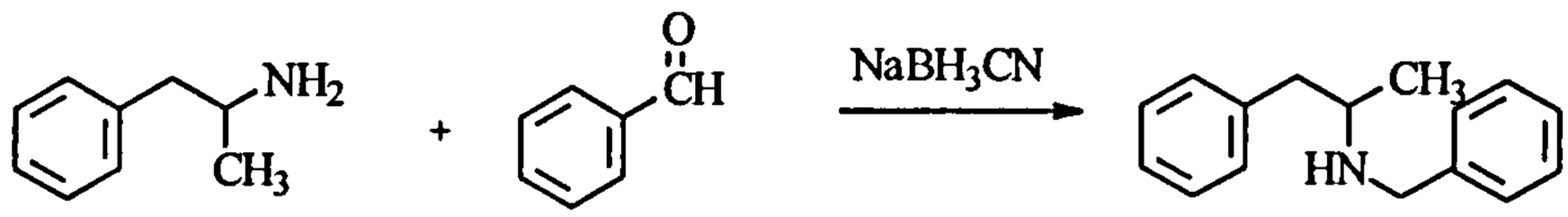
Scheme 5: Synthesis of imine compounds.

5.2.3.2 Amine compounds

Benzylamphetamine

In the synthesis of benzylamphetamine **88**, the mixture of 1.0 g (0.0075 mol) of amphetamine base, 0.80 g (0.0075 mol) of benzaldehyde and 0.54 g (0.0087 mol) of

$NaBH_3CN$ was stirred in 50 ml of MeOH at room temperature overnight (Scheme 6). The pH was measured and adjusted to pH 7.0 by adding HCl, if necessary.



Amphetamine **33** Benzaldehyde **42** Benzylamphetamine **88**

Scheme 6: Synthesis of benzylamphetamine.

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 alkalised with NaOH pellets and extracted with CH_2Cl_2. The organic solvent was evaporated. The oily benzylamphetamine product was purified by column chromatography. The CH_2Cl_2/MeOH gradient, starting from 100% CH_2Cl_2 and ending up to 100% MeOH, was used for elution. The pure benzylamphetamine oil yield was 0.2 g (12%).

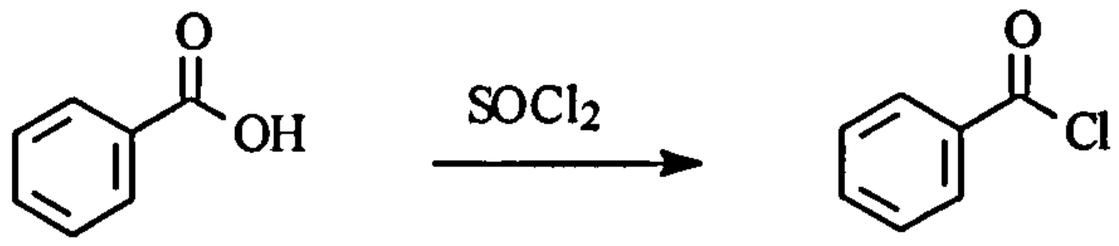
5.2.3.3 Amide compounds

N-Acetylamphetamine

Acetylchloride **118** (3.0 g, 0.037 mol) was dissolved in THF (300 ml) and amphetamine base (5.0 g, 0.037 mol) added (Scheme 9, Reaction I). The reaction mixture was stirred at room temperature overnight. The THF was evaporated. The resulting oily residue was dissolved in water and acidified with HCl. The water phase was extracted with CH_2Cl_2 and the organic solvent was evaporated. The yield of N-acetylamphetamine **77**, which was 95% pure, was 3.0 g (0.017 mol, 46%). To purify the product, N-acetylamphetamine was refluxed in petroleum benzene and ethanol was added until all products were dissolved. The mixture was cooled down and placed in a fridge and afterwards in a freezer. The product was filtered and dried. The yield of pure product was 1.5 g (23%).

Benzoylamphetamine

Benzoic acid **119** (5.1 g, 0.042 mol) and thionylchloride ($SOCl_2$) (9.2 ml, 0.126 mol) were refluxed in toluene for 3.5 hours resulting in benzoyl chloride **120** (Scheme 7). The solvent was evaporated under reduced pressure. Benzene was added and evaporated to remove the resulting water from the reaction mixture. The process was repeated twice.



Benzoic acid **119** Benzoyl chloride **120**

Scheme 7: Synthesis of benzoyl chloride.

In an analogous way to the synthesis of N-acetylamphetamine, benzoyl chloride **120** (5.4 g) was dissolved in THF and amphetamine base (5.8 g) added (Scheme 9, Reaction II). The mixture was stirred overnight at the room temperature. The reaction was finished and the oily product dissolved in acidic water. The mixture was extracted with CH_2Cl_2 and the regenerated organic phase washed with $NaHCO_3$ to remove inactive starting material and other acidic compounds. The yield of solid product was 6.1 g (66%). To purify the benzoylamphetamine **87,** the product was refluxed in petroleum benzene as above. The yield of pure benzoylamphetamine was 3.7 g (40%).

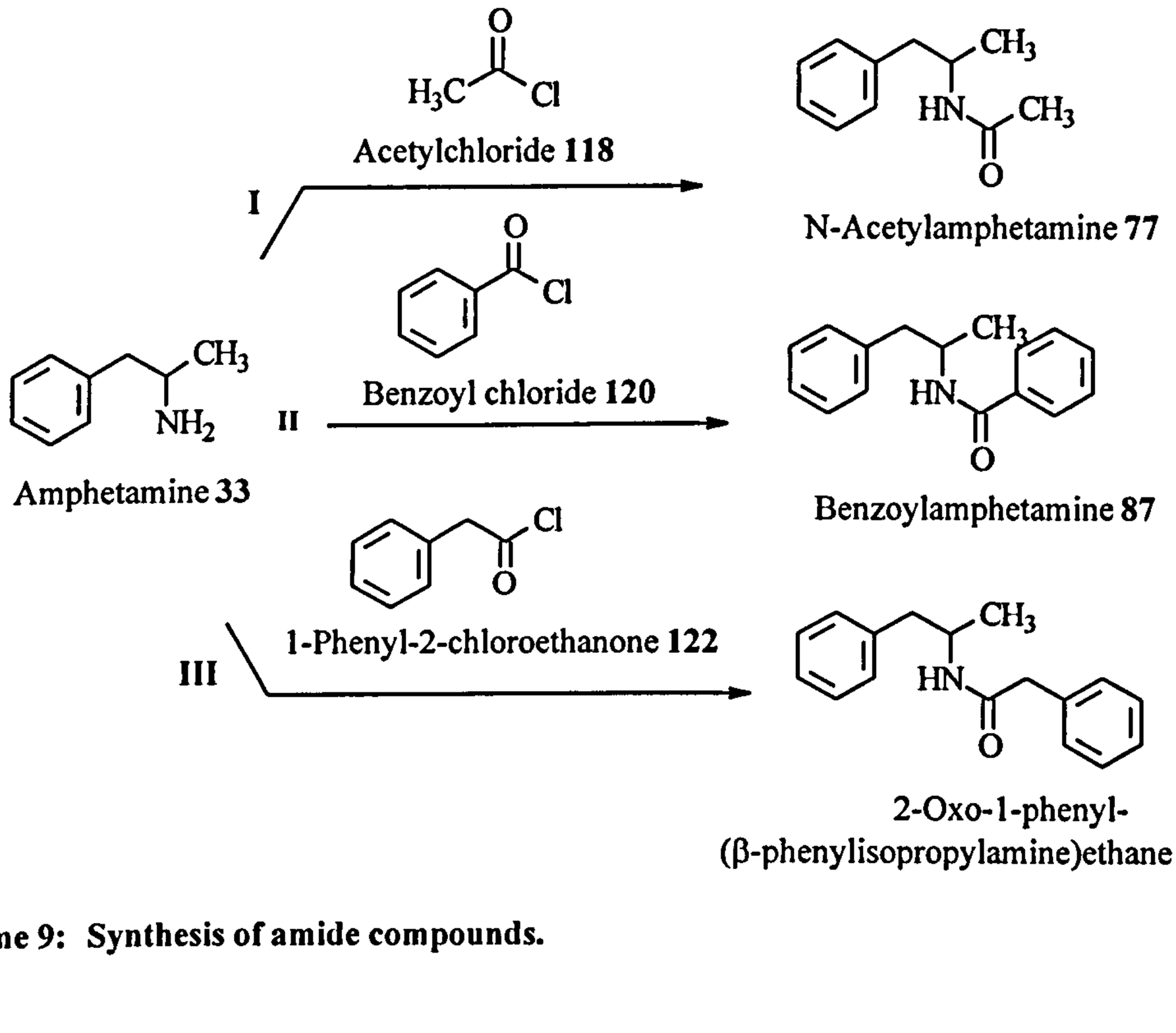
2-Oxo-1-phenyl-(β-phenylisopropylamine)ethane

2-oxo-1-phenyl-(β-phenylisopropylamine)ethane (2-oxo **115**) was synthesised utilising phenylacetic acid as a starting material. Phenylacetic acid **121** (4.4 g, 0.032 mol) was dissolved in toluene and $SOCl_2$ (7 ml, 0.097 mol) added (Scheme 8). The mixture was refluxed for 6 hours and the solvent was evaporated.

Phenylacetic acid **121** 1-Phenyl-2-chloroethanone **122**

Scheme 8: Synthesis of 1-phenyl-2-chloroethanone.

The resulting reaction mixture **122** was dissolved in THF and amphetamine base (4.4 g) added (Scheme 9, Reaction III). The mixture was treated in a similar method to the synthesis of benzoylamphetamine **87.** The yield of solid 2-oxo was 4.8 g (0.019 mol, 65%). The product was purified as above and the yield of pure product was 2.0 g (0.008 mol, 27%).



Scheme 9: Synthesis of amide compounds.

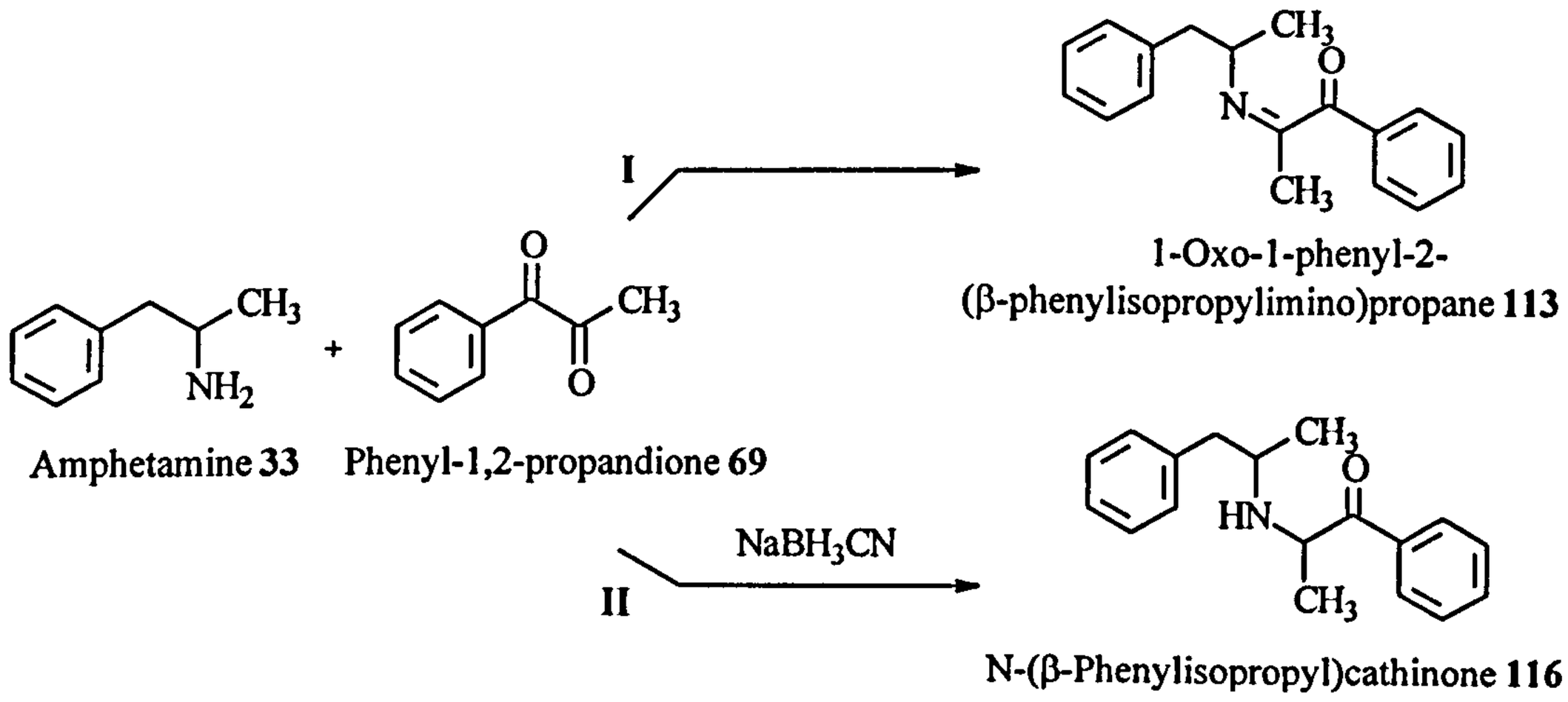
5.2.3.4 Oxo compounds

1-Oxo-1-phenyl-2-(β-phenylisopropylimino)propane

1-Phenyl-1,2-propandione **69** (1.2 g, 0.0082 mol) and amphetamine base (1.2 g, 0.0088 mol) were stirred in methanol (200 ml) at room temperature overnight (Scheme 10, Reaction I). The solvent was evaporated, yielding 1-oxo-1-phenyl-2-(β-phenylisopropylimino)propane (1-oxo **113**) (1.4 g, 0.0052 mol, 64%). The purity of the product was 86%, but any subsequent attempt to purify the product resulted in product decomposition.

N-(β-Phenylisopropyl)cathinone

In the synthesis of N-(β-phenylisopropyl)cathinone (cathinone **116**) 1-oxo-1-phenyl-2-(β-phenylisopropylimino)propane was prepared as an intermediate product. 1-Phenyl-1,2-propandione **69** (0.56 g, 0.0038 mol) and amphetamine base (0.52 g, 0.0038 mol) was stirred overnight in methanol. 0.5 g (0.008 mol) of $NaBH_3CN$ was added and stirring proceeded for 1 hour (Scheme 10, Reaction II). MeOH was evaporated and the reaction finished by adding water and HCl. The reaction mixture was stirred for 2 days. The acid mixture was washed with CH_2Cl_2. The organic phase was evaporated and the yield of cathinone product was 1.6 g. The compound was too unstable to purify.

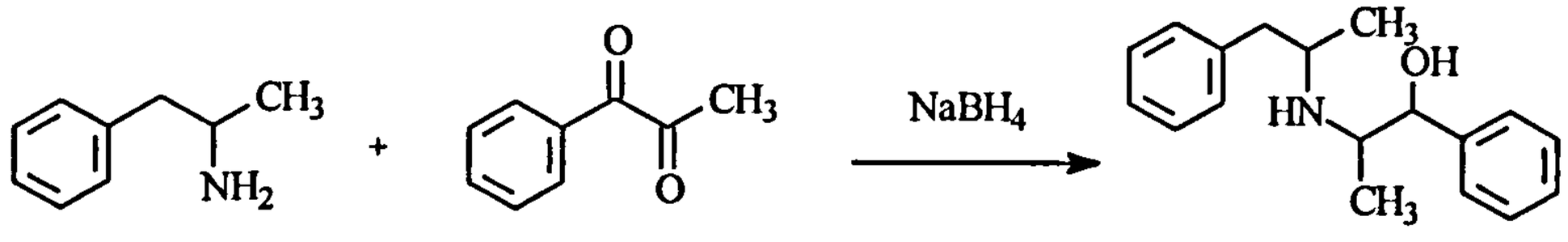


Scheme 10: Synthesis of oxo compounds.

5.2.3.5 Hydroxyl compounds

N,β-Hydroxy-N,N-di(β-phenylisopropyl)amine

N,β-Hydroxy-N,N-di(β-phenylisopropyl)amine (cathinol **117**) was synthesised via a three step reaction. In the first step, 1-oxo-1-phenyl-2-(β-phenylisopropylimino)-propane **113** was prepared, which was reduced to N-(β-phenylisopropyl)cathinone **117** in the second step (Scheme 10). 1.2 g (0.0089 mol) of amphetamine base and 1.1 g (0.0074 mol) of phenyl-1,2-propandione **69** were stirred in 200 ml of MeOH and 0.7 g (0.011 mol) of $NaBH_3CN$ added. The carbonyl group of cathinone was reduced to the corresponding alcohol by adding $NaBH_4$ (0.5 g, 0.013 mol) and stirring for 2 days (Scheme 11). The MeOH was evaporated and the reaction quenched by addition of water, HCl and triethylamine (2 ml). The reaction mixture was extracted with CH_2Cl_2 and the solvent evaporated. The oily cathinol (1.0 g, 0.0037 mol, 41%) was purified by washing the product with saturated $NaHCO_3$ solution.



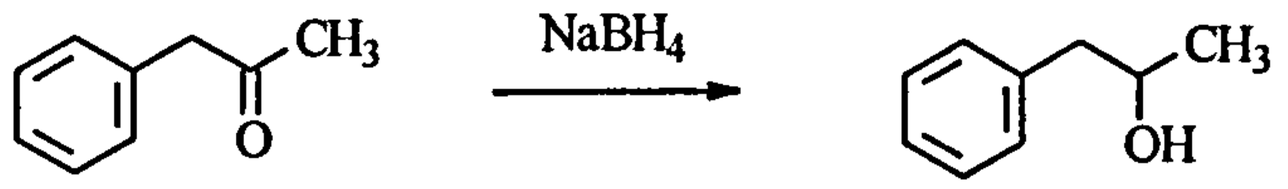
Amphetamine **33** Phenyl-1,2-propandione **69** N,β-Hydroxy-N,N-di(β-phenylisopropyl)amine **117**

Scheme 11: Synthesis of N,β-hydroxy-N,N-di(β-phenylisopropyl)amine.

The product that contained two diastereoisomers of cathinol was dissolved in ether, and white crystals formed. The insoluble crystals were filtered and dried. Recrystallisation gave one pure diastereoisomer (0.15 g).

1-Phenyl-2-propanol

BMK was reduced to the corresponding alcohol **99** by stirring 1.01 g (0.0075 mol) of BMK with 0.33 g (0.0087 mol) of $NaBH_4$ in 30 ml of 2-propanol (Scheme 12). The reaction was quenched by adding water and concentrated HCl. The reaction mixture was stirred overnight. The solvent was evaporated and the mixture extracted with CH_2Cl_2. The solvent was evaporated and 1-phenyl-2-propanol was obtained.



BMK 39 1-Phenyl-2-propanol 99

Scheme 12: Synthesis of 1-phenyl-2-propanol.

5.2.4 Stability of synthesised impurities

The stability of N-acetylamphetamine **77**, aldimine **97**, benzoylamphetamine **87**, benzylamphetamine **88**, 2-oxo **115** and cathinol **116** was studied in a standard impurity mixture with pure amphetamine base and in a synthesised reductive amination amphetamine matrix. The stability was studied at 6 different time delays, 0, 4, 12, 24, 48 and 96 hours (t_0, …, t_{96}). The influence of different organic solvents and storage conditions was also determined. Isooctane, toluene, CH_2Cl_2, diethyl ether, ethyl acetate and ethanol were used as organic solvents. The samples were stored in daylight at 25°C and 8°C.

Sample preparation

A stock solution in the standard impurity mixture was prepared by dissolving 10 mg of each impurity in 10 ml of CH_2Cl_2. 100 mg of amphetamine base was dissolved in 10 ml of CH_2Cl_2 to prepare amphetamine solution. 10 µl of stock solution and 10 µl of amphetamine solution was mixed and diluted into 1 ml with different organic solvents. Each solvent contained tetracosane (internal standard, i.s.) at concentration of 10 µg/ml. The mixture was split into six vials for injection at 6 time delays.

The stability of the impurities in the reductive amination amphetamine matrix was studied using the same solvents as in the standard mixture, excluding ethanol. In the sample preparation, 300 mg of amphetamine was dissolved in 3 ml of 63.2 mM [46] phosphate buffer. The pH of the solvent was adjusted to pH 7 with NaOH solution. The mixture was extracted with 1.2 ml of organic solvent, which contained i.s. at

concentration of 10 µg/ml. Correspondingly, the extract was split into 6 vials for injection at the 6 time delays.

Relative response factors (RRF), i.e. the ratio of peak area of target impurities to peak area of internal standard, were calculated for each time delay in each of the different solvents. Furthermore, the values were normalised by calculating the ratio of the peak area of each time delay relative to the peak area of time delay t_0. The mean value (x), standard deviation (s) and relative standard deviation (RSD) were calculated from these normalised values by using equations 16 - 18 [164].

$$\bar{x} = \frac{\sum_i^n x_i}{n} \qquad s = \sqrt{\frac{\sum_i^n (x_i - \bar{x})^2}{n-1}} \qquad RSD = \frac{s}{\bar{x}} \times 100\,\% \qquad \textbf{(16-18)}$$

5.3 Results and discussion

5.3.1 Synthesis of amphetamine

Synthesis of amphetamine was carried out using different amines and reducing agents. The more selective $NaBH_3CN$ gave a better yield compared to $NaBH_4$ which reduced part of the BMK to the corresponding alcohol, 1-phenyl-2-propanol. In the scaling up, the use of $NaBH_3CN$ was more complicated. Using large amounts of reducing agent, the reaction was difficult to quench. The reaction mixture needed to be stirred for a few days to avoid the formation of a complex mixture. Ammonium acetate seemed to be a more suitable amine in the production of reductive amination amphetamine than the volatile ammonia. Even a 10-fold excess of ammonia was not enough to produce a good yield of amphetamine. Using $NaBH_4$ and ammonia all of the BMK was reduced to alcohol even if a Shiff's base was formed beforehand. Typical reductive amination impurity profiles using $NaBH_3CN$ and ammonium acetate are shown in Figure 19. A profile of a seized street sample is also shown. The target compounds were labelled using the same numbers as in Figure 17.

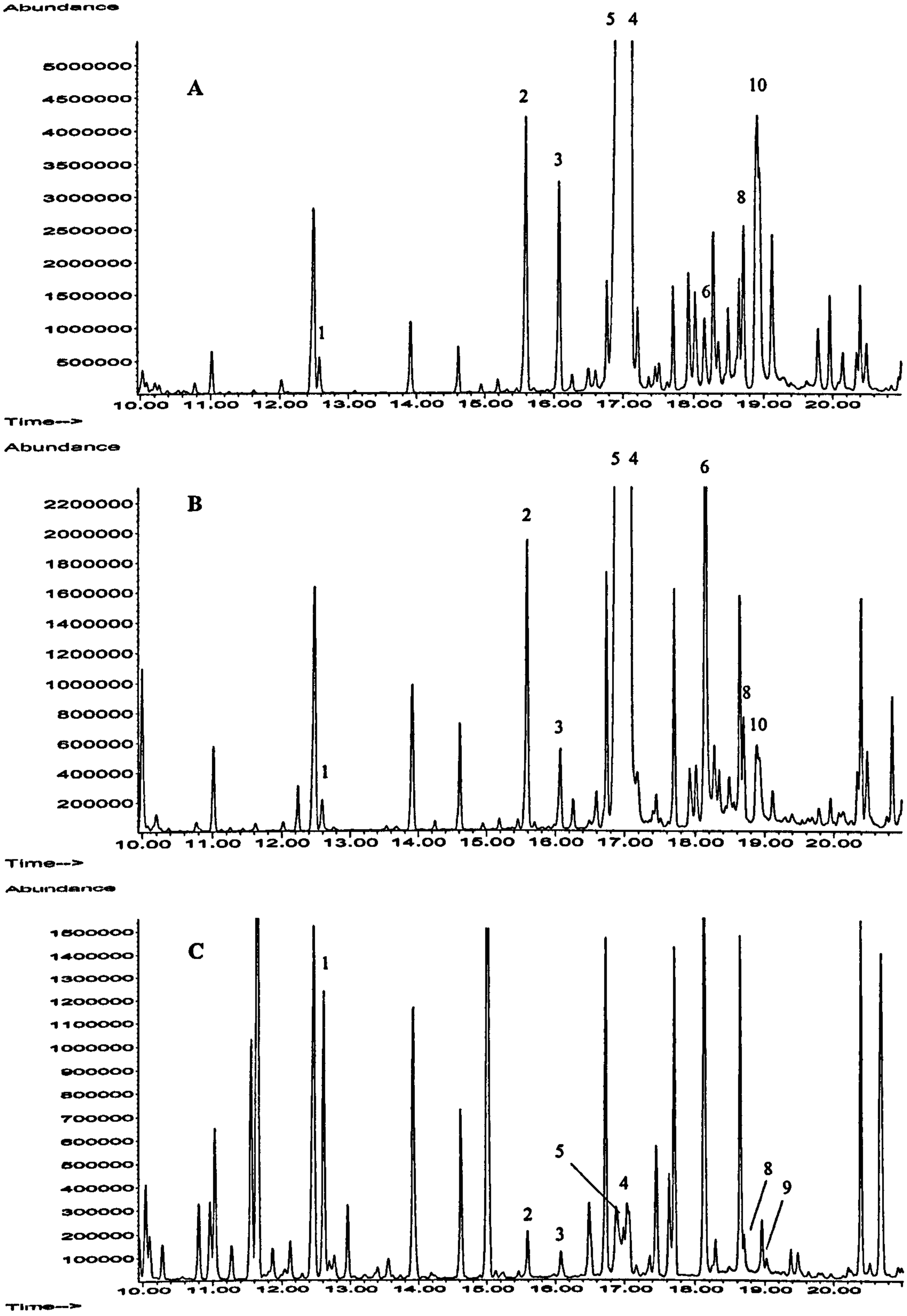


Figure 19: **Comparison of reductive amination batches. A) Synthesised amphetamine batch 1, B) synthesised amphetamine batch 2 and C) seized amphetamine batch. Peak identification: 1) N-acetylamphetamine, 2) aldimine, 3) benzylamphetamine, 4) DPIA, 5) ketimine, 6) 1-oxo, 8) benzoylamphetamine, 9) 2-oxo and 10) cathinone.**

5.3.2 Synthesis of impurities

The standard impurities were synthesised and the structures confirmed by GC-MS, UV, FTIR and NMR techniques. The results were compared to available literature values. The spectrometric data is available for aldimine [165], benzylamphetamine and benzoylamphetamine [166,167], N-acetylamphetamine [168], 2-oxo [169] and 1-phenyl-2-propanol [170]. Other impurities have not been identified before, and reference values are not available. The mass spectrum and retention time of each synthesised standard impurity was compared with data obtained from the analysis of street samples and the respective GC-MS data. The differences in the retention times are a consequence of possible modifications in the GC-MS method during the study.

N-(β-Phenylisopropyl)benzaldimine

The aldimine was synthesised using the excess of amphetamine. All of the benzaldehyde was reacted and aldimine was formed (Figure 20). The purity of the aldimine was improved with a gentle distillation. Due to the instability of the imine compound the aldimine was, however, impossible to purify completely from amphetamine. All spectroscopic data is available and shown below.

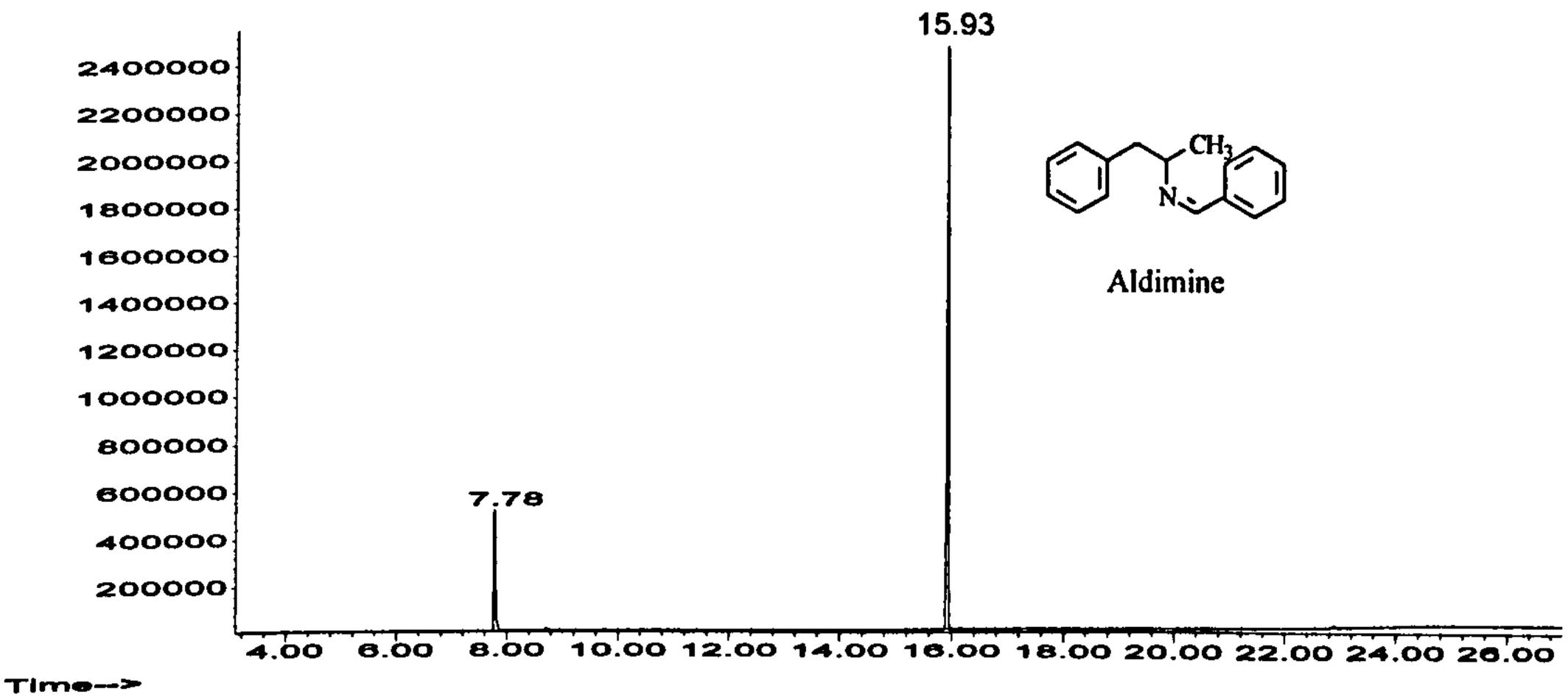


Figure 20: Chromatogram of aldimine (t=15.93 min). Unreacted amphetamine at 7.78 min.

MS: m/z = 222 $[M-H]^+$ 0.3%, 133 $[M-C_7H_6]^+$ 10%, 132 $[M-C_7H_7]^+$ 100%, 117 $[M-C_7H_8N]^+$ 5%, 105 $[C_7H_7N]^+$ 27%, 91 $[C_7H_7]^+$ 21%, 77 $[C_6H_5]^+$ 9%.

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

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

^{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, Ph), 8.01 (1H, s, N=CH).

^{13}C NMR: δ = 22.24 (CH_3), 44.59 (CH_2), 68.20 (CH), 125.97 (C-4'), 126.24 (C-4''), 128.04 (C-3,5'), 128.48 (C-3,5''), 129.25 (C-2,6'), 129.69 (C-2,6''), 136.37 (C-1'), 139.35 (C-1''), 159.30 (N=CH).

N-(β-Phenylisopropyl)benzyl methyl ketimine

The ketimine was synthesised in an analogous way to the aldimine with the excess of amphetamine. In addition to ketimine, extra impurities formed, namely aldimine and N-acetylamphetamine. Moreover, the starting material was left in the final product. Ketimine gives a bad, tailing chromatographic peak at retention time 18.24 min (Figure 21).

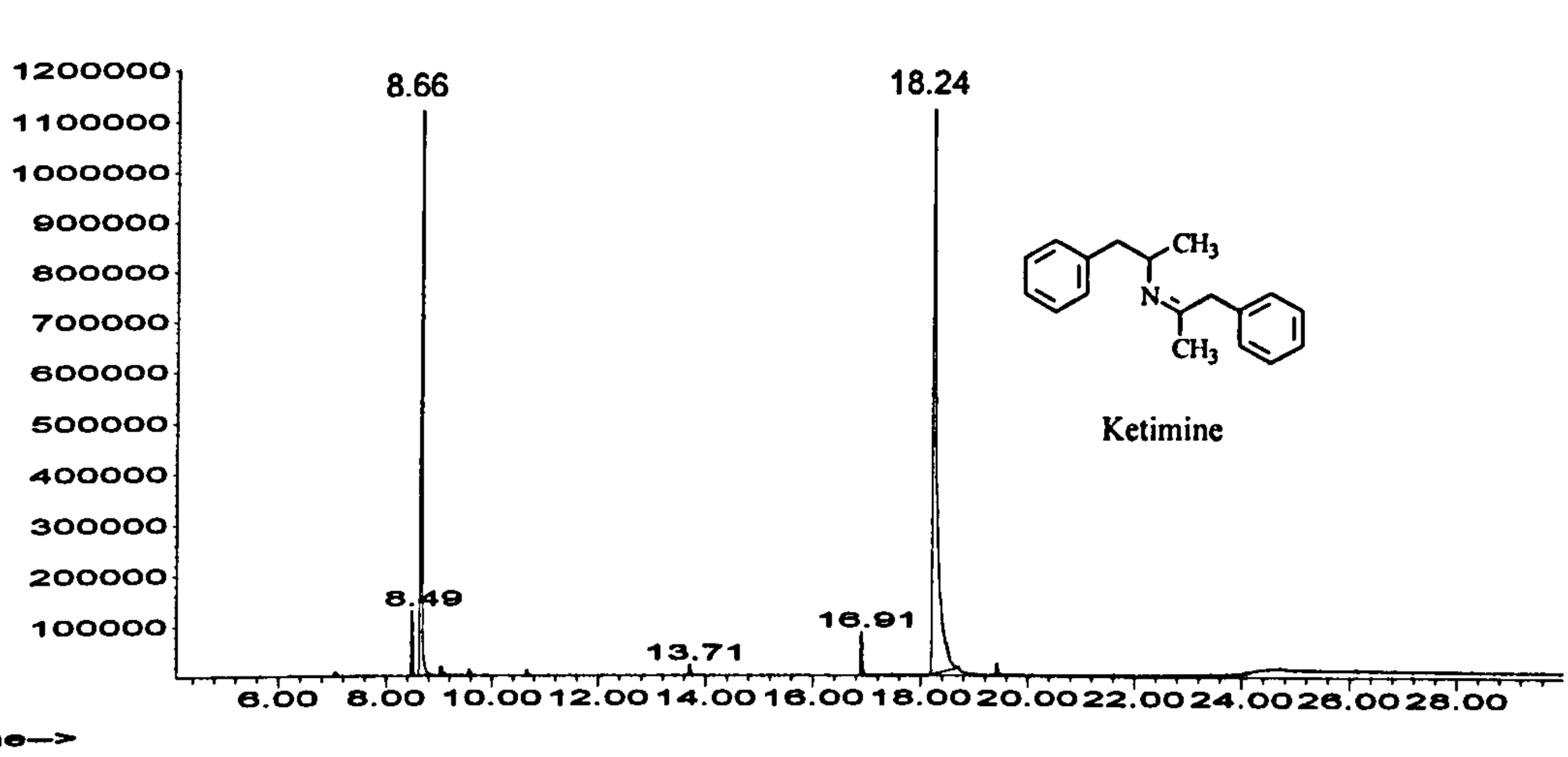


Figure 21: Chromatogram of ketimine (t=18.24 min). Unreacted BMK at 8.49 min and the excess of amphetamine at 8.66 min, N-acetylamphetamine at 13.71 min and aldimine at 16.91 min.

It was difficult to remove the amphetamine from the ketimine. During the distillation, ketimine started to polymerise. During storage the ketimine also decomposed back to the starting materials, amphetamine and BMK. Because of the unstability of ketimine, all spectrometric data is not available.

MS: m/z = 161 $[M\text{-}C_7H_6]^+$ 8%, 160 $[M\text{-}C_7H_7]^+$ 68%, 119 $[PhCH_2CHCH_3]^+$ 45%, 91 $[C_7H_7]^+$ 100%.

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

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

^1H and ^{13}C NMR: Compound is unstable and too impure to obtain NMR data.

Benzylamphetamine

Benzylamphetamine is a reduced form of aldimine. The double bond of aldimine was reduced with $NaBH_3CN$. Unreacted aldimine could be still found in the final product (Figure 22).

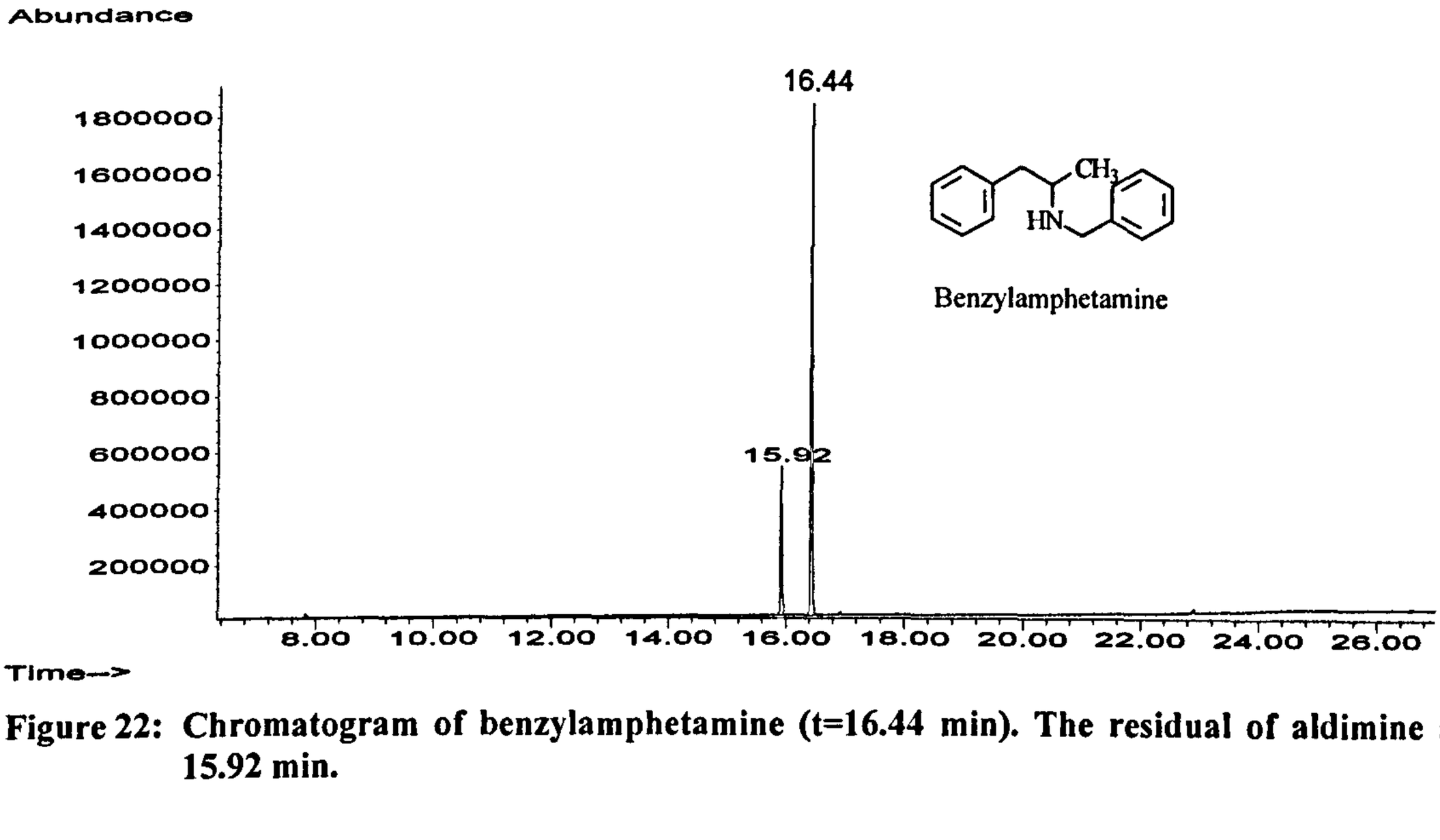


Figure 22: Chromatogram of benzylamphetamine (t=16.44 min). The residual of aldimine at 15.92 min.

Benzylamphetamine was found to be a stable compound and it was possible to purify it by column chromatography. The pure standard compound was separated and spectrometric analyses performed. The spectrometric data is shown below.

MS: m/z = 224 $[M-H]^+$ 0.2%, 134 $[M-C_7H_7]^+$ 63%, 91 $[C_7H_7]^+$ 100%, 77 $[C_6H_5]^+$ 3%, 65 $[C_5H_5]^+$ 2%.

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

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

^{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, Ph).

^{13}C NMR: δ = 20.21 (CH_3), 43.60 (CH_2), 51.29 (CH), 53.70 (NH-CH_2), 126.15 (C-4'), 126.80 (C-4''), 127.98 (C-3,5'), 128.56 (C-3,5''), 129.30 (C-2,6'), 129.30 (C-2,6''), 139.42 (C-1'), 140.54 (C-1'').

N-Acetylamphetamine

The basic structure is similar in amide compounds, namely N-acetylamphetamine, benzoylamphetamine and 2-oxo compounds. The amphetamine structure with carboxyl groups next to the nitrogen was equal in all substances. The functional group in the compounds were methyl, phenyl and benzyl group, respectively. All these compounds were in solid form and they could be purified by recrystallisation. The chromatogram of N-acetylamphetamine is shown in Figure 23. Amphetamine was not detected after purification. The spectrometric data for N-acetylamphetamine is shown below.

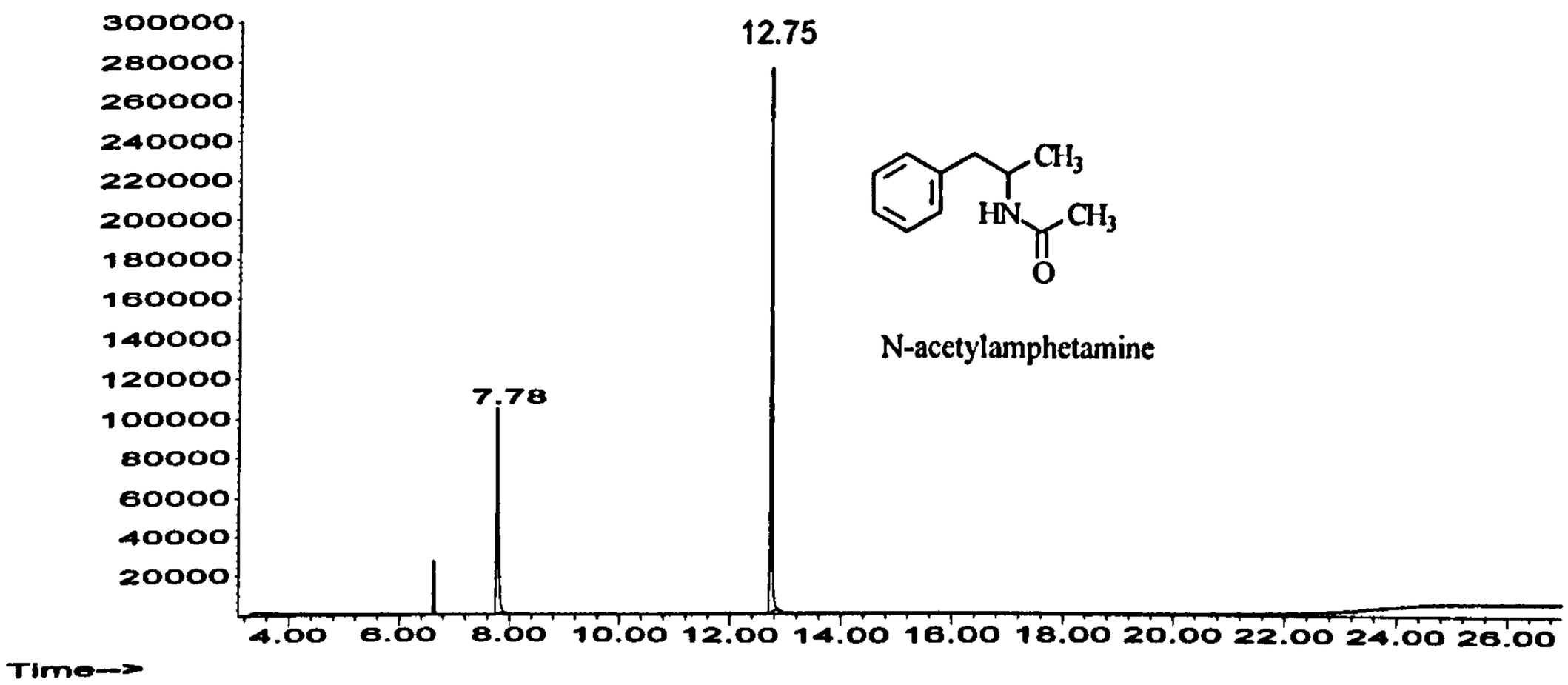


Figure 23: Chromatogram of N-acetylamphetamine (t=12.75 min). The residual of amphetamine at 7.78 min.

MS: m/z = 177 $[M]^+$ 1%, 134 $[M\text{-}CH_3CO]^+$ 1%, 118 $[M\text{-}CH_3CONH_2]^+$ 25%, 91 $[C_7H_7]^+$ 22%, 86 $[CH_3CHNHCOCH_3]^+$ 41%, 44 $[CH_3CHO]^+$ 100% and 44 $[CH_3CHNH_2]^+$ 23%.

FTIR: γ_{max} (cm^{-1}): 1653 (C=0), 155, 701, 747, 1372, 507, 3250 (NH amide), 2968, 608, 1298.

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

^{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 (10H, m, Ph).

^{13}C NMR: δ = 19.93 (CH_3), 23.93 ($\underline{C}H_3$-CO), 42.43 (CH_2), 46.13 (CH), 126.4 (C-4'), 128.37 (C-3,5'), 138.05 (C-1'), 169.38 (CO).

Benzoylamphetamine

Benzoylamphetamine was synthesised via benzoylchloride. A small amount of by-product was left in the final product before purification (Figure 24). The mass spectrum of benzoylchloride is shown in Figure 26. Benzoylamphetamine was purified and spectrometric data collected. The data is shown below.

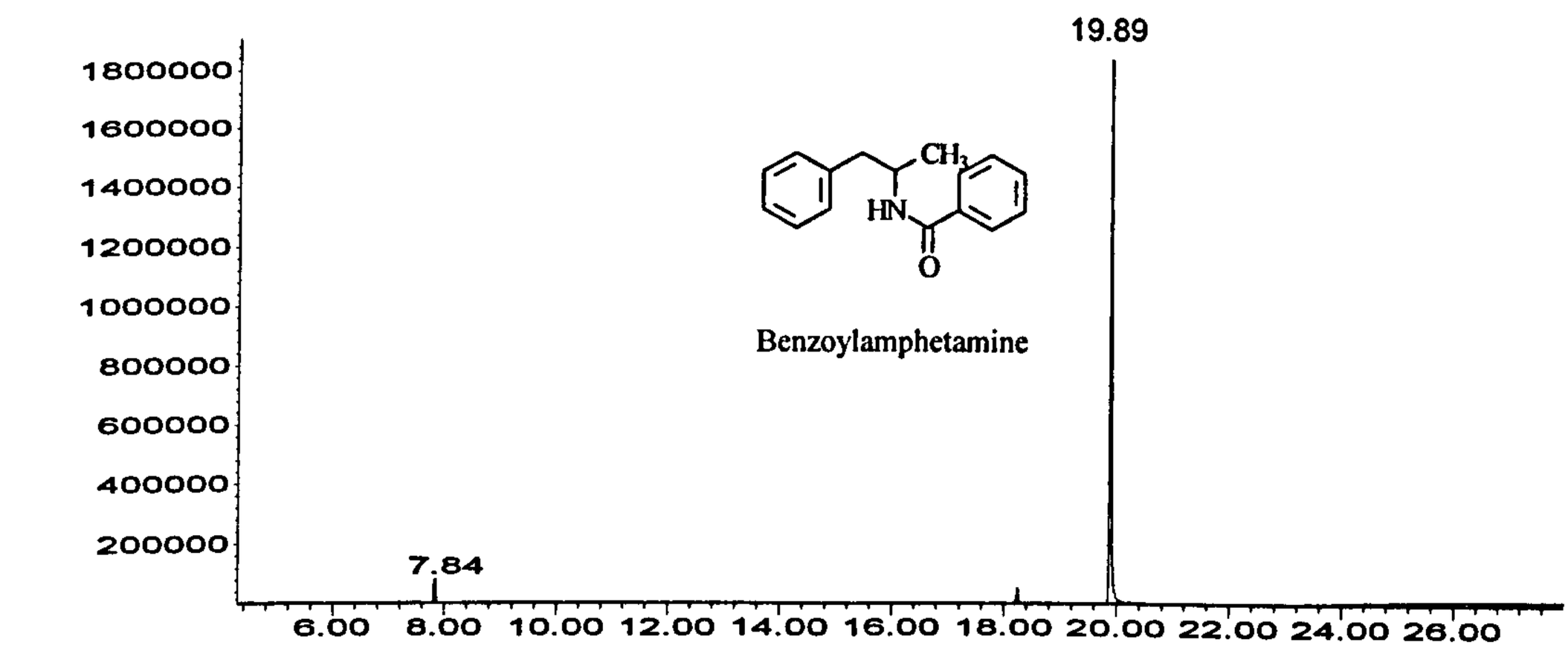


Figure 24: Chromatogram of benzoylamphetamine (t=19.89 min). The by-product benzoylchloride at 7.84 min.

MS: m/z = 239 $[M]^+$ 0.3%, 148 $[M-C_7H_7]^+$ 43%, 118 $[PhCHCHCH_3]^+$ 8%, 105 $[PhCO]^+$ 100%, 91 $[C_7H_7]^+$ 13%, 77 $[C_6H_5]^+$ 39%.

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

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

^{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, Ph).

^{13}C NMR: δ = 19.99 (CH_3), 42.39 (CH_2), 46.49 (CH), 126.53 (C-4'), 126.79 (C-4''), 128.44 (C-3,5'), 128.53 (C-3,5''), 129.55 (C-2,6'), 131.32 (C-2,6''), 134.89 (C-1'), 137.86 (C-1'').

2-Oxo-1-phenyl-(β-phenylisopropylamine)propane

The 2-oxo compound was synthesised in an analogous way to benzoylamphetamine. Unreacted phenylacetic acid was obtained in the final product (Figure 25). The mass spectrum of phenylacetic acid is shown in Figure 26. The 2-oxo was recrystallised and spectrometric data collected for solid material. The data is shown below.

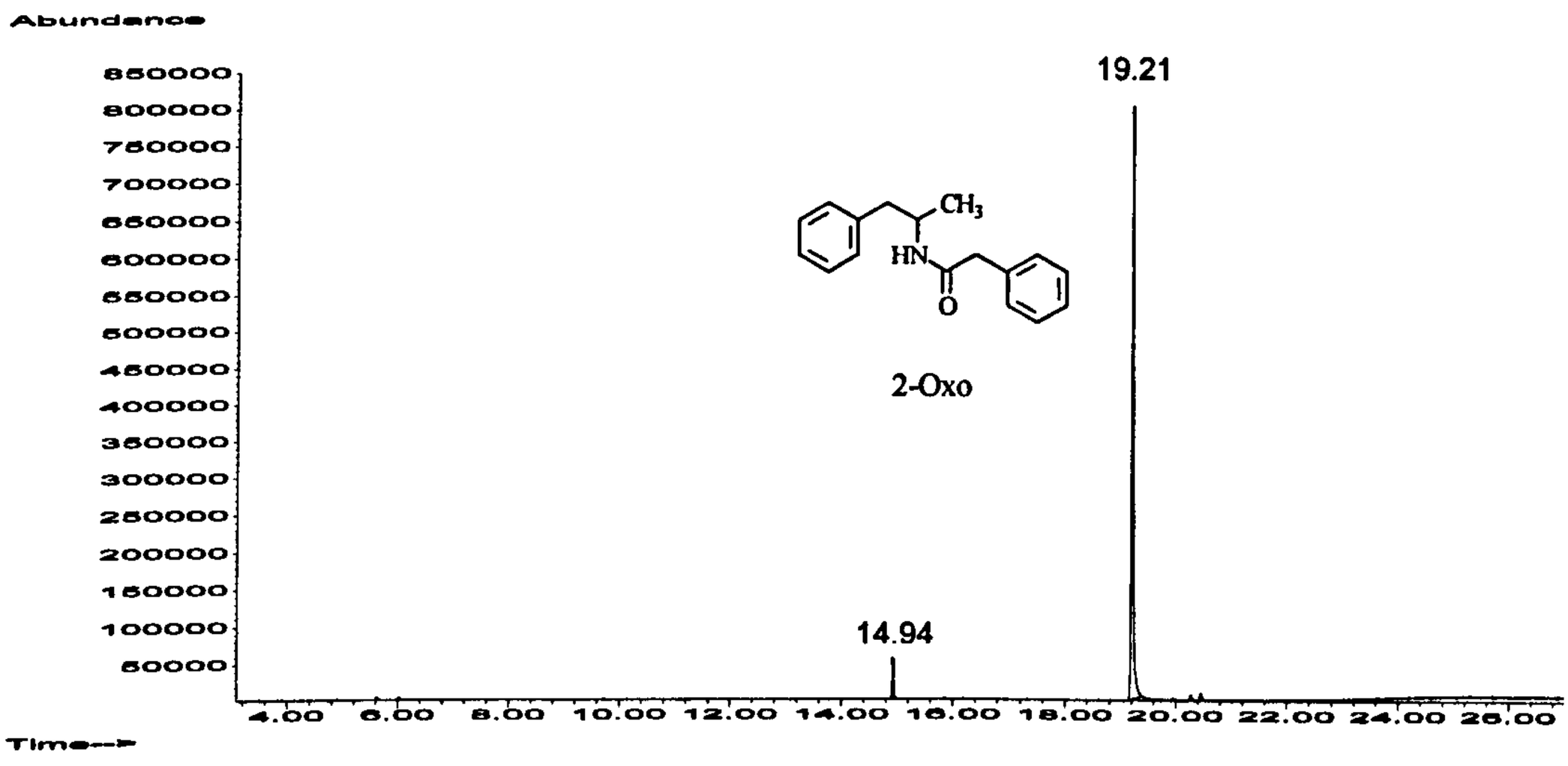


Figure 25: Chromatogram of 2-oxo (t=19.21 min). Phenylacetic acid at 14.94 min.

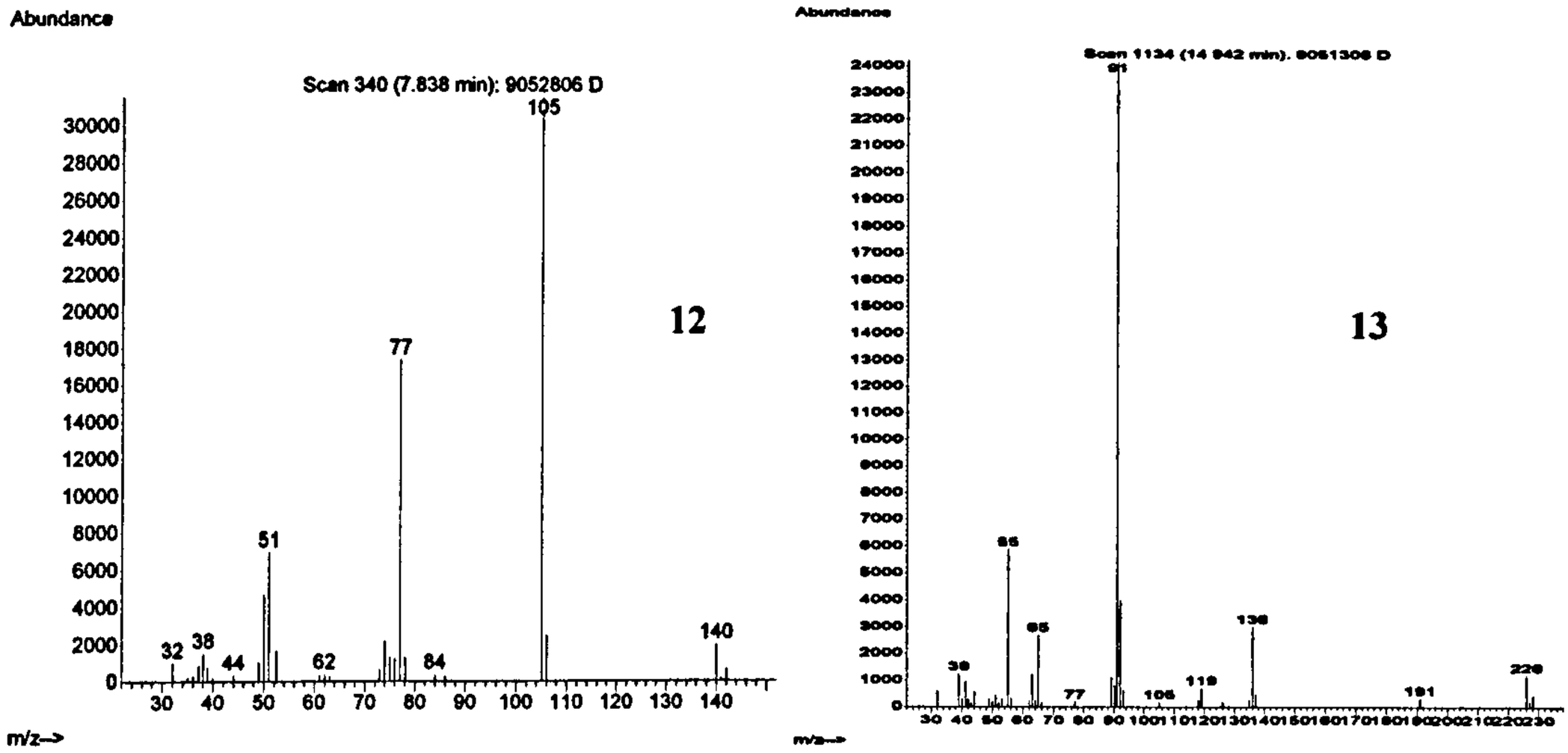


Figure 26: Mass spectra of extra compounds found in benzoylamphetamine and 2-oxo chromatograms. Peak identification: 12) benzoylchloride and 13) phenylacetic acid.

MS: m/z = 253 $[M]^+$ 2%, 162 $[M\text{-}C_7H_7]^+$ 33%, 119 $[PhCH_2CO]^+$ 58%, 91 $[C_7H_7]^+$ 100%, 77 $[C_6H_5]^+$ 29%, 65 $[C_5H_5]^+$ 6%.

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

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

^{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, $CO\text{-}CH_2$), 4.25 (1H, m, CH), 5.20 (1H, d, NH, J=6.6Hz), 7.01-7.33 (10H, m, Ph).

^{13}C NMR: δ = 19.98 (CH_3), 42.17 (CH_2), 46.04 (CH), 126.39 (C-4'), 127.27 (C-4''), 128.34 (C-3,5'), 128.99 (C-3,5''), 129.37 (C-2,6'), 129.44 (C-2,6''), 134.90 (C-1'), 137.61 (C-1'').

<u>1-Oxo-1-phenyl-2-(β-phenylisopropylimino)propane</u>

The 1-oxo compound has a double bond from nitrogen making it unstable. There were still some starting materials left in the final product (Figure 27). An unknown impurity appears at 18.53 min. The mass spectra of 1-phenyl-1,2-propandione and the unknown substance A are shown in Figure 28.

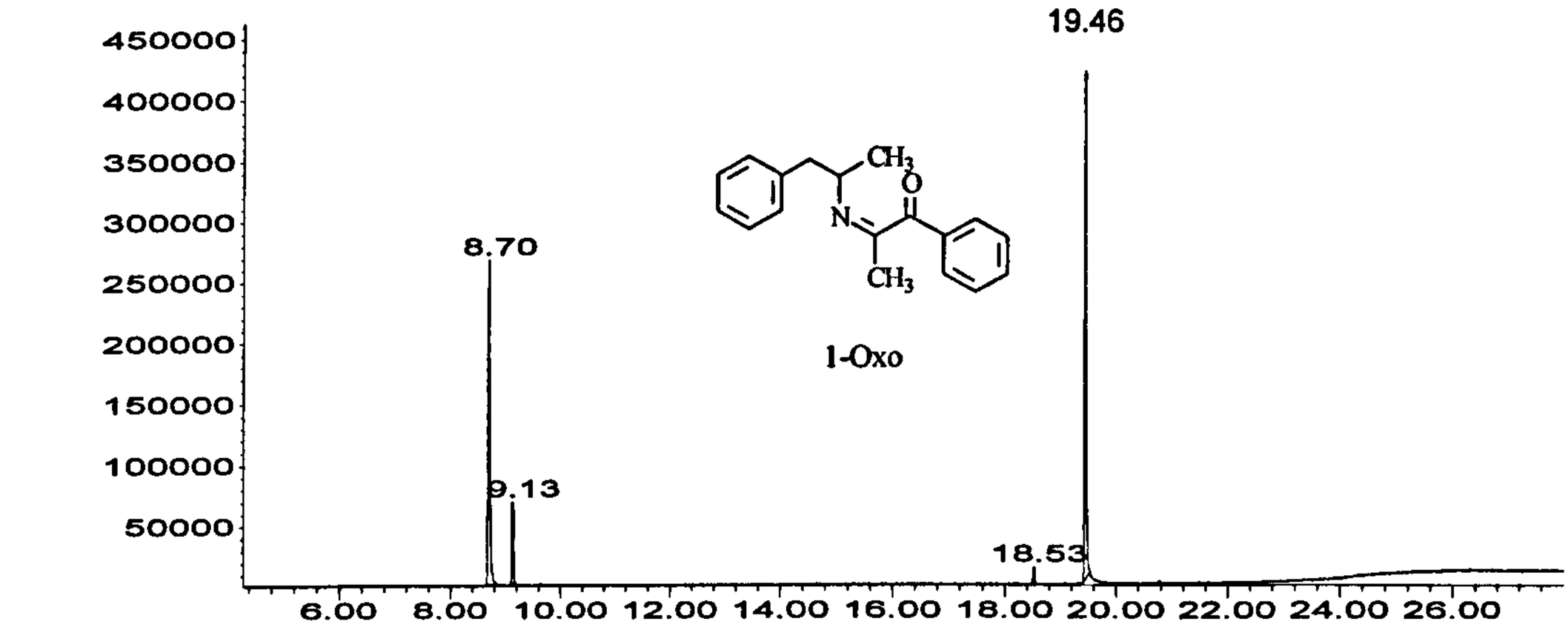


Figure 27: Chromatogram of 1-oxo (t=19.46 min). Amphetamine at 8.70 min, 1-phenyl-1,2-propanedione at 9.13 min and unknown impurity A at 18.53 min.

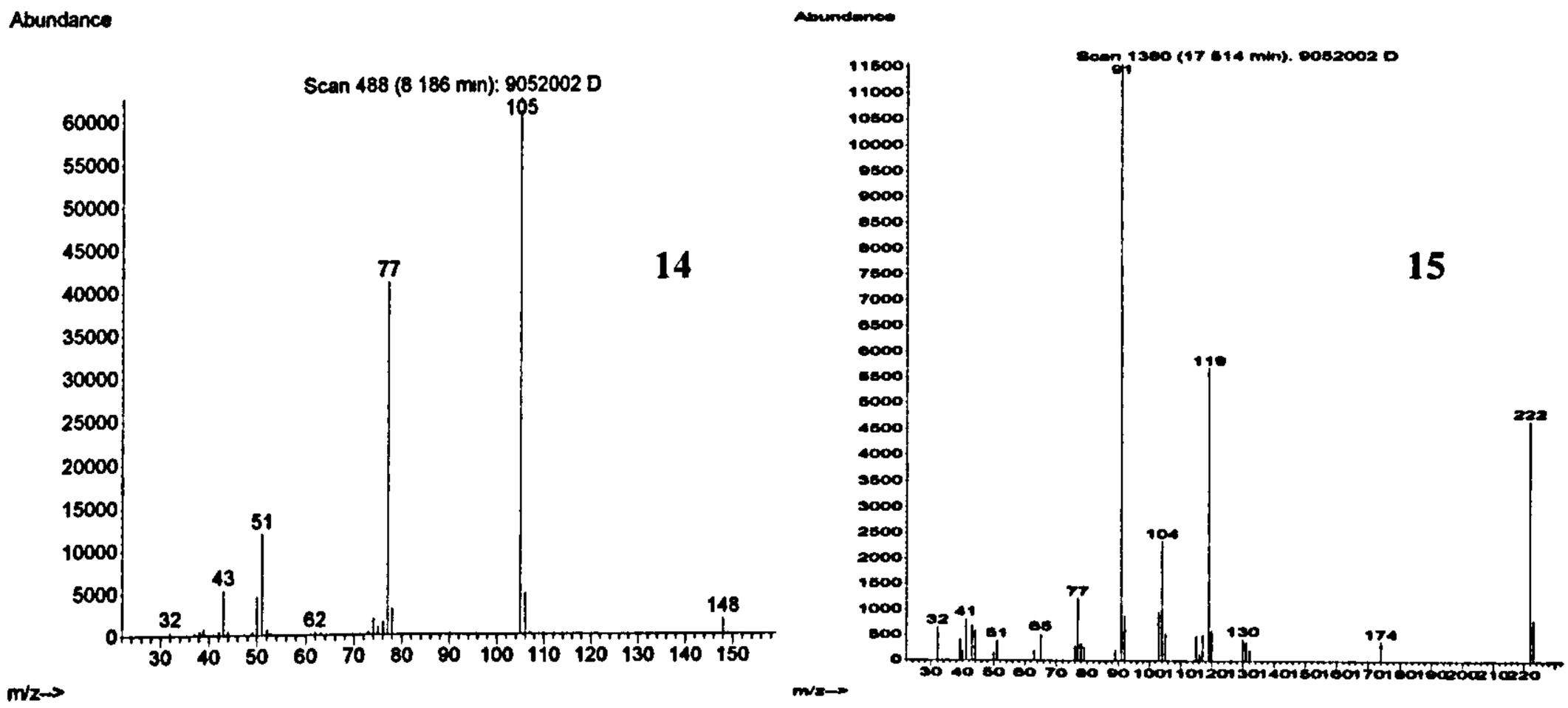


Figure 28: Mass spectra of extra compounds found in 1-oxo chromatogram. Peak identification: 14) 1-phenyl-1,2-propandione and 15) unknown substance A.

An attempt was made to purify the product by distillation and column chromatography but more impurities were detected afterwards. The spectrometric data for 1-oxo is shown below.

MS: m/z = 174 $[M\text{-}C_7H_7]^+$ 3%, 160 $[M\text{-}C_6H_5CO]^+$ 33%, 119 $[C_6H_5CH_2CHCH_3]^+$ 58%, 105 $[C_6H_5CO]^+$ 27%, 91 $[C_7H_7]^+$ 100%, 77 $[C_6H_5]^+$ 29%.

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

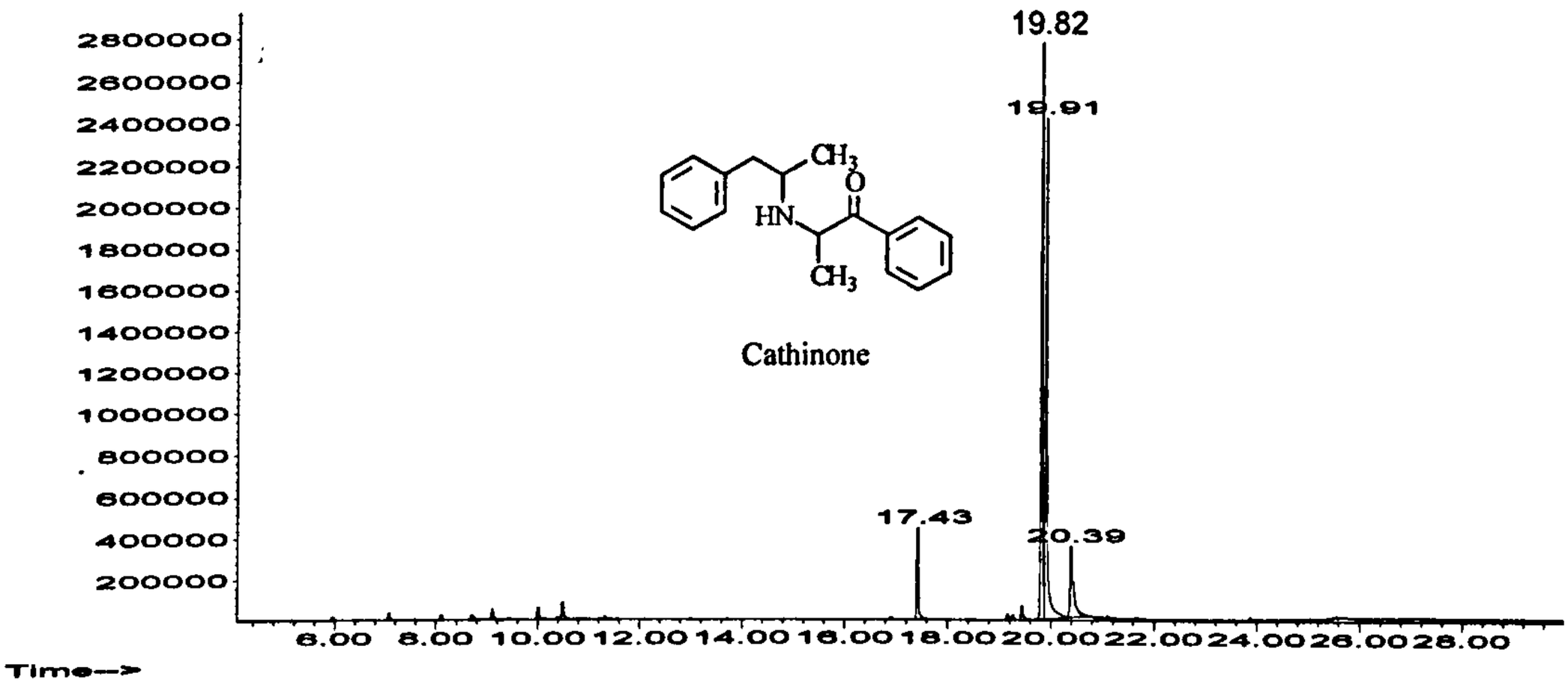
UV: λ_{max} (nm): [MeOH] 249, [0.1 M NaOH] 249 and [0.1 M H_2SO_4] 249.

^{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, Ph).

^{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 (Ph), 164.40 (C=N), 194.19 (C=O).

N-(β-Phenylisopropyl)cathinone

Cathinone is a reduced form of 1-oxo. Two isomers of cathinone were detected by GC-MS at 19.82 min and 19.91 min (Figure 29). Moreover benzylamphetamine and the reduced form of cathinone, cathinol, was detected at 20.39 min.



Cathinone

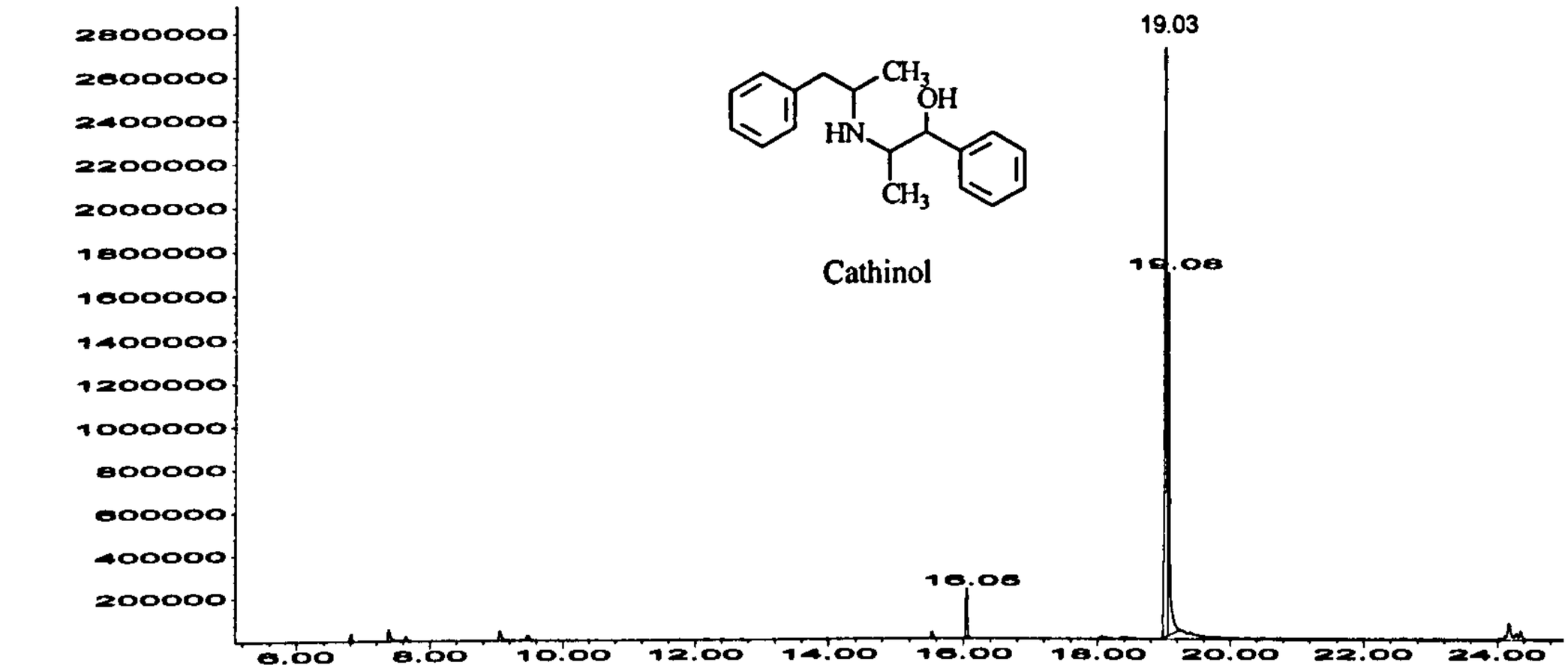
Figure 29: Chromatograph of cathinone (two isomers t=19.82 min and 19.91 min). Benzylamphetamine at 17.43 min and cathinol at 20.39 min.

The yield of cathinone was too low for any spectrometric analysis. It was also unstable which made the handling of the compound difficult. Only MS data is available.

MS: m/z = 266 $[M-H]^+$ 0.2%, 176 $[M-C_7H_6]^+$ 28%, 162 $[M-C_7H_5O]^+$ 100%, 119 $[C_8H_7O]^+$ 24%, 105 $[C_7H_5O]^+$ 17%, 91 $[C_7H_7]^+$ 69%, 77 $[C_6H_5]^+$ 17%, 44 $[CH_3CHNH_2]$ 23%.

N-β-Hydroxy-N,N-di(β-phenylisopropyl)amine

Cathinol was prepared from cathinone by the reduction of the ketone group to the corresponding alcohol. Two isomers of cathinol were detected (Figure 30). The compound was purified by recrystallisation. After recrystallisation only one isomer was left. The spectrometric data was collected for this pure isomer. In theory, there are two diastereomers within one isomer due to four chiral centres. These two diastereomers could be detected by NMR.



Cathinol

Figure 30: Chromatogram of cathinol (two isomers t=19.03 min and 19.08 min). Benzylamphetamine at 16.05 min.

MS: m/z = 268 $[M-H]^+$ 1%, 178 $[M-C_7H_7]^+$ 8%, 162 $[M-C_7H_5O]^+$ 100%, 119 $[C_8H_7O]^+$ 15%, 105 $[C_7H_5O]^+$ 2%, 91 $[C_7H_7]^+$ 34%, 77 $[C_6H_5]^+$ 5%, 44 $[CH_3CHNH_2]$ 6%.

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

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

^1H 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, Ph).

^{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 (C-2,6''), 126.40 (C-4'), 126.99 (C-4''), 127.99 (C-3,5''), 128.37 (C-3,5'), 129.20 (C-2,6'), 138.90 (C-1'), 141.30 (C-1'').

1-Phenyl-2-propanol

1-Phenyl-2-propanol is the reduced form of BMK. The compound is also commercially available. It is a common impurity for an incomplete reduction process. In amphetamine synthesis via $NaBH_4$ reduction some of the ketone group of BMK reduced to alcohol. The purity of synthesised 1-phenyl-2-propanol was over 99%. The spectrometric data is shown below.

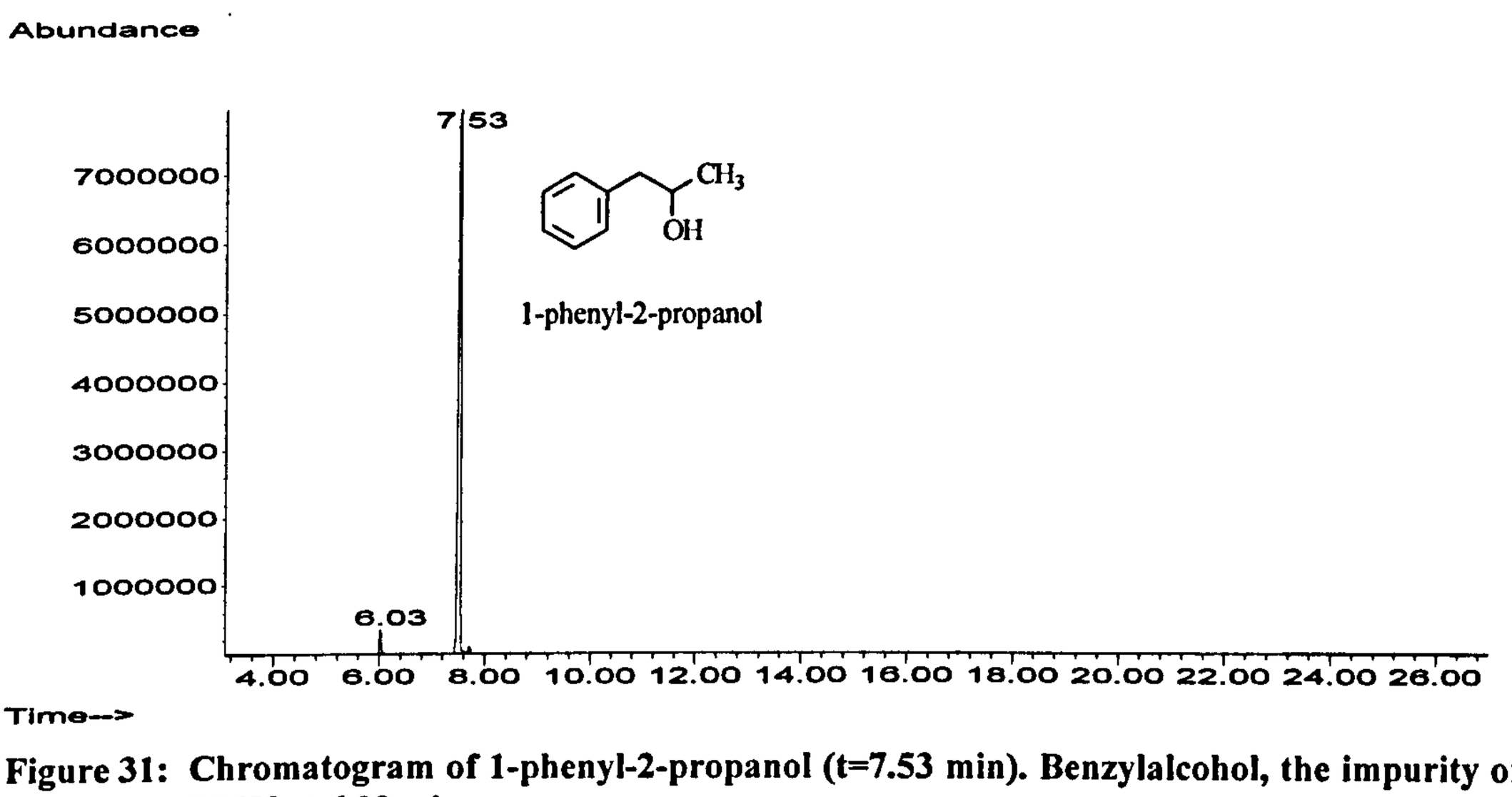


Figure 31: Chromatogram of 1-phenyl-2-propanol (t=7.53 min). Benzylalcohol, the impurity of BMK at 6.03 min.

MS: m/z = 136 $[M]^+$ 3%, 92 $[PhCH_3]^+$ 100%, 91 $[C_7H_7]^+$ 71%, 65 $[C_5H_5]^+$ 14%.

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

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

^{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, Ph).

^{13}C NMR: δ = 22.68 (CH_3), 45.17 (CH_2), 68.77 (CH), 126.36 (C-4'), 128.43 (C-3,5'), 129.33 (C-2,6'), 138.49 (C-1').

5.3.3 Stability of synthesised impurities

From previous studies it is known that ketimine and 1-oxo decompose during short storage in a freezer under nitrogen. Thus, a study of stability of these compounds was not undertaken in the standard impurity mixture. Acetylamphetamine, aldimine, benzylamphetamine, benzoylamphetamine, 2-oxo and cathinol were well separated chromatographicly in the standard mixture.

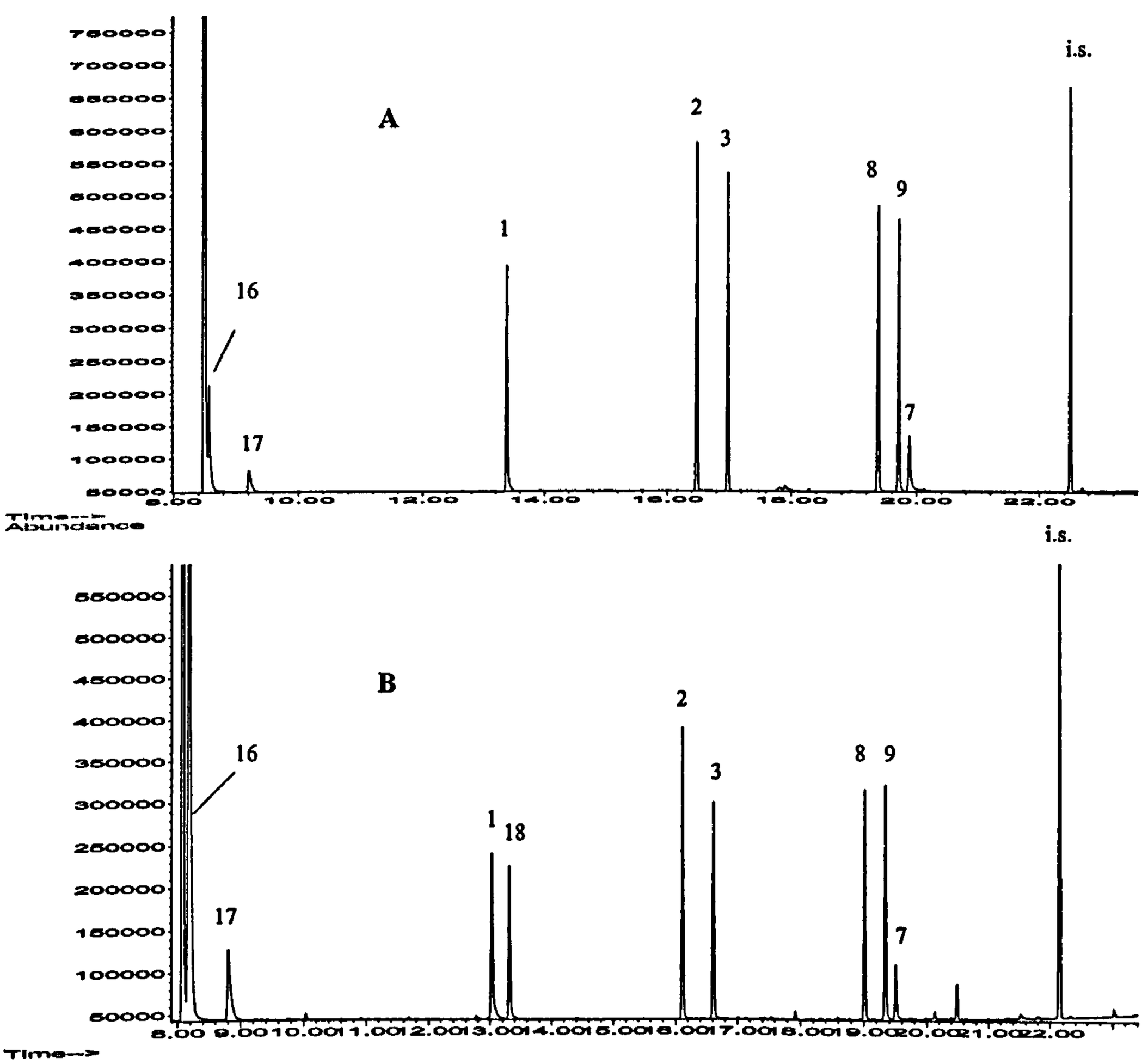
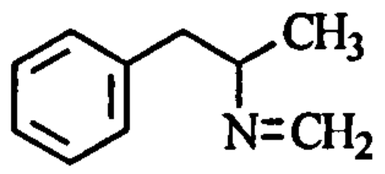
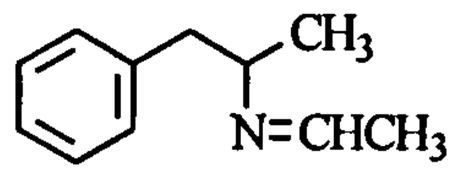


Figure 32: Chromatogram of the standard mixture A) in isooctane and B) in diethyl ether. Peak identification: 1) N-acetylamphetamine, 2) aldimine, 3) benzylamphetamine, 7) cathinol, 8) benzoylamphetamine, 9) 2-oxo, 16) amphetamine methyl imine, 17) amphetamine ethyl imine and 18) BHT.

Two extra impurity peaks were also detected in each chromatogram (Figure 32). The proposed compounds are amphetamine methyl imine **123** and amphetamine ethyl imine **124** (Figure 33). Moreover, one more extra peak was detected in the diethyl ether chromatograms. The compound was found to be a stabiliser, 2,6-di-tert-butylhydroxytoluene (BHT **101**), which can be found in the commercial solvent. The mass spectra of substances are shown in Figure 34.

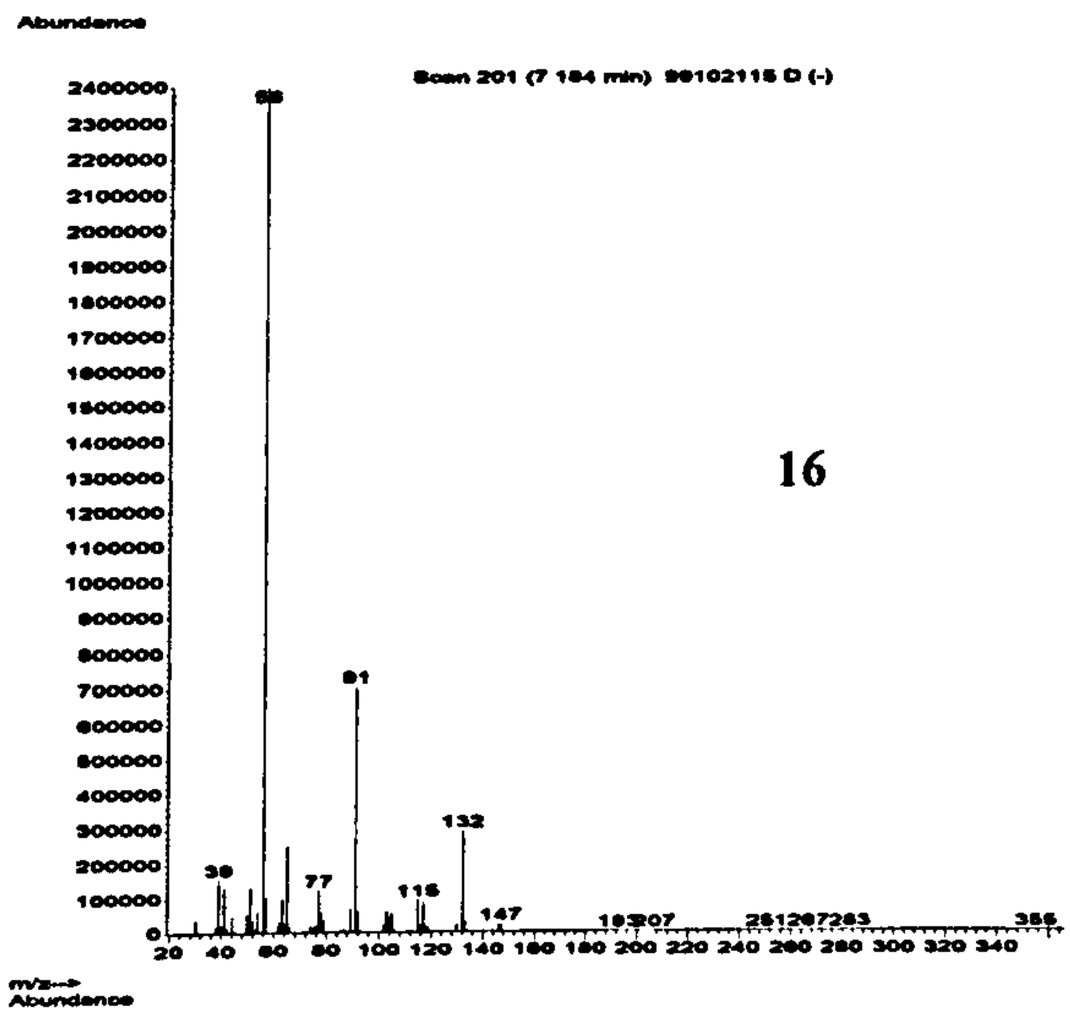


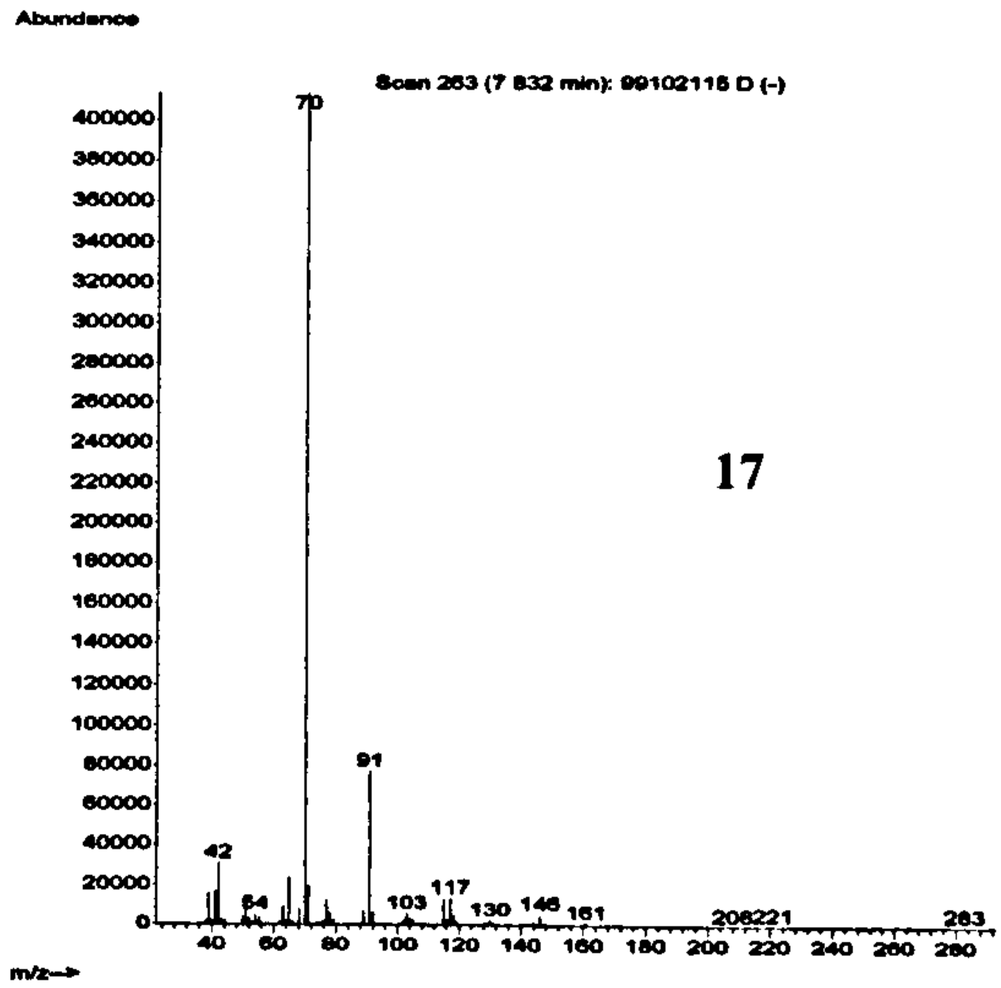
Amphetamine methyl imine **123**



Amphetamine ethyl imine **124**

Figure 33: Proposed structural formulas of identified extra compounds found in the standard mixture.





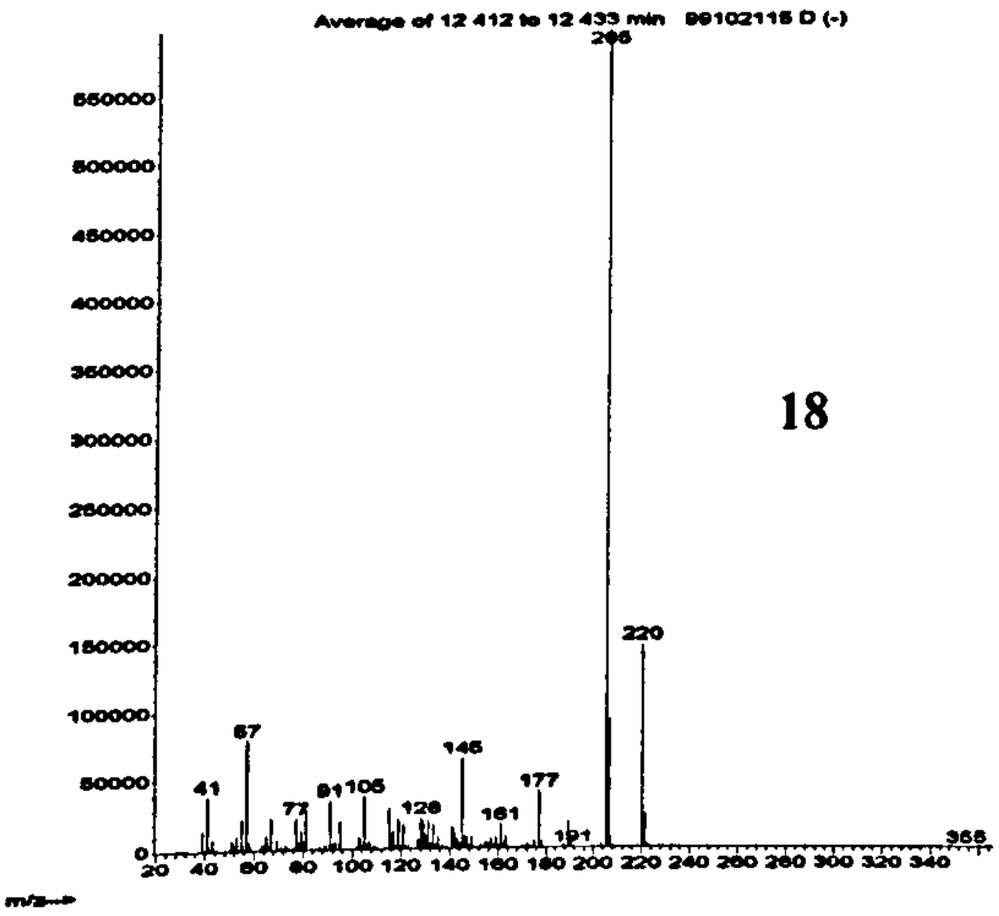


Figure 34: Mass spectra of extra compounds found in the standard mixture. Peak identification: 16) amphetamine methyl imine, 17) amphetamine ethyl imine and 18) BHT.

As it can be seen from the Figure 32, the concentration of amphetamine methyl imine increased in the samples prepared in diethyl ether. The same occurrence was detected in dichloromethane. The compounds could be found to be from the reaction of amphetamine with the solvent.

There were some problems during the study. Diethyl ether condensed outside the insert vials at 8°C and therefore the results are not reliable. Cathinol was not detected in samples prepared in ethyl acetate at 25°C (Figure 35). The OH group has probably reacted with acetate group of the solvent and formed the compounds including ester or ether group. The mass spectra of unknown substances B and C are shown in Figure 36.

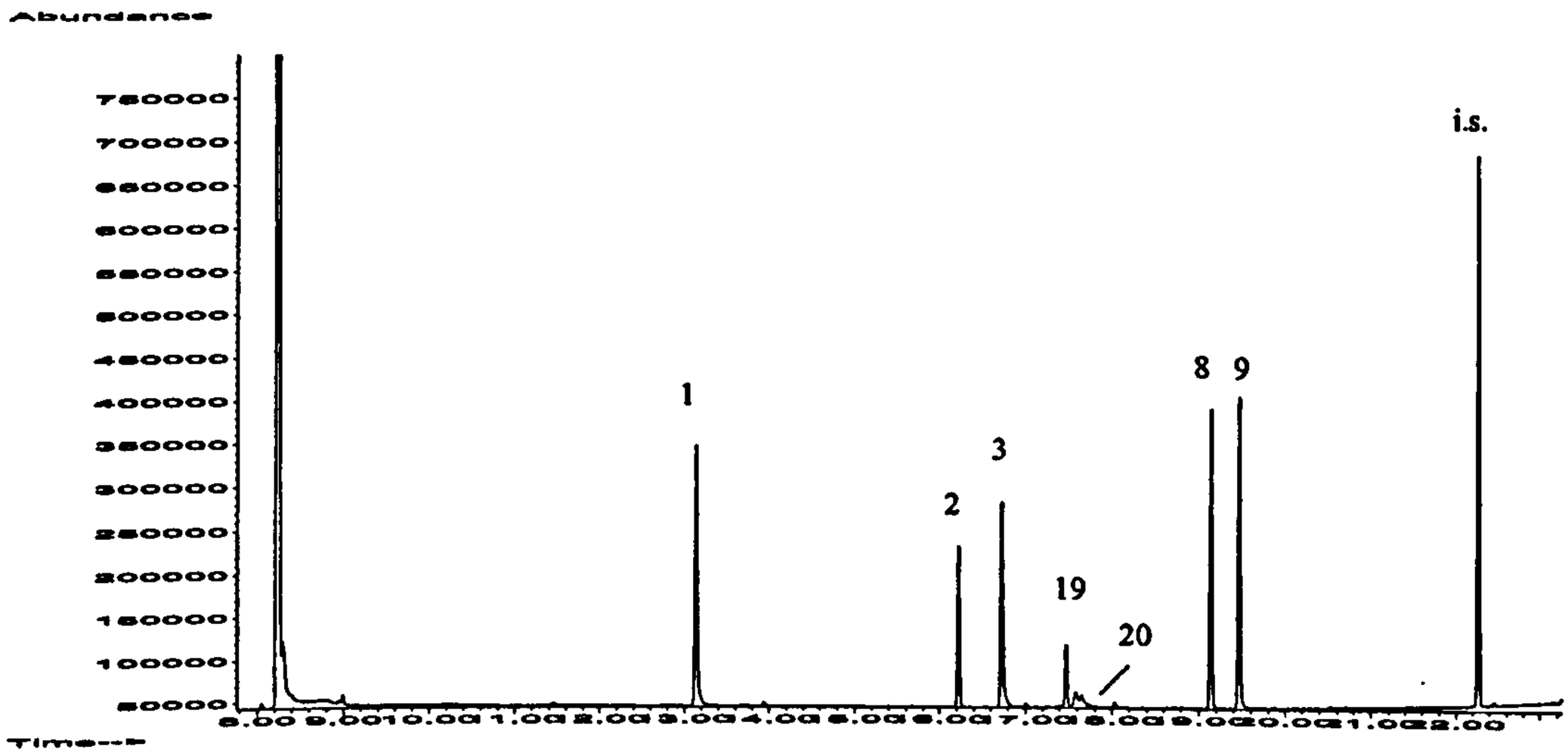


Figure 35: Chromatogram of the standard mixture in ethyl acetate. Peak identification: 1) N-acetylamphetamine, 2) aldimine, 3) benzylamphetamine, 8) benzoylamphetamine, 9) 2-oxo, 19) unknown B and 20) unknown C.

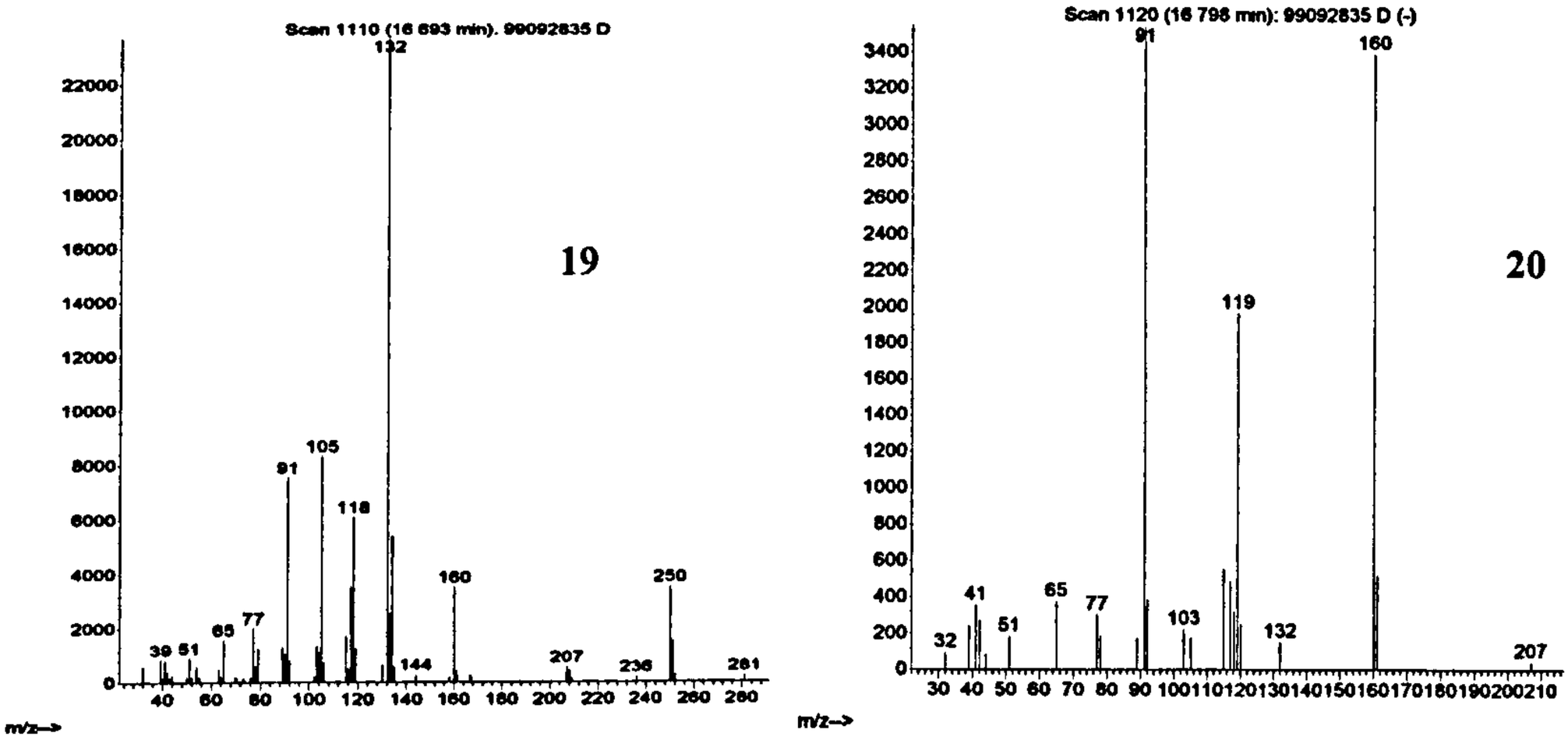


Figure 36: Mass spectra of extra compounds found in the standard mixture in ethyl acetate. Peak identification: 19) unknown compound B and 20) unknown compound C.

The results of stability study are given in Table 6, as RSD values.

Table 6: Stability of impurities in standard mixture given as RSD values in different solvents and at different temperatures.

Synthetic mixture														
	Isooctane 25°C							Isooctane 8°C						
	0h	4h	12h	24h	48h	96h	RSD	0h	4h	12h	24h	48h	96h	RSD
Acetylamphet.	1.000	0.998	1.000	0.996	0.980	0.941	2%	1.000	1.008	0.970	1.004	0.992	0.964	2%
Aldimine	1.000	0.984	0.963	0.972	0.980	0.985	1%	1.000	0.992	0.962	0.999	0.990	0.988	1%
Benzylamphet.	1.000	0.992	1.022	1.008	0.996	1.014	1%	1.000	1.016	0.968	1.001	1.004	0.970	2%
Benzoylamphet.	1.000	0.992	1.005	0.999	0.996	0.994	0%	1.000	1.005	0.963	0.994	0.995	0.961	2%
2-oxo	1.000	0.995	1.009	1.004	0.995	0.985	1%	1.000	1.003	0.962	0.993	0.987	0.952	2%
Cathinol	1.000	1.210	1.437	1.332	1.301	1.448	12%	1.000	1.070	1.006	1.039	1.023	0.986	3%
Mean							**3%**							**2%**
	Diethyl ether 25°C							**Diethyl ether 8°C**						
Acetylamphet.	1.000	0.977	1.009	1.000	1.024	1.027	2%	1.000	0.982	1.098	1.027	1.174	1.221	8%
Aldimine	1.000	0.986	1.002	0.961	0.977	0.958	2%	1.000	0.974	1.130	0.988	1.133	1.301	11%
Benzylamphet.	1.000	1.009	1.039	1.024	0.974	1.086	3%	1.000	0.975	1.100	0.982	1.107	1.156	7%
Benzoylamphet.	1.000	0.976	0.988	0.999	1.019	1.045	2%	1.000	0.951	1.047	0.976	1.109	0.953	6%
2-oxo	1.000	0.978	0.992	1.010	1.022	1.045	2%	1.000	0.942	1.023	0.975	1.106	0.885	7%
Cathinol	1.000	1.230	1.356	1.493	n.d.	n.d.	14%	1.000	1.005	1.046	1.059	1.127	0.827	9%
Mean							**4%**							**8%**

Table 6: Cont'd / ...

Synthetic mixture														
	Ethyl acetate 25°C							Ethyl acetate 8°C						
	0h	4h	12h	24h	48h	96h	RSD	0h	4h	12h	24h	48h	96h	RSD
Acetylamphet.	1.000	0.983	0.986	0.955	0.947	0.947	2%	1.000	0.999	0.993	1.001	1.013	1.002	1%
Aldimine	1.000	0.531	0.517	0.492	0.467	0.400	35%	1.000	0.993	0.989	1.003	1.002	0.982	1%
Benzylamphet.	1.000	0.972	1.030	0.988	1.030	0.928	4%	1.000	0.991	0.989	0.973	0.959	1.004	2%
Benzoylamphet.	1.000	1.001	1.006	1.001	1.012	1.011	0%	1.000	0.995	1.005	1.000	1.000	1.017	1%
2-oxo	1.000	1.001	1.008	1.002	1.012	1.009	0%	1.000	0.995	1.010	1.007	1.010	1.025	1%
Cathinol	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.000	1.028	1.055	1.094	1.099	0.909	6%
Mean							**8%**							**2%**
	Dichloromethane 25°C							Dichloromethane 8°C						
Acetylamphet.	1.000	0.993	1.021	1.057	1.041	1.099	3%	1.000	0.964	0.990	0.998	1.025	1.003	2%
Aldimine	1.000	0.999	1.019	1.023	1.036	1.009	1%	1.000	0.969	0.997	1.009	1.017	1.049	2%
Benzylamphet.	1.000	1.010	1.000	1.009	0.994	1.051	2%	1.000	0.990	1.003	1.007	0.979	0.978	1%
Benzoylamphet.	1.000	1.004	1.002	1.013	1.005	1.049	2%	1.000	0.939	0.950	0.928	0.953	0.952	2%
2-oxo	1.000	1.003	1.002	1.021	1.008	1.057	2%	1.000	0.943	0.940	0.905	0.943	0.937	3%
Cathinol	1.000	1.138	1.110	1.313	1.259	0.489	26%	1.000	1.456	1.279	1.124	1.724	1.650	19%
Mean							**6%**							**5%**
	Toluene 25°C							Toluene 8°C						
Acetylamphet.	1.000	0.952	0.904	0.935	0.986	0.892	4%	1.000	1.052	1.006	1.013	1.315	1.037	10%
Aldimine	1.000	0.940	0.908	0.932	0.970	0.930	3%	1.000	1.014	0.986	0.984	1.236	1.047	8%
Benzylamphet.	1.000	0.969	0.934	0.953	0.982	0.922	3%	1.000	1.061	1.042	1.025	1.229	1.038	7%
Benzoylamphet.	1.000	0.991	0.978	0.986	1.001	0.972	1%	1.000	1.041	1.039	1.023	1.052	1.016	2%
2-oxo	1.000	1.003	0.990	0.993	1.006	0.979	1%	1.000	1.049	1.044	1.034	1.068	1.026	2%
Cathinol	1.000	1.153	1.121	1.111	1.133	1.073	5%	1.000	1.135	1.173	1.121	1.272	1.112	7%
Mean							**3%**							**6%**
	Ethanol 25°C							Ethanol 8°C						
Acetylamphet.	1.000	0.993	0.985	0.994	0.980	1.003	1%	1.000	1.021	1.034	1.040	1.017	1.040	1%
Aldimine	1.000	0.890	0.912	0.905	0.856	0.780	7%	1.000	1.034	1.027	1.027	0.999	1.014	1%
Benzylamphet.	1.000	1.030	1.023	1.018	1.021	1.031	1%	1.000	1.010	1.031	1.020	1.001	0.983	2%
Benzoylamphet.	1.000	1.011	0.998	1.006	1.002	1.010	0%	1.000	0.992	1.011	0.994	0.980	0.979	1%
2-oxo	1.000	1.027	1.006	1.006	1.013	1.015	1%	1.000	0.994	1.015	1.000	0.980	0.995	1%
Cathinol	1.000	1.413	1.407	1.283	1.483	1.482	12%	1.000	0.989	0.996	0.990	0.989	0.978	1%
Mean							**4%**							**1%**

n.d. Not detected.

The mean values in Table 5 ranged from 1% to 8% which indicates that the reductive amination impurities are generally stable in the different solvents. If the results are studied in more detail, it can be seen that the impurities correlate with one another. For example, at the same time as N-acetylamphetamine decomposed to amphetamine in isooctane, the concentration of aldimine increased (Figure 37).

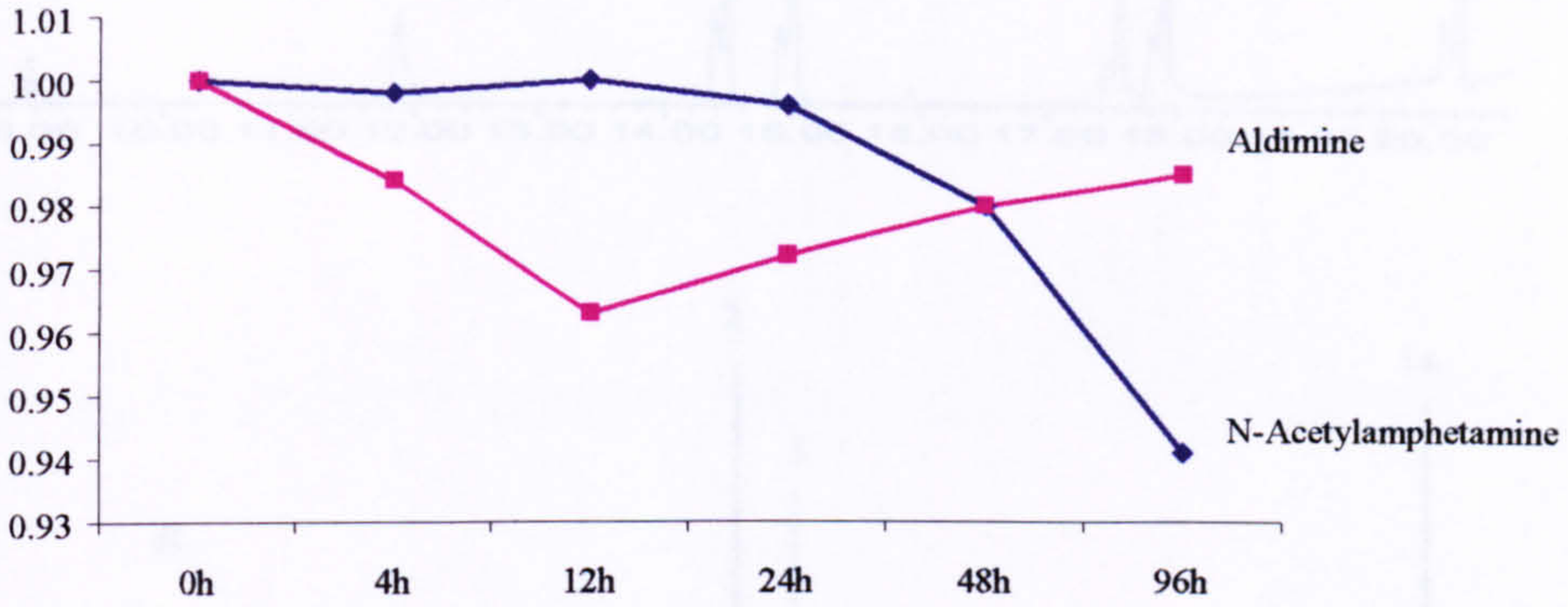


Figure 37: Stability of aldimine and N-acetylamphetamine in isooctane.

Peak splitting

Peak splitting occurred for some of the compounds when toluene was used as the solvent (Figure 38). In literature, chromatographic problems have been studied in detail and the same phenomenon found [171]. It has been pointed out that the peak distortion by band broadening in space tends to be poorly reproducible.

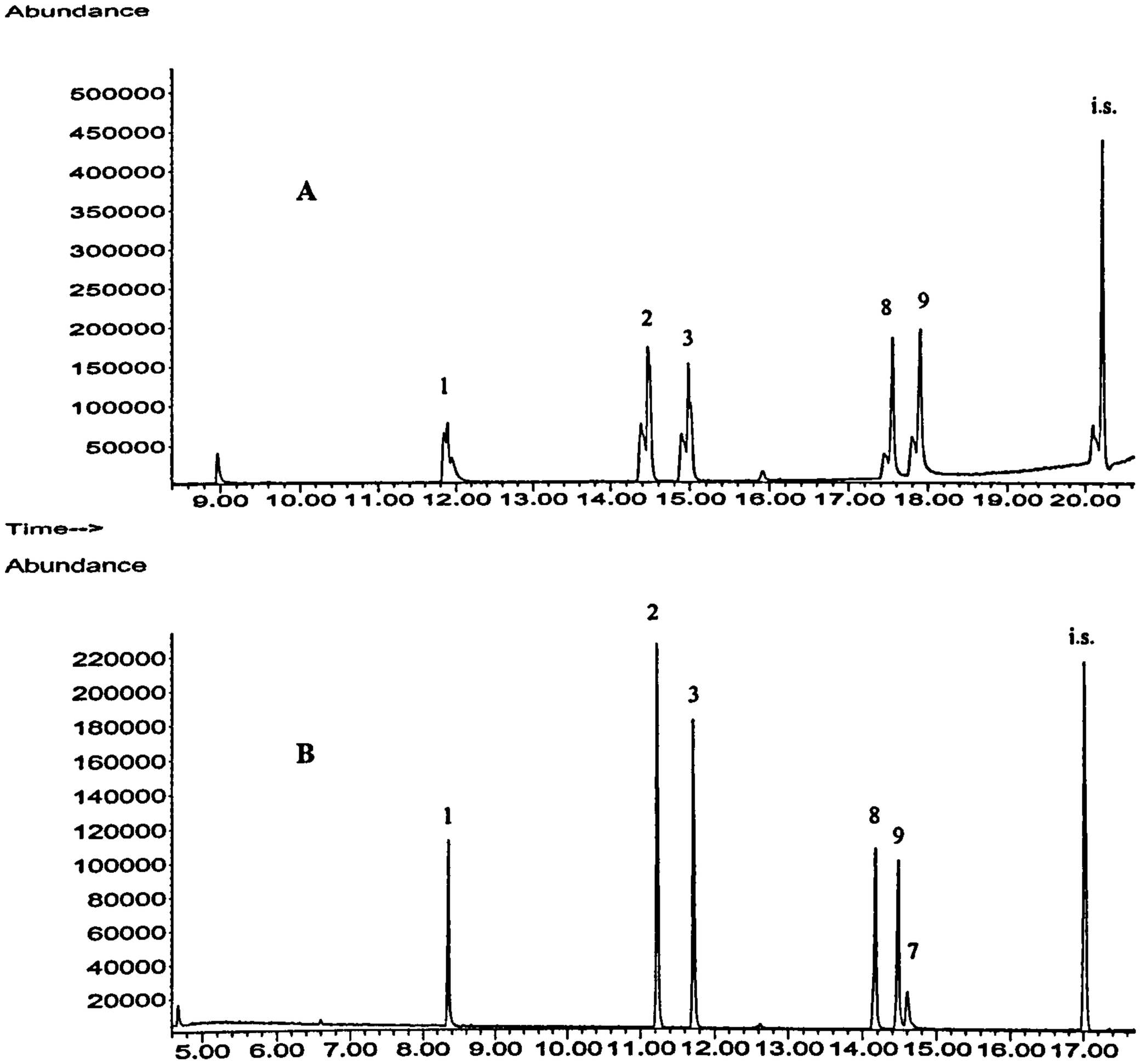


Figure 38: GC-MS chromatogram of the standard mixture in toluene using initial temperature A) at 60°C and B) at 90°C. Peak identification: 1) N-acetylamphetamine, 2) aldimine, 3) benzylamphetamine, 7) cathinol, 8) benzoylamphetamine and 9) 2-oxo.

From the chromatographic standpoint, the initial temperature should be only 20°C below the boiling point of the solvent. The initial temperature used at 60°C was too low for toluene that caused peak distortion. By increasing temperature to 90°C the compounds exhibited better chromatographic behaviour. The stability study of the synthetic mixture in toluene was repeated at 90°C with good results. These new results are shown in Table 6.

Acetylamphetamine, aldimine, benzylamphetamine and benzoylamphetamine were identified from synthesised amphetamine and the stability of these compounds was

studied. Chromatograms of impurities extracted with isooctane and toluene are shown in Figure 39.

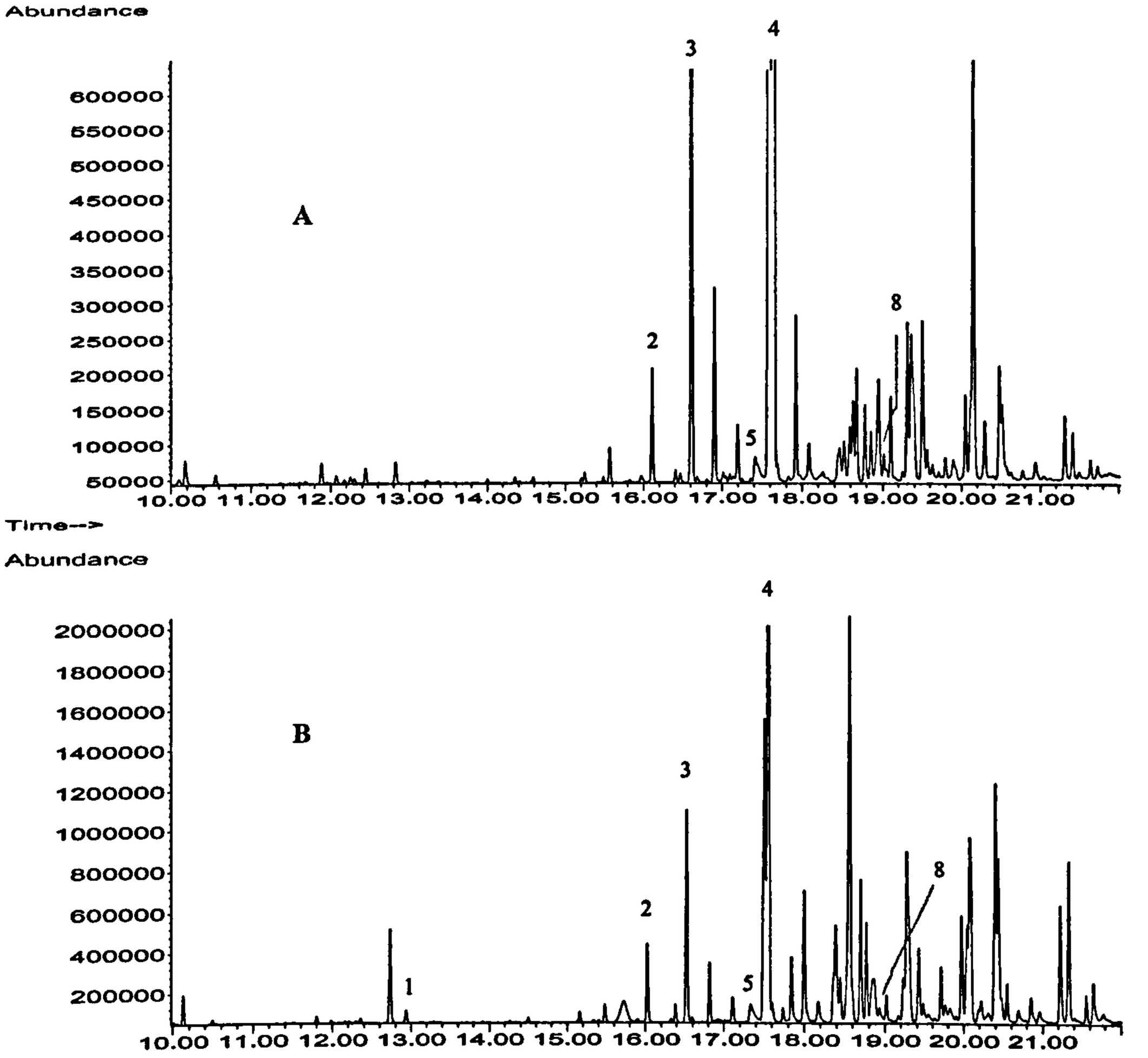


Figure 39: Chromatogram of the synthesised amphetamine A) in isooctane and B) in toluene. Peak identification: 1) N-acetylamphetamine, 2) aldimine, 3) benzylamphetamine 4) DPIA, 5) ketimine and 8) benzoylamphetamine.

The concentrations of some impurities were low and therefore identification and integration of peaks were not accurate. At 25°C aldimine and benzoylamphetamine were co-eluted with unknown peaks in CH_2Cl_2, and these impurities could not be used in the stability study. The ratios of normalised relative response factors to t_0 were calculated and the stability of impurities is given in RSD values in Table 7.

Table 7: Stability of impurities in amphetamine matrix given in RSD values in different solvents and at temperatures.

Amphetamine matrix						
	Isooctane		**Diethyl ether**		**Ethyl acetate**	
	25°C	8°C	25°C	8°C	25°C	8°C
Acetylamphetetamine	n.d.	n.d.	8%	10%	6%	6%
Aldimine	9%	4%	9%	38%	10%	35%
Benzylamphetetamine	2%	1%	4%	8%	1%	2%
Benzoylamphetamine	6%	1%	7%	10%	1%	14%
2-oxo	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Cathinol	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Mean	**6%**	**2%**	**7%**	**19%**	**8%**	**14%**
	Toluene		**Dichloromethane**		**Ethanol**	
Acetylamphetetamine	6%	n.a.	3%	5%	n.a.	n.a.
Aldimine	8%	n.a.	n.d.	7%	n.a.	n.a.
Benzylamphetetamine	4%	n.a.	7%	1%	n.a.	n.a.
Benzoylamphetamine	5%	n.a.	n.d.	0%	n.a.	n.a.
2-oxo	n.d.	n.a.	n.d.	n.d.	n.a.	n.a.
Cathinol	n.d.	n.a.	n.d.	n.d.	n.a.	n.a.
Mean	**6%**	**n.a.**	**5%**	**4%**	**n.a.**	**n.a.**

n.d. Not detected.
n.a. The data is not available.

5.4 Conclusion

Ten typical reductive amination impurities were synthesised. These compounds offer significant information about the synthetic route. Unfortunately, some target compounds, namely aldimine, ketimine and 1-oxo were too unstable to be utilised in profiling. Using non-polar capillary columns, Ultra-1 and Ultra-2, amphetamine and 1-phenyl-2-propanol co-eluted, and thus could not be separately quantified.

The GC method was modified as split peaks were obtained when toluene was used as the solvent, with an initial oven temperature at 60°C. The initial temperature was raised to 90°C which was also used in the further studies. Regardless of problems related to toluene and diethyl ether, significant differences in the results between different solvents and temperatures were not observed. The greatest individual deviation in the stability was observed for cathinol. Generally, the reductive

amination impurities appeared to be stable in all solvents for at least 96 hours. A similar conclusion was made regarding impurities from other synthetic routes. Diethyl ether and dichloromethane were difficult to handle in practice, and therefore these solvents were discarded from the further studies. The best organic solvents for further studies were chosen to be isooctane and toluene.

6 OPTIMISATION OF GC METHOD

6.1 Introduction

Total selectivity of a chromatographic assay is a result of four factors: (i) sample preparation, (ii) sample introduction, (iii) chromatography and (iv) detection. In this chapter, the optimisation of sample introduction, chromatographic separation processes and detection were carried out for reductive amination amphetamine impurities. In the sample introduction, different injection techniques were evaluated. The composition of extracts of street drugs is unknown and therefore vaporising injection techniques should be used. Different injection techniques were evaluated using a cool on-column as a reference technique; the latter is known to be free of discrimination problems. The actual injection techniques evaluated were split and splitless injections.

Several stationary phases were evaluated in the optimisation of the chromatographic separation process. The choice of column was based on the total separation power, resolution of analytes and inertness (peak tailing). The evaluation of the columns was made in stages, based upon batches of results and thus, different properties observed for the columns in different studies. The used GC conditions are described in detail in each chapter. In detection study, FID and MSD in Scan and SIM modes were studied.

The development of a harmonised amphetamine profiling method included a study of Leuckart and nitrostyrene impurities by other laboratories involved in the project. The results have been shown for reductive amination amphetamine, but the final conclusion takes all of the results into consideration. The sample preparation is discussed in Chapter 7.

6.2 Characterising performance of a chromatographic method

Fundamental aspects

The absolute retention time is the sum of adjusted retention time ($t_R^{'}$) and dead time (t_0) (equation 19) [172]. The former is the time an analyte spends in the stationary phase and the latter is the time to elute an unretained compound. The dead time can be defined as the ratio of the length of the column, L, and the average linear carrier gas velocity, $\bar{u}$, (equation 20). The retention factor (capacity factor), k, is the ratio of these times (equation 21). Capacity has only a minor effect on the resolution if a temperature programme is used.

$$t_R = t_R^{'} + t_0 \qquad t_0 = \frac{L}{\bar{u}} \qquad k = \frac{t_R - t_0}{t_o} = \frac{t_R^{'}}{t_0} \qquad \textbf{(19-21)}$$

The column efficiency can be defined using the plate height, H, (equation 22) or the effective plate number, N_{eff}, (equation 23), where w_b is the peak width at the baseline and $w_{½}$ peak width at half height. Efficiency is, however, often given as theoretical plate number, N, where absolute retention time is used. Thus, the equation 23 can be re-written into equation 24.

$$H = \frac{L}{16} \times \left(\frac{w_b}{t_R^{'}} \right)^2 = \frac{L}{5.545} \times \left(\frac{w_{½}}{t_R^{'}} \right)^2 \qquad \textbf{(22)}$$

$$N_{eff} = \frac{L}{H} = 5.545 \times \left(\frac{t_R^{'}}{w_{½}} \right)^2 \qquad N = \frac{L}{H} = 5.545 \times \left(\frac{t_R}{w_{½}} \right)^2 \qquad \textbf{(23-24)}$$

Efficiency is maximised by using as long and narrow a column as possible. However, this has disadvantages, namely a smaller sample capacity with narrower internal diameter and longer analysis time with a longer column. This results in broader peaks and thus decreasing sensitivity due to smaller signal to noise ratio. Maximum efficiency can be achieved only if the column is operated under a linear carrier gas

flow corresponding to the minimum plate height, H_{min}. For helium as a carrier gas the gas flow is approx. 25 cm/s and for hydrogen 35 cm/s (Figure 40).

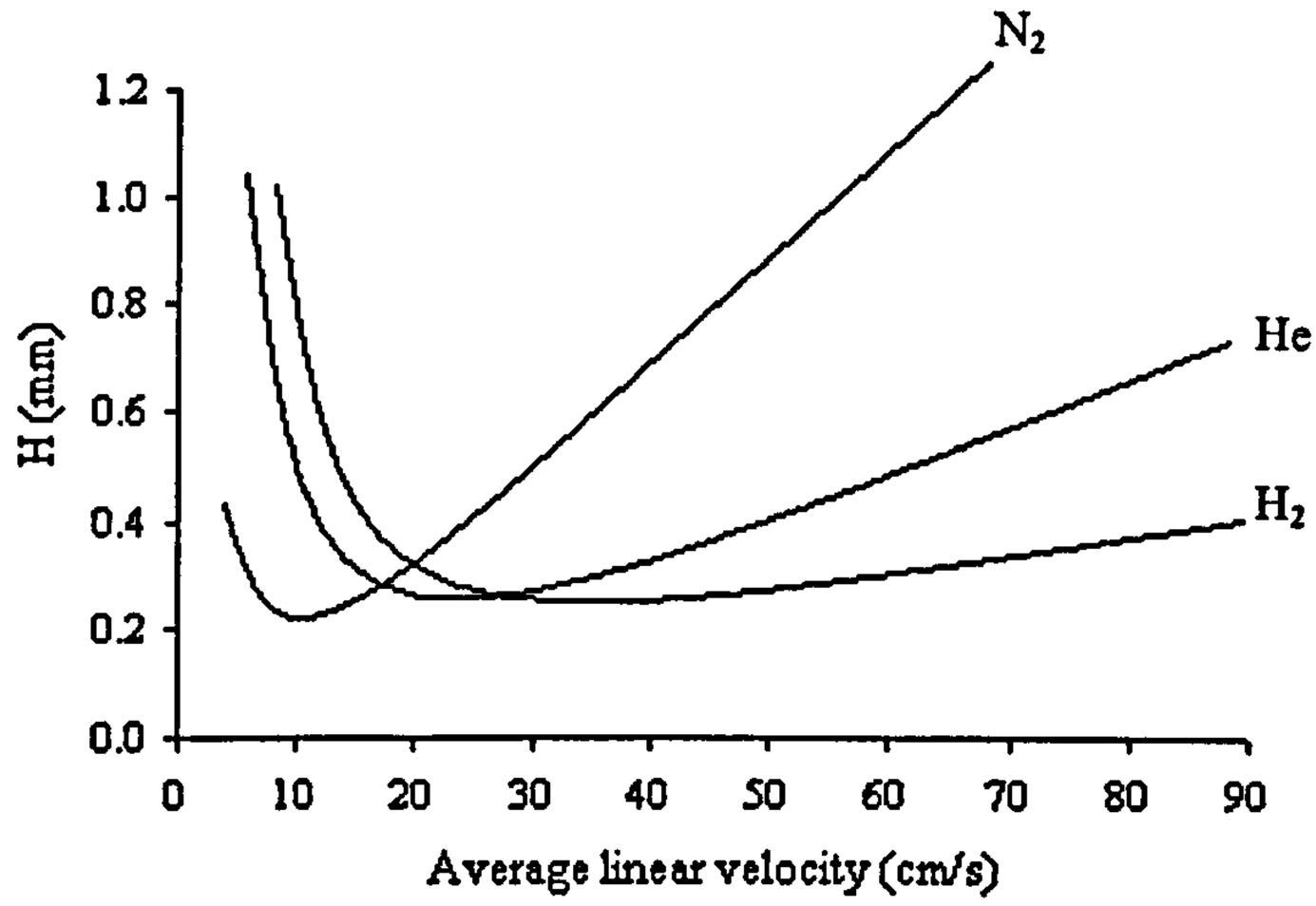


Figure 40: Van Deemter curve of plate height, H, against average linear velocity, $\overline{u}$, for a GC column using H_2, He, N_2 as carrier gas [173].

Selectivity, α, is defined using the ratio of adjusted retention times of two analytes (equation 25) [172].

$$\alpha = \frac{t'_{R,2}}{t'_{R,1}} = \frac{k_2}{k_1} \qquad (25)$$

Selectivity can be tuned by stationary phase selection and oven temperature optimisation. Polar stationary phases usually give the best selectivity, but have poor retention index reproducibility and stability at high temperatures.

Chromatographic resolution, R_S, can be determined by the means of the terms given above, i.e efficiency, selectivity and capacity (equation 26).

$$R_s = \frac{\sqrt{N}}{4} \times \frac{\alpha - 1}{\alpha} \times \frac{k}{k+1} \qquad (26)$$

Stability of the chromatographic system

In qualitative and quantitative analysis, it is important to demonstrate the stability of the chromatographic system. Normally, this is measured (i) within day (repeatability) and (ii) between days (reproducibility). The average value (the arithmetic mean, $\bar{x}$) and the standard deviations, s, are calculated as shown in equations 16 and 17 [164]. The relative standard deviation (RSD) or coefficient of variation (CV), is a measure of relative variability [164,174]. It is used in the comparison of the precision of results, and calculated by equation 18.

Calibration and quantification

Calibration of instrumentation is one of the prerequisites of analytical measurements. Linearity should be established and the regression equation calculated. The calibration curves are plotted using detector response, e.g. peak area against the concentration of the drug. The method of least squares is used for the calculation of the regression equation [164]. It is important to ensure that only the linear part of the calibration curve is employed. A linear regression of the general form is described as $y = a + bx$. In linear regression, a straight line is drawn though the observed data points. A sloped regression line will indicate for each value the independent variable x_i an estimated value of the dependent variable. The estimated value of y_i is designated by $\hat{y}_i$. The regression equation therefore is $\hat{y} = a + bx$. The concentration of the drug, i.e. the component, x_0, can be defined with the regression equation when observed y value of sample is y_0.

$$x_0 = \frac{y_0 - a}{b} \tag{27}$$

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 [175,176]. The individual calibration points have values y_1, y_2, ..., y_n, and mean value $\bar{y}$. The deviation of an observation y from the regression line is $(y - \hat{y})$. As the

line goes through the point $\bar{x}$ and $\bar{y}$, the regression equation can be written as follows:

$$\bar{y} = a + b\bar{x} \quad \Rightarrow \quad a = \bar{y} - b\bar{x} \tag{28}$$

Therefore

$$\begin{aligned} \hat{y} &= a + bx = (\bar{y} - b\bar{x}) + bx = \bar{y} + b(x - \bar{x}) \\ \Rightarrow \quad \hat{y} - \bar{y} &= b(x - \bar{x}) \end{aligned} \tag{29}$$

where the term $(\hat{y}_i - \bar{y})$ is known as the deviation, estimated y from mean of y, and the sum of squares is defined $\sum(\hat{y}_i - \bar{y})^2$. The sum of squares $\sum(y_i - \hat{y}_i)^2$ is the error of observed y from estimated y. The term $\sum(y_i - \bar{y})^2$ is known as the 'sum of squares about $\bar{y}$' (SS_{yy}) and it can be defined by the sum of two terms referred to above. This can be proved as follows:

$$(\hat{y}_i - \bar{y}) + (y_i - \hat{y}_i) = \hat{y}_i - \bar{y} + y_i - \hat{y}_i = y_i - \bar{y} \tag{30}$$

The Pearson product-moment coefficient of correlation, r, is calculated using equation 31. The value of r varied $-1 \leq r \leq 1$. Values of r obtained in instrumental analysis are normally very close to 1.

$$r = \frac{SS_{xy}}{\sqrt{SS_{xx} \times SS_{yy}}} = \frac{\sum(x_i - \bar{x}) \times (y_i - \bar{y})}{\sqrt{\sum(x_i - \bar{x})^2 \times \sum(y_i - \bar{y})^2}} \tag{31}$$

The coefficient of determination (R^2) is defined using equation 32. The term of sum of square y-residuals (SSE) $\sum(y_i - \hat{y}_i)^2$ should be as small as possible if the curve is a good fit to the data points. The value of R^2 varied $0 \leq R^2 \leq 1$ [175,176].

$$R^2 = \frac{SS_{yy} - SSE}{SS_{yy}} = \frac{\sum(y_i - \bar{y})^2 - \sum(y_i - \hat{y}_i)^2}{\sum(y_i - \bar{y})^2} = \frac{\sum(\hat{y}_i - \bar{y})^2}{\sum(\hat{y}_i - \bar{y})^2 + \sum(y_i - \hat{y}_i)^2} \tag{32}$$

In the straight-line graph, $R^2 = r^2$, where r is the product-moment correlation coefficient.

Theoretical detection limits

The line of regression calculated in the previous section will be used to estimate the limit of detection of the analytical procedure. The limit of detection can be described as the lowest concentration of the analyte that can be detected with a predetermined level of confidence [175]. The limits of detection of an analyte may be described as concentration which gives an instrumental signal (y) significantly different from the 'blank' signal. The limit of detection is the analyte concentration giving a signal equal to the blank signal, y_B, plus three standard deviations of the blank, s_B, (equation 33).

$$y - y_B = 3 \times s_B \quad \Rightarrow \quad y = y_B + 3 \times s_B \tag{33}$$

A fundamental assumption of the unweighted least-squares method is that points on the calibration plot have a normally distributed variation with a standard derivation estimated by $s_{y/x}$. Therefore $s_{y/x}$ can be used in place of s_B in the estimation of the limit of detection. Thus, the equation can be written as described in equation 34 [175]. The statistic $s_{y/x}$ is calculated by using equation 35 which utilises the y-residuals, $(y_i - \hat{y}_i)$.

$$y = y_B + 3 \times s_{y/x} \qquad s_{y/x} = \sqrt{\frac{\sum_i (y_i - \hat{y}_i)^2}{n-2}} \tag{34-35}$$

6.3 Experimental

Quality control

Stability of the GC-FID unit was controlled throughout the experiment. A Grob test mixture containing alkanes, fatty acid methyl esters, alcohols, acids and amines was used as a quality control test sample. The individual compounds were namely 1-octanol, 2-ethylhexanoic acid, 2,6-dimethylphenol, 2,6-dimethylaniline, dicyclohexylamine, decanoic, undecanoic and dodecanoic acid methyl esters and alkanes C_{10} - C_{18}, excluding C_{11}. The chemicals were obtained from Sigma (St.

Louis, the USA) except 1-octanol, 2-ethylhexanoic acid, dicyclohexylamine and 2,6-dimethylaniline were purchased from Aldrich and decanoic acid methyl ester from Fluka.

Control charts were established on the collected data. Data obtained from first 20 measurements were utilised to determine the mean value, warning limits (two times standard deviation, 2s) and action limits (three times standard deviation, 3s). These establish an acceptance criteria for the values of the following parameters: (i) absolute peak area, (ii) absolute retention time, (iii) efficiency, (iv) selectivity and (v) inertness (peak symmetry). The stability of sample introduction and of carrier gas flow was determined with absolute peak area and absolute retention time. The stability of chromatographic separation process was studied by calculating efficiency, selectivity and peak tailing.

6.3.1 Sample introduction

Sample preparation

A new reductive amination amphetamine batch was synthesised. 8.0 g (0.059 mol) of BMK and 40 g (0.53 mol) of ammonium acetate were stirred in MeOH at room temperature for 3 hours. 3.8 g (0.059 mol) of $NaBH_3CN$ was added and stirred overnight. The reaction was quenched by evaporation of the solvent. The residue was dissolved in water/acetone and acidified with concentrated HCl and stirred for two days. The mixture was washed with CH_2Cl_2. The water phase was alkalised with NaOH and extracted with CH_2Cl_2. The organic solvent was evaporated and amphetamine oil was obtained. The oil was crystallised to amphetamine sulphate by addition of 37% sulphuric acid and MeOH.

For sample preparation, 300 mg of reductive amination amphetamine was dissolved in 3 ml of phosphate buffer (63.2 mM) [46], shaken for 30 min and the pH adjusted to 7.0. 1.2 ml of isooctane was added, containing 10 μg/ml internal standard (tetracosane obtained from Aldrich). The extraction was repeated four times and the extracts combined.

In the sample introduction study impurity peaks were identified and relative response factors (RRF) calculated. RRF values from split and splitless injections were compared to the reference values from cool on-column injection, i.e. the relative deviation was calculated (equation 36).

$$\text{Relative deviation (\%)} = \frac{\text{RRF(split/splitless)} - \text{RRF(cool on - column)}}{\text{RRF(cool on - column)}} \times 100\ \% \quad \textbf{(36)}$$

GC method

The GC-MS-FID instrument was equipped with a cool on-column inlet. The sample introduction study was carried out using two 25 m (L) x 0.2 mm (i.d.), coated with 0.33 μm film of 5% phenyl methyl silicone columns (Ultra-2). The columns were connected into injection port with 2.5 m (L) x 0.33 mm (i.d.) uncoated pre-column and the Y-shape splitter. Three different injection techniques, namely cool on-column, split and splitless were used. In the oven-track mode, the temperature of the cool on-column inlet was 3°C higher than the column temperature throughout the column temperature program. The temperature program started with 1 min isothermal hold at 60°C, followed by a linear ramp (10°C/min) to the final temperature of 300°C for 10 min. In split injection samples were introduced using a split purge flow of 30.9 ml/min which produces a split ratio 1:20. In splitless mode the split purge valve opened after 1 min. In split and splitless modes the injection temperatures were 220, 240, 260 and 280°C. The detector temperatures for FID and MSD were 310°C in all runs. Three replicate injections for each injection technique and at each temperature were performed.

6.3.2 Chromatographic separation process

In the chromatographic separation process several columns and stationary phases were evaluated using the same amphetamine extract prepared for the sample introduction study. The columns were obtained from Agilent Technology. The chosen column for the experiment are listed below:

- 25 m (L) x 0.20 mm (i.d.), d_f 0.33 μm of dimethyl silicone (HP Ultra-1)
- 25 m (L) x 0.20 mm (i.d.), d_f 0.33 μm of 5% phenyl methyl silicone (HP Ultra-2)
- 25 m (L) x 0.20 mm (i.d.), d_f 0.31 μm of 50% diphenyl dimethyl silicone (HP-50+)
- 30 m (L) x 0.25 mm (i.d.), d_f 0.25 μm of 50% phenyl methyl silicone (DB-17MS)
- 25 m (L) x 0.20 mm (i.d.), d_f 0.33 μm of 35% diphenyl dimethyl silicone (HP-35)
- 30 m (L) x 0.25 mm (i.d.), d_f 0.25 μm of 35% phenyl methyl silicone (DB-35MS)

Six different oven temperature programs were used ranging from 2 to 12 °C/min, i.e. 2, 4, 6, 8, 10 and 12 °C/min. Split injection at 260°C was used with other GC conditions as in the sample introduction. Separation power was described by the means of number of integrated peaks in each column. Pre- and post-resolutions and peak symmetry were collected for each target compounds from GC-FID chromatograms utilising GC-FID ChemStation Software Rev. A.06.03. The MSD was used to check the purity of the peak.

6.3.3 Detection

In the detection study, repeatability, reproducibility, sensitivity, selectivity and linearity were determined for FID, MSD in Scan and SIM modes, utilising the Grob control sample. In the repeatability study, the Grob mix was injected twenty times. Correspondingly, in the reproducibility study the Grob mix was injected once on twenty different days. The peak areas relative to an internal standard (eicosane, C_{20}) were used in the calculations. Eicosane was obtained from Aldrich. The RSD values were calculated by equation 18.

Three replicates of different concentrations of the Grob mixture were injected in a sensitivity study. The lowest concentration, at which repeatability was acceptable, was obtained. The following concentrations were studied: 0.1 µg/ml, 0.05 µg/ml, 0.01 µg/ml and 0.005 µg/ml. Selectivity was determined utilising absolute peak areas and RSD values of four replicates. The peak areas were weighted and normalised using equations 2 and 3. RSD values were calculated utilising these normalised peak areas. In the linearity study, the Grob mixture was run at five different concentrations covering a range of five decades of concentration from 0.01 µg/ml to 100 µg/ml having the internal standard at constant concentration. The correlation coefficient was calculated (equation 31) and linearity was checked (equation 32).

Sample preparation

An internal standard (eicosane, C_{20}) solution was prepared in toluene at a concentration of 10.0 µg/ml. The Grob test mixture containing 1-octanol, 2,6-dimethylphenol, 2,6-dimethylaniline, dicyclohexylamine, decanoic, undecanoic and dodecanoic acid methyl esters and alkanes, mainly C_{12}, C_{13}, C_{15} - C_{19} and C_{24} was prepared at concentration of 10 µg/ml. The 1:10 dilution with the internal standard solution, i.e. 1.0 µg/ml solution, was used for repeatability, reproducibility and selectivity parts of the study. A dilution series was used for sensitivity and linearity.

GC method

The detection study was carried out using two 30 m (L) x 0.25 mm (i.d.), coated with 0.25 µm of 35% phenyl methyl silicone (DB-35MS) capillary columns. The columns were connected into injection port with two 2.5m (L) x 0.25 mm (i.d.) uncoated pre-columns and the divider. Splitless injection was used with an injection temperature of 250°C. 2 µl was injected into each column. The oven temperature programme was started from 90°C (1 min), rated 8°C/min to a final temperature of 300°C, held for 10 min. The temperatures of the detectors were 310°C. Simultaneous detection with FID and MSD in Scan and SIM modes was evaluated. Three replicates of each extraction

condition were performed. In order to avoid systematic error the extractions were performed in random order.

6.4 Results and discussion

6.4.1 Sample introduction

The cool on-column injection technique was used as "absolute" sample introduction technique. The same amphetamine extract was also analysed using splitless and split injection techniques at different injection temperatures. The chromatograms for each injection technique at 260°C are shown in Figure 41.

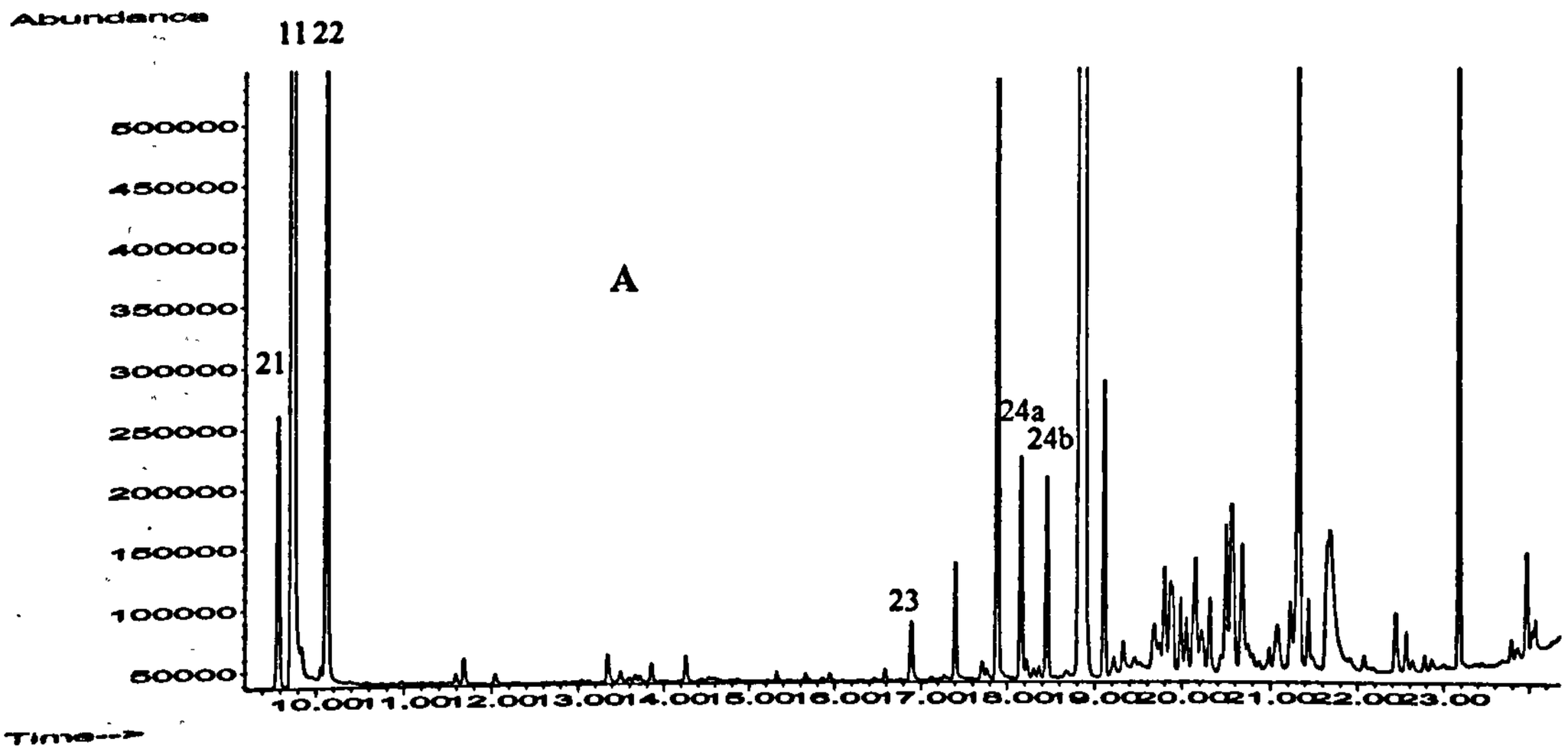


Figure 41: Comparison of different sample introduction techniques. Chromatogram of A) cool on-column (whole), B) cool on-column (snapshot), C) split mode and D) splitless mode. Peak identification: 1) N-acetylamphetamine, 2) aldimine, 3) benzylamphetamine 4) DPIA, 5) ketimine, 8) benzoylamphetamine, 11) 1-phenyl-2-propanol, 21) BMK, 22) 1-phenyl-1,2-propandione, 23) 1,2-diphenylethanone, 24a) and 24b) 3,4-diphenyl-3-buten-2-one.

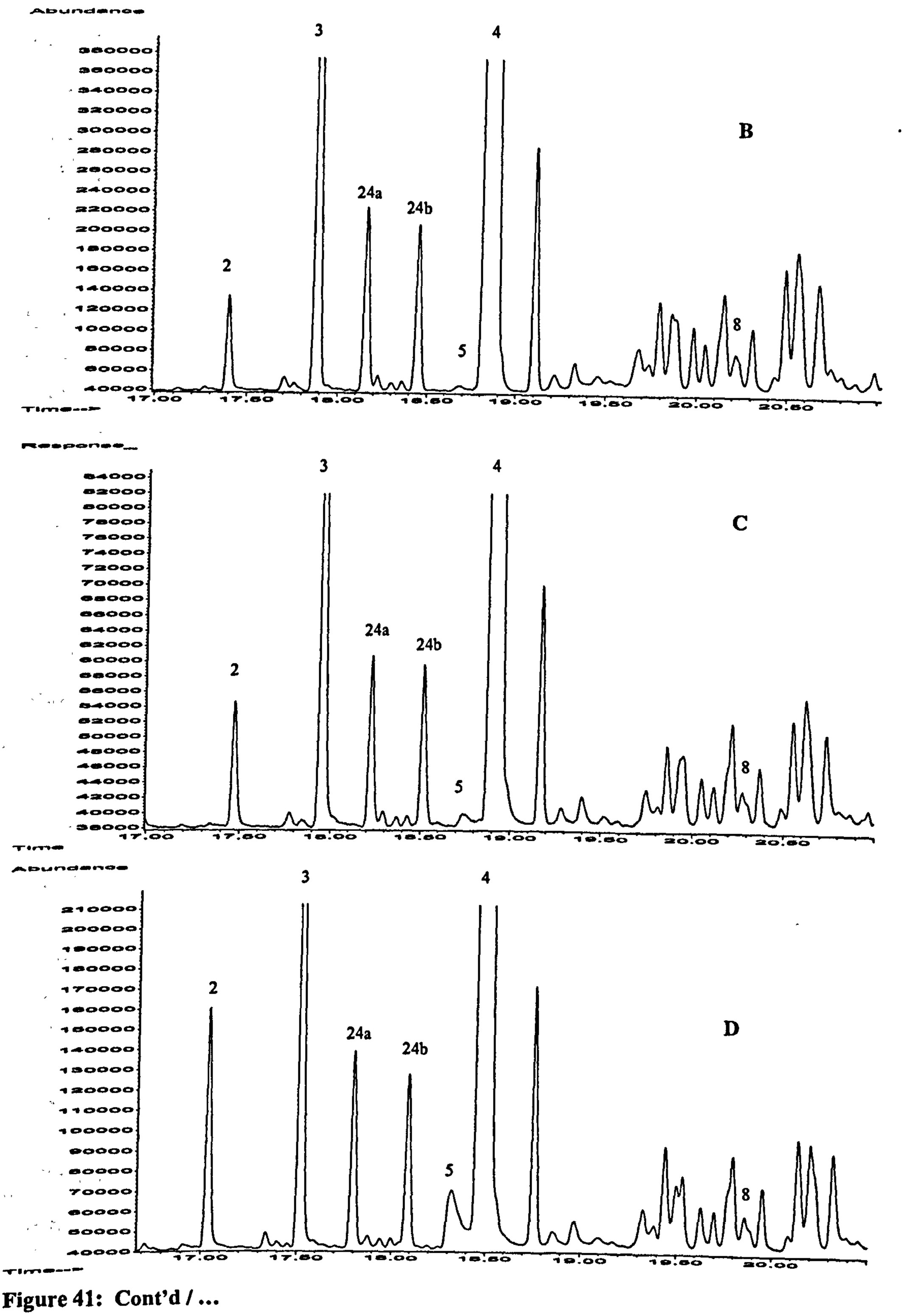
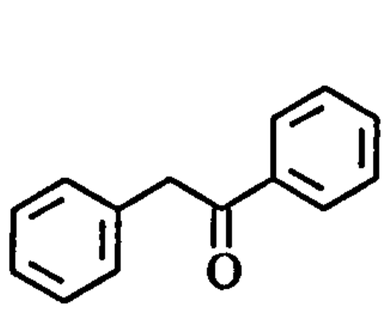
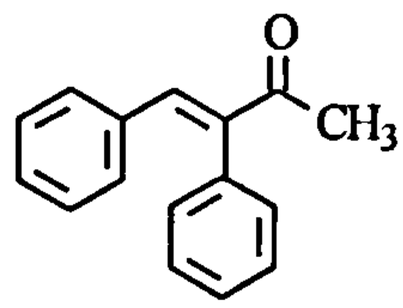


Figure 41: Cont'd / ...

Impurity peaks were identified and response factors (RRF) calculated. RRF values from split and splitless injections were compared to the reference values from cool on-column injection, i.e. the relative deviations were calculated (equation 36). In addition to the synthesised impurities aldimine, benzylamphetamine and benzoylamphetamine few other impurities were identified and their RSD values calculated. Of these compounds, BMK, DPIA, 1-phenyl-1,2-propandione had already been identified earlier, and two new compounds, 1,2-diphenylethanone **125** and two isomers of 3,4-diphenyl-3-buten-2-one **126** were chosen for the sample introduction study. The mass spectra of 1,2-diphenylethanone and 3,4-diphenyl-3-buten-2-one are shown in Figure 43.



1,2-diphenylethanone **125**



3,4-diphenyl-3-buten-2-one **126**

Figure 42: Structural formulas of identified extra compounds.

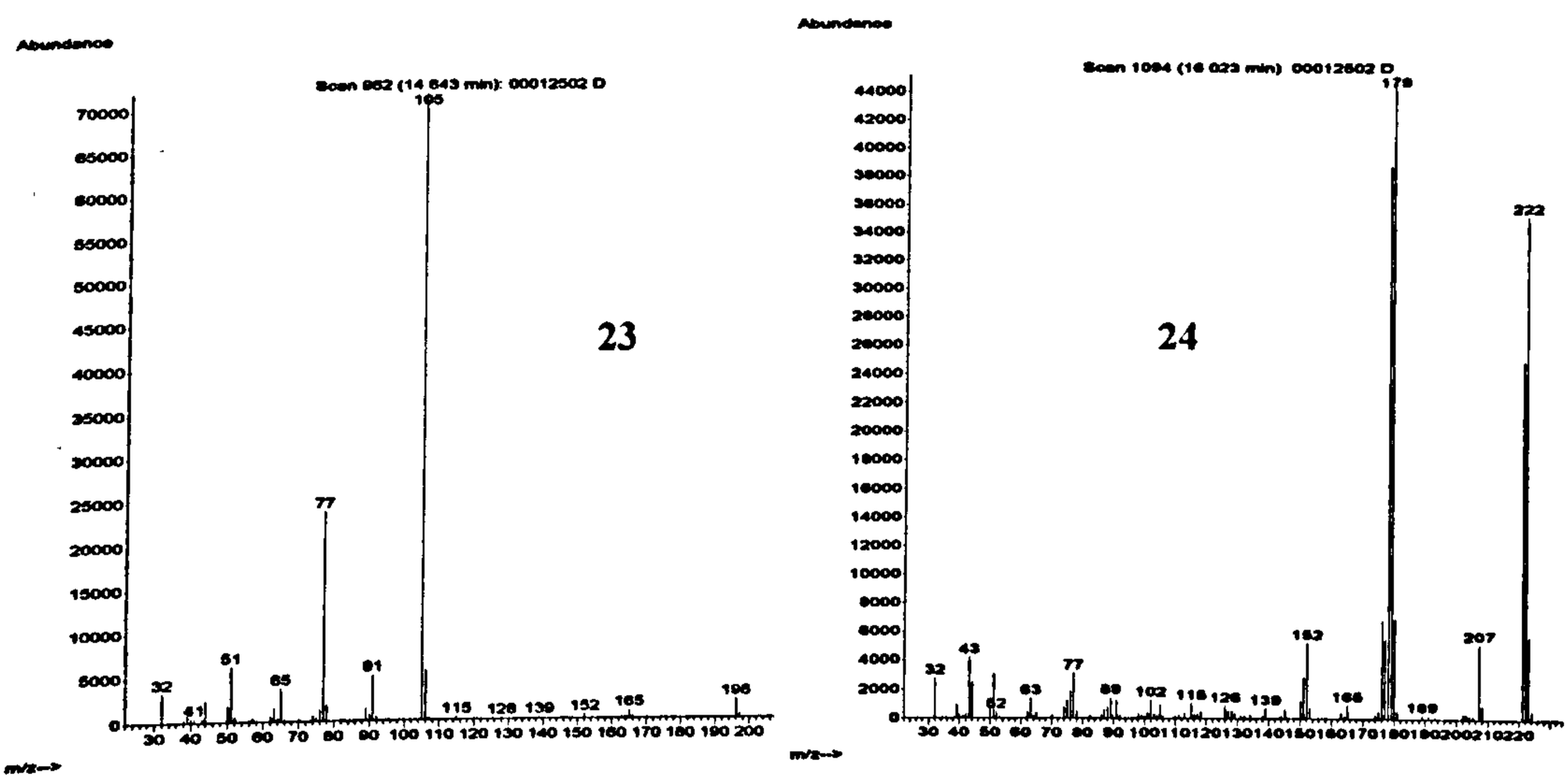


Figure 43: Mass spectra of extra compounds chosen in the sample introduction study. Peak identification: 23) 1,2-diphenylethanone and 24) 3,4-diphenyl-3-buten-2-one.

The results of sample introduction are shown in Table 8. 1-Phenyl-2-propanol co-eluted with amphetamine on the Ultra-2 column.

Table 8: RSD and relative deviation results for impurities in the sample introduction study.

RSD									
	Cool on-column	Splitless				Split			
		220°C	240°C	260°C	280°C	220°C	240°C	260°C	280°C
BMK	1.46%	3.14%	1.09%	4.62%	3.37%	0.54%	0.84%	2.51%	2.48%
1-Phenyl-2-propanol	1.31%	3.05%	2.88%	6.01%	2.64%	1.23%	0.85%	1.57%	1.64%
1-Phenyl-1,2-propandione	1.57%	3.70%	1.52%	4.47%	2.33%	1.70%	1.44%	2.74%	2.78%
1,2-Diphenylethanone	2.26%	1.68%	1.42%	2.19%	0.68%	3.44%	0.39%	2.02%	2.01%
Aldimine	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%
3,4-Diphenyl-3-buten-2-one (1)	1.16%	1.53%	1.11%	2.31%	0.96%	0.88%	1.29%	2.32%	2.33%
3,4-Diphenyl-3-buten-2-one (2)	1.31%	2.92%	0.92%	2.68%	0.79%	1.30%	0.73%	2.05%	1.98%
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%
Mean reductive amination	**1.43%**	**2.32%**	**1.53%**	**3.54%**	**1.66%**	**1.54%**	**0.84%**	**1.95%**	**1.94%**
Mean all impurities	**1.32%**	**2.57%**	**2.15%**	**3.57%**	**3.02%**	**1.98%**	**1.35%**	**2.23%**	**2.63%**

Relative deviation								
	Splitless				Split			
	220°C	240°C	260°C	280°C	220°C	240°C	260°C	280°C
BMK	18.77%	19.43%	24.48%	25.53%	19.79%	9.04%	0.78%	0.13%
1-Phenyl-2-propanol	22.01%	23.99%	18.93%	6.44%	10.45%	3.35%	15.02%	18.75%
1-Phenyl-1,2-propandione	21.26%	27.41%	24.59%	4.04%	24.63%	10.60%	3.24%	4.58%
1,2-Diphenylethanone	2.48%	1.16%	7.19%	7.56%	17.87%	8.09%	2.00%	1.06%
Aldimine	68.11%	80.91%	108.4%	126.2%	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%
3,4-Diphenyl-3-buten-2-one (1)	8.60%	2.54%	5.54%	4.08%	12.89%	4.03%	4.70%	5.05%
3,4-Diphenyl-3-buten-2-one (2)	26.15%	22.41%	21.31%	16.10%	22.83%	13.94%	2.25%	6.17%
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%
Mean reductive amination	**19.70%**	**19.09%**	**21.35%**	**17.35%**	**21.47%**	**13.59%**	**8.71%**	**10.43%**
Mean all impurities	**15.62%**	**14.53%**	**16.70%**	**15.61%**	**20.04%**	**15.67%**	**12.96%**	**13.49%**

In general, the best repeatability for reductive amination impurities was obtained with split injection at 240°C. The smallest relative deviation was obtained also with split injection at 260°C. The results were equal as all reactions were taken into consideration. Overall, splitless injection provides slightly higher error than split injection. The differences were, however, insignificant between the split and splitless injections. The concentration of impurities in street amphetamine samples can be

sometimes very low. To avoid the loss of detection of impurity peaks the splitless injection was chosen for further studies.

6.4.2 Chromatographic separation process

The carrier gas velocity was calibrated for each column to 25 cm/s. Separation power, inertness (peak tailing), purity and resolution of target compounds were evaluated for each of the column and temperature program. The separation power was investigated for Ultra-1, Ultra-2 and HP-50+ columns. In the determination of separation power, "Initial Area Reject" of the ChemStation integrator was adjusted using 5% from the area of the internal standard (tetracosane, C_{24}) in order to avoid the integration of small peaks. With some temperature programmes, the internal standard co-eluted with other target compounds. Co-elution occurred with the 1-oxo compound at 4°C/min for HP-50+. Hence, another peak which was well separated at each temperature program was chosen as the "internal standard compound" to calculate the initial area reject value. Separation power is given in Table 9 by the number of integrated peaks in each chromatogram, for an identical extract.

Table 9: Separation power on all columns given by the number of integrated peaks.

T-program (°C/min)	Ultra-1	Ultra-2	HP-50+
2	62	84	61
4	80	91	63
6	72	73	58
8	67	73	62
10	63	72	55
12	58	68	56

The best separation power in terms of number of resolved peaks was obtained with slow temperature programmes. The Ultra-2 column gave the best separation power compared to other columns. Resolution and inertness were investigated for each column with all temperature programs. Chromatograms for each column at 8°C/min are shown in Figure 44.

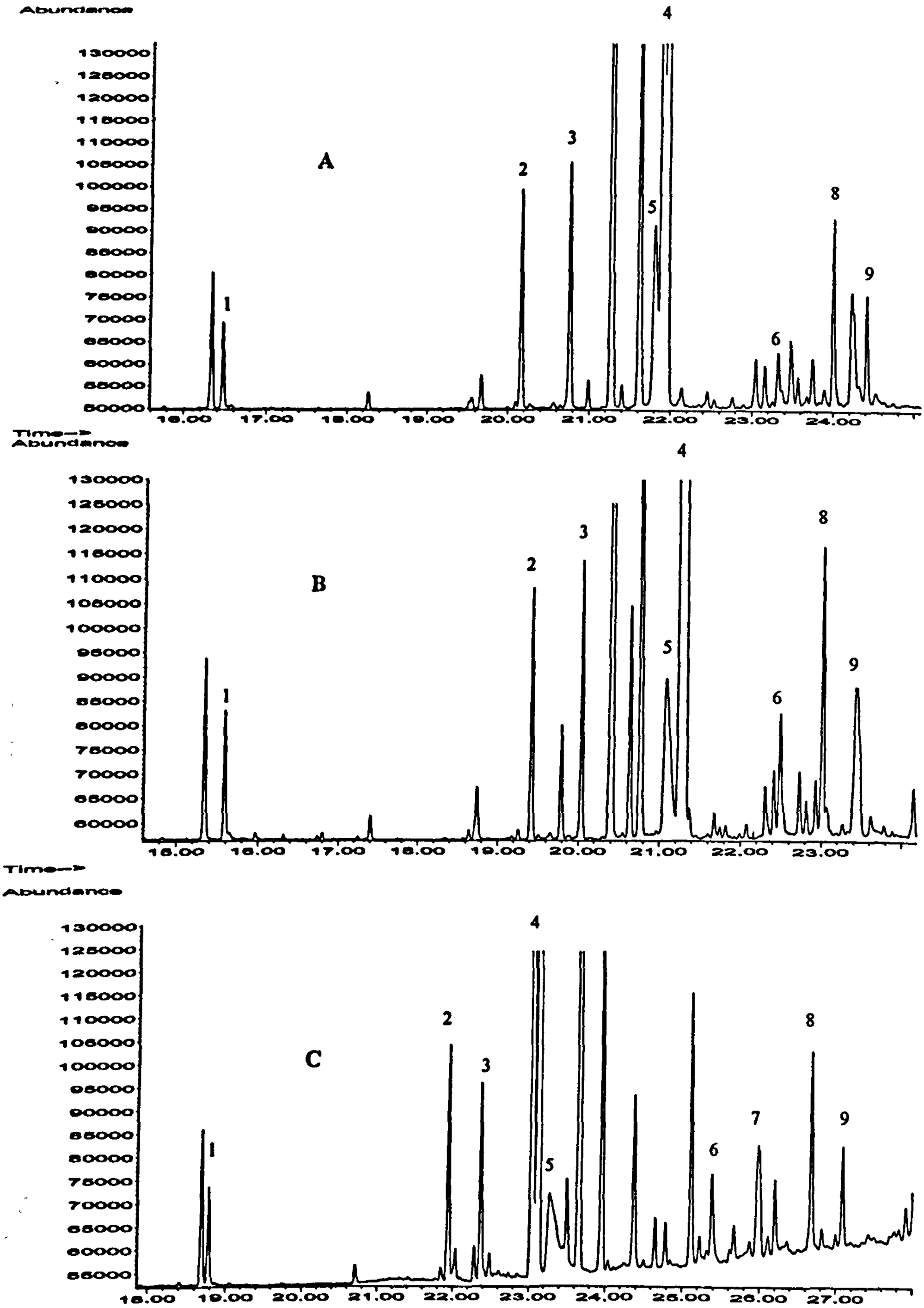


Figure 44: Comparison of different columns at 8°C/min. A) Ultra-2, B) Ultra-1 and C) HP-50+. Peak identification: 1) N-acetylamphetamine 2) aldimine, 3) benzylamphetamine, 4) DPIA, 5) ketimine, 6) 1-oxo, 7) cathinol, 8) benzoylamphetamine and 9) 2-oxo.

The data was processed using the GC ChemStation program. The resolution of the columns was established as the average of number of target peaks with resolution > 1.0 that indicates that the two peaks differ by 95% (Table 10).

Table 10: Pre- and post resolution and resolution > 1 calculated for each column at different temperature program.

		Ultra-1		Ultra-2		HP-50+	
T-program (°C/min)	Compound	Pre-resolution	Post-resolution	Pre-resolution	Post-resolution	Pre-resolution	Post-resolution
2	Acetylamphetamine	1.921	2.046	5.154	3.575	4.089	51.135
	Aldimine	3.033	1.471	12.303	1.990	2.037	2.910
	Benzylamphetamine	2.178	3.583	2.608	5.395	2.938	3.322
	Ketimine	2.665	2.387	2.673	1.403	1.352	1.132
	DPIA (1)	2.387	1.571	1.403	1.688	12.676	2.119
	DPIA (2)	1.571	2.338	1.688	3.354	2.119	1.352
	1-oxo	0.900	1.095	0.302	0.608	1.386	1.206
	Benzoylamphetamine	2.135	1.388	2.565	2.155	10.083	3.247
	2-oxo	1.545	0.633	3.132	3.278	1.035	3.521
	Cathinol	-	-	-	-	1.568	1.455
Resolution >1 Reduc.amin.		**80%**		**80%**		**100%**	
Resolution >1 All impurities		**83%**		**83%**		**100%**	
4	Acetylamphetamine	3.182	1.148	3.727	3.263	2.777	30.697
	Aldimine	3.036	1.912	10.798	2.619	1.904	2.230
	Benzylamphetamine	1.928	1.917	3.652	5.272	2.191	1.611
	Ketimine	1.375	1.983	1.630	1.015	1.127	1.368
	DPIA (1)	1.983	1.454	1.015	1.426	3.456	1.838
	DPIA (2)	1.454	1.768	1.426	3.303	1.840	1.127
	1-oxo	1.763	1.741	3.452	0.867	1.079	1.769
	Benzoylamphetamine	1.486	0.955	2.216	1.295	1.254	1.220
	2-oxo	1.789	0.580	1.082	2.253	1.375	0.9310
	Cathinol	-	-	-	-	1.491	0.840
Resolution >1 Reduc.amin.		**80%**		**85%**		**91%**	
Resolution >1 All impurities		**83%**		**92%**		**92%**	
6	Acetylamphetamine	2.390	1.188	3.139	2.662	2.097	1.999
	Aldimine	3.371	1.740	1.012	2.078	1.841	1.934
	Benzylamphetamine	1.425	1.425	2.849	4.557	1.921	1.207
	Ketimine	0.973	1.798	1.980	0.990	1.052	1.455
	DPIA (1)	1.798	1.248	0.990	1.327	3.402	1.666
	DPIA (2)	1.252	1.325	1.327	2.740	1.666	1.052
	1-oxo	1.421	1.065	0.758	1.893	0.922	3.684
	Benzoylamphetamine	1.618	1.037	2.111	1.057	2.397	0.958
	2-oxo	1.298	0.511	1.351	1.957	1.309	4.962
	Cathinol	-	-	-	-	1.510	2.333
Resolution >1 Reduc.amin.		**80%**		**75%**		**91%**	
Resolution >1 All impurities		**92%**		**92%**		**83%**	
8	Acetylamph.	4.879	1.009	2.646	1.529	1.697	1.713
	Aldimine	3.278	1.991	1.252	1.651	1.685	1.747
	Benzylamph.	2.770	4.441	2.084	4.373	1.628	2.167
	Ketimine	0.873	1.662	1.980	0.977	1.079	1.177
	DPIA (1)	1.663	1.162	0.977	1.181	2.204	1.555
	DPIA (2)	1.165	1.134	1.181	3.048	1.555	1.079
	1-oxo	1.345	1.566	1.046	0.800	0.897	3.321
	Benzoylamph.	1.499	0.816	1.806	1.525	1.573	1.470
	2-oxo	0.744	1.728	1.331	1.334	1.419	1.420
	Cathinol	-	-	-	-	2.735	2.328
Resolution >1 Reduc.amin.		**75%**		**75%**		**95%**	
Resolution >1 All impurities		**83%**		**92%**		**92%**	

Table 10: Cont'd /...

		Ultra-1		Ultra-2		HP-50+	
T-program (°C/min)	Compound	Pre-resolution	Post-resolution	Pre-resolution	Post-resolution	Pre-resolution	Post-resolution
10	Acetylamphetamine	4.365	1.230	2.237	1.307	1.430	1.693
	Aldimine	1.077	2.136	1.319	1.563	1.538	1.506
	Benzylamphetamine	2.496	1.203	1.561	4.070	1.374	1.864
	Ketimine	0.844	1.625	1.396	0.963	1.011	1.179
	DPIA (1)	1.626	1.021	0.964	1.107	2.117	1.411
	DPIA (2)	1.024	1.213	1.107	2.670	1.415	1.011
	1-oxo	1.127	3.913	1.140	0.935	0.858	1.074
	Benzoylamphetamine	1.250	0.897	1.735	1.261	1.913	1.739
	2-oxo	0.936	1.504	1.131	1.366	1.094	1.397
	Cathinol	-	-	-	-	1.554	2.258
Resolution >1 Reduc.amin.		**75%**		**75%**		**95%**	
Resolution >1 All impurities		**83%**		**92%**		**92%**	
12	Acetylamphetamine	4.068	3.267	1.967	0.972	1.148	32.451
	Aldimine	3.162	2.080	1.248	1.426	1.447	1.399
	Benzylamphetamine	1.097	6.341	0.805	3.830	1.240	1.564
	Ketimine	0.708	1.510	1.897	0.946	1.033	1.031
	DPIA (1)	1.457	0.922	0.948	1.023	3.282	1.310
	DPIA (2)	0.922	2.542	1.023	2.672	1.313	1.033
	1-oxo	1.010	3.800	1.142	1.074	0.477	0.687
	Benzoylamphetamine	1.177	2.184	1.603	1.206	0.719	1.397
	2-oxo	0.689	1.319	2.001	1.142	1.583	1.670
	Cathinol	-	-	-	-	1.471	2.339
Resolution >1 Reduc.amin.		**70%**		**70%**		**86%**	
Resolution >1 All impurities		**92%**		**83%**		**75%**	

The more polar HP-50+ column appeared to give the best resolution for reductive amination compounds. The results indicated that it was the most suitable for all amphetamine types. The HP-50+ column was chosen for further studies. However, significant bleeding of the stationary phase of the column was observed as compared to other stationary phases, for example, Ultra-2 (Figure 45).

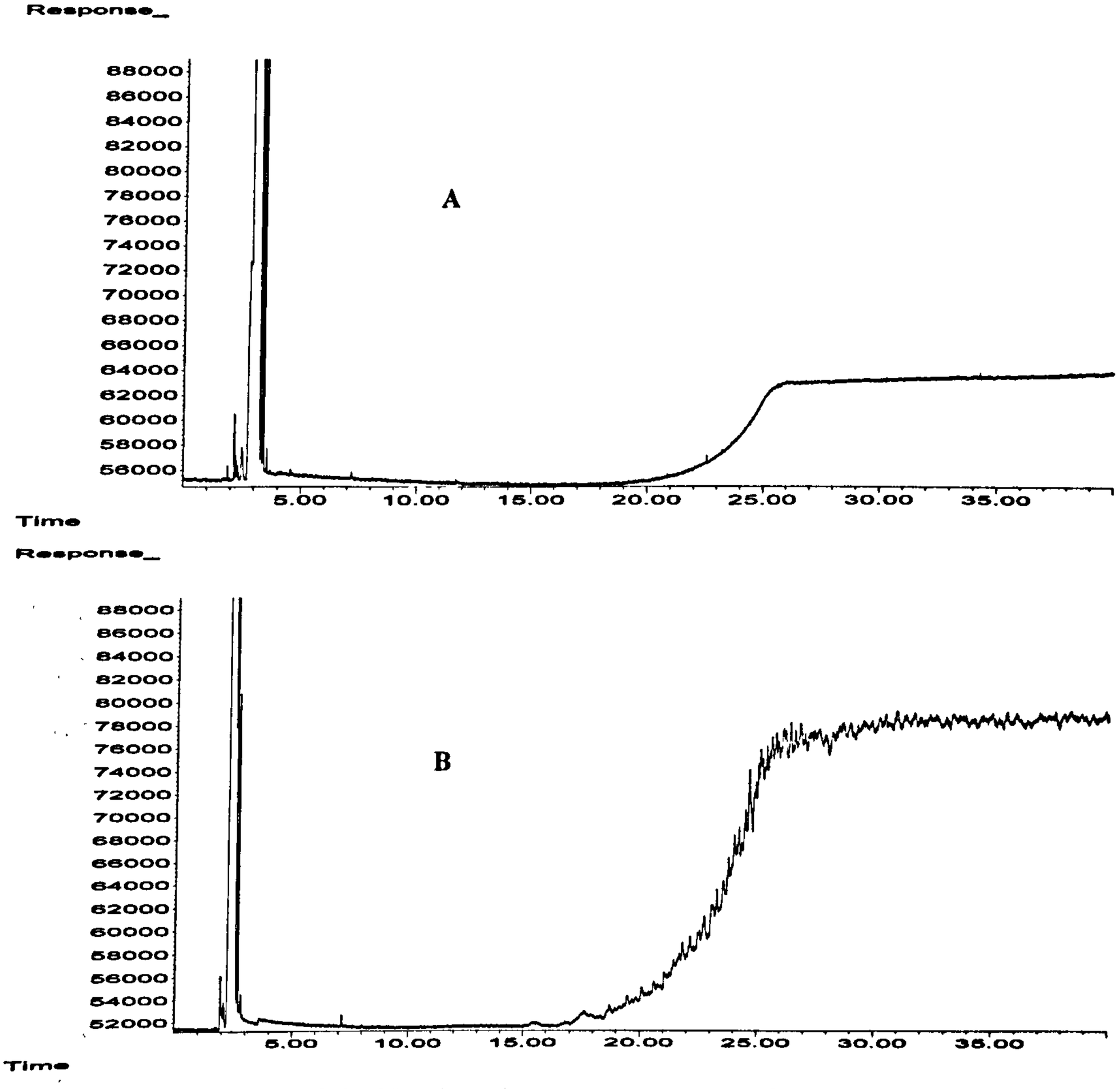
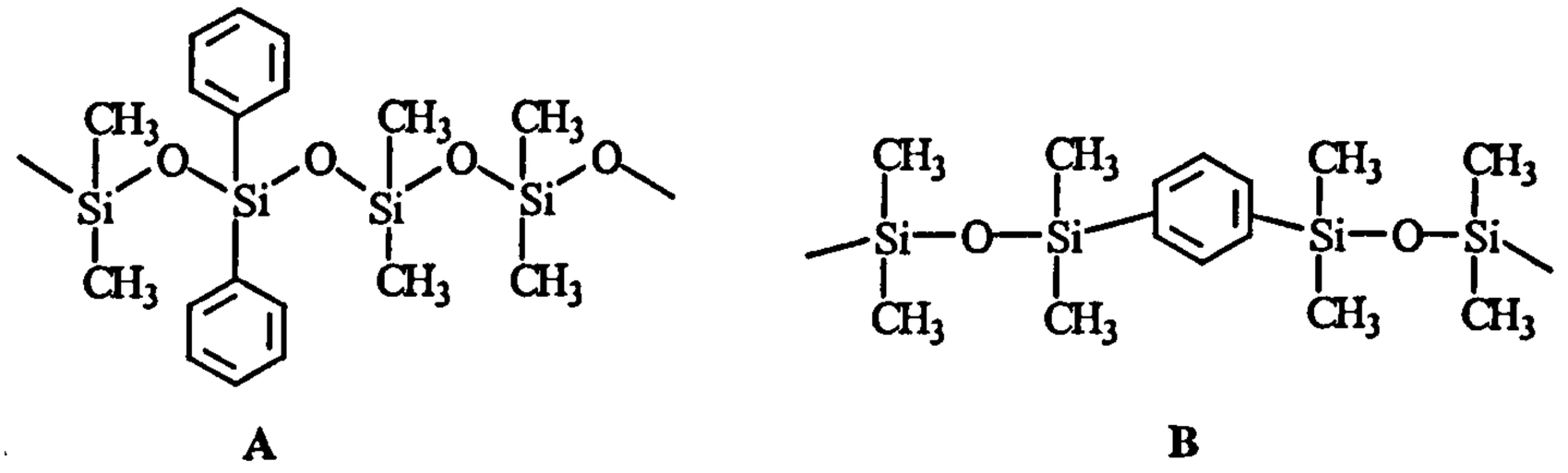


Figure 45: Comparison of the bleeding of different stationary phases in a blank sample by A) Ultra-2 and B) HP-50+.

Therefore, alternative columns were examined. The first choice for an alternative column was DB-17MS, which was specially prepared for GC-MS work and equivalent to the stationary phase of HP-50+. The MS columns are known to bleed less due to the different processing of the stationary phase. The structure of the stationary phase in a normal phenyl methyl silicone column and in a corresponding MS type column is shown in Figure 46 [177].



A B

Figure 46: Structural formulas of stationary phase in A) normal phenyl methyl silicone column and B) corresponding MS type column.

Additionally, 35% phenyl methyl silicone columns, namely HP-35 and DB-35MS were studied. Resolution and inertness were evaluated for HP-35, DB-35MS, DB-17MS and HP-50+ as a comparison, using temperature program rates of 8, 10 and 12°C/min. Note that the test sample is different than in earlier experiments. Chromatograms of the alternative columns are shown in Figure 47.

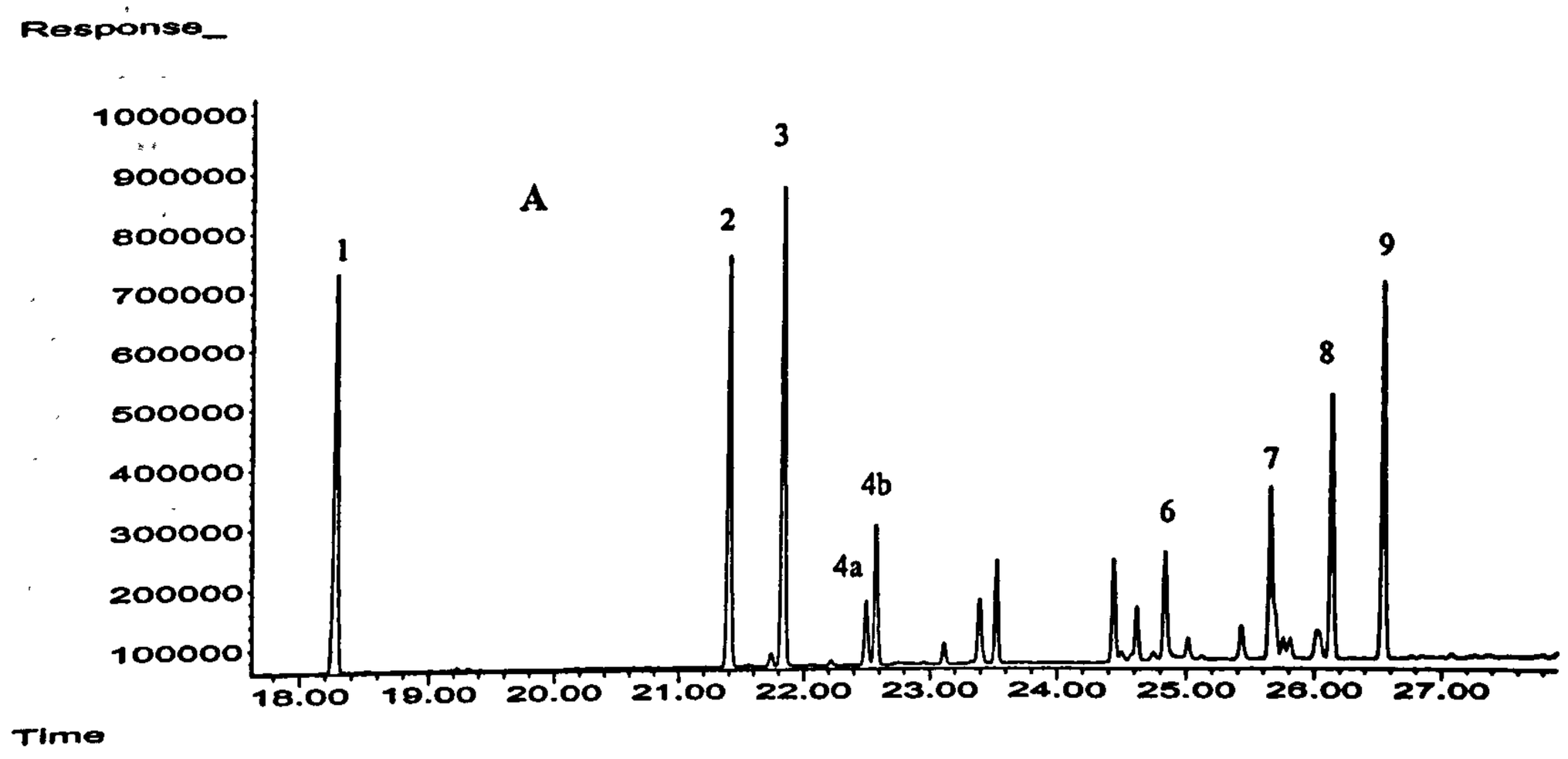


Figure 47: Comparison of alternative columns at 8°C/min. A) HP-50+, B) DB-17MS, C) HP-35 and D) DB-35MS. Peak identification: 1) Acetylamphetamine 2) aldimine, 3) benzylamphetamine, 4a and 4b) DPIA, 6) 1-oxo, 7) cathinol, 8) benzoylamphetamine and 9) 2-oxo.

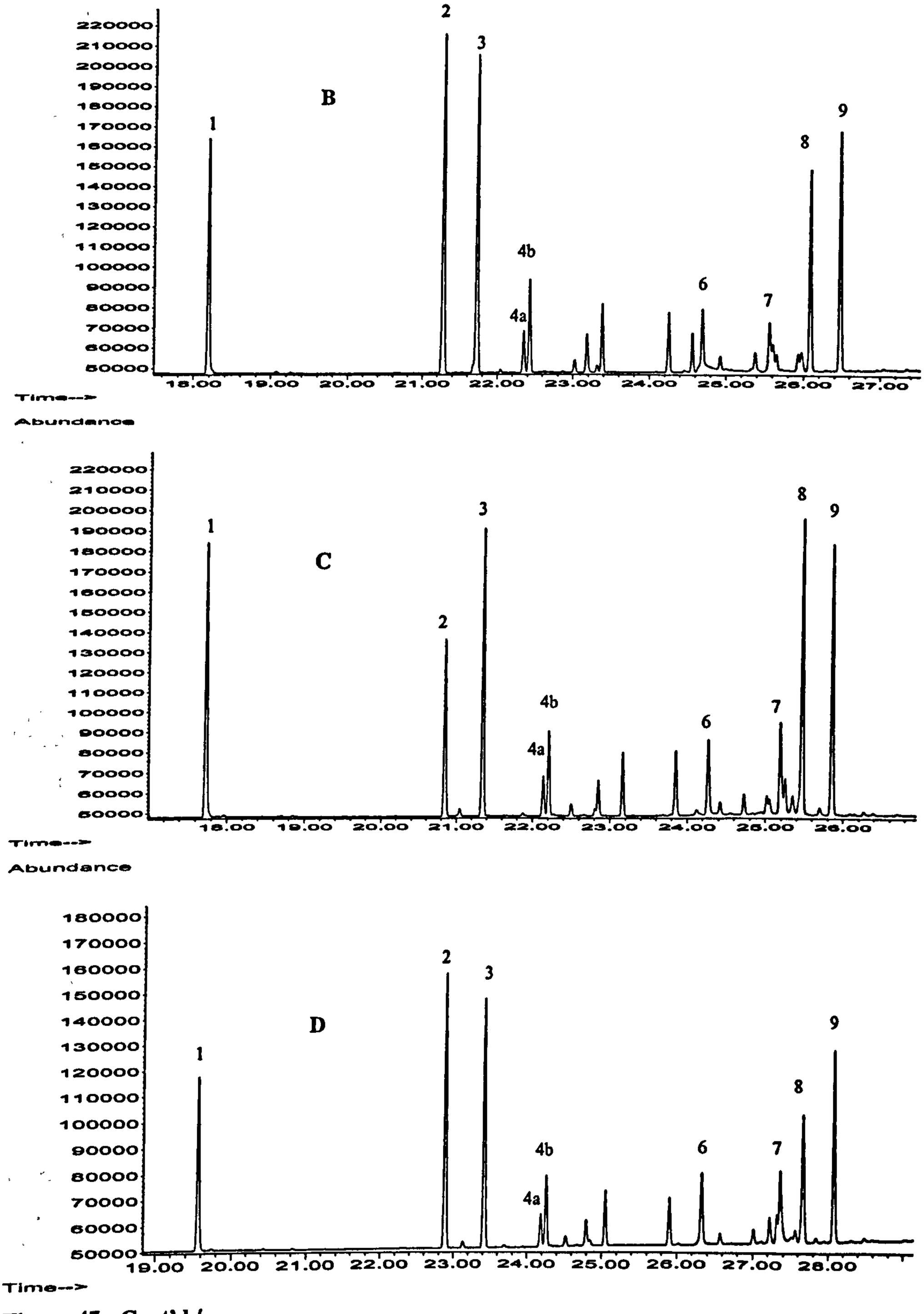


Figure 47: Cont'd / ...

The resolution results of alternative columns are summarised in Table 11.

Table 11: Pre- and post resolution and resolution > 1 calculated for each alternative column at different temperature program.

		HP-50+		DB-35MS		HP-35		DB-17MS	
T-program (°C/min)	Compound	Pre-resolution	Post-resolution	Pre-resolution	Post-resolution	Pre-resolution	Post-resolution	Pre-resolution	Post-resolution
8	Acetylamph.	13.752	4.67	18.758	3.364	15.302	4.473	14.712	16.961
	Aldimine	24.625	2.703	39.123	4.611	44.804	4.111	39.803	9.081
	Benzylamph	1.848	4.529	5.455	5.200	6.105	11.268	9.081	6.652
	DPIA (1)	5.483	1.455	9.219	1.357	5.486	1.428	6.311	1.617
	DPIA (2)	1.460	7.200	1.355	4.881	1.433	6.056	1.623	9.389
	1-oxo	1.436	3.23	5.938	4.467	2.521	2.870	2.705	4.497
	Benzoylamph.	1.515	1.725	1.767	3.187	2.284	4.386	1.98	3.776
	2-oxo	3.306	3.009	4.769	4.362	3.083	4.593	3.258	16.638
	Cathinol	1.371	1.800	0.805	1.988	2.581	1.166	3.236	0.758
Resolution >1 Reduc.amin.		**100%**		**100%**		**100%**		**100%**	
Resolution >1 All impurities		**95%**		**91%**		**94%**		**91%**	
10	Acetylamph.	24.51	56.493	18.528	3.631	15.720	1.215	14.479	1.331
	Aldimine	56.717	6.348	33.311	4.523	29.901	4.014	37.825	5.317
	Benzylamph.	1.701	6.563	5.150	4.144	5.691	9.954	4.011	4.167
	DPIA (1)	6.176	1.426	9.820	1.331	6.289	1.320	7.015	1.442
	DPIA (2)	1.424	9.937	1.331	4.641	1.320	1.287	1.450	9.108
	1-oxo	1.303	3.186	3.050	4.241	2.117	3.008	4.013	4.238
	Benzoylamph.	1.657	7.318	1.483	3.103	1.867	4.349	1.930	7.291
	2-oxo	7.366	9.468	4.367	3.556	2.918	4.100	7.291	2.488
	Cathinol	3.299	1.411	2.406	1.755	2.191	2.167	2.524	0.566
Resolution >1 Reduc.amin.		**100%**		**100%**		**100%**		**100%**	
Resolution >1 All impurities		**95%**		**94%**		**91%**		**93%**	
12	Acetylamph.	24.064	54.13	17.714	3.682	14.147	1.314	14.276	1.371
	Aldimine	54.391	6.101	10.121	4.009	34.628	3.936	25.914	8.305
	Benzylamph.	1.451	5.604	4.495	3.182	5.252	5.049	8.278	4.356
	DPIA (1)	6.542	1.338	10.196	1.228	6.415	1.252	1.178	1.428
	DPIA (2)	1.337	9.311	1.226	4.298	1.252	1.244	1.428	9.116
	1-oxo	1.159	3.235	2.905	4.128	2.249	2.836	1.666	3.586
	Benzoylamph.	1.700	7.290	1.267	2.924	1.696	4.375	1.572	7.008
	2-oxo	7.241	8.939	1.511	3.184	2.811	3.936	7.008	2.107
	Cathinol	2.899	1.241	2.275	1.622	1.876	2.429	2.242	0.488
Resolution >1 Reduc.amin.		**100%**		**100%**		**100%**		**95%**	
Resolution >1 All impurities		**91%**		**92%**		**85%**		**91%**	

Good resolution can be obtained with all alternative columns since the resolutions were similar to those obtained on HP-50+. Also, the bleeding of the alternative stationary phases was less than with HP-50+.

In addition to resolution, inertness was determined. Inertness was calculated such that an inverse (1/x) was first taken for symmetric values smaller than 1.0 and average was thereafter calculated. The results are shown in Table 12. As a

consequence of these data, the DB-35MS was chosen as the final column of the harmonised amphetamine profiling method.

Table 12: Inertness of each column at different temperature program.

Reductive amination impurities						
T-program (°C/min)	Ultra-1	Ultra-2	HP-50+	HP-35	DB-35MS	DB-17MS
2	1.29	1.64	1.18	-	-	-
4	1.21	1.13	1.09	-	-	-
6	1.21	1.42	**1.07**	-	-	-
8	1.23	1.14	1.08	1.19	1.12	1.08
10	1.25	1.18	1.21	1.12	1.16	1.13
12	1.24	1.21	1.11	1.14	1.21	1.27
All impurities						
T-program (°C/min)	Ultra-1	Ultra-2	HP-50+	HP-35	DB-35MS	DB-17MS
2	2.27	2.82	2.16	-	-	-
4	2.31	2.25	2.03	-	-	-
6	2.23	2.20	1.89	-	-	-
8	2.11	2.33	1.61	1.46	**1.24**	1.56
10	2.00	2.07	1.67	1.35	1.38	1.35
12	2.04	1.99	1.67	1.42	1.30	1.40

6.4.3 Detection

The Grob mixture contained two co-eluting pairs. Pentadecane was co-eluted with undecanoic acid methyl ester by FID. By MSD in Scan mode these compounds were not baseline separated. Correspondingly, hexadecane and dodecanoic acid methyl ester were not resolved to baseline by FID and MSD. In SIM mode only undecanoic and dodecanoic acid methyl esters were chromatographed from the co-eluting pairs. The Grob chromatograms recorded by each detector are shown in Figure 48.

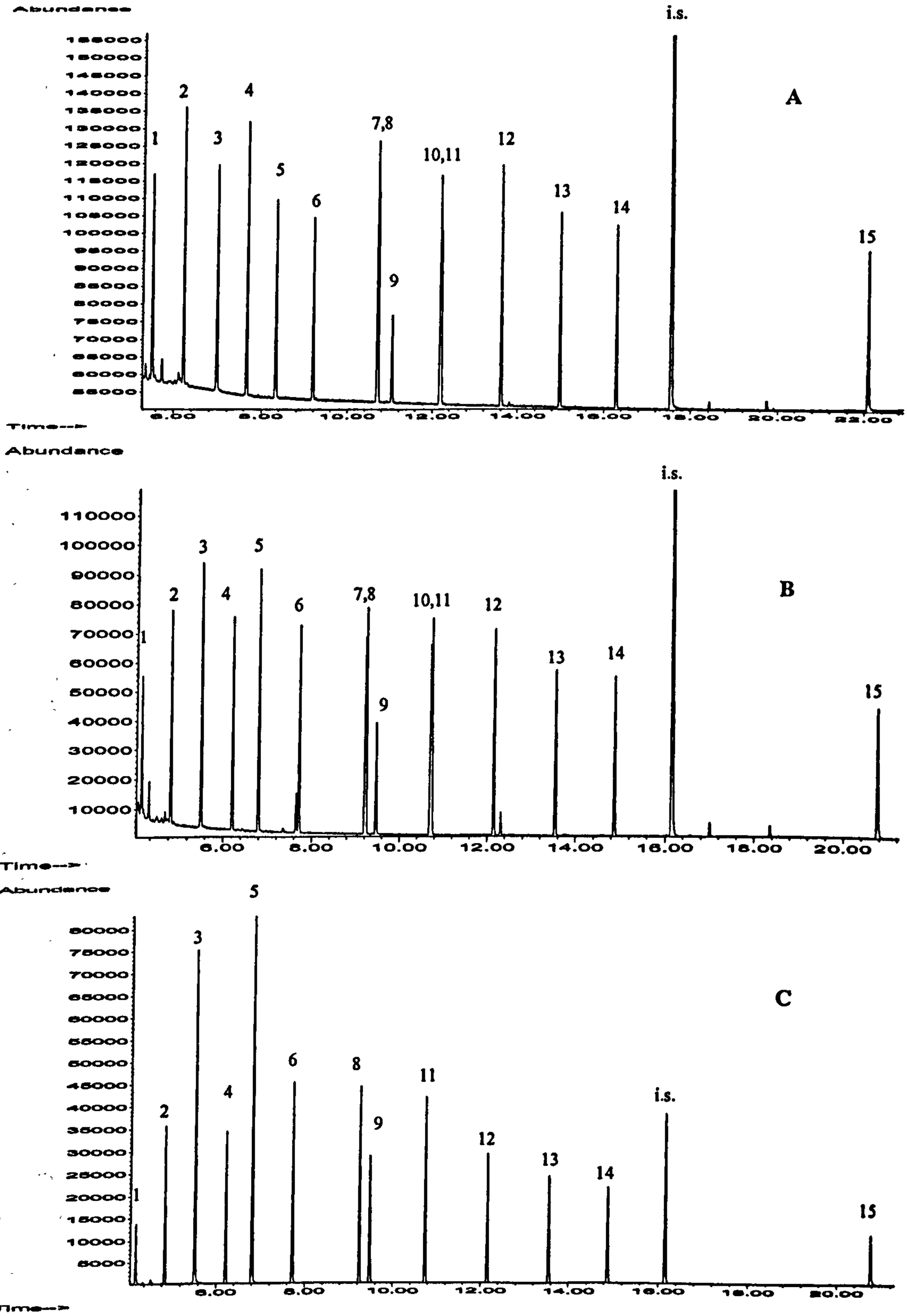


Figure 48: **Chromatogram of the Grob mixture detected by A) FID, B) MSD in Scan mode and C) MSD in SIM mode. Peak identification: 1) 1-octanol, 2) C_{10}, 3) 2,6-dimethylphenol, 4) C_{13}, 5) 2,6-dimethylaniline, 6) decanoic acid methyl ester, 7) C_{15}, 8) undecanoic acid methyl ester, 9) dicyclohexylamine, 10) C_{16}, 11) dodecanoic acid methyl ester, 12) C_{17}, 13) C_{18}, 14) C_{19} and 15) C_{24}.**

Repeatability and reproducibility

Repeatability and reproducibility were determined for each detector. RSD values were calculated between 20 replicates during the day and between days. The results of the repeatability and reproducibility study are summarised in Table 13.

Table 13: RSD values for repeatability and reproducibility study.

	Repeatability			Reproducibility		
	FID	MSD/ Scan	MSD/ SIM	FID	MSD/ Scan	MSD/ SIM
1-Octanol	3%	5%	4%	2%	6%	7%
Dodecane (C_{12})	2%	5%	4%	3%	6%	7%
2,6-dimethylphenol	3%	4%	4%	2%	5%	7%
Tridecane (C_{13})	2%	4%	4%	2%	5%	6%
2,6-dimethyl aniline	3%	4%	4%	2%	5%	7%
Decanoic acid methyl ester	2%	4%	4%	6%	5%	6%
Undecanoic acid methyl ester	5%	3%	4%	1%	5%	6%
Dicyclohexylamine	5%	6%	5%	16%	15%	18%
Dodecanoic acid methyl ester	2%	3%	4%	2%	5%	6%
Heptadecane (C_{17})	1%	4%	4%	1%	5%	5%
Octadecane (C_{18})	1%	5%	4%	1%	4%	5%
Nonadecane (C_{19})	1%	4%	4%	1%	4%	5%
Tetracosane (C_{24})	2%	17%	7%	2%	7%	7%
Mean	**2%**	**5%**	**4%**	**3%**	**6%**	**7%**
Mean (dicyclohexylamine excluded)				**2%**	**5%**	**6%**

During the study, unexpected tailing was observed for high boiling point alkanes in the MS trace. The problem was located in the MSD ion source, and indeed the installation of "Ultra Ion source" parts solved the problem completely. The tailing problem is described in detail in Chapter 6.4.4. The reproducibility study was repeated and good results were obtained with each detector. RSD values of repeatability and reproducibility ranged from 2% to 6%. Significant individual deviation occurred for dicyclohexylamine.

Sensitivity

In the sensitivity study, the lowest concentration was determined as the lowest concentration still producing acceptable repeatable results. The results are shown in Table 14.

Table 14: RSD results of sensitivity study.

	0.005 µg/ml			0.01 µg/ml			0.05 µg/ml			0.10 µg/ml		
	FID	MSD/ Scan	MSD/ SIM	FID	MSD/ Scan	MSD/ SIM	FID	MSD/ Scan	MSD/ SIM	FID	MSD/ Scan	MSD/ SIM
Dodecane (C_{12})	25%	n.d.	10%	15%	12%	3%	4%	13%	3%	0.1%	5%	2%
2,6-dimethylphenol	22%	n.d.	6%	5%	16%	2%	6%	12%	2%	0.0%	6%	3%
Tridecane (C_{13})	14%	n.d.	15%	11%	10%	6%	15%	22%	3%	0.0%	4%	2%
2,6-dimethyl aniline	52%	n.d.	5%	20%	11%	1%	6%	16%	2%	0.0%	7%	4%
Decanoic acid ester	9%	n.d.	4%	19%	7%	1%	19%	14%	2%	0.3%	6%	3%
Undecanoic acid ester	7%	n.d.	6%	8%	7%	2%	7%	20%	2%	0.0%	6%	3%
Dicyclohexylamine	44%	n.d.	26%	53%	43%	25%	16%	30%	8%	0.1%	14%	5%
Dodecanoic acid ester	52%	n.d.	4%	64%	27%	5%	52%	18%	1%	0.1%	7%	2%
Heptadecane (C_{17})	28%	n.d.	11%	25%	24%	7%	9%	18%	1%	0.0%	9%	1%
Octadecane (C_{18})	14%	n.d.	15%	24%	27%	9%	39%	17%	2%	0.0%	5%	1%
Nonadecane (C_{19})	26%	n.d.	11%	5%	32%	8%	4%	27%	1%	0.0%	3%	1%
Tetracosane (C_{24})	8%	n.d.	14%	7%	30%	10%	11%	12%	1%	0.1%	4%	1%
Mean	**25%**	**n.d.**	**11%**	**21%**	**20%**	**6%**	**16%**	**18%**	**2%**	**0.1%**	**6%**	**2%**
Mean of alkanes	**19%**	**n.d.**	**13%**	**15%**	**22%**	**7%**	**14%**	**18%**	**2%**	**0.1%**	**5%**	**1%**

n.d. not detected.

The lowest concentrations for FID and MSD in Scan and SIM modes were 0.1 µg/ml, 0.1 µg/ml and 0.01 µg/ml, respectively. Significant differences were not detected in the results if only alkanes were taken into account compared to all compounds.

Selectivity

In the selectivity study, the absolute peak areas of heptadecane and octadecane relative to tridecane and correspondingly, the ratios of absolute peak areas of undecanoic and dodecanoic acid methyl esters relative to decanoic acid methyl ester were determined. Moreover, repeatability between four replicates was calculated utilising normalised peak areas. The results are shown in Table 15.

Table 15: Absolute peak areas and their RSD values of Grob mixture compounds. The values are mean values from four replicates.

	Absolute peak area			RSD		
	FID	MSD/ Scan	MSD/ SIM	FID	MSD/ Scan	MSD/ SIM
1-Octanol	874073	57865	83135	2%	2%	1%
Dodecane (C_{12})	1145892	177764	260961	1%	2%	1%
2,6-dimethylphenol	1111790	332051	469951	1%	1%	1%
Tridecane (C_{13})	1194132	181203	268244	1%	1%	0%
2,6-dimethyl aniline	1027393	371418	521451	1%	1%	1%
Decanoic acid methyl ester	852731	270399	391574	2%	1%	1%
Undecanoic acid methyl ester	**1930607**	**268845**	**391079**	**1%**	**1%**	**1%**
Dicyclohexylamine	419864	299473	371153	17%	18%	27%
Dodecanoic acid methyl ester	**733685**	**255486**	**369129**	**2%**	**2%**	**1%**
Heptadecane (C_{17})	1180290	176320	262584	1%	1%	2%
Octadecane (C_{18})	966104	142339	212126	1%	1%	1%
Nonadecane (C_{19})	884091	127527	187348	2%	2%	1%
Tetracosane (C_{24})	804253	122310	177416	3%	5%	4%
Mean				**2%**	**3%**	**3%**
Mean (dicyclohexylamine excluded)				**1 %**	**2 %**	**1 %**

The absolute peak areas of tridecane, heptadecane and octadecane relative to the peak area of tridecane were 1.0:0.9:1.0 with each detector. These alkanes were an example of separated peaks. When using the FID mode, the ratios of decanoic, undecanoic and dodecanoic acid methyl esters to decanoic acid methyl ester were 1.0:2.3:0.9, respectively. The results indicated non-separated peaks, i.e. pentadecane was co-eluted with undecanoic acid methyl ester. For Scan and SIM modes the ratios were 1.0:1.0:0.9. This indicated that even if the peaks were not separated to the baseline, the correct absolute peak areas were detected. Moreover, on MSD the RSD values were as good for co-eluting compounds as they were for fully separated compounds.

Linearity

In the linearity study, the Pearson product-moment coefficient of correlation, r, and the coefficient of determination, R^2, were defined with equation 31 and equation 32, respectively. In a straight-line graph, $R^2 = r^2$. As an example the r^2 and R^2 values have been calculated for 2,6-dimethylphenol below.

Table 16: Parameters in calculations of Pearson product-moment coefficient of correlation, r, and coefficient of determination, R^2, for 2,6-dimethylphenol.

Conc. (x)	Observed RRF (y_i)	x^2	y^2	x × y	Calculated RRF($\hat{y}_i$)	Residual ($y_i - \hat{y}_i$)	$(y_i - \hat{y}_i)^2$	$\hat{y}_i - \bar{y}$	$(\hat{y}_i - \bar{y})^2$
0.01	0.002	0.00	0.00	2E-05	-0.12	0.12	0.01	-3.21	10.33
0.05	0.006	0.00	0.00	0.0003	-0.11	0.12	0.01	-3.21	10.29
0.10	0.012	0.01	0.00	0.0012	-0.11	0.12	0.01	-3.20	10.24
1.00	0.13	1.00	0.02	0.1251	0.03	0.10	0.01	-3.06	9.39
5.00	0.66	25.00	0.44	3.302	0.64	0.03	0.00	-2.46	6.05
10.0	1.36	100.0	1.85	13.607	1.39	-0.03	0.00	-1.70	2.90
25.0	3.34	625.0	11.13	83.407	3.66	-0.33	0.11	0.57	0.32
50.0	7.03	2500	49	351.73	7.45	-0.41	0.17	4.35	18.96
100	15.31	10000	234	1530.7	15.02	0.29	0.08	11.92	142.18
$\Sigma = 191.16$ $\bar{x} = 21.24$	$\Sigma = 27.84$ $\bar{y} = 3.09$	$\Sigma = 13251$	$\Sigma = 297$	$\Sigma = 1983$			$\Sigma = 0.42$		$\Sigma = 210.66$

$$r = \frac{1391.5}{\sqrt{9190.8 \times 211.1}} = 0.999015 \qquad \Rightarrow \quad r^2 = 0.998032$$

$$R^2 = \frac{210.66}{210.66 + 0.42} = 0.998032 \qquad \Rightarrow \quad r^2 = R^2$$

The calculations indicated that 2,6-dimethylphenol was linear within a concentration range from 0.01 µg/ml to 100 µg/ml analysed by FID. The parameters were also calculated for other compounds. The results are shown in Table 17.

Table 17. Results of linearity study for each Grob compound.

	r			$r^2 = R^2$		
	FID	MSD/ Scan	MSD/ SIM	FID	MSD/ Scan	MSD/ SIM
Dodecane (C_{12})	0.96493	0.99470	0.99185	0.93109	0.98943	0.98376
Octanol	0.99889	0.99999	0.99944	0.99779	0.99999	0.99887
2,6-dimethylphenol	0.99902	0.99998	0.99948	0.99803	0.99997	0.99897
Tridecane (C_{13})	0.99913	0.99976	0.99887	0.99825	0.99952	0.99774
2,6-dimethyl aniline	0.99904	0.99998	0.99948	0.99807	0.99997	0.99895
Decanoic acid methyl ester	0.99907	0.99988	0.99901	0.99815	0.99976	0.99802
Undecanoic acid methyl ester	0.99913	0.99976	0.9988	0.99826	0.99953	0.99761
Dicyclohexylamine	0.99892	0.99999	0.99934	0.99785	0.99998	0.99869
Dodecanoic acid methy lester	0.99834	0.99941	0.99778	0.99668	0.99882	0.99556
Heptadecane (C_{17})	0.99912	0.99965	0.99825	0.99825	0.99931	0.99651
Octadecane (C_{18})	0.99907	0.99907	0.99838	0.99814	0.99814	0.99676
Nonadecane (C_{19})	0.99899	0.99977	0.99863	0.99798	0.99954	0.99727
Tetracosane (C_{24})	0.99871	0.99978	0.99851	0.99742	0.99955	0.99702

6.4.4 Peak tailing

During the experiments a peak tailing problem was observed for the quality control sample, i.e. the Grob mixture. At first, the high boiling hydrocarbons (C_{19} and C_{24}) in the Grob mixture began to have increasingly tailing peak shape in the MSD trace. Thereafter, the tailing was equally well detected in the FID trace. The pre-columns were then changed resulting in symmetrical peaks. This tailing effect only takes place for samples such as the modified Grob mixture (Figure 49). Nonadecane in the amphetamine extract has a perfect peak shape, which indicates that numerous amines in the amphetamine extract seem to deactivate the system.

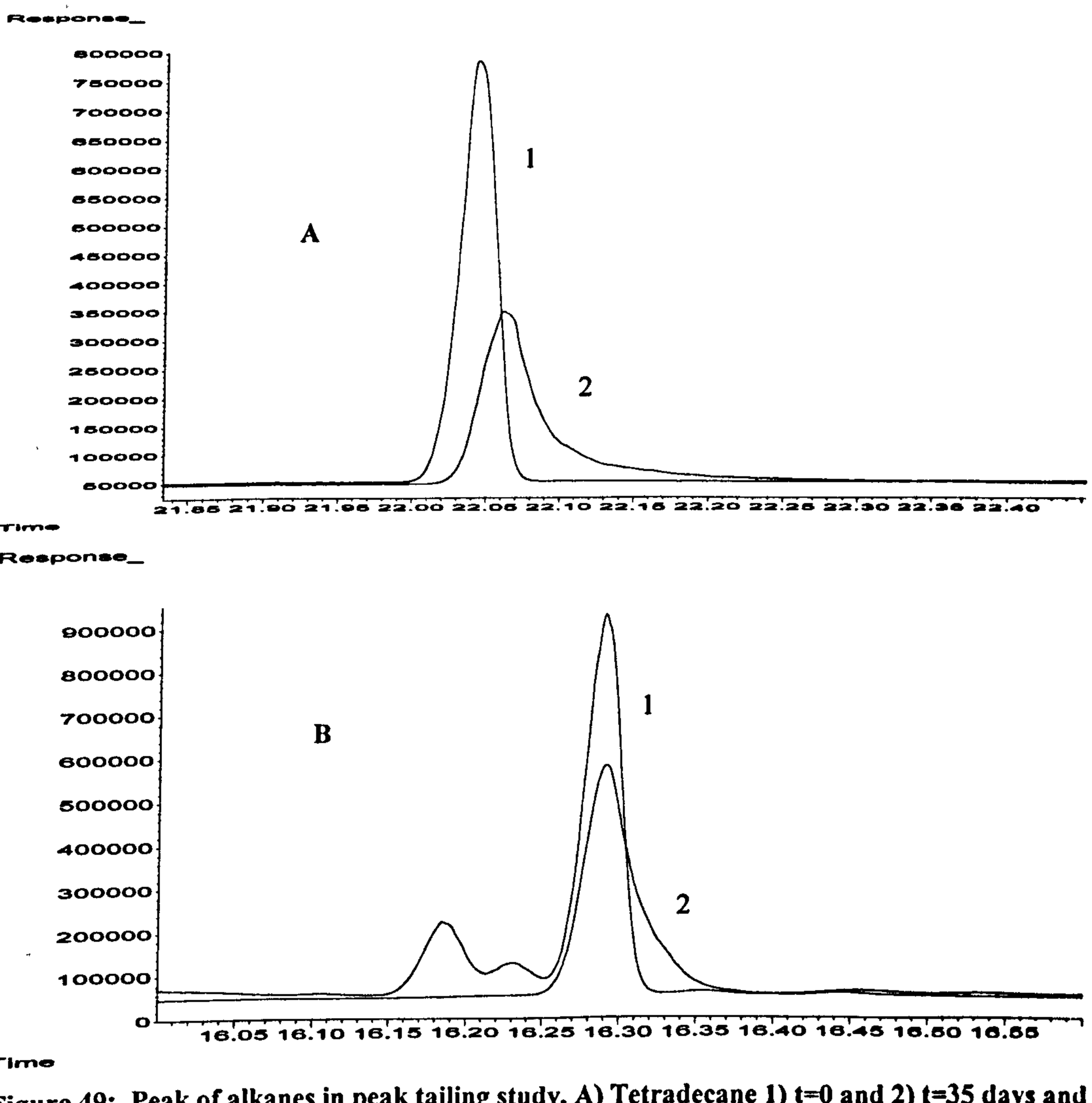


Figure 49: Peak of alkanes in peak tailing study. A) Tetradecane 1) t=0 and 2) t=35 days and B) nonadecane t=35 days 1) in amphetamine extract and 2) in the Grob mixture.

More experiments were done to resolve the reason for the tailing problem. In the next step, it was found that the tailing phenomenon was independent of the stationary phase. It was, however, discovered that the peak symmetry could be improved using non-deactivated press fits instead of deactivated ones. Symmetrical peaks were obtained thereafter for a few injections, but the problem appeared again. More research was undertaken and finally the problem was discovered to be at the MSD. With the aid of Agilent Technologies, Finland, three parts, namely the repeller, ion source and plate were replaced with "Ultra ion source" parts. Using these special parts the tailing disappeared completely (Figure 50). This was ensured by injecting several samples. The results showed that the peak symmetry was permanently as good as it was in the beginning.

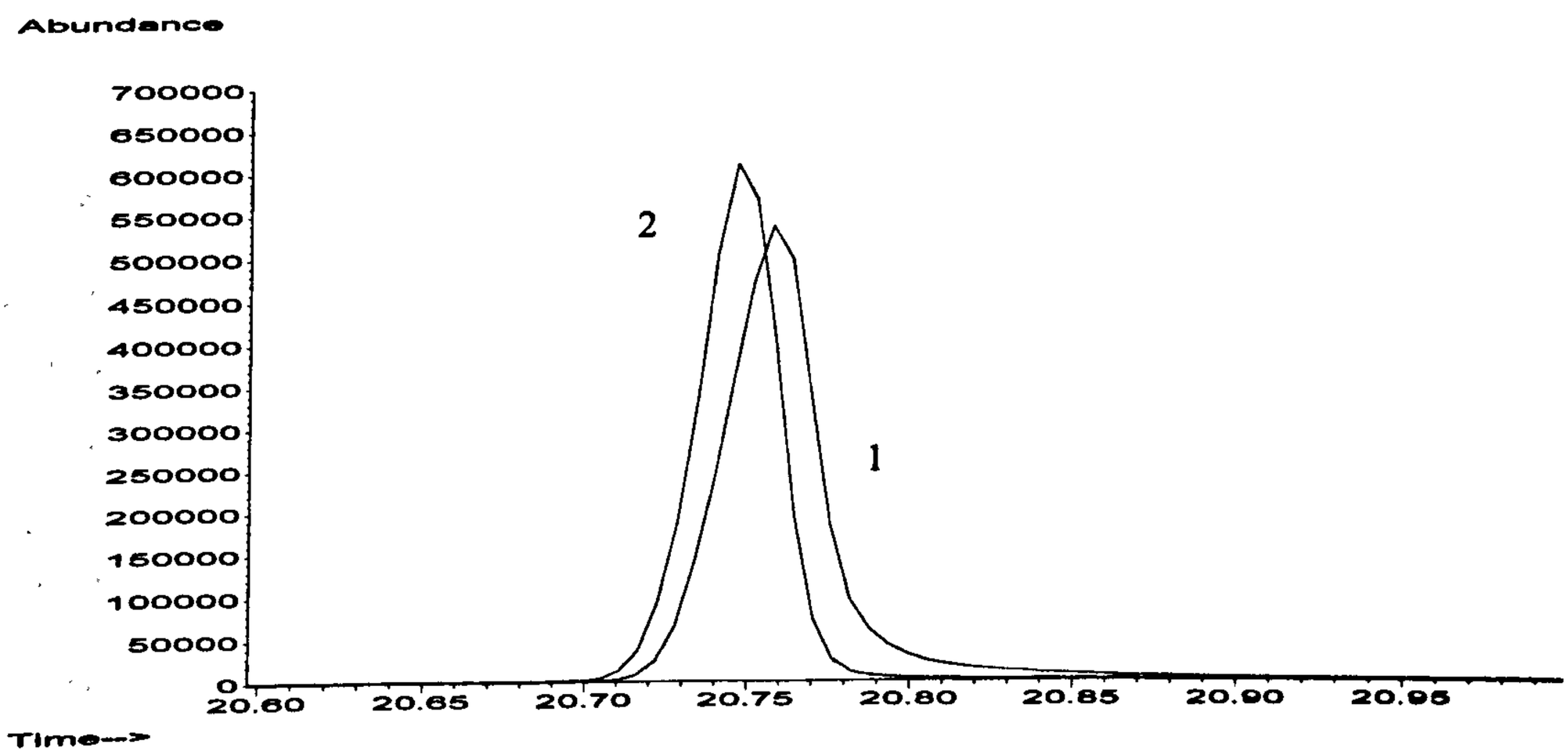


Figure 50: Peak of tetradecane 1) with the standard ion source parts, 2) with the Ultra ion source parts.

6.5 Conclusion

In general, the development of a GC method starts with a non-polar methyl silicone column. Therefore, the optimisation of sample introduction was studied with the HP Ultra-2 column. The best repeatability was obtained with split injection at 240°C. For other inlet temperatures the standard deviation was only slightly higher. The smallest

relative deviation was also obtained with split injection. Split injection gave the best results at 260°C. Splitless injection provides somewhat higher error and relative deviation than split injection. The best temperature would be 240°C for splitless techniques. The differences were, however, insignificant between split and splitless injections. The concentration of impurities in street amphetamine samples can sometimes be very low. To avoid the loss of detection of impurity peaks the splitless injection at 250°C was chosen for further studies. However, the sample preparation technique for next study was split injection at 260°C.

In general, the best separation power in terms of number of resolved peaks for each column was obtained with slow temperature programmes, 2 and 4°C/min. Ultra-2 gave the best separation power compared to other columns. Differences in separation power between columns and between other temperature programmes were not significant. The choice of the column was made by taking the results of all synthetic routes into consideration. The best resolution was found to be difficult to determine. For most columns the best resolution was obtained with slow temperature programs. The best overall resolution for the target compounds was obtained with the more polar HP-50+ column; as much as 100% of the target compounds could be separated with resolution > 1.0 at 2°C/min. Unfortunately, this temperature programme is unpractical due to the long running time. There were not significant differences in the inertness between columns. The choice of the column for next sample preparation studies was HP-50+ at 8°C/min. However, due to significant bleeding of 50% diphenyl dimethyl stationary phase, alternative columns, namely DB-17MS, HP-35 and DB-35MS were studied as well. The results indicated good resolution and insignificant bleeding with these three columns. The final choice of the column was DB-35MS.

Repeatability and reproducibility were acceptable for each detector, FID and MSD in Scan and SIM mode. The results ranged from 2% to 6%. In the sensitivity study, the lowest concentrations with good repeatability for FID and MSD in Scan and SIM mode were 0.1 µg/ml, 0.1 µg/ml and 0.01 µg/ml, respectively. SIM mode was ten times more sensitive than FID and Scan mode. The results of the selectivity study

indicated a good repeatability even for co-eluting peak pairs. In conclusion, the highly selective MSD was also very repeatable. The Pearson product-moment coefficient of correlation, r, and the coefficient of determination, R^2, were defined for each detector to determine linearity. The results indicated that each detector was linear over five decades, from 0.01 μg/ml to 100 μg/ml. FID was found to provide a good overall performance with the exception of selectivity. Thus, the final choice of detector was the more selective MSD in Scan mode.

7 OPTIMISATION OF SAMPLE PREPARATION

7.1 Introduction

Liquid-liquid extraction (LLE)

Liquid-liquid extraction (LLE) is useful for separating analytes from the sample matrix by partitioning the sample between two phases [178]. Phase one is often aqueous and the second an organic solvent. Hydrophilic compounds prefer the polar aqueous phase, whereas hydrophobic compounds will be found mainly in the organic solvent. Analytes extracted into the organic solvent can often be injected directly onto an HPLC or GC column. Some practical problems are associated with the LLE including emulsion formation, analytes bound to high molecular weight compounds (e.g. drugs to proteins) and mutual solubility of the two phases.

In LLE, it is of utmost importance that the buffer of choice has (i) high dissolving power and (ii) high buffer capacity. The buffer has its best buffer capacity at pH = pK_a of the salt [179]. In the current application, the former is required in order to dissolve an amount of amphetamine which is large enough to allow sufficient detection sensitivity for trace level concentrations of synthesis impurities. This is extremely important since a partly dissolved sample inevitably has a different profile from that of an entirely dissolved sample. Recent trends in the characteristics of street amphetamines have been shown that very pure amphetamine containing very few impurities occurs more often than before and therefore a method with high sensitivity and selectivity is required.

Street amphetamine can be very acidic or basic due to the batch to batch variation of the drug. High buffer capacity is required to avoid the need for laborious adjustment of the pH to the default value. An ideal situation would be one where no pH adjustment is required after having dissolved amphetamine in the buffer.

Solid phase extraction (SPE)

Solid phase extraction (SPE) is a simple technique to use and a number of disposable extraction columns with different sorbent materials are available. Most of the sorbents are based on chemically modified silica particles. However, usually silica-based columns should not use be used at > pH 8 because of rapid dissolution of the silica support and a resulting collapse of the column bed [178]. New demands for sample preparation led to the development of a new, specially designed polymeric sorbent which performs optimally for reserved-phase SPE [180]. These polymeric sorbents have 2 - 3 times more surface area and a greater capacity than the silica-based columns. Octyl-bonded (C_8) and octadecyl-bonded silica (C_{18}) are typical non-polar sorbents whereas cyanopopyl (CN) and aminopropyl (NH_2) represent polar sorbents. Mixed-mode columns are also available. These columns contain both non-polar and strong cation ($-SO_3^-$) or anion exchange ($-NH_3^+$) functional groups [181]. SPE offers many benefits and advantages including high recovery of analytes, high concentration of sample, highly purified extracts, ease of automation and reduction in organic solvent consumption [181]. It is the most powerful technique currently available for rapid and selective sample preparation.

In LLE, the solvent acts as one of the phases participating in the partition process. The SPE stationary phase, such as C_{18} 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 power of the elution solvent should be as good 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 a polar solvent is used. In conclusion, selection criteria for the organic solvent used in LLE and in SPE are rather different.

SPE is similar to LLE technique in that both are based on a 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 from the organic solvent used in LLE. Elution of retained impurities from a SPE column is partitioning chromatography and thus the elution solvent should be re-evaluated for SPE.

In this thesis LLE and SPE techniques will be evaluated to find the best sample preparation method for the amphetamine profiling. Method development for LLE can be divided into a number of different parts including (i) optimisation of type, pH and concentration of buffer, (ii) optimisation of type of solvent and (iii) optimisation of buffer and solvent volumes. Moreover, the influence of matrix effects is studied. Most parameters are interdependent which means that hundreds of experiments would be necessary to evaluate everything. Therefore, several parameters are studied at the same time. In SPE, the type of SPE column has a significant influence on the extraction power and therefore different columns are evaluated as well. The best type of buffer from LLE will be chosen for the SPE study. Moreover, equally with LLE buffer and solvent volumes were optimised.

7.2 Experimental

7.2.1 Materials and methods

Materials

Adulterants, namely lactose and caffeine 99% were obtained from Merck and Aldrich, respectively. Tris(hydroxymethyl)aminoethane (Trizma®) base and citric acid monohydrate were obtained from Sigma. Buffer chemicals sodiumhydrogen phosphate ($Na_2HPO_4 \cdot 7\ H_2O$) and tri-sodium citrate dihydrate ($C_6H_5O_7Na_3 \cdot 2\ H_2O$) were obtained from Fluka. Sodium dihydrogen phosphate monohydrate ($NaH_2PO_4 \cdot H_2O$) was obtained from Merck.

GC-method

The analyses were performed using the GC-MSD-FID instrument system. Two 25 m (L) x 0.25 mm (i.d.), coated with 0.25 µm of 50% diphenyl dimethyl silicone (HP-50+) capillary columns were connected with a 2.5 m (L) x 0.32 mm (i.d.) uncoated and deactivated pre-column and the Y-shape splitter. Split injection was used with a split flow 1:62 and the injection temperature was 250°C. 2 µl was injected into each column. The oven temperature program was started from 90°C for 1 min, increased at 8°C/min and the final temperature was 300°C for 10 min. Temperatures of detectors were 310°C. Three replicates of each extraction condition were analysed. In order to avoid systematic error, the extractions were performed in a non-sequential order.

7.2.2 Liquid-liquid extraction (LLE)

7.2.2.1 Optimisation of buffer and solvent

Each buffer was evaluated at a pH which was close to its pK_a value. The pH range 6.0 - 8.0 was covered. Three buffers and three organic solvents were studied. The buffers used were citrate, phosphate and Tris, prepared at 0.1 M and 1.0 M concentrations at pH 6.2 (citrate, $pK_a = 6.4$), pH 7.0 (phosphate, $pK_a = 7.2$) and pH 7.9 (Tris, $pK_a = 8.1$). Thus, the difference between pK_a and pH was 0.2 pH units in all cases. The solvents used were isooctane, toluene and ethyl acetate.

Buffer preparation

In the preparation of 0.1 M citrate buffer, two solutions were prepared. In solution A, 21.0 g of citric acid was dissolved in 1 litre of distilled water [182]. In solution B, 29.4 g of $C_6H_5O_7Na_3 \cdot 2\ H_2O$ was dissolved in 1 litre of distilled water. 140 ml of solution A and 856 ml of solution B were mixed. The pH was adjusted exactly to pH 6.20 by adding at first 1.0 M and thereafter 0.1 M NaOH or HCl. This results in 0.1

M citrate buffer. A 1.0 M solution was prepared accordingly using concentrations that were 10 times higher.

In the preparation of 0.1 M phosphate buffer, two solutions were prepared. In solution A, 3.8 g of $NaH_2PO_4 \cdot H_2O$ was dissolved in 1 litre of distilled water [182]. In solution B, 26.8 g of $Na_2HPO_4 \cdot 7\ H_2O$ was dissolved in 1 litre of distilled water. 390 ml of solution A and 610 ml of solution B were mixed. pH was adjusted exactly to pH 7.00 as described above. 1 M buffer was prepared by dissolving 138.0 g of $NaH_2PO_4 \cdot H_2O$ in ca. 900 litre of distilled water. The pH was adjusted exactly to the desired pH by adding at first 10 M and thereafter 1 M NaOH. The flask was filled up to the 1.0 litre volume and pH checked and readjusted if necessary.

In the preparation of 0.1 M Tris buffer, 2.11 g of Trizma base was dissolved in 1 litre distilled water (solution A) [182]. 0.1 M HCl solution was prepared (solution B). 50 ml of solution A and 32.0 ml of solution B were mixed. The pH was adjusted exactly to pH 7.90 by adding firstly 1.0 M and then 0.1 M NaOH or HCl. Tris buffer at concentration 1 M was prepared by dissolving 121.1 g of Trizma base in 1 litre of distilled water. At first, 37% HCl was added and thereafter 1.0 M HCl drop by drop to adjust pH exactly to the wanted pH. The flask was filled up to the 1.0 litre volume and pH checked and readjusted, if necessary.

Sample preparation

For the optimisation of extraction procedure, amphetamine was synthesised via reductive amination routes using $NaBH_3CN$ as described in Chapter 6.3.1. Due to the low concentration of target compounds in the home-made amphetamine, a few mg of pure impurities as a mixture in dichloromethane, was added drop by drop before preparing the amphetamine sulphate. The resulting amphetamine was fully homogenised. This sample will be referred to as TEST-1 sample. Part of the TEST-1 sample was mixed in 10:20:20 ratios (%, w/w) with lactose and caffeine and homogenised thoroughly. This sample will be referred to as TEST-2 sample.

200 mg of TEST-1 was weighed in a glass test tube. 2 ml of buffer was added and shaken thoroughly for 30 min using a mechanical shaker. The pH was adjusted with NaOH or HCl. 200 µl or 400 µl (ethyl acetate) of solvent, containing tetracosane at 10 µg/ml, was added and shaken thoroughly for 30 min. The samples were centrifuged for 5 min. The organic phase was placed in an autosampler vial (with insert vial) and analysed. TEST-2 sample was prepared as above. 400 mg of TEST-2 was dissolved in 4 ml of buffer. 400 µl or 800 µl (ethyl acetate) of solvent was added.

Blank samples were prepared as above, but naturally without amphetamine. The blank samples were analysed after every four "real" samples. The Grob test mix was analysed in the beginning, in the middle and at the end of the sample sequence to check the stability of the chromatographic system.

Buffer capacity

Several seized amphetamine samples were used to study the buffer capacity. 200 mg of sample was dissolved in 4 ml of different buffers, namely 0.1 M phosphate (pH 7.0), 0.5 M phosphate (pH 7.2) and 1 M Tris (pH 8.1). The pH was measured with a pH meter. Measured pH value minus pH of the buffer, Δ pH, was calculated.

7.2.2.2 Optimisation of extraction procedure and matrix effect

For the optimisation of the extraction procedure, comparison between one-step and three-step extraction with utilising 200 µl and 600 µl of solvent was made. Moreover, influence of the buffer volume was investigated as well. The volumes evaluated were 2 ml and 8 ml. The buffer/solvent pairs were Tris/toluene and phosphate/isooctane. Samples were prepared as above using the TEST-2 sample. The three replicates were made for each condition. Aliquots of constant volume were combined in the case of three-step extraction.

The matrix effect was studied with different amount of amphetamine, i.e. 15%, 50% and 100% mixed with caffeine such that the total amount of the sample was 200 mg. Tris and phosphate buffers with isooctane and toluene solvents were evaluated. One-step and three-step extraction, as well as small volume (4 ml) and large volume (8 ml) extractions, were undertaken. Extraction was carried out as in previous experiments.

7.2.3 Solid phase extraction (SPE)

Solid phase extraction (SPE) was carried out using a Gilson Aspec XL (Villiers-le-Bel, France) solid phase extraction system (Figure 51). This automated extractor allows extremely precise control over all extraction parameters and therefore it provides data with a very good repeatability and reproducibility. The results from a preliminary study indicated that a polymeric sorbent column Oasis® HBL obtained from Waters and a mixed-mode HCX column (C_8 + cation exchanger) from the International Sorbent Technology (IST) company gave the highest recovery of the analytes. Thus, these columns were chosen for the further study. In the optimisation of the extraction procedure, the analytes were eluted with ethyl acetate and toluene, i.e. with two solvents with different eluotropic values. Further, different buffer volumes were studied.

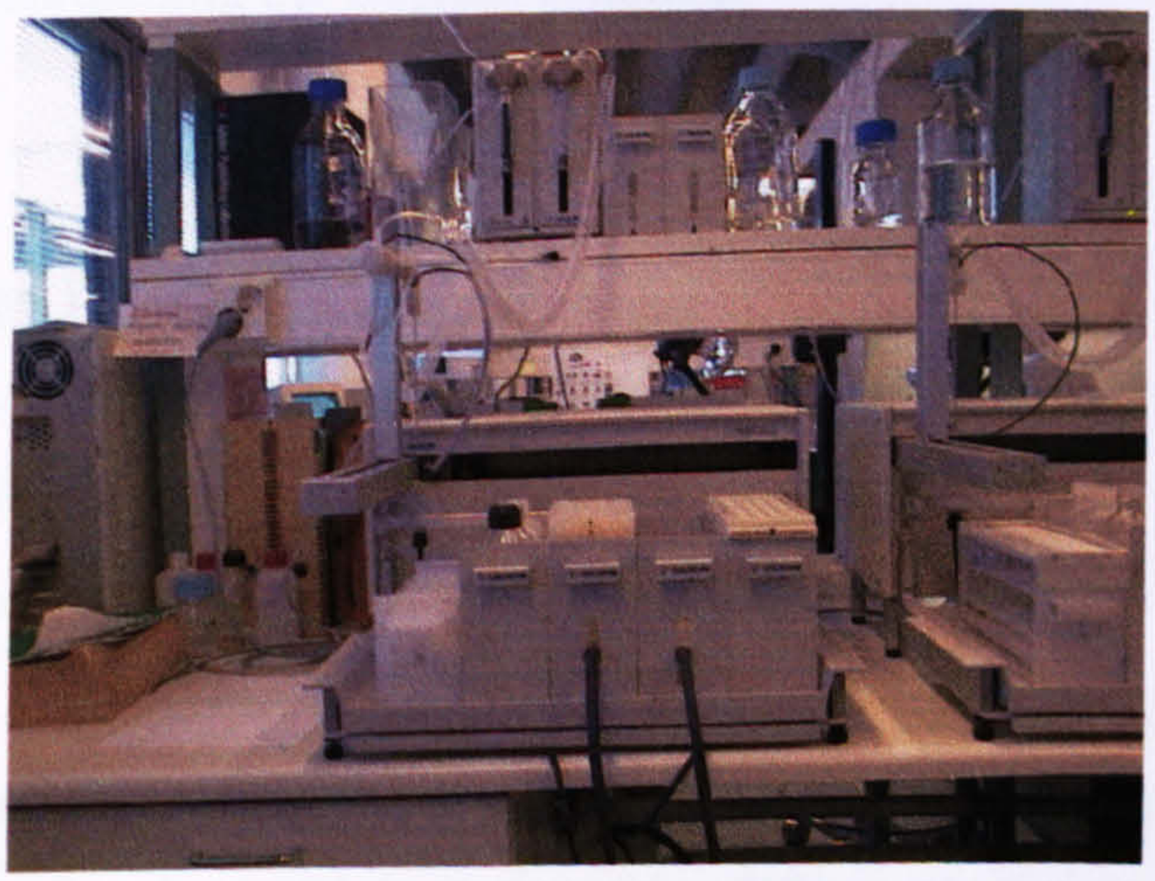


Figure 51: Gilson Aspec XL solid phase extraction system.

Sample preparation I

200 mg of the TEST-2 sample was dissolved in 4 ml of 1 M Tris buffer (pH 8.1). The SPE column was conditioned with 1 ml of MeOH and 1 ml of 1 M Tris buffer. The sample was loaded onto the column using a flow rate of 1 ml/min. The column was washed with 2 ml of water, dried with nitrogen blowing using a flow rate of approx. 60 ml/min for 10 min. The analytes were eluted using 3 times 200 µl of solvent (toluene or ethyl acetate), each fraction was collected separately in appropriate test tubes. The extracts were placed into vials, which contained 20 µl of internal standard (eicosane) solution at a concentration of 0.1 mg/ml.

Sample preparation II

The TEST-2 sample was dissolved in 4 ml of buffer and the solution loaded onto the column. The eluate was collected and extracted separately using the LLE method. The SPE column was washed with water, which was collected and extracted separately using the LLE method. Analytes were eluted from the SPE column with three times 200 µl of ethyl acetate and once with 200 µl of MeOH. All fractions were collected separately and placed in GC vials, which contained 20 µl of internal standard (eicosane) solution at 0.1 mg/ml concentration. The eluate from sample loading and the wash water fractions were LLE extracted with 200 µl of toluene and placed in GC vials containing the internal standard.

Sample preparation III

The influence of different washing solvents was studied utilising Leuckart type amphetamine samples. The sample solution (200 mg/4 ml of Tris) was loaded onto the Oasis column, which was then washed with 2 ml of water in the first case and, as a comparison, with 2 ml of Tris buffer. Analytes were eluted three times with 200 µl of ethyl acetate as above. The same sample was also extracted using LLE method as described earlier. RRF values obtained from the SPE method were compared with those obtained using this method.

Reference sample preparation in LLE

The LLE method was used as a reference method. 200 mg of TEST-2 sample was dissolved in 4 ml of Tris buffer (pH 8.1) and extracted with 200 µl of toluene. The extract was placed in a GC vial, which contained 20 µl of internal standard (eicosane) solution at concentration of 0.1 mg/ml.

7.2.3.1 Test tube effect

Influence of test tubes and shaking time in LLE

200 mg of Leuckart type amphetamine sulphate was weighed in new and in used glass test tubes and in polypropylene tubes. 4 ml of Tris buffer was added and the solutions shaken for 10 min. 200 µl of toluene containing 10 µg/ml of internal standard was added and the mixtures shaken for 5 min and, as a comparison, for 30 min. Five replicates for each extraction were prepared. RRF values and standard deviation of replicates were calculated. Thereafter, the ratios of results representing different shaking times were calculated for each test tube and shaking time. Moreover, old glass test tubes were compared to new tubes.

Influence of test tubes and shaking system in SPE

Used glass tubes and new ones with Teflon caps were studied in SPE. The samples were prepared as described in Sample preparation I. Moreover, different shaking systems were studied. Firstly, the tubes were shaken in vertical position and secondly, the tubes were placed horizontally in the shaking system.

Influence of methanol in test tube effect and on the entire SPE procedure

200 mg of the Leuckart type amphetamine was dissolved in 4 ml of 1 M Tris buffer by shaking for 30 min. The solution was poured into a second test tube and 200 µl of toluene added. 4 ml of Tris buffer and 200 µl of toluene were added to the first test tube. The test tubes were shaken for 30 min. Aliquots of toluene were taken in GC

vials, which contained 20 µl of internal standard, namely eicosane at 0.1 mg/ml concentration. The experiment was then repeated with Tris buffer containing 10% of MeOH. The influence of MeOH addition to the entire SPE procedure was studied by loading amphetamine/Tris solution into the Oasis column. The column was washed with water. Analytes were eluted using three times of 200 µl of ethyl acetate. The extracts were placed in GC vials containing internal standard. The experiment was repeated with Tris buffer containing 10% of MeOH.

7.3 Results and discussion

7.3.1 Liquid-liquid extraction

7.3.1.1 Optimisation of buffer and solvent

At first, solubility, i.e. the dissolution power of the buffers was investigated. The TEST-1 sample was completely soluble in all buffers. The test sample (TEST-2) was not completely soluble in citrate and phosphate buffers due to the high amount of caffeine. The dissolution power of Tris buffer was better but not complete. In a homogeneity study of the amphetamine batches peak areas of target compounds were measured and relative response factors (RRF) calculated. The peak purity of target compounds was checked using the MSD. RSD values between replicates were acceptable: 3% for TEST-1 and 6% for TEST-2 sample. The dissolving problem might be the reason for the high deviation of homogeneity results from the TEST-2 sample.

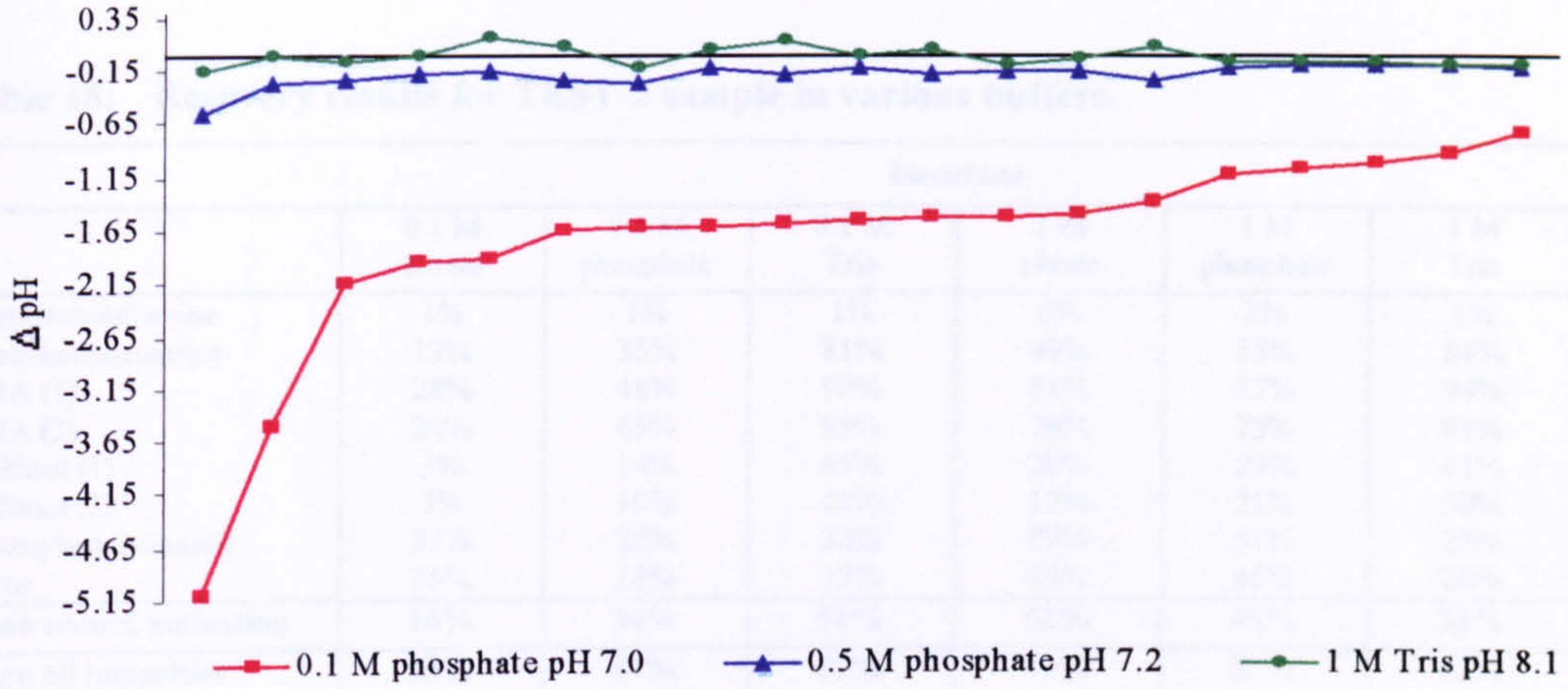


Figure 52: The pH change of a number of seized amphetamine samples when dissolved in different buffers.

The buffer capacity was studied with different types of amphetamines. To conclude results of this experiment, it was clear that the Tris buffer, partly due to its higher concentration, had better buffer capacity (Figure 52).

In the optimisation of the buffer/solvent system, the RRF values for ethyl acetate were corrected to 200 µl for TEST-1 and to 400 µl for TEST-2 from the originally used 400 and 800 µl. Additionally, the results for TEST-2 were multiplied by a factor of 5 because the amphetamine concentration of the TEST-2 sample was 20%. The values were normalised by calculating RRF/maximum RRF from each condition resulting directly in recovery values. Thereafter an average of the target compounds was calculated for each condition. The highest mean value indicates the best recovery (Table 18).

Table 18: Recovery results for TEST-2 sample in various buffers.

	Isooctane					
	0.1 M citrate	0.1 M phosphate	0.1 M Tris	1 M citrate	1 M phosphate	1 M Tris
Acetylamphetamine	1%	1%	1%	6%	2%	1%
Benzylamphetamine	13%	35%	81%	49%	58%	84%
DPIA (1)	28%	48%	90%	81%	77%	94%
DPIA (2)	24%	45%	88%	76%	75%	91%
Cathinol (1)	3%	14%	65%	20%	29%	61%
Cathinol (2)	3%	10%	46%	12%	21%	50%
Benzoylamphetamine	33%	22%	22%	89%	51%	25%
2-oxo	26%	18%	17%	84%	45%	20%
Mean reduct. amination	16%	24%	51%	52%	45%	53%
Mean all impurities	58%	57%	69%	49%	57%	65%
	Toluene					
	0.1 M citrate	0.1 M phosphate	0.1 M Tris	1 M citrate	1 M phosphate	1 M Tris
Acetylamphetamine	29%	23%	19%	100%	50%	21%
Benzylamphetamine	62%	78%	53%	86%	93%	77%
DPIA (1)	80%	85%	53%	92%	97%	80%
DPIA (2)	76%	82%	53%	90%	95%	78%
Cathinol (1)	42%	73%	47%	88%	98%	86%
Cathinol (2)	36%	77%	0%	77%	82%	69%
Benzoylamphetamine	100%	75%	48%	94%	96%	79%
2-oxo	100%	79%	49%	93%	95%	78%
Mean reduct. amination	66%	72%	40%	90%	88%	71%
Mean all impurities	74%	76%	78%	68%	78%	78%
	Ethyl acetate					
	0.1 M citrate	0.1 M phosphate	0.1 M Tris	1 M citrate	1 M phosphate	1 M Tris
Acetylamphetamine	89%	71%	82%	28%	91%	53%
Benzylamphetamine	37%	63%	100%	14%	54%	0%
DPIA (1)	44%	70%	100%	15%	55%	67%
DPIA (2)	41%	66%	100%	16%	54%	51%
Cathinol (1)	35%	62%	100%	16%	60%	58%
Cathinol (2)	19%	45%	73%	11%	46%	62%
Benzoylamphetamine	92%	76%	94%	21%	60%	54%
2-oxo	91%	77%	93%	21%	61%	62%
Mean reduct. amination	56%	66%	(93%)	18%	60%	51%
Mean all impurities	76%	78%	(91%)	52%	76%	83%

The chromatograms for different buffer/solvent systems are shown in Figure 53 in the same scale.

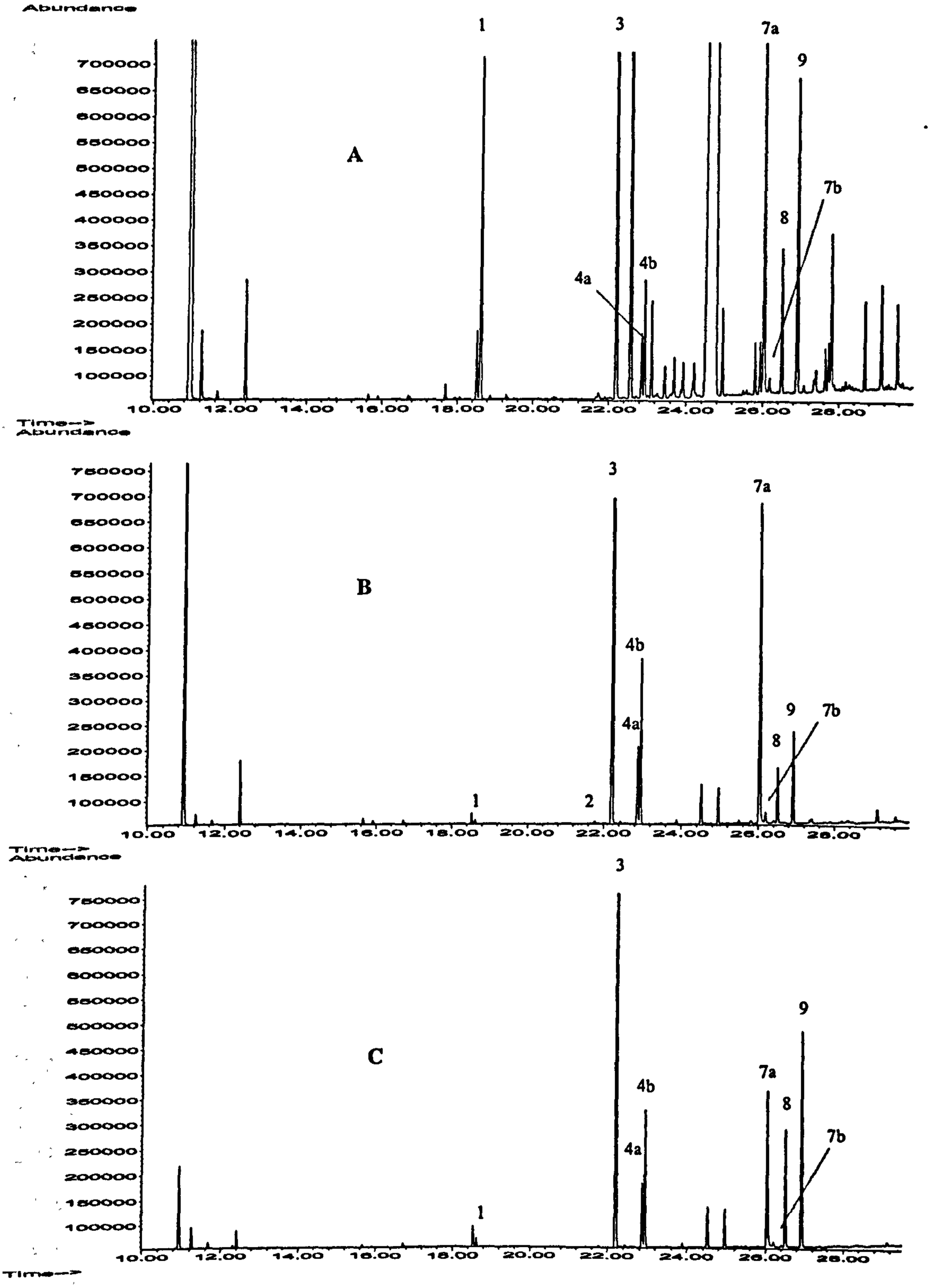


Figure 53: **Comparison of different buffers and solvents. The TEST-2 sample extracted with A) 1 M Tris/ethyl acetate, B) 1 M Tris/isooctane, C) 1 M phosphate/isooctane D) 1 M Tris/toluene and E) 1 M phosphate/toluene. Peak identification: 1) N-acetylamphetamine, 3) benzylamphetamine, 4a) and 4b) DPIA, 7a) and 7b) cathinol, 8) benzoylamphetamine and 9) 2-oxo.**

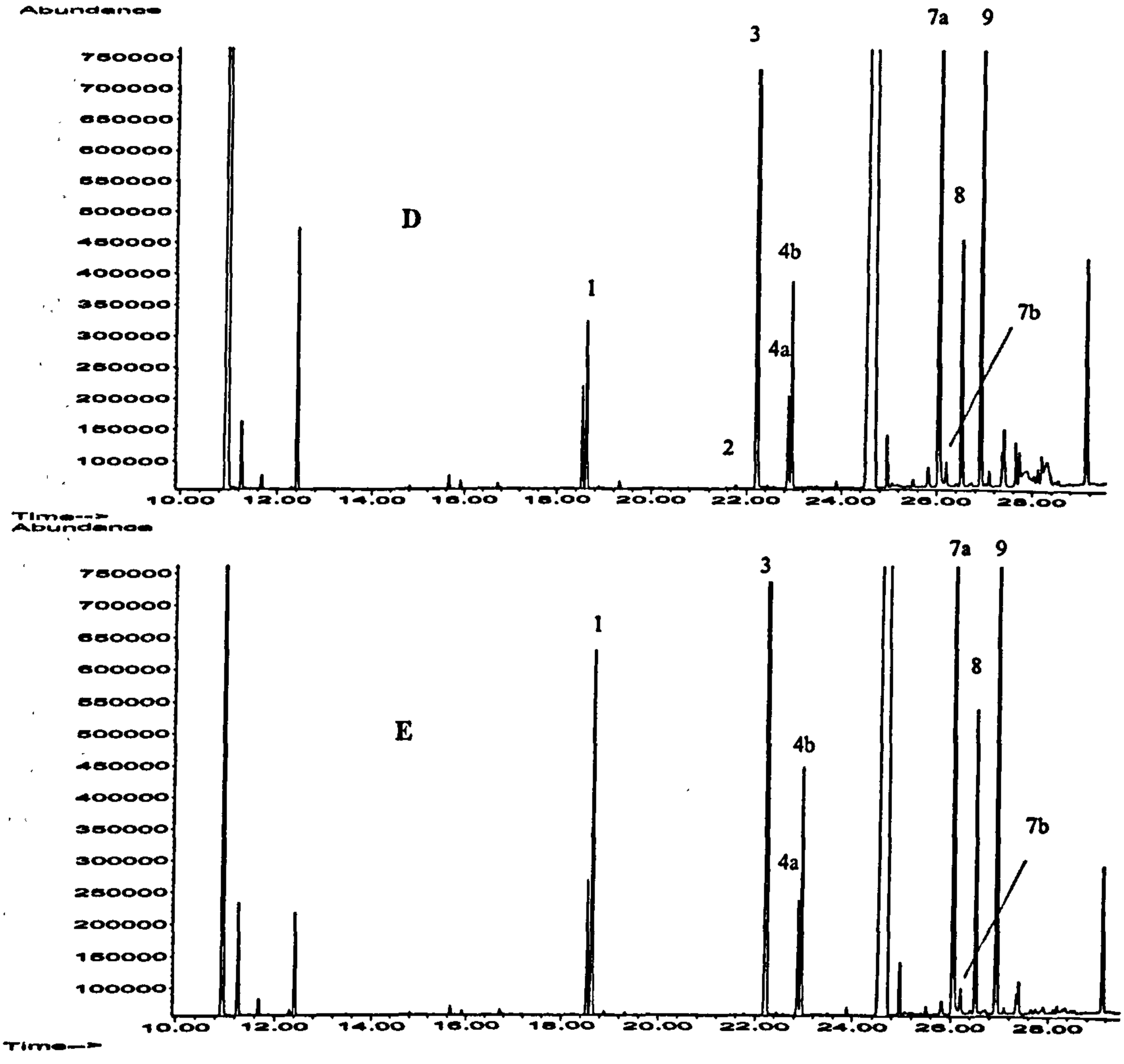


Figure 53: Cont'd / ...

Basic compounds, such as amphetamine, benzylamphetamine, DPIA and cathinol, extract better at a high pH, at pH 7.9. For the neutral compounds, such as N-acetylamphetamine, benzoylamphetamine and 2-oxo citrate at pH 6.20 and phosphate at pH 7.0 seem to perform better. Even though ethyl acetate gives the best recovery, isooctane and toluene were chosen for further studies due to the high water solubility of ethyl acetate. The results show that toluene gives somewhat better recovery than isooctane.

7.3.1.2 Optimisation of extraction procedure and matrix effect

Total recovery and RSD of results from the optimisation of the extraction procedure and matrix effect obtained with different methods were studied with the aim of finding the conditions that provide the highest recovery and the best repeatability. The sensitivity was calculated by dividing the RRF value of certain compounds under certain conditions by the maximum value for that compound. For each condition the mean value was calculated for all compounds to illustrate the overall performance. Recovery was calculated by dividing the RRF value by maximum value and taking the volume of solvent into consideration. The highest mean values indicate the best sensitivity and recovery. The lowest RSD value indicates the best repeatability of (three) replicates.

The RRF values were normalised against the amount of amphetamine for the calculation of the matrix effects. All experiments were made first using 8 ml buffer volume. Thereafter, the four best conditions were chosen and corresponding extractions repeated also for a 4 ml buffer volume. The results are summarised in Table 19. Recovery and repeatability results of the matrix effect study are summarised in Table 20 and Table 21, respectively.

Table 19: Recovery and repeatability results of extraction procedure.

RECOVERY

	Tris/toluene								phosphate/isooctane							
	2 ml + 200 µl	2 ml + 600 µl	2 ml + 3x200 µl	2 ml + 3x600 µl	8 ml + 200 µl	8 ml + 600 µl	8 ml + 3x200 µl	8 ml + 3x600 µl	2 ml + 200 µl	2 ml + 600 µl	2 ml + 3x200 µl	2 ml + 3x600 µl	8 ml + 200 µl	8 ml + 600 µl	8 ml + 3x200 µl	8 ml + 3x600 µl
Acetylamphetamine	17%	42%	48%	100%	6%	18%	19%	47%	2%	7%	7%	5%	1%	2%	2%	18%
Benzylamphetamine	78%	82%	96%	98%	73%	55%	96%	90%	63%	70%	100%	67%	39%	28%	79%	72%
DPIA (1)	76%	81%	94%	99%	72%	54%	94%	91%	66%	70%	100%	66%	45%	30%	82%	71%
DPIA (2)	75%	79%	93%	95%	71%	53%	93%	89%	64%	70%	100%	66%	45%	31%	83%	71%
Cathinol (1)	79%	80%	94%	90%	68%	52%	93%	83%	50%	70%	100%	68%	22%	25%	58%	84%
Cathinol (2)	79%	82%	92%	100%	69%	58%	93%	97%	52%	67%	97%	67%	23%	24%	59%	79%
Benzoylamphetamine	68%	78%	90%	96%	62%	52%	92%	88%	53%	69%	100%	72%	32%	29%	74%	84%
2-oxo	68%	79%	92%	100%	60%	52%	92%	89%	50%	60%	97%	71%	28%	27%	67%	83%
Mean reduc. amin.	**68%**	**76%**	**87%**	**97%**	**60%**	**49%**	**84%**	**84%**	**50%**	**60%**	**88%**	**60%**	**29%**	**24%**	**63%**	**70%**
Mean all impurities	**66%**	**76%**	**89%**	**94%**	**57%**	**63%**	**79%**	**84%**	**39%**	**49%**	**61%**	**57%**	**31%**	**35%**	**52%**	**60%**

REPEATABILITY

	Tris/toluene								phosphate/isooctane							
	2 ml + 200 µl	2 ml + 600 µl	2 ml + 3x200 µl	2 ml + 3x600 µl	8 ml + 200 µl	8 ml + 600 µl	8 ml + 3x200 µl	8 ml + 3x600 µl	2 ml + 200 µl	2 ml + 600 µl	2 ml + 3x200 µl	2 ml + 3x600 µl	8 ml + 200 µl	8 ml + 600 µl	8 ml + 3x200 µl	8 ml + 3x600 µl
Acetylamphetamine	5%	5%	2%	3%	0%	5%	2%	4%	2%	2%	3%	6%	9%	2%	6%	4%
Benzylamphetamine	6%	5%	2%	3%	6%	26%	3%	4%	3%	2%	3%	7%	17%	5%	7%	4%
DPIA (1)	5%	5%	1%	3%	7%	24%	3%	4%	3%	2%	4%	8%	21%	5%	8%	5%
DPIA (2)	5%	6%	2%	3%	6%	26%	3%	4%	3%	2%	4%	7%	20%	6%	7%	4%
Cathinol (1)	5%	6%	2%	3%	6%	26%	3%	5%	0%	2%	4%	9%	12%	4%	9%	4%
Cathinol (2)	4%	5%	3%	2%	6%	23%	4%	1%	2%	2%	4%	8%	14%	0%	8%	6%
Benzoylamphetamine	3%	5%	3%	4%	5%	26%	3%	4%	1%	3%	6%	9%	12%	5%	9%	4%
2-oxo	3%	5%	4%	3%	5%	25%	3%	4%	1%	7%	4%	9%	11%	4%	9%	4%
Mean reduc. amin.	**5%**	**5%**	**2%**	**3%**	**5%**	**21%**	**3%**	**4%**	**2%**	**3%**	**4%**	**8%**	**15%**	**4%**	**8%**	**4%**
Mean all impurities	**7%**	**5%**	**5%**	**7%**	**8%**	**10%**	**8%**	**7%**	**8%**	**5%**	**5%**	**7%**	**11%**	**6%**	**9%**	**7%**

The chromatograms of the three best conditions are shown in Figure 54.

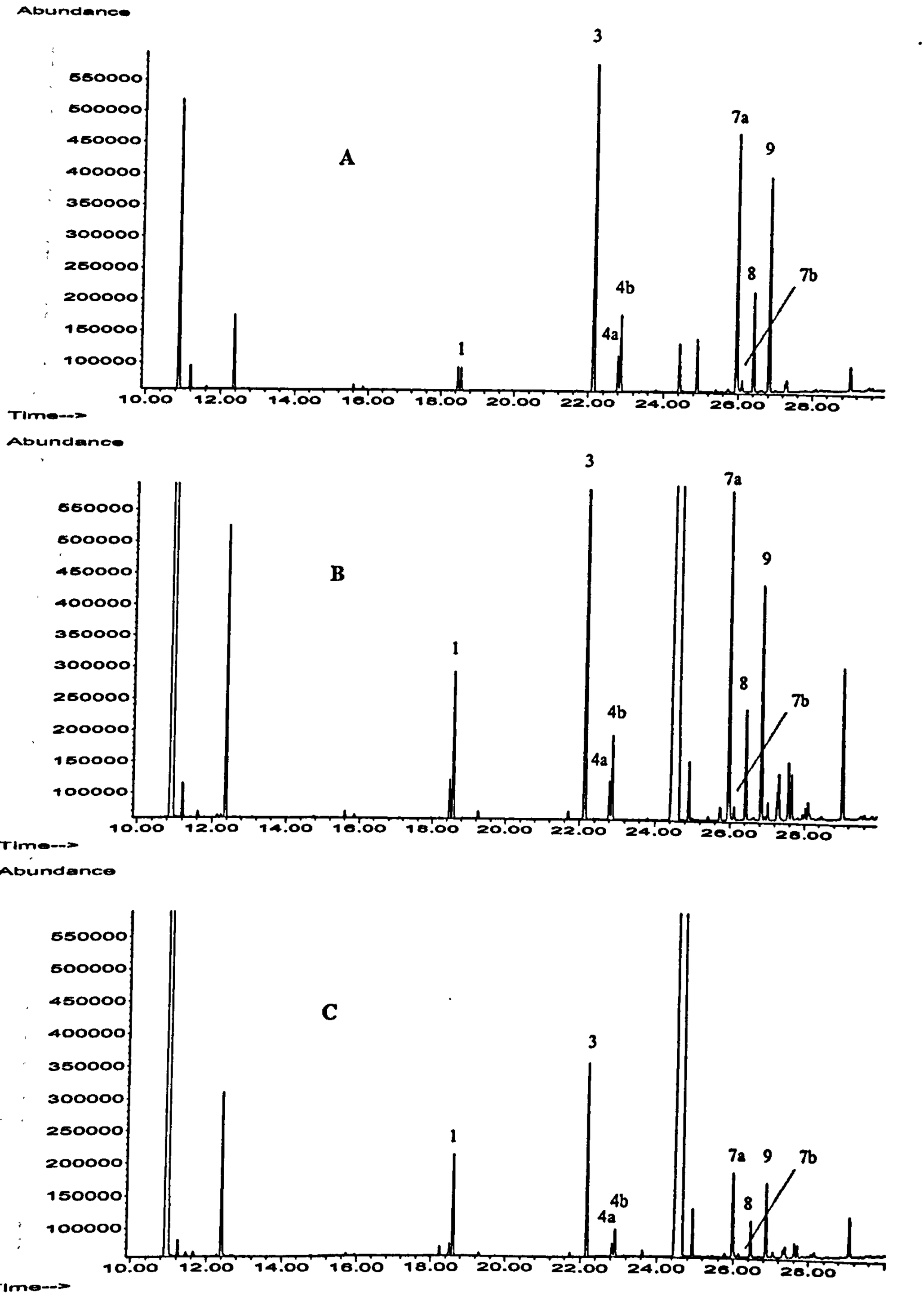


Figure 54: Comparison of different extraction procedures. The TEST-2 sample extracted with A) 2 ml of 1 M phosphate/3x200 µl of isooctane, B) 2 ml of 1 M Tris/3x200 µl of toluene and C) 2 ml of 1 M Tris/3x600 µl of toluene.

Table 20: Recovery results of matrix study.

RECOVERY																							
	8 ml Tris/tolu, 1-step			8 ml phoshate/isooct, 1-step			8 ml Tris/isooct, 1-step			8 ml phoshate/tolu, 1-step			8 ml Tris/tolu, 3-step			8 ml phosphte/isooct, 3-step			8 ml Tris/isooct, 3-step			8 ml phosphate/tolu, 3-step		
[mg]	30	100	200	30	100	200	30	100	200	30	100	200	30	100	200	30	100	200	30	100	200	30	100	200
Acetylamphet	4%	4%	5%	1%	1%	1%	0%	0%	0%	17%	16%	19%	13%	13%	14%	2%	2%	2%	0%	0%	0%	47%	44%	42%
Benzylamphet	57%	46%	56%	22%	42%	31%	13%	37%	35%	39%	31%	31%	89%	70%	65%	61%	69%	65%	33%	60%	42%	69%	69%	62%
DPIA (1)	55%	45%	55%	23%	50%	33%	15%	41%	37%	38%	30%	30%	86%	67%	62%	63%	73%	65%	35%	62%	43%	66%	66%	60%
DPIA (2)	57%	46%	56%	24%	50%	35%	16%	42%	40%	40%	32%	33%	89%	70%	65%	65%	75%	67%	36%	64%	46%	70%	69%	63%
Cathinol (1)	46%	44%	60%	11%	17%	18%	6%	17%	21%	31%	29%	33%	76%	66%	68%	35%	39%	49%	19%	37%	35%	58%	64%	62%
Cathinol (2)	42%	40%	65%	4%	13%	18%	4%	16%	22%	11%	24%	36%	26%	55%	70%	16%	17%	47%	4%	19%	30%	18%	46%	64%
Benzoylamphet	51%	42%	52%	23%	28%	28%	3%	6%	8%	41%	33%	36%	88%	69%	66%	59%	57%	60%	12%	17%	18%	73%	68%	66%
2-oxo	49%	42%	52%	21%	24%	25%	3%	5%	6%	42%	33%	36%	88%	68%	66%	55%	52%	57%	9%	13%	14%	74%	69%	66%
Mean red.ami.	**41%**	**35%**	**45%**	**15%**	**22%**	**21%**	**5%**	**13%**	**14 %**	**34%**	**28%**	**31%**	**71%**	**57%**	**56%**	**42%**	**44%**	**47%**	**15%**	**26%**	**22%**	**64%**	**63%**	**60%**
Mean		**41%**			**19%**			**11%**			**31%**			**61%**			**44%**			**21%**			**62%**	
Mean all impurities		**68%**			**51%**			**52%**			**66%**			**75%**			**61%**			**47%**			**74%**	

	4 ml Tris/tolu, 1-step			4 ml phoshate/isooct, 1-step			4 ml Tris/isooct, 1-step			4 ml phoshate/tolu, 1-step			4 ml Tris/tolu, 3-step			4 ml phosphate/isooct, 3-step			4 ml Tris/isooct, 3-step			4 ml phosphate/tolu, 3-step		
[mg]	30	100	200	30	100	200	30	100	200	30	100	200	30	100	200	30	100	200	30	100	200	30	100	200
Acetylamphet	16%	7%	8%	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	34%	28%	26%	41%	22%	25%	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	100%	66%	70%
Benzylamphet	76%	56%	44%	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	38%	61%	52%	100%	71%	59%	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	97%	74%	80%
DPIA (1)	74%	54%	42%	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	38%	59%	50%	100%	68%	57%	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	98%	72%	77%
DPIA (2)	76%	55%	44%	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	38%	61%	52%	100%	70%	59%	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	97%	74%	79%
Cathinol (1)	71%	56%	47%	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	36%	59%	54%	100%	73%	64%	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	97%	76%	84%
Cathinol (2)	53%	72%	56%	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	35%	74%	64%	85%	91%	83%	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	84%	95%	100%
Benzoylamphet	70%	48%	42%	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	40%	58%	52%	99%	66%	60%	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	100%	71%	80%
2-oxo	70%	47%	42%	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	40%	58%	52%	99%	67%	61%	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	100%	71%	81%
Mean red.ami.	**60%**	**43%**	**36%**							**38%**	**53%**	**47%**	**88%**	**60%**	**54%**							**99%**	**72%**	**79%**
Mean		**47%**									**46%**			**67%**									**83%**	
Mean all impurities		**75%**			**63%**			**69%**			**70%**			**77%**			**68%**			**64%**			**83%**	

n.a. The data is not available.

Table 21: Repeatability results of matrix study.

REPEATABILITY																							
	8 ml Tris/tolu, 1-step			8 ml phoshate/isooct, 1-step			8 ml Tris/isooct, 1-step			8 ml phoshate/tolu, 1-step			8 ml Tris/tolu, 3-step			8 ml phosphte/isooct, 3-step			8 ml Tris/isooct, 3-step			8 ml phosphate/tolu, 3-step		
[mg]	30	100	200	30	100	200	30	100	200	30	100	200	30	100	200	30	100	200	30	100	200	30	100	200
Acetylamphet	3%	1%	16%	18%	0%	3%	0%	16%	29%	16%	17%	0%	3%	2%	1%	28%	1%	0%	0%	13%	32%	7%	8%	13%
Benzylamphet	16%	7%	19%	31%	8%	13%	91%	21%	30%	31%	38%	8%	8%	8%	1%	27%	4%	4%	61%	20%	42%	13%	8%	2%
DPIA (1)	16%	6%	19%	32%	11%	13%	91%	22%	28%	31%	37%	8%	8%	8%	1%	26%	5%	3%	61%	19%	40%	13%	8%	1%
DPIA (2)	16%	7%	19%	34%	11%	11%	85%	21%	23%	30%	35%	7%	8%	8%	1%	28%	5%	3%	59%	19%	35%	13%	7%	1%
Cathinol (1)	19%	8%	19%	22%	4%	5%	81%	21%	17%	30%	37%	7%	11%	9%	1%	26%	2%	19%	52%	19%	27%	12%	8%	7%
Cathinol (2)	7%	17%	20%	11%	7%	9%	0%	34%	21%	41%	35%	9%	6%	15%	2%	1%	4%	33%	141%	20%	44%	31%	30%	15%
Benzoylamphet	9%	3%	18%	38%	8%	8%	47%	8%	22%	24%	37%	4%	6%	6%	1%	30%	3%	6%	34%	8%	29%	10%	10%	3%
2-oxo	7%	4%	18%	35%	8%	7%	48%	8%	23%	24%	37%	4%	6%	6%	1%	31%	3%	8%	35%	7%	29%	11%	9%	4%
Mean red.ami.	**12%**	**7%**	**19%**	**28%**	**7%**	**9%**	**74%**	**19%**	**24%**	**28%**	**34%**	**6%**	**7%**	**8%**	**1%**	**25%**	**3%**	**9%**	**63%**	**16%**	**35%**	**14%**	**11%**	**6%**
Mean		**12%**			**14%**			**39%**			**23%**			**5%**			**13%**			**38%**			**10%**	
Mean all impurities		**9%**			**6%**			**11%**			**11%**			**5%**			**9%**			**23%**			**6%**	

	4 ml Tris/tolu, 1-step			4 ml phoshate/isooct, 1-step			4 ml Tris/isooct, 1-step			4 ml phoshate/tolu, 1-step			4 ml Tris/tolu, 3-step			4 ml phosphate/isooct, 3-step			4 ml Tris/isooct, 3-step			4 ml phosphate/tolu, 3-step		
[mg]	30	100	200	30	100	200	30	100	200	30	100	200	30	100	200	30	100	200	30	100	200	30	100	200
Acetylamphet	2%	2%	2%	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	45%	3%	1%	4%	1%	1%	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	6%	2%	5%
Benzylamphet	3%	1%	3%	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	67%	3%	2%	3%	1%	1%	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	7%	4%	7%
DPIA (1)	3%	1%	4%	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	64%	3%	2%	3%	1%	1%	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	6%	4%	7%
DPIA (2)	3%	1%	3%	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	65%	3%	2%	2%	1%	1%	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	7%	4%	7%
Cathinol (1)	7%	1%	2%	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	64%	0%	1%	4%	0%	0%	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	7%	4%	2%
Cathinol (2)	19%	2%	2%	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	83%	2%	1%	10%	2%	1%	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	5%	7%	8%
Benzoylamphet	3%	2%	4%	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	59%	2%	3%	2%	1%	1%	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	9%	3%	7%
2-oxo	2%	2%	3%	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	59%	2%	3%	2%	1%	1%	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	9%	2%	7%
Mean red.ami.	**3%**	**2%**	**3%**							**59%**	**2%**	**2%**	**3%**	**1%**	**1%**							**8%**	**3%**	**6%**
Mean		**3%**									**21%**			**1%**									**5%**	
Mean all impurities		**4%**			**3%**			**3%**			**7%**			**3%**			**2%**			**4%**			**5%**	

n.a. The data is not available.

One-step extraction gave a clearly better sensitivity than three-step extraction due to the higher volume of the solvent in the latter case. The highest recovery was obtained with the Tris/toluene system, 2 ml buffer volume and three-step extraction method with 600 µl solvent volume. The results were equal for reductive amination amphetamine and for all amphetamine types. There were no significant differences in repeatability of results between the Tris/toluene and phosphate/isooctane systems.

In the matrix effect study, 4 ml buffer volume gave a better recovery compared with 8 ml. Pure amphetamine gave a better repeatability than the adulterated amphetamine sample, which indicates the occurrence a of matrix effect. Repeatability was somewhat better with 4 ml buffer volume.

7.3.2 Solid phase extraction

The target compounds were identified and relative response factors (RRF) calculated for each target compound. The recovery was calculated for the sum of the RRF of the three different fractions at each condition. The 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 (Table 22). Repeatability results are also shown in Table 22.

Table 22: Recovery and repeatability results of SPE for sum of three fractions.

RECOVERY									
	LLE	SPE: HCX column				SPE: Oasis column			
	4 ml Tris+tolu	4 ml Tris+et.ac.	8 ml Tris+et.ac.	4 ml Tris+tolu	8 ml Tris+tolu	4 ml Tris+et.ac.	8 ml Tris+et.ac.	4 ml Tris+tolu	8 ml Tris+tolu
Acetylamphetamine	100%	31%	5%	5%	2%	13%	10%	8%	7%
Benzylamphetamine	81%	77%	65%	99%	82%	82%	93%	98%	100%
DPIA (1)	72%	75%	63%	100%	83%	75%	83%	88%	89%
DPIA (2)	69%	73%	61%	100%	83%	71%	78%	85%	85%
Cathinol (1)	86%	81%	67%	83%	67%	96%	100%	99%	93%
Cathinol (2)	70%	67%	55%	76%	62%	87%	91%	100%	92%
Benzoylamphetamine	86%	15%	8%	11%	7%	85%	100%	81%	84%
2-oxo	90%	21%	13%	15%	8%	87%	100%	79%	82%
Mean reduc.aminat.	**82%**	**55%**	**42%**	**61%**	**49%**	**75%**	**82%**	**80%**	**79%**
Mean all impurities	**70%**	**61%**	**47%**	**43%**	**33%**	**72%**	**71%**	**73%**	**81%**
REPEATABILITY									
	LLE	**SPE: HCX column**				**SPE: Oasis column**			
	4 ml Tris+tolu	4 ml Tris+et.ac.	8 ml Tris+et.ac.	4 ml Tris+tolu	8 ml Tris+tolu	4 ml Tris+et.ac.	8 ml Tris+et.ac.	4 ml Tris+tolu	8 ml Tris+tolu
Acetylamphetamine	8%	16%	7%	10%	4%	5%	14%	8%	16%
Benzylamphetamine	8%	2%	6%	7%	15%	7%	3%	16%	5%
DPIA (1)	8%	3%	5%	5%	13%	8%	5%	13%	5%
DPIA (2)	8%	3%	5%	5%	13%	8%	5%	14%	6%
Cathinol (1)	6%	2%	7%	9%	11%	10%	6%	11%	7%
Cathinol (2)	5%	4%	6%	6%	8%	10%	6%	12%	6%
Benzoylamphetamine	7%	17%	11%	8%	4%	5%	4%	14%	5%
2-oxo	7%	16%	12%	10%	5%	5%	3%	13%	6%
Mean reduc.aminat.	**7%**	**8%**	**7%**	**8%**	**9%**	**7%**	**6%**	**13%**	**7%**
Mean all impurities	**9%**	**17%**	**14%**	**12%**	**13%**	**12%**	**6%**	**18%**	**7%**

The best recovery for reductive amination impurities was obtained with Oasis column using 8 ml of Tris buffer and ethyl acetate for elution of the analytes. The same system also gave the best repeatability. The recovery was as good as with the LLE method. The HCX column performed poorly in the extraction of the benzoylamphetamine and 2-oxo. In general, all SPE columns/methods tested in this experiment are not suitable for N-acetylamphetamine.

The chromatograms of the first fraction of the 8 ml of Tris/200 µl of toluene system using different SPE columns are shown in Figure 55. The chromatogram of the LLE system, Tris (4ml)/toluene (200 µl) is shown as a reference.

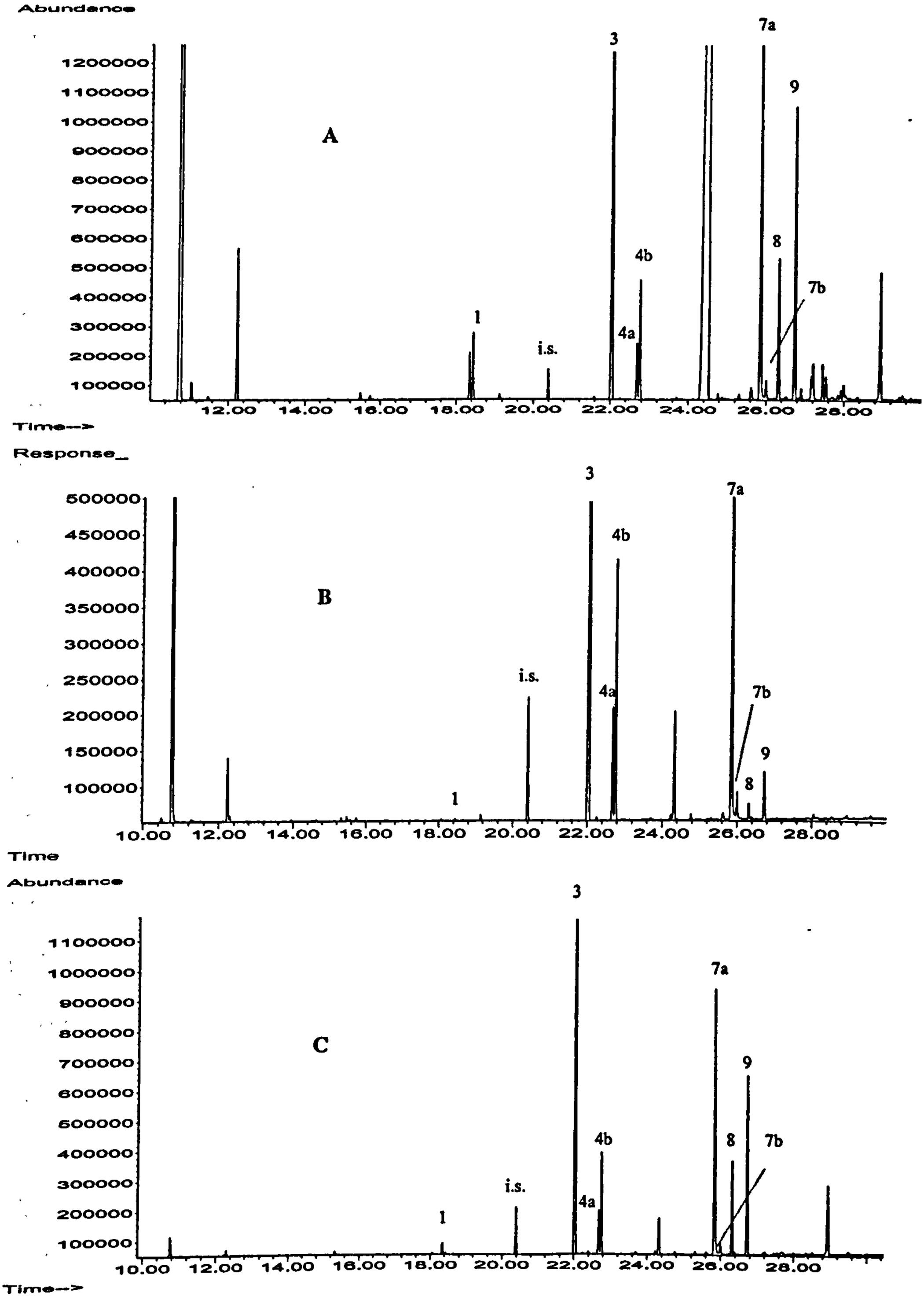


Figure 55: **Comparison of extraction techniques. A) LLE, B) SPE using HCX column and C) SPE using Oasis column. Peak identification: 1) N-acetylamphetamine, 3) benzylamphetamine, 4) DPIA, 7) cathinol, 8) benzoylamphetamine and 9) 2-oxo.**

To obtain more information concerning the SPE procedure, the overall recovery of each target compound in different SPE fractions was measured. The samples were prepared as described in the "Sample preparation II" and the "Reference sample preparation in LLE". The results are shown in Table 23. Moreover, SPE technique compared to LLE.

Table 23: Decomposition of the compounds in different SPE fractions and comparison of SPE and LLE.

HCX column							
	1. extract (et.ac.)	2. extract (et.ac.)	3. extract (et.ac.)	4. extract (MeOH)	Elute (tolu)	Wash (tolu)	SPE/LLE
Acetylamphetamine	2%	3%	3%	2%	41%	49%	13%
Benzylamphetamine	76%	3%	0%	0%	0%	20%	93%
DPIA (1)	81%	8%	2%	1%	0%	8%	97%
DPIA (2)	85%	9%	1%	0%	0%	5%	98%
Cathinol (1)	48%	1%	1%	0%	0%	50%	93%
Cathinol (2)	60%	0%	0%	0%	0%	40%	96%
Benzoylamphetamine	4%	0%	0%	0%	1%	95%	17%
2-oxo	5%	0%	0%	0%	1%	94%	23%
Mean	**45%**	**3%**	**1%**	**0%**	**5%**	**45%**	**66%**
Oasis column							
	1. extract (et.ac.)	2. extract (et.ac.)	3. extract (et.ac.)	4. extract (MeOH)	Elute (tolu)	Wash (tolu)	SPE/LLE
Acetylamphetamine	85%	0%	0%	0%	6%	8%	13%
Benzylamphetamine	99%	1%	0%	0%	0%	0%	100%
DPIA (1)	99%	1%	0%	0%	0%	0%	102%
DPIA (2)	99%	1%	0%	0%	0%	0%	101%
Cathinol (1)	99%	0%	0%	0%	0%	0%	111%
Cathinol (2)	100%	0%	0%	0%	0%	0%	124%
Benzoylamphetamine	98%	1%	0%	1%	0%	0%	100%
2-oxo	99%	1%	0%	0%	0%	0%	96%
Mean	**97%**	**1%**	**0%**	**0%**	**1%**	**1%**	**93%**

Some reductive amination impurities were missing when SPE was used. For reductive amination impurities, the HCX column gave only a 66% recovery compared with the LLE method. The Oasis column gave nearly the same result as the LLE method. The target compounds extracted through the HCX column were found at a great extent in the "wash" fraction, i.e. from the water fraction. As much as 45%

of all impurities, and in particular benzoylamphetamine and 2-oxo, were lost when washing the column with water.

Attempts to resolve the washing solution problems described above were made by replacing water with Tris buffer. The experiment was undertaken only for Leuckart type amphetamine. The samples were prepared as described in Sample preparation III. RRF values obtained from the SPE method were compared with values from the LLE method. The results are summarised in Table 24.

Table 24: Influence of different washing solvents in SPE measured with Oasis column.

	Water as washing solvent/LLE			Buffer as washing solvent/LLE		
	1. extract	2. extract	3. extract	1. extract	2. extract	3. extract
4-Methyl-5-phenylpyrimidine	63%	1%	0%	56%	50%	0%
4-Benzylpyrimidine	59%	0%	0%	52%	0%	0%
DPPA	137%	2%	1%	104%	2%	1%
DPIA (1)	135%	2%	1%	117%	2%	1%
DPIA (2)	139%	1%	1%	123%	1%	0%
DPIMA (1)	132%	1%	1%	115%	1%	0%
DPIMA (2)	132%	3%	2%	116%	2%	0%
2,6-Dimethyl-3,5-diphenylpyridine	31%	6%	0%	18%	0%	0%
2,4-Dimethyl-3,5-diphenylpyridine	37%	7%	0%	20%	7%	0%
"Pyridine 7"	58%	3%	0%	35%	0%	0%
DPIF (1)	95%	1%	0%	50%	1%	0%
DPIF (2)	97%	1%	1%	51%	1%	0%
Mean	**93%**	**2%**	**0%**	**71%**	**6%**	**0%**

The results show clearly that water performs better than the Tris buffer. The Tris buffer especially dissolves more pyridine and DPIF compounds than water. Also the second fraction contained more impurities when buffer was used as the washing solvent.

7.3.2.1 Test tube effect

Influence of methanol in test tube effect in SPE

Results obtained above in Table 24 do not completely explain the loss of target compounds during the SPE. A straightforward conclusion can be made that more than 70% of pyridines and DPIF compounds could stick to the wall of the test tube while amphetamine solution is being poured onto the SPE column. This explains the poor recovery for these compounds in SPE. This phenomenon, referred to as the "test tube effect", can be explained as most of the target compounds are partly or entirely insoluble in the buffer solution. To overcome this problem, MeOH addition with the aim of improving solubility of the target compounds was therefore tested. However, use of an overly high concentration of MeOH may cause breakthrough in SPE. Thus, the influence of MeOH addition on the entire SPE procedure was examined. The results are shown in Table 25. Mean values indicated the percentage left in the test tubes.

Test tubes and shaking system in SPE

The influence of different test tubes and shaking systems in SPE was studied by comparing used glass tubes with new ones. The test tubes were studied using a vertical shaking system. In the study of different shaking systems the used test tubes were used. In the first shaking system, the tubes were shaken in vertical position with the result that an oily layer could be seen on the top of the aqueous phase. Secondly, the tubes were placed horizontally in the shaking system. In this case the oily layer could not be seen. Target compounds left in a test tube were calculated for a system applying Tris buffer alone and for Tris with 10% MeOH. Results are shown in Table 25.

Table 25: Influence of addition of MeOH on the entire SPE procedure and in test tube effect using different shaking position and type of test tubes.

Test tube effect with 0% MeOH					
	Used glass test tube	New glass test tube	Vertical shaking	Horizontal shaking	
4-Methyl-5-phenylpyrimidine	2%	1%	4%	2%	
4-Benzylpyrimidine	2%	2%	4%	3%	
DPPA	2%	2%	4%	2%	
DPIA (1)	5%	2%	10%	3%	
DPIA (2)	4%	2%	9%	3%	
DPIMA (1)	15%	6%	29%	7%	
DPIMA (2)	17%	6%	33%	7%	
2,6-Dimethyl-3,5-diphenylpyridine	66%	36%	74%	33%	
2,4-Dimethyl-3,5-diphenylpyridine	66%	36%	72%	33%	
"Pyridine 7"	56%	25%	65%	25%	
DPIF (1)	54%	20%	74%	21%	
DPIF (2)	55%	21%	74%	21%	
Mean	**29%**	**13%**	**38%**	**13%**	

Test tube effect with 10% MeOH					Entire SPE procedure
	Used glass test tube	New glass test tube	Vertical shaking	Horizontal shaking	10% MeOH / 0% MeOH
4-Methyl-5-phenylpyrimidine	2%	1%	3%	2%	71%
4-Benzylpyrimidine	2%	2%	4%	2%	68%
DPPA	2%	1%	3%	2%	85%
DPIA (1)	2%	1%	3%	2%	89%
DPIA (2)	2%	1%	3%	2%	90%
DPIMA (1)	0%	1%	4%	2%	93%
DPIMA (2)	5%	1%	5%	2%	94%
2,6-Dimethyl-3,5-diphenylpyridine	34%	10%	33%	16%	102%
2,4-Dimethyl-3,5-diphenylpyridine	34%	9%	35%	14%	69%
"Pyridine 7"	27%	5%	23%	10%	88%
DPIF (1)	16%	3%	11%	4%	95%
DPIF (2)	16%	3%	12%	4%	99%
Mean	**12%**	**3%**	**12%**	**5%**	**87%**

MeOH addition significantly reduced the test tube effect for pyridines and DPIF compounds. 10% MeOH concentration is sufficient to remove the pyridines from the test tube. The same improvement can be also obtained if new borosilicate glass test tubes or the horizontal shaking system are used. The test tube effect was decreased by over a third, i.e. from 29% to 13% and 38% to 13%, respectively. Chromatograms in Figure 56 illustrate the influence of new shaking system and MeOH addition in impurities left in the old test tubes.

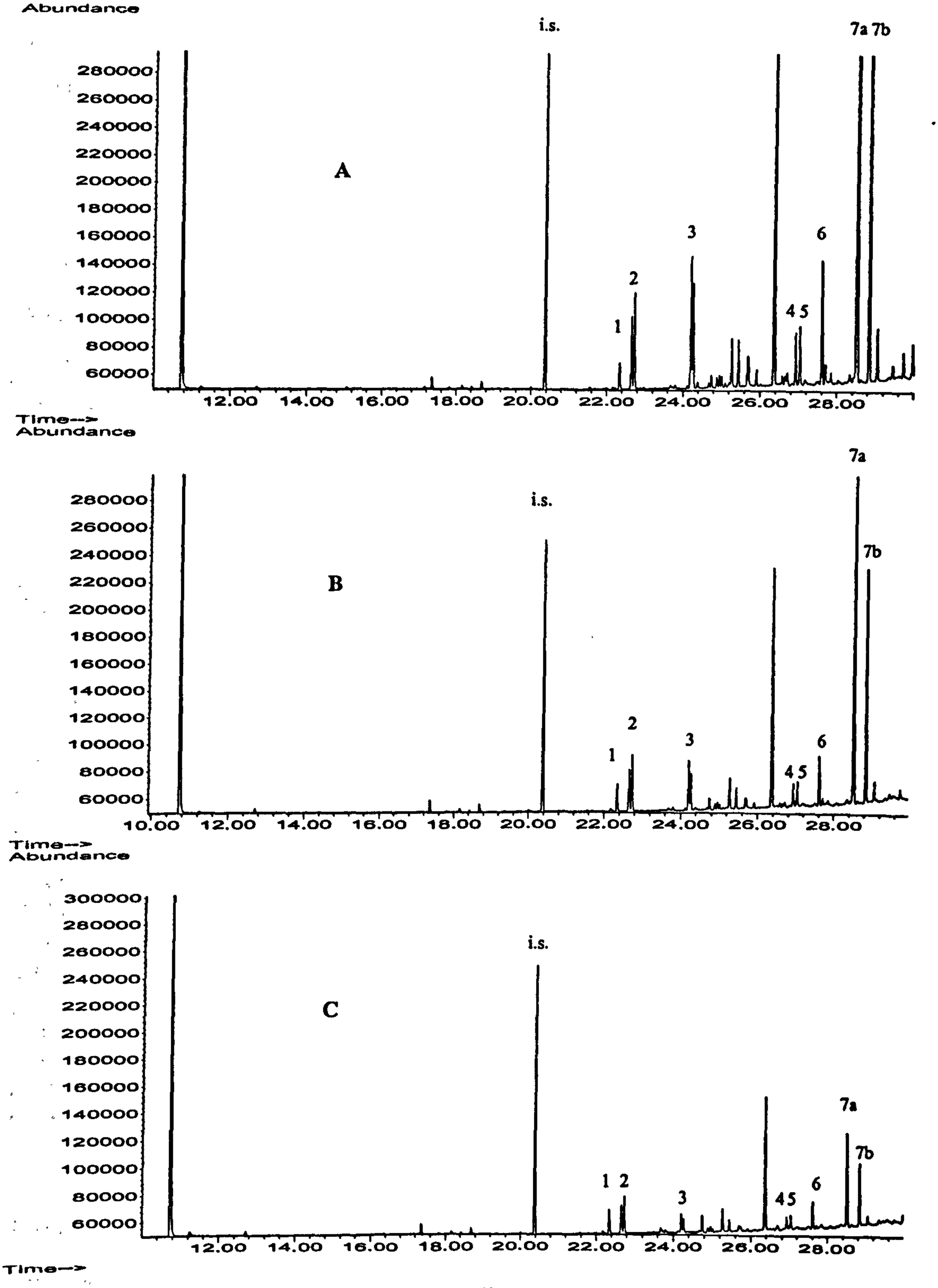


Figure 56: **Comparison of test tube effect using different shaking systems. A) Vertical shaking system with 0% MeOH, B) horizontal shaking system with 0% MeOH and C) horizontal shaking system with 10% MeOH. Peak identification: 1) DPPA, 2) DPIA, 3) DPIMA, 4) 2,6-dimethyl-3,5-diphenylpyridine, 5) 2,4-dimethyl-3,5-diphenyl-pyridine, 6) "pyridine 7", 7a) and 7b) DPIF.**

In conclusion, combination of new tubes and 10% MeOH addition cause a 10-fold decrease in the test tube effect. As a consequence of MeOH addition, some target compounds were, however, partly lost during sample loading onto the SPE column (Table 24).

Test tubes and shaking times in LLE

Influence of different test tubes and shaking times was also studied in LLE. Hitherto 30 min shaking time has been used to dissolve the amphetamine sulphate into solvent and for extraction. The procedure is very time consuming. In this study the influence of shorter shaking time was studied. The results are shown in Table 26.

Table 26: Comparison of different shaking times and test tubes in LLE.

	New glass tube 5min/30min	Used glass tube 5min/30min	Polyprop. tube 5min/30min	Used tube/New tube (30min)	Polyprop /New tube (30 min)
4-Methyl-5-phenylpyrimidine	100%	101%	103%	100%	94%
4-Benzylpyrimidine	101%	101%	82%	102%	137%
N-Formylamphetamine	101%	103%	96%	101%	99%
DPPA	100%	100%	95%	102%	99%
DPIA (1)	100%	100%	96%	102%	103%
DPIA (2)	100%	100%	96%	102%	103%
DPIMA (1)	100%	99%	96%	101%	102%
DPIMA (2)	100%	100%	96%	101%	102%
2,6-Dimethyl-3,5-diphenylpyridine	99%	96%	96%	100%	112%
2,4-Dimethyl-3,5-diphenylpyridine	100%	100%	118%	100%	107%
"Pyridine 7"	98%	98%	92%	98%	104%
DPIF (1)	100%	100%	91%	101%	109%
DPIF (2)	100%	101%	89%	101%	109%
Mean	**100%**	**100%**	**97%**	**101%**	**106%**

From the results it is clear that 5 min shaking time gave same results as 30 min shaking time. Accordingly, no difference was observed between the used and new glass test tubes. For polypropylene tubes, 30 min shaking time gave better results than 5 min shaking time. Repeatability was good under all conditions; RSD was only 2 - 5%. In conclusion, 10 min shaking time was chosen for dissolving and extracting the amphetamine sulphate in further experiments.

7.4 Conclusion

The results were better for high concentration buffers (1 M) than for low concentration (0.1 M) buffers in all cases. Citrate was the best buffer for reductive amination impurities. In general, most of the amphetamine compounds are, however, more basic. In the development of a harmonised method for amphetamine profiling this needs to be taken into account. Some important target compounds might still be left in the aqueous phase using citrate buffer at pH 6.2. Therefore citrate was discarded from forthcoming studies.

In conclusion, by taking the practical aspects as well as the results into consideration, the best LLE performance was obtained with a system applying Tris and toluene. The same system applying phosphate buffer gives nearly the same results. Of the SPE columns, the Oasis column appeared to perform better than the HCX column. In general, the repeatability of the LLE methods was better than that of the SPE method due to significant test tube effect in SPE sample preparation. 10 min shaking time was sufficient in the extraction process. The final choice of sample preparation technique was LLE applying 4 ml of 1 M Tris (pH 8.1) and 200 µl of toluene.

8 COMPARISON STUDY

8.1 Introduction

Four laboratories in total participated in the development of a harmonised method for the profiling of amphetamine. Several experiments were performed as described in previous chapters in order to find the best conditions for the GC analysis with a good inter-laboratory reproducibility. Repeatability and reproducibility were defined as the degree to which profiles from the same sample analysed within one day and between different days respectively matched. A corresponding experiment has been done for heroin between three laboratories [89]. In the study intra-laboratory reproducibility was sufficient, but reproducibility was poor between different laboratories. The results showed the comparisons at the international level are best carried out in a central laboratory. It is, however, known that street heroin is more difficult material from the profiling point of view than synthetic drugs such as amphetamine. The ideal situation would be that amphetamine profiling could be performed at national laboratories utilising a common international database.

Visual comparison is a frequently used method for the comparison analysis in some laboratories. The entire chromatograms of two samples are compared. It is, however, rather laborious and more importantly "coarse" as the result is based on subjective evaluation only. It is therefore often reasonable to first compare the samples utilising numerical classification methods, e.g. distance methods. Additionally, the visual comparison can be performed in questionable cases. Frequently utilised distance methods are described in more detail in Chapter 2.7.1. Classification or grouping of samples is a method for finding similarities or dissimilarities within data sets. Several cluster methods can be used for the classification of large data set. The methods have been described in detail in Chapter 2.7.2.

8.2 Experimental

8.2.1 Intra- and inter-laboratory study

Three reductive amination amphetamine batches were synthesised. Each batch was spiked with pure impurities due to the absence of the target compounds or the concentration of these compounds being to low. Amphetamine batches were diluted with caffeine and lactose resulting in amphetamine at ca. 20% concentration. In addition, one sample was prepared in order to compare results between diluted and undiluted samples. Part of the batch 1 was diluted further to 1:10. Each sample was analysed at different time delays: 0, 7, 14, 28, 42 and 56 days (t_0, ... , t_{56}). Six replicates were analysed for each batch a chosen day to determine the variation within a day (repeatability). The variation between days (reproducibility) was calculated using the values of different days.

Sample preparation (the optimised method)

200 mg of amphetamine was dissolved in 4 ml of 1 M Tris (pH 8.10) and shaken for 10 minutes. 200 µl of toluene, which contained eicosane at concentration of 10 µg/ml, was added and shaken for further 10 min. The tubes were centrifuged for 2 - 3 min at ca. 2500 rpm to separate the phases. An aliquot of the toluene layer was taken and placed in a GC vial containing insert vial.

GC analysis (the optimised method)

Two 30 m (L) x 0.25 mm (i.d.), coated with 0.25 µm of 35% phenyl methyl silicone (DB-35MS) capillary columns were connected with the 2.5m (L) x 0.25 mm (i.d.) pre-column and the divider. Splitless injection and a tapered splitless liner were used. The injector temperature was 250°C. The temperature of detectors was 310°C. 2 µl was injected into each column. The oven temperature program was 8°C/min. For GC-FID program the retention time of internal standard was set at 16.30 min using the retention time locking (RTLock) macro at the ChemSation software. Peak areas

of the target compounds and the internal standard recorded by the FID were measured and relative response factors (RRF) calculated.

Quality control

Each sequence was started with two blank samples for checking extraction system purity. The stability of the GC-MSD-FID unit was controlled throughout the experiment using the Grob mixture. For repeatability control, Leuckart type amphetamine was analysed in the beginning and after each sequence.

8.2.2 Comparison study

Three new batches of amphetamine using the reductive amination synthetic route were synthesised. Each of these batches was prepared so that the different amphetamine concentrations were 5, 40 and 100%. This was achieved by dilution with appropriate amounts of adulterants, i.e. caffeine and lactose. Different concentrations of the same batch were produced to see if the intensity of the impurity profile has any influence on the comparison study. Seized amphetamine samples were also analysed to establish a test database. 100 casework samples, which were presumed to be reductive amination amphetamines, were collected. In addition to standard impurity substances, some new compounds were taken into consideration. The new substances were identified on the basis of the Wiley275 Rev. D.02.00 MS library (Agilent Technology, Palo Alto, CA, the USA).

The reductive amination compounds and the target compounds from other amphetamine reactions were collected into "Quantitation Database Globals" tool of MS ChemStation software. The tool was utilised to identify the target compounds from chromatograms and to give a quantitation report. The quantitation report contains retention times and the peak area response of target compounds. The data was collected from FID and MSD, but in this experiment only MSD data, utilising the peak areas of target ions, was used.

The peak areas of target compounds were combined from the quantitation reports. To avoid a large peak dominating the profile, the peak response was divided by corresponding standard deviation of the whole data set (equation 2). The influence of the peaks on the distance method is then independent of the peak size. It was also necessary to normalise the responses because seized samples originating from the same batch may have a different amphetamine concentration. To compensate the influence of different concentration the weighted peak area was divided by the sum of the weighted peak areas (equation 3). The importance of equal weighting and normalisation has been also pointed out in the previous amphetamine profiling study [27].

Different distance and classification methods were used in the data handling. In this experiment, Euclidean distance, the Pearson correlation coefficient and Cosine function methods were studied. Identified peaks were utilised to find similarities or dissimilarities between the analysed samples. The results from the Euclidean distance were introduced as relative values, i.e. the individual distance was divided by maximum value of horizontal values and multiplied by 100. The models were carried out with SPSS for Windows software package Rev.10.1.3.

8.3 Results and discussion

8.3.1 Intra-and inter-laboratory study

Repeatability and reproducibility were measured as described in Chapter 6.3.3. The chromatograms of the reductive amination amphetamine batches analysed by three different laboratories, namely NBI, SKL and IPSC are shown in Figure 57.

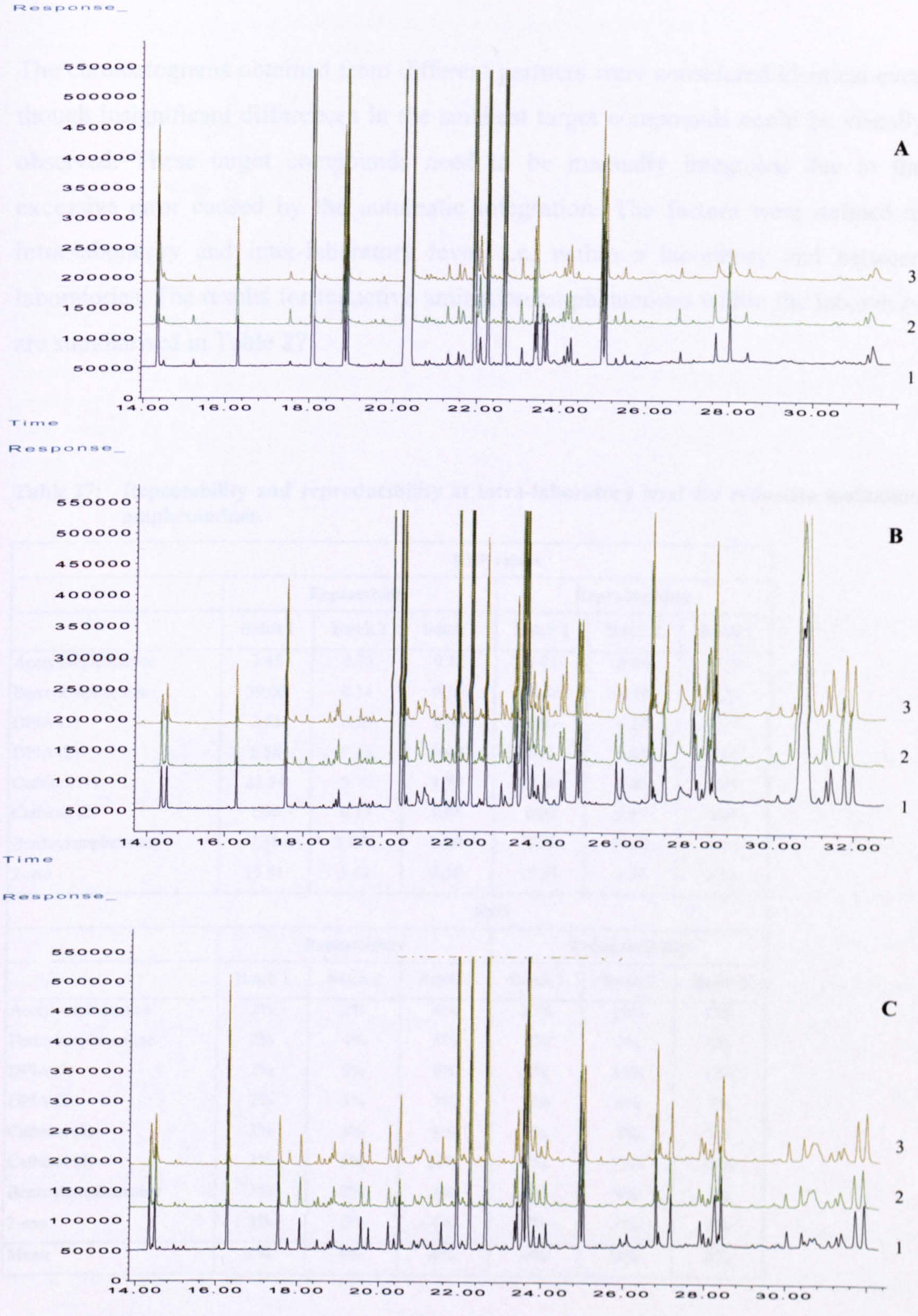


Figure 57: Chromatograms of synthesised reductive amination amphetamines analysed by NBI (1), IPSC (2) and SKL (3). A) Batch 1, B) batch 2 and C) batch 3.

The chromatograms obtained from different partners were considered identical even though insignificant differences in the smallest target compounds could be visually observed. These target compounds need to be manually integrated due to the excessive error caused by the automatic integration. The factors were defined at intra-laboratory and inter-laboratory level, i.e. within a laboratory and between laboratories. The results for reductive amination amphetamines within the laboratory are summarised in Table 27.

Table 27: Repeatability and reproducibility at intra-laboratory level for reductive amination amphetamines.

RRF values						
	Repeatability			Reproducibility		
	Batch 1	Batch 2	Batch 3	Batch 1	Batch 2	Batch 3
Acetylamphetamine	3.45	0.75	0.32	3.73	0.86	0.37
Benzylamphetamine	39.00	0.14	0.16	38.85	0.14	0.17
DPIA (1)	2.53	0.23	0.04	2.50	0.22	0.04
DPIA (2)	5.58	0.43	0.09	5.52	0.40	0.10
Cathinol (1)	21.54	5.70	1.79	21.39	5.40	1.75
Cathinol (2)	1.06	0.17	0.05	1.00	0.17	0.05
Benzoylamphetamine	7.38	15.52	1.60	7.25	14.53	1.57
2-oxo	15.81	3.62	2.38	15.51	3.38	2.33
RSD						
	Repeatability			Reproducibility		
	Batch 1	Batch 2	Batch 3	Batch 1	Batch 2	Batch 3
Acetylamphetamine	2%	2%	6%	11%	12%	13%
Benzylamphetamine	2%	4%	5%	2%	3%	4%
DPIA (1)	2%	9%	8%	2%	11%	12%
DPIA (2)	2%	3%	7%	2%	6%	7%
Cathinol (1)	3%	3%	5%	3%	8%	7%
Cathinol (2)	3%	6%	13%	9%	13%	11%
Benzoylamphetamine	3%	2%	4%	2%	9%	5%
2-oxo	3%	2%	4%	2%	9%	6%
Mean	2%	4%	6%	4%	9%	8%

Intra- and inter-laboratory results for each amphetamine batch are shown in Table 28.

Table 28: Intra- and inter-laboratory results for all amphetamine types.

	Intra-laboratory		Inter-laboratory
	Repeatability	Reproducibility	Reproducibility
Reductive amination batch 1	2%	4%	4%
Reductive amination batch 2	4%	9%	12%
Reductive amination batch 3	6%	8%	7%
Leuckart batch 1	10%	8%	9%
Leuckart batch 2	6%	7%	10%
Leuckart batch 3	5%	13%	6%
Leuckart batch 4	3%	5%	7%
Leuckart batch 5	6%	10%	8%
Leuckart batch 6	3%	9%	11%
Nitrostyrene batch 1	6%	12%	11%
Nitrostyrene batch 2	10%	7%	11%
Nitrostyrene batch 3	8%	8%	10%
Mean	**5%**	**8%**	**9%**

The table above (Table 28) indicates the variability obtained for 12 samples by three participating laboratories. The RSD values illustrate the sum of different errors, namely instrument error, sample preparation error and inhomogeneity of the samples. The results indicate good repeatability and reproducibility at intra- and inter-laboratory levels. The repeatability and reproducibility at intra-laboratory level ranged from 2% to 10% and 4% to 13%, respectively. At inter-laboratory level the variability of the same sample analysed over 6 days (in a two month period) ranged from 4% to 12%. In general, there were insignificant differences between intra- and inter-laboratory results. The developed profiling method can be considered excellent in terms of repeatability and reproducibility, also at the inter-laboratory level.

8.3.2 Comparison study

Several synthesised amphetamine batches at different concentrations and 100 seized street samples were analysed and the results introduced into a database. The target compounds with MSD ions - a target ion (Tgt) and two qualifiers (q1 and q2), and retention times for FID and MSD are given in Table 29. The structure of most compounds is detailed in Chapter 3.3.

Table 29: Compounds utilised in the harmonised profiling method.

#	Impurities	MSD ions (Tgt, q1, q2)	MSD t_R (min)	FID t_R (min)	Synthetic route
1	2-Methyl-3-phenylaziridine	132, 133, 117	6.96	8.63	Nitrost.
2	1-Phenyl-2-propaneoxime (1)	149, 131, 132	8.87	10.39	Nitrost.
3	1-Phenyl-2-propaneoxime (2)	131, 116, 130	8.94	10.48	Nitrost.
4	4-Methyl-5-phenylpyrimidine	170, 169, 102	11.80	13.45	Leuck.
5	N-Propylbenzamide	105, 77, 163	12.38	13.93	Nitrost.
6	4-Benzylpyrimidine	169, 170, 115	12.43	14.07	Leuck.
7	N-Acetylamphetamine	118, 117, 86	12.78	14.36	Aminat., Leuck.
8	N-Formylamphetamine	118, 72, 117	13.03	14.64	Leuck.
9	1,2-Diphenethylamine	106, 79, 107	15.29	16.87	Nitrost.
10	N,N-Dibenzylamine	106, 92, 91	15.56	17.12	Aminat., Nitrost.
11	1,2-Diphenylethanone	105, 77, 196	16.24	17.78	Aminat., Nitrost.
12	Benzylamphetamine	134, 91, 135	16.54	18.09	Aminat., Leuck.
13	DPPA	120, 121, 103	16.85	18.38	Leuck.
14, 15	DPIA (1) and (2)	162, 163, 119	17.41 and 17.52	18.88 and 19.00	Aminat., Leuck.
16	α-Methyl-diphenethylamine	148, 105, 119	17.64	19.11	Aminat., Nitrost.
17, 18	DPIMA (1) and (2)	176, 177, 119	18.92 and 19.00	20.39 and 20.47	Leuck.
19	Unknown substance D (2)	160, 143, 128	19.84	21.30	Leuck.
20	1,3-Dimethyl-2-phenylnaphthalene	232, 217, 215	20.04	21.48	Leuck.
21	Unknown substance D (3)	143, 160, 128	20.53	22.00	Leuck.
22	Cathinol	162, 163, 161	20.54	21.96	Aminat.
23	1-Benzyl-3-methylnaphthalene	232, 217, 215	20.70	22.14	Leuck.
24	Unknown substance D (4)	143, 160, 128	20.77	22.23	Leuck.
25	Benzoylamphetamine	105, 148, 149	20.82	22.26	Aminat., Leuck.
26	Unknown substance E (2)	120, 143, 160	21.10	22.51	Leuck.
27	2-Oxo	162, 163, 118	21.27	22.67	Aminat., Leuck.
28	2,6-Dimethyl-3,5-diphenylpyridine	259, 258, 260	21.46	22.85	Leuck.
29	2,4-Dimethyl-3,5-diphenylpyridine	259,258, 260	21.60	23.01	Leuck.
30	"Pyridine 7 and 14" (co-eluted)	258, 259, 260	22.08	23.47	Leuck.
31	"Pyridine 272"	272, 273, 258	22.26	23.64	Leuck.
32	2,6-Diphenyl-3,5-dimethylpyridine	258, 259, 244	23.01	24.39	Leuck.
33, 34	DPIF (1) and (2)	190, 191, 119	23.26 and 23.63	24.64 and 24.99	Leuck.

Some compounds mentioned in Table 29, for example, DPIA and benzylamphetamine were found from amphetamines synthesised via different synthesis routes. As additional findings, two or more isomers were detected for some substances. The first isomers of unknown compound D and E were not utilised as they co-eluted with other compounds. The structures of three pyridines could not be identified and they were named as pyridine 7, 14 and 272. In total 34 different substances were listed for reductive amination, Leuckart and nitrostyrene reactions. The number of substances for different reaction types were 9, 18 and 7, respectively. The GC-MSD chromatogram of the control sample is shown in Figure 58.

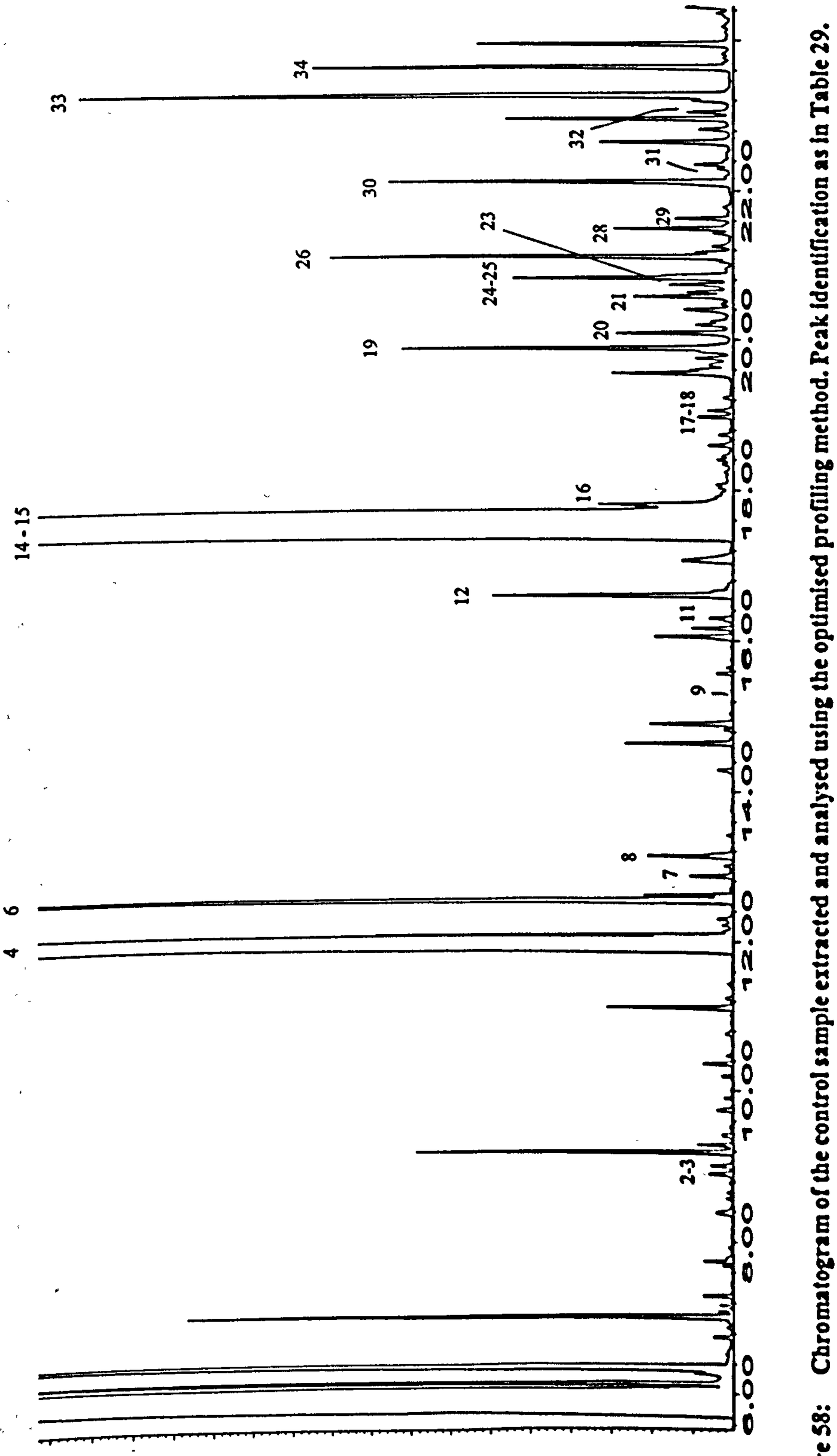


Figure 58: Chromatogram of the control sample extracted and analysed using the optimised profiling method. Peak identification as in Table 29.

The 34 peaks identified above were utilised to find similarities and dissimilarities between the analysed samples. The distances were calculated using the Euclidean distance, the Pearson correlation coefficient and the Cosine function methods. The distances were calculated for a 100% synthesised sample relative to diluted samples, other synthesised batches (100%) and all street samples. The results were introduced as distances and RSD values. The results from the distance models are shown in Table 30.

Table 30: Results of different distance methods. Each 100% reductive amination batch was compared to diluted samples, other synthesised batches (100%) and all dataset.

100% Batch 1						
	Euclidean		Cosine		Pearson	
	Distance	RSD	Distance	RSD	Distance	RSD
40% Batch 1	5.49	13%	0.53	27%	0.58	27%
5% Batch 1	7.75	35%	1.12	55%	1.22	55%
100% Batch 2	68.64	1%	84.43	0%	93.20	0%
100% Batch 3	71.27	1%	82.08	0%	89.81	0%
All dataset	57.40	33%	81.55	22%	92.22	21%
100% Batch 2						
	Euclidean		Cosine		Pearson	
	Distance	RSD	Distance	RSD	Distance	RSD
40% Batch 2	1.72	21%	0.04	42%	0.04	44%
5% Batch 2	9.44	18%	0.58	33%	0.57	33%
100% Batch 3	16.22	3%	3.36	4%	3.61	4%
All dataset	58.86	38%	78.65	22%	88.29	22%
100% Batch 3						
	Euclidean		Cosine		Pearson	
	Distance	RSD	Distance	RSD	Distance	RSD
40% Batch 3	0.93	19%	0.01	23%	0.01	22%
5% Batch 3	4.01	13%	0.08	23%	0.07	22%
All dataset	60.63	34%	82.49	22%	91.52	22%

Once the different distance methods were compared, it can be seen that the Cosine and Pearson models performed better than the Euclidean distance. The distances between linked samples, i.e. undiluted and diluted samples, were much smaller than they were when using the Euclidean distance method. Correspondingly, much longer

distance between unlinked samples occurred using the Cosine and Pearson models. It is clear that the similarity between 100% and 40% samples was better than between 100% and 5% samples. The short distance between synthesised batches 2 and 3 indicated some similarities. In general, each synthesised batch was significantly different compared to all street samples. Naturally, individual similarities can be found.

In addition to the batch level comparison, HCA and PCA models were used for classification. The HCA is based on the results of distance method. The results are presented as a dendrogram. The part of dendrogram measured by the Cosine function method is shown in Figure 59.

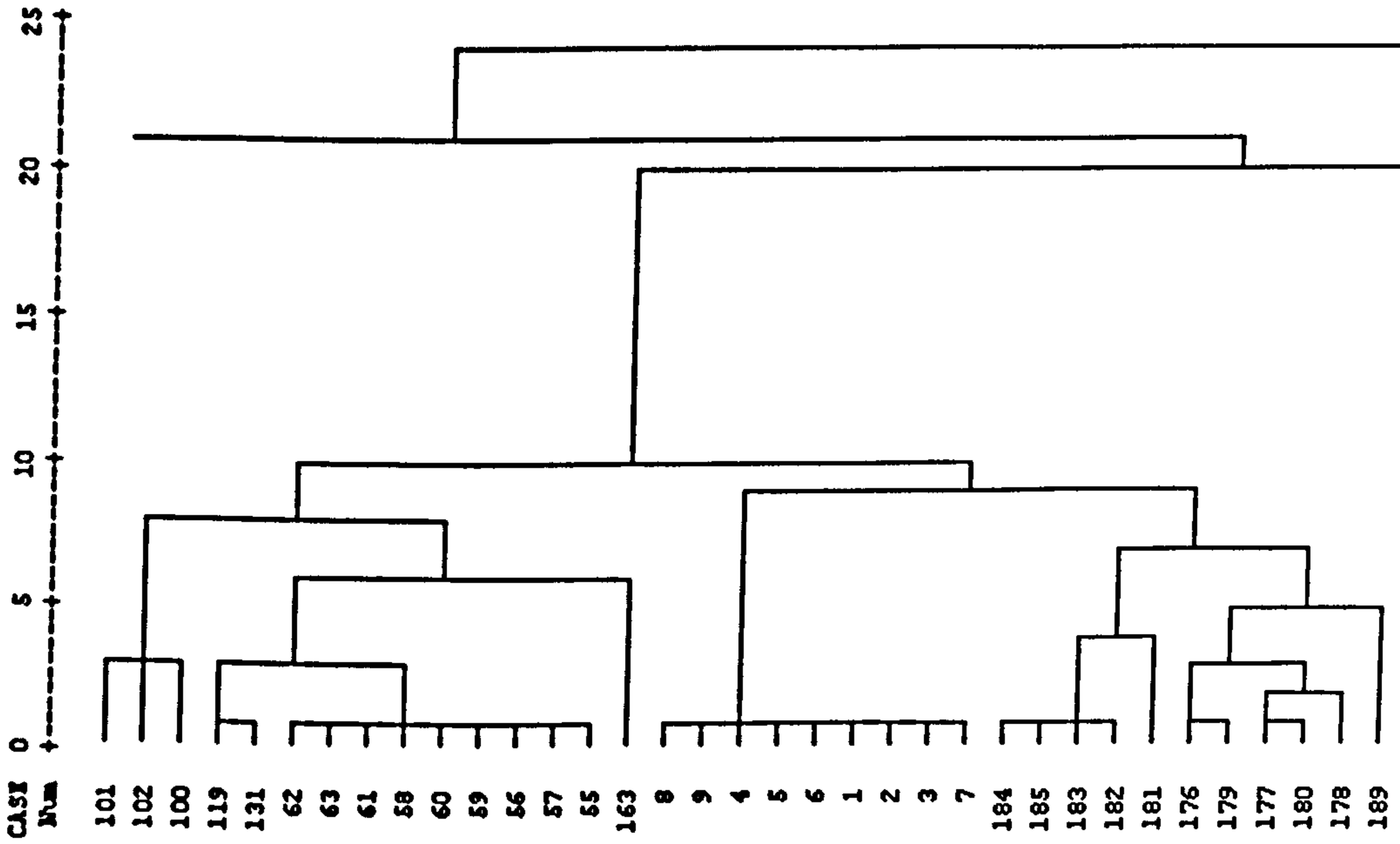


Figure 59: Part of a dendrogram measured by Cosine function method.

A strong link between sample numbers 1 - 9 and 55 - 63 was observed. The linkage is clear as there are three replicates of 100% (1 - 3), of 40% (4 - 6) and of 5% (7 - 9) reductive amination amphetamine of the same batch. Correspondingly, samples 55 - 63 originate from the same Leuckart batch.

The PCA was carried out with two different software packages. The SPSS was used to obtain numerical data and using the Unscrambler software data was able to present this data graphically. Part of the SPSS results is shown in Table 31. Over 200 analysed samples were grouped into 25 groups and the highest values indicate a group number and a link between samples. In this example (reductive amination batches 2 and 3) the samples have a linkage between one another, but the linkage does not exist between other groups.

Table 31: Part of classification results obtained with SPSS.

	Class number														
Sample	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
100% Batch 2	97%	-3%	-1%	-3%	-6%	0%	0%	4%	6%	3%	21%	-2%	0%	0%	0%
100% Batch 2	97%	-3%	-1%	-3%	-7%	0%	0%	4%	6%	4%	21%	-2%	0%	0%	0%
100% Batch 2	97%	-3%	-1%	-3%	-7%	0%	0%	4%	6%	2%	20%	-2%	0%	0%	0%
40% Batch 2	97%	-3%	-1%	-3%	-6%	0%	0%	4%	6%	5%	20%	-1%	0%	0%	0%
40% Batch 2	97%	-3%	-1%	-3%	-7%	0%	0%	4%	6%	5%	20%	-1%	0%	0%	0%
40% Batch 2	97%	-3%	-1%	-3%	-6%	0%	0%	4%	6%	5%	19%	-1%	0%	0%	0%
5% Batch 2	98%	-2%	-1%	-2%	-6%	1%	1%	3%	7%	2%	13%	-1%	0%	1%	0%
5% Batch 2	99%	-2%	-1%	-2%	-5%	1%	1%	3%	7%	1%	13%	-1%	0%	1%	0%
5% Batch 2	97%	-2%	0%	-2%	-6%	0%	0%	4%	6%	3%	19%	-2%	0%	1%	0%
100% Batch 3	99%	2%	4%	-4%	-4%	1%	9%	3%	7%	-5%	-1%	-2%	3%	0%	1%
100% Batch 3	99%	2%	4%	-4%	-4%	1%	9%	3%	7%	-5%	0%	-2%	3%	0%	0%
100% Batch 3	99%	2%	5%	-4%	-4%	1%	9%	3%	7%	-5%	0%	-2%	3%	0%	1%
40% Batch 3	99%	2%	4%	-4%	-4%	1%	8%	3%	7%	-5%	1%	-2%	3%	0%	1%
40% Batch 3	99%	2%	4%	-4%	-4%	1%	8%	3%	7%	-5%	0%	-2%	3%	0%	1%
40% Batch 3	99%	2%	4%	-4%	-4%	1%	8%	3%	7%	-5%	0%	-2%	3%	0%	1%
5% Batch 3	99%	2%	4%	-3%	-3%	1%	8%	3%	8%	-5%	-2%	-4%	3%	0%	1%
5% Batch 3	99%	2%	3%	-3%	-4%	1%	8%	3%	8%	-5%	-1%	-4%	2%	1%	1%
5% Batch 3	99%	2%	3%	-3%	-4%	1%	7%	3%	8%	-5%	-1%	-4%	2%	0%	1%

The grouping of synthesised amphetamine batches using the Unscrambler is shown in Figure 60.

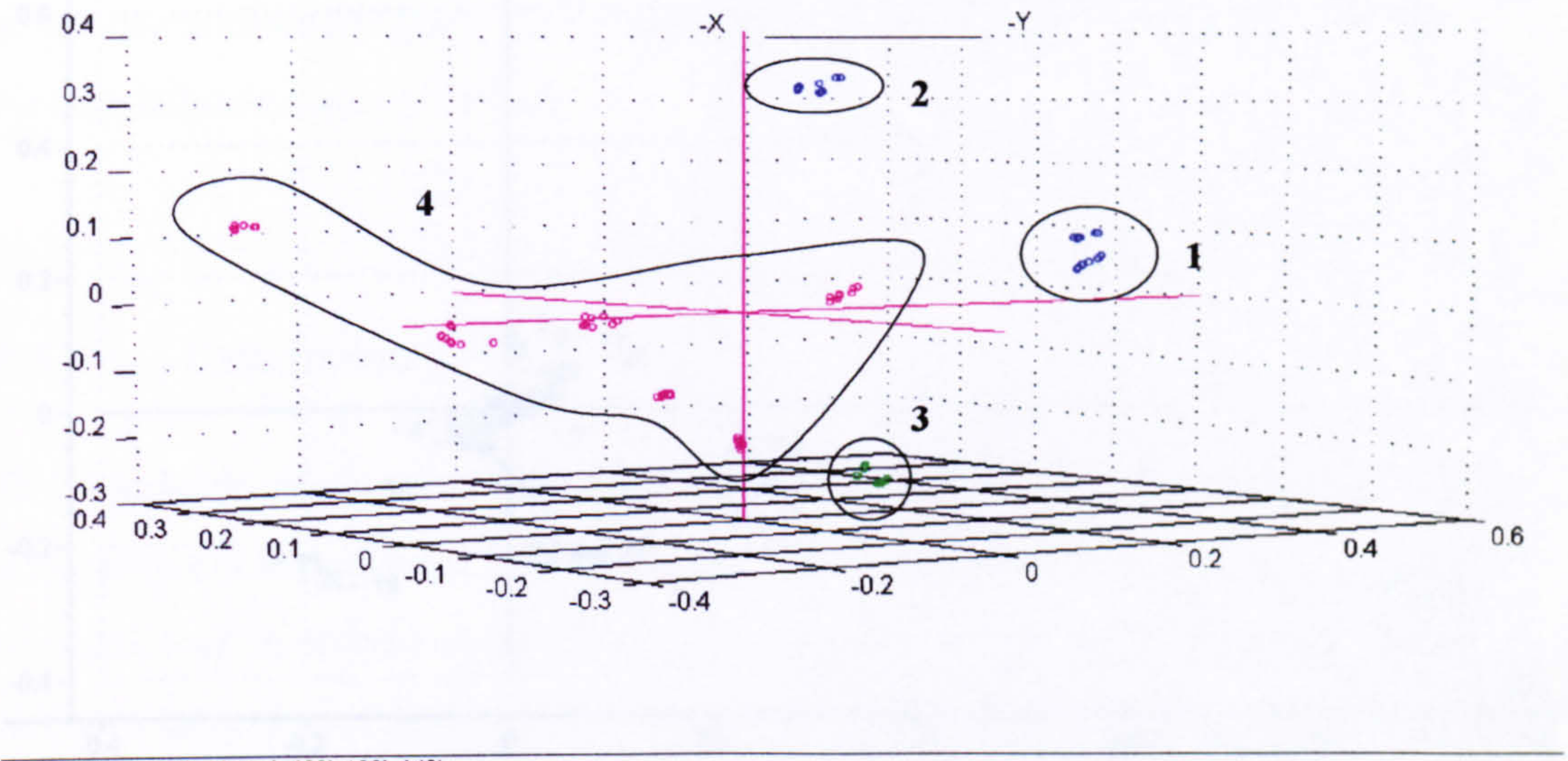


Figure 60: PCA results for synthesised amphetamine batches. The samples have been classified into four groups (1 - 4).

Four separated groups could be identified in the graphical illustration. The two reductive amination batches (batches 2 and 3) were similar and belong into the group 1. These samples were also grouped into the same class in the Table 31. The third reductive amination batch (batch 1) was significantly different. The samples belong into the group 2. The nitrostyrene samples are separated from other samples and grouped into the group 3. The largest, dispersed group 4 includes Leuckart samples.

The Unscrambler software enables also to detect the correlation between impurities. A short distance in the graphical illustration in Figure 61 indicates a significant correlation.

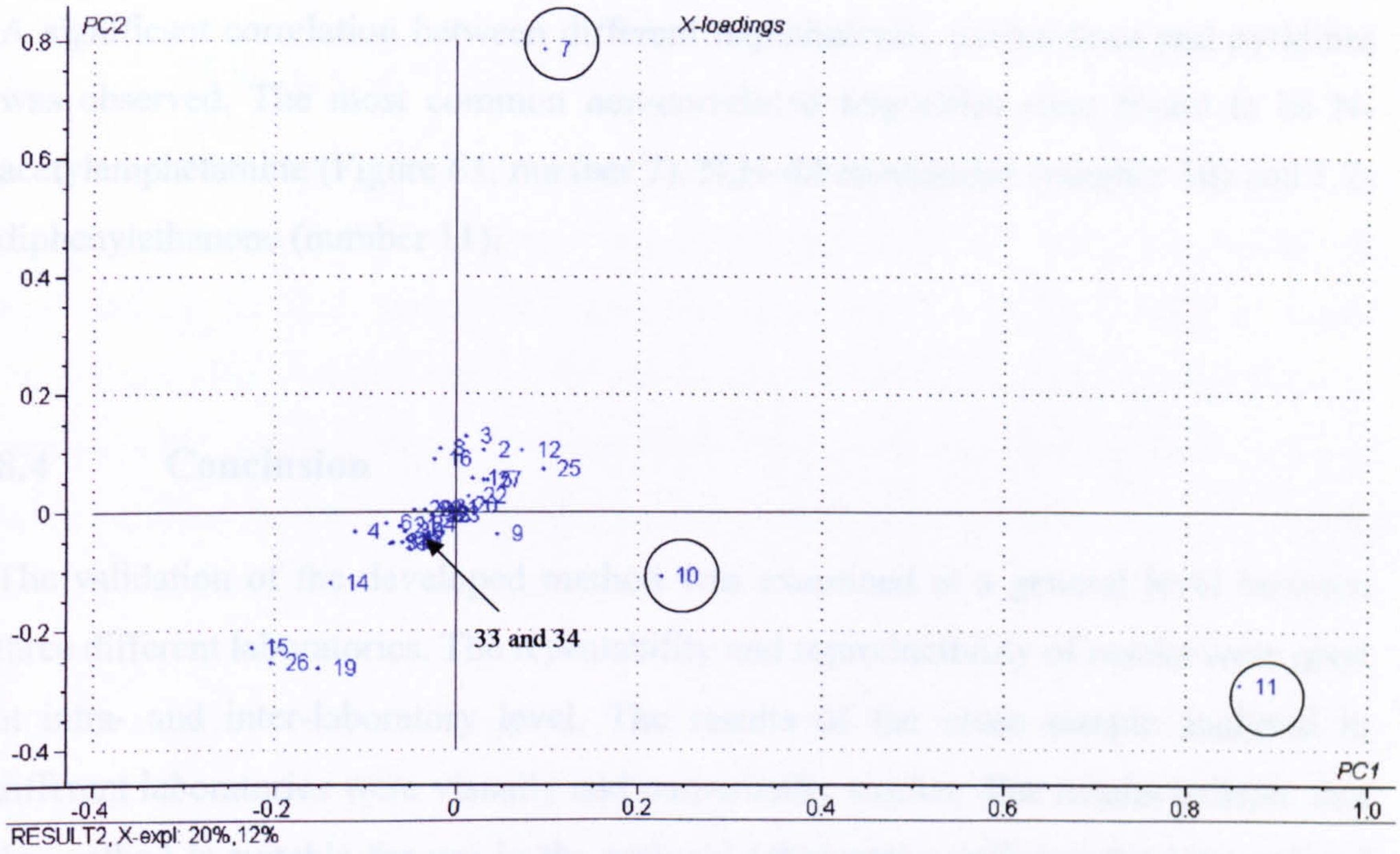


Figure 61: Illustration of correlation between impurities. Non-correlated impurities have been circled.

All isomers are naturally significantly correlated, for example, two isomers of DPIF (33 and 34). Numerical values can also be calculated. The correlation coefficient for two isomers of DPIF was 0.994739. The result is also shown as a correlation curve (Figure 62).

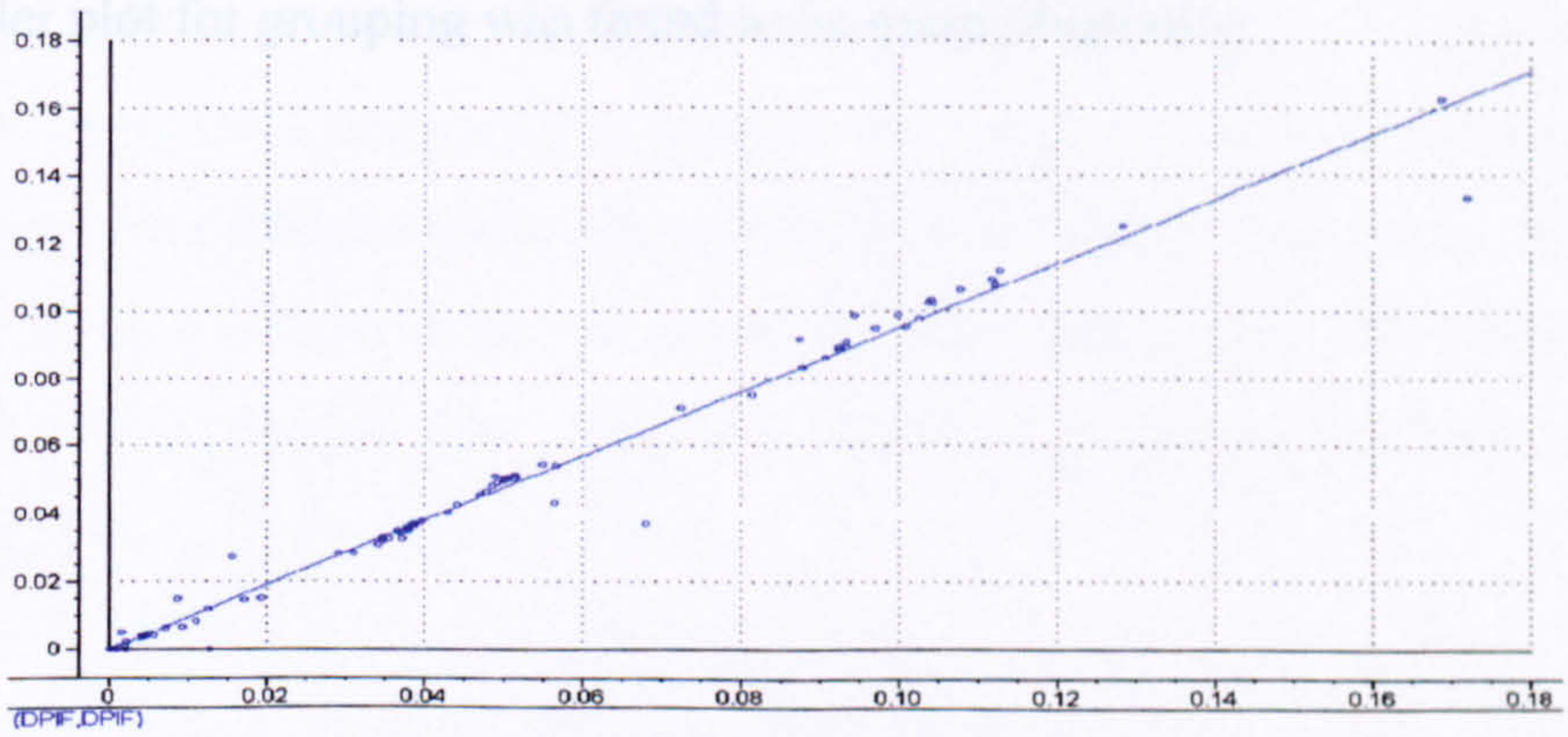


Figure 62: Correlation between two isomers of DPIF.

A significant correlation between different naphthalenes, pyrimidines and pyridines was observed. The most common non-correlated impurities were found to be N-acetylamphetamine (Figure 61, number 7), N,N-dibenzylamine (number 10) and 1,2-diphenylethanone (number 11).

8.4 Conclusion

The validation of the developed method was examined at a general level between three different laboratories. The repeatability and reproducibility of results were good at intra- and inter-laboratory level. The results of the same sample analysed in different laboratories were visually and numerically similar. The results indicate that the method is suitable for use in the national laboratories utilising the international database.

In general, several distance methods are available to perform the batch level comparison. The Euclidean distance method was found to be very sensitive for changes, i.e. a small modification has a significant influence on the results. The Pearson and Cosine methods gave equal results. Different synthetic routes were picked up by PCA. The classification results were presented graphically and numerically. In practice, the large dendrograms are difficult to handle. The Unscrambler plot for grouping was found to be more illustrative.

9 FINAL CONCLUSION

A chemical impurity profiling method compatible with amphetamines produced by three different synthesis routes was developed. It was discovered that the optimisation of GC parameters and the extraction conditions were of equal importance. The main advantages of the developed method include (i) suitability for non-Leuckart amphetamines and (ii) high repeatability at inter-laboratory level, which enables the profiling to be performed in different laboratories and the data to be introduced into a common database.

The development of the new profiling method was based on standard impurities. Ten standard impurities typical to reductive amination amphetamine were synthesised. Unfortunately, most of these compounds can also be found in other type of amphetamines, hence these compounds cannot be considered as route specific compounds. Moreover, the stability study showed the unsuitability of some specific reductive amination impurities for the profiling method.

The difference between the injection techniques was not significant. Some differences were observed between different injection temperatures. Results for both split and splitless injections were rather similar to those of the cool on-column injection. In conclusion, both vaporising techniques could be used for the sample introduction. The cool on-column technique cannot be used for real world samples as they contain non-volatile material. The main advantage of the splitless injection is that the target compounds can be detected at low concentration and thus splitless at 250°C was finally chosen as the sample introduction technique. Coincidentally, the same injection technique has also been used in previous profiling methods [39,51,158,159].

Non-polar capillary columns, non-linear column temperature programs and long analysis times have frequently been used in previous amphetamine profiling methods [39]. The choice of the analytical column in the present study was based on the good resolution of the target compounds, high separation power and the good stability of

the stationary phase of the column. The main advantage of choosing the DB-35MS column was that it fulfils all requirements at the linear temperature program at 8°C/min. Good separation power and resolution were achieved in feasible analysis time (37 min).

Using the splitless injection technique requires the use of a pre-column. However, it was found that the lifetime of an ordinary pre-column was limited as non-volatile compounds gradually migrate through the pre-column and get stuck to the wall of the analytical column causing peak tailing. Use of a thin-film DB-35MS as a pre-column performed similarly as a retention gap, but it possessed a significantly longer lifetime.

The choice of the detection technique was complex. FID is usually considered as the first choice for a profiling method because of its high reproducibility. The more selective MSD, however, offers many advantages over the FID. Peak identification, which is based on, in addition to the retention times, the specific mass spectra is more reliable. Even the use of the retention time locking does not enable easy peak identification without the structural information of analytes available from the MSD. Differences in the repeatability and reproducibility results between FID and MSD were found to be insignificant, the final choice for the detection technique was MSD. The final GC method has been summarised in detail in Appendix 1.

In previous amphetamine profiling methods, LLE technique, applying a low concentration phosphate buffer at pH 7.0, has regularly been used. This requires laborious and slow adjustment of pH after dissolving the amphetamine. This problem was solved nicely by choosing a high concentration (1 M) buffer. No significant differences between the performance between buffers were found. As most amphetamine impurities are very basic, better recovery can be obtained at a higher pH. Therefore Tris buffer at pH 8.10 was the final choice.

Non-polar organic solvents are commonly used in existing amphetamine profiling methods. These solvents indeed have some benefits, including poor extraction power of amphetamine and caffeine. In this study, it was found that the extraction of most amphetamine impurities was better with more polar solvents. On the other hand, co-extraction of amphetamine and caffeine was found to have an insignificant influence in the analysis. Therefore, the best overall performance was achieved with toluene, which was chosen as the extraction solvent for the harmonised method. The final sample preparation method has been summarised in detail in Appendix 2.

The SPE was also proven to be a competitive sample preparation technique. The differences between the tested SPE columns were significant. The Oasis column gave similar results to those obtained with the LLE technique. The SPE technique was, however, finally discarded due to the unexpected problems caused by the test tube effect.

The main objective of the study - development of a harmonised amphetamine profiling method - was completely achieved. This was proven when the new method was tested with real street samples. The repeatability and reproducibility results at intra- and inter-laboratory levels indicate that the harmonised method is fully capable of producing interchangeable results.

Evaluation of the statistical data handling methods was carried out only at a general level. The tested distance and classification methods were based on 34 target compounds. Differences between the distance methods were insignificant. Each method could be used for statistical data handling. The tested PCA was found out to be the preferred classification method.

It is essential to study new statistical methods in more detail in the near future. After an extensive study, the number of target compounds could be reduced by discarding some correlating compounds. This would also simplify the method.

At the time of writing, the harmonised profiling method has been tested and used by only three laboratories. These laboratories have an excellent know-how regarding the application and troubleshooting of this method. The method will be introduced to other forensic laboratories in the near future. However, it is of utmost importance to sufficiently train the personnel to use the method once it has been introduced to other laboratories.

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The GC parameters in the harmonised amphetamine profiling method:

Oven:	Initial temperature:	90°C
	Initial time:	1 min
	T-program:	8 °C/min
	Final temperature:	300°C
	Final time:	10 min
	Run time:	37 min
Inlet:	Mode:	Splitless
	Initial temperature:	250°C
	Purge time:	1 min
	Gas saver flow:	20 ml/min
	Gas saver time:	1.5 min
	Total flow:	60 ml/min
	Purge flow:	57 ml/min
Column:	Length:	30 m
	Diameter:	0.25 mm
	Film thickness:	0.25 µm
	Stationary phase:	35% phenyl methyl silicone (DB-35MS)
Pre-column:	Length:	3 m
	Diameter:	0.25 mm
	Film thickness:	0.10 µm
	Stationary phase:	35% phenyl methyl silicone (DB-35MS)
	Mode:	Constant flow
	Carrier gas:	He
	Average velocity:	25 cm/s (at 90°C)
	Outlet:	MSD
	Outlet pressure:	Vacuum
Detector:	Temperature:	310°C
	Tune file:	STUNE.U
	Acquisition mode:	Scan
	Solvent delay:	4 min
	Mass range:	40 – 300
	Threshold:	50
	Sample #:	3, A/D samples 8
	MS Quadupole temperature:	150°C
	MS Source temperature:	230°C
Autosampler:	Injection volume:	1 µl
	Syringe size:	10 µl
	Velocity delay:	1 seconds
	Plunger speed:	Fast

Sample preparation in harmonised amphetamine profiling method:

Sample:	Type: Amount	Amphetamine 200 mg
Buffer:	Type: Concentration: Volume: pH:	Tris 1 M * 4 ml 8.10
Organic solvent:	Type: Volume:	Toluene 200 µl
Extraction procedure:	One-step	
Shaking time:	10 min	
Centrifugation:	Speed: Time:	ca. 2500 rpm 2 – 3 min.

* Tris buffer at concentration 1 M was prepared by dissolving 121.1 g of Trizma base in 1 litre distilled water. At first, 37 % HCl was added and thereafter 1.0 M HCl drop by drop to adjust pH exactly to pH 8.10. The flask was filled up to the 1.0 litre volume and pH checked and readjusted if necessary.

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Development of a predictive model for batch membership of street samples of heroin

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Abstract

Street samples (n=31) of heroin were analysed by gas chromatography with flame ionisation detection to determine opiate, noscapine and papaverine content. Using this data, the chromatograms obtained could be resolved into eight groups by visual examination of the data. The concentrations of opiates were significantly correlated (P<0.05) with the exception of the pairs 6-*O*-monoacetylmorphine/noscapine and morphine/6-*O*-monoacetylmorphine. This precludes the use of simple cluster analysis for determining and predicting the relationship of different street samples. Application of Fisher's linear discriminant analysis to the data set indicated that 91.9% of the samples could be discriminated including pairs which could not be discriminated by eye. A blind trial (n=2) resulted in the correct assignment to street sample. Application of such methods may provide, in the future, a powerful tool for the prediction of batch membership of drugs at the street level.

Keywords: Batch membership; Drug profiling; Heroin; Fisher's linear discriminant modelling; Opiates

1. Introduction

During the course of drug analysis, including the analysis of heroin, it may be necessary to (i) identify any controlled substances present in a mixture, (ii) quantify the controlled substances present and (iii) where necessary, determine whether two or more samples once formed a larger batch of drug [1]. This latter is particularly important

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when attempting to prosecute a case of alleged trafficking or supply of drugs, including heroin, where determination of the identical nature of drug samples, or otherwise, can be of vital importance [1]. This is especially significant because heroin is produced in a batchwise process and no two batches of heroin are chemically identical.

Heroin is a complex mixture of compounds which result from obtaining opium resin from the field poppy *Papaver somniferum* L., the subsequent recovery of morphine from the opium resin and the acetylation of the morphine into the target molecule, diamorphine [2], in addition to materials which have been added to 'cut' the heroin. It is the morphine, 6-*O*-monoacetylmorphine, diamorphine, codeine, acetylcodeine, papaverine and noscapine content which is of greatest significance in heroin comparisons [1,3] since whilst adulterants including caffeine, sugars and local anaesthetics [4] may be added to a sample, thus changing the absolute amounts of these compounds present in the sample, the relative amounts of the compounds native to the heroin batch will remain constant, provided that one or more of the adulterants does not contain any of these compounds.

There are a large number of methods described for the quantitative determination of the alkaloidal content of heroin samples ([5] and references contained therein). Whilst both HPLC and GC methods can be used, GC offers greater resolution of complex mixtures and by-passes the problems associated with the disposal of large volumes of organic waste. If GC methods are to be used, particularly for the quantitative determination of the opiate alkaloids, derivatisation with, for example, silylating reagents should be employed [6]. This avoids many of the problems associated with GC analysis of heroin samples, including transacetylation, sorption of the alkaloids onto the chromatographic system and differences in response to salt or free base of the drug. Additionally, such treatment means that extracts of opium and heroin can be analysed directly, without further processing [7].

The principle objective of chemical impurity profiling methods is to determine whether or not two or more drug samples once formed a larger batch. However, such comparisons of drug (including heroin) samples is a two-stage process [8]. The first is the chemical impurity profiling of the sample, methods for which have been well developed [9,10]. Capillary gas chromatographic methods following extraction and derivatisation of the samples has afforded chromatograms from which much information concerning the impurity profile of the drug can be derived.

The second stage is interpretation of the data [8]. A number of different methods have been applied to obtain such information, including straightforward comparison of heroin impurity profiles on paper [9], and studies of ratios of selected compounds in the materials being compared [10]. Additionally, computerised methods and cluster analyses have been used to determine similarities between samples [7]. The use of Euclidean distances requires that the parameters used for developing the dendrogram are independent of each other [11]. Further, whilst such calculations describe associations between two or more samples, a predictive approach would allow data from a particular sample to be compared directly to a database without the need for recalculation of the Euclidean distances. Such an approach has already been demonstrated to be of use in predicting country of origin of heroin street samples [12].

In this paper, we describe a method for the numerical comparison of street heroin samples from Glasgow based upon Fischer's linear discriminant analysis [13] following

analysis of the opiate, papaverine and noscapine content of the samples. Further, it is demonstrated that the model can be used to predict the batches to which samples are related, at the level of street seizure.

2. Materials and methods

2.1. Chemicals

Diamorphine hydrochloride, 6-*O*-monoacetylmorphine hydrochloride, morphine hydrochloride, codeine phosphate, acetylcodeine hydrochloride, noscapine and papaverine were obtained from McFarlane Smith (Edinburgh, UK). *N,O-bis*-Trimethylsilylacetamide (BSA) was obtained from Sigma (Poole, Dorset, UK). *N*-Docasane ($C_{22}H_{46}$) was obtained from Alltech (USA). Nitrogen, hydrogen and air were supplied by BOC. All other chemicals and solvents used were analytical reagent grade or better. Street samples of heroin, the identity of which had been confirmed (Janhunen, K., unpublished data) were obtained from the Procurator Fiscal's Office, Glasgow.

2.2. Sample derivatisation and gas chromatographic analysis

All samples were derivatised with BSA prior to gas chromatographic (GC) analysis with flame ionisation detection (FID).

Standard solutions were prepared at appropriate concentrations in methanol (1 mg/ml to establish the chromatographic system, 0.125–1 mg/ml for quantitative analysis), whilst street samples were dissolved at 5 mg/ml in methanol. These latter were centrifuged at 5000×*g* for 1 min to remove insoluble suspended material. A 50-μl aliquot of the solution to be derivatised was placed in a GC derivatisation vial and the solvent removed under a stream of nitrogen. An aliquot (50 μl) of 10% (v/v) BSA in *n*-hexane containing docasane at 1.0 mg/ml was added to the sample and the vial was sealed. The mixture was allowed to react at 60°C for 1 h, and the resulting derivatives analysed using GC–FID.

The derivatives were analysed using a Carlo Erba Strumentazioni HRGC 5300 GC system, fitted with a BP-1 column (12 m×0.22 mm i.d., 0.25 μl layer thickness) with nitrogen carrier gas at a pressure of 0.7 kg/cm^2. The injection temperature was maintained at 280°C, the detector temperature at 300°C, the injection volume 1 μl and the split ratio 50:1. The column was held at 150°C for 2 min and then the temperature raised to 300°C at 9°C/min. This temperature was held for 5 min and the system then cycled to the starting temperature. Data was recorded using a Nelson ATD converter and Nelson Chromatography System software v3.1, at a scan rate of 10 scans/s.

2.3. Sample identification

The identity of the components of the heroin was determined by comparison of the relative retention time of the separated analytes in the street samples with the relative retention times of the standards, each against the internal standard, *n*-docasane.

2.4. Sample quantification

For each compound to be used in the profiling process, linearity of detector response to the analytes was established prior to the chemical profiling. Solutions of the compound were prepared at 0.125, 0.25, 0.5, 0.75 and 1.0 mg (as salt)/ml in methanol and derivatised as above. The samples were chromatographed in duplicate, with blanks between concentrations to establish that 'carry-over' had not occurred. The regression equations of detector response against drug concentration was calculated for each drug, using the method of least squares [14], with the concentration of drug corrected to free base form. The suitability of the application of a straight line was tested using the analysis of residuals [15] and the theoretical limits of determination obtained were for each drug under these analytical conditions [16].

2.5. Investigation of correlation coefficients of opiate drugs, noscapine and papaverine

Having established that the detector response was linear over a dynamic range suitable for the analysis of the street samples, the next process in the development of a means of comparing samples was to determine whether or not the concentrations of the opiates, noscapine and papaverine were significantly correlated or not at $P<0.05$. The concentrations of morphine, monoacetylmorphine, diamorphine, codeine, acetylcodeine, noscapine and papaverine were determined in each of the 31 street samples. Spearman's rank correlation coefficient [17] was calculated for each compound pair, using Systat for Windows v3.1, which employs the formula

$$\rho = 1-6\Sigma d^2/(n(n^2-1))$$

where d is the difference in the ranks of the concentrations of the two drugs and n is the number of data points. In order to determine whether or not the correlation was significant or not, the t_s test statistic was calculated, using the formula [18]

$$t_s = \frac{\rho}{\sqrt{\frac{1-\rho^2}{n-2}}} = \rho\sqrt{\frac{n-2}{1-\rho^2}}$$

for each value of the correlation coefficient which was then tested for significance at the 95% level by comparison to tabulated values from Student's t tables [19].

2.6. Comparison of street samples of heroin

Since the aim of this study was to develop predictive models using Fisher's linear discriminant analysis, the first step was to determine whether this would be of value, that is, that it would allow greater discrimination of the samples than can be achieved by comparing the chromatograms by eye. The chromatograms of each of the street samples were compared visually. The number of different groups into which the chromatograms fell, on examination by eye, were recorded.

The same data set was subjected to mathematical analysis. Fisher's linear discriminant analysis was applied to the data using the software package SPSS for Windows, v6.1 using stepwise analysis and treating each variable as equally weighted. Such analysis generates models of the form

$$\xi_1 = \alpha_1 \times x_1 + \beta_1 \times y_1 + \ldots$$
$$\xi_2 = \alpha_2 \times x_2 + \beta_2 \times y_2 + \ldots$$

where ξ_i are the calculated discriminant value for a particular sample, α_i, β_i, ... are the discriminant coefficients calculated from the data set, and x_i, y_i, etc., are the drug concentrations. The drug sample is assigned to the group for which the discriminant coefficients multiplied by the drug concentrations results in the highest calculated discriminant value.

In order to demonstrate that the discriminant models developed above could be used to predict batch membership of street samples, a blind trial was undertaken. Two street samples were extracted, derivatised and the drug content analysed as above. The drug concentrations were fed into the discriminant models developed above, and the predicted batch membership determined. This was compared to the batch to which the samples actually belonged to determine the applicability of the models to this type of use.

3. Results and discussion

3.1. Sample identification

The separation of the opiates achieved is shown in Fig. 1 and the relative retention

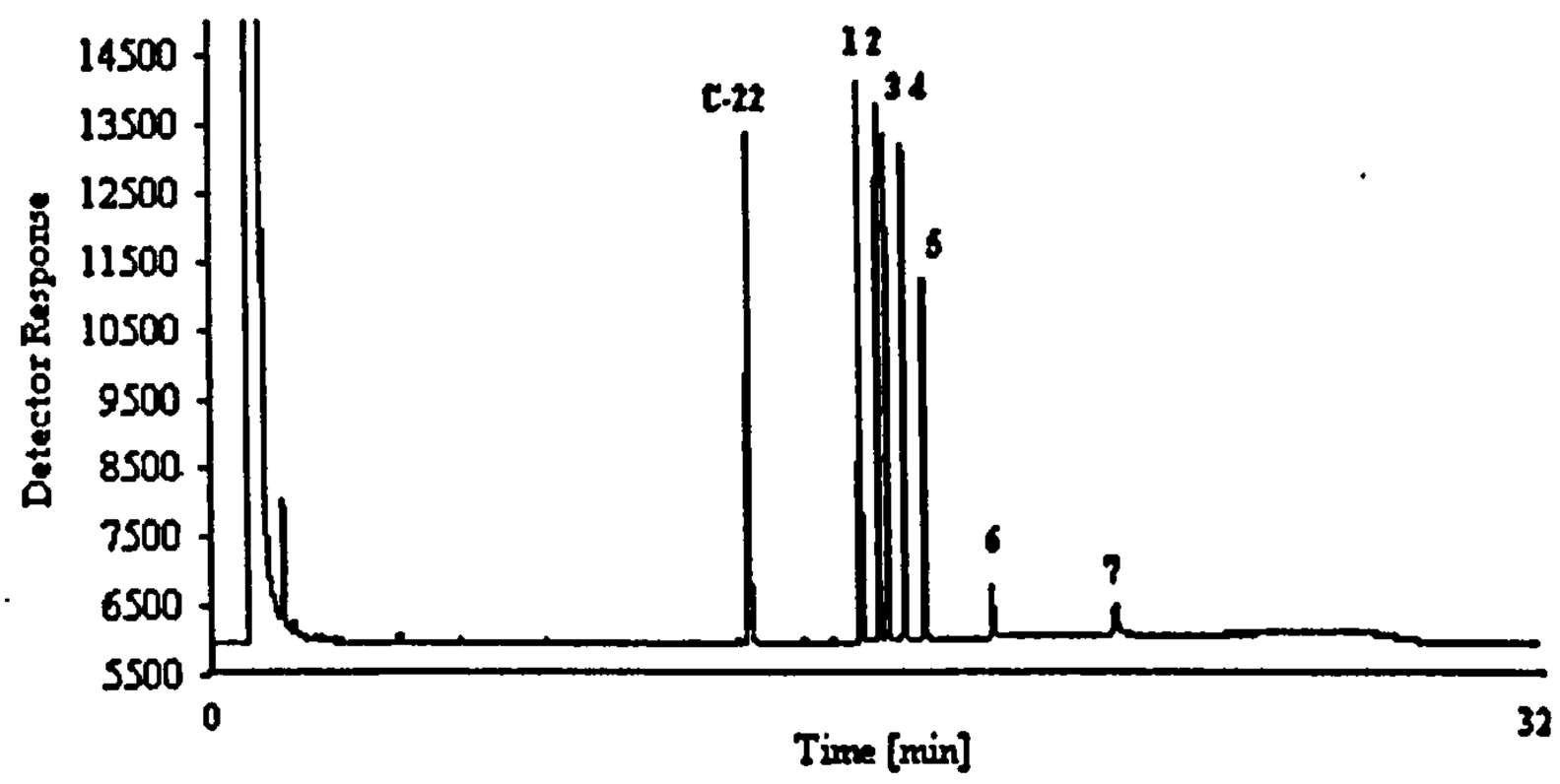

Fig. 1. Separation of opiates by GC after derivatisation using *N,O-bis*-trimethylsilylacetamide. Peak identification: (1) codeine; (2) acetylcodeine; (3) morphine; (4) 6-monoacetylmorphine; (5) diamorphine; (6) papaverine; and (7) noscapine.

Table 1
Relative retention times of opiate drugs following derivatisation with *N,O-bis*-trimethylsilyl acetamide and separation by GC–FID[a]

Drug	Relative retention time
Codeine	1.20
Acetylcodeine	1.23
Morphine	1.25
6-*O*-monoacetylmorphine	1.27
Diamorphine	1.32
Papaverine	1.45
Noscapine	1.68

[a] Absolute retention time of *n*-docasane, 13.8 min.

times against *n*-docasane are given in Table 1. The total analysis time for each sample was 32 min.

3.2. Sample quantification

The regression equations and theoretical limits of determination for the drugs under these analytical conditions are given in Table 2. The detector response was linear to each of the compounds under analysis under the conditions described. The system was sufficiently sensitive for the analysis of casework samples.

3.3. Correlation coefficients of opiate drugs, papaverine and noscapine

The opiate content of the 31 street samples was determined and the correlation of the compounds investigated using Spearman's rank correlation coefficient. Codeine was not detected in any of the samples. The full data set is not shown here, for brevity, but can be obtained from the authors. The correlation coefficients calculated from this data are given in Table 3. Of the pairs tested, only 6-*O*-monoacetylmorphine/noscapine and 6-*O*-monoacetylmorphine/morphine were not significantly correlated at the 5% level of significance. This is perhaps not surprising, given the process by which heroin is made [2].

Table 2
Regression equations for the GC–FID analysis of the heroin alkaloids derivatised with *N,O-bis*-trimethylsilylacetamide and theoretical limits of determination

Drug	Equation	Limit of determination (μg on column)
Codeine	$Y=1.274X+0.020$	0.17
Acetylcodeine	$Y=0.828X+0.019$	0.15
Morphine	$Y=1.122X-0.001$	0.11
6-*O*-monoacetylmorphine	$Y=0.862X+0.013$	0.12
Diamorphine	$Y=0.656X+0.005$	0.13
Papaverine	$Y=0.697X-0.057$	
Noscapine	$Y=0.098X-0.026$	

Table 3
Spearman's rank correlation coefficients for the alkaloids detected in the 31 street samples of heroin

	Acetylcodeine	6-*O*-monoacetyl morphine	Diamorphine	Papaverine	Noscapine
Acetylcodeine	1				
6-*O*-monoacetyl morphine	0.306	1			
Diamorphine	0.975	0.301	1		
Papaverine	0.782	0.218	0.755	1	
Noscapine	0.802	0.118	0.797	0.755	1
Morphine	0.245	0.009	0.197	0.380	0.286

This precludes the use of simple Euclidean distances as a means of examining relationships of street samples to each other and another method is required.

3.4. Comparison of street samples of heroin

The chromatograms obtained following analysis of the street samples were compared visually. These samples could only be divided into eight groups on the basis of comparison by eye. Fig. 2 illustrates the chromatograms from two street samples that were part of a multi-sample seizure, that could not be discriminated visually. These samples contained acetylcodeine, morphine, 6-*O*-monoacetylmorphine, diamorphine and

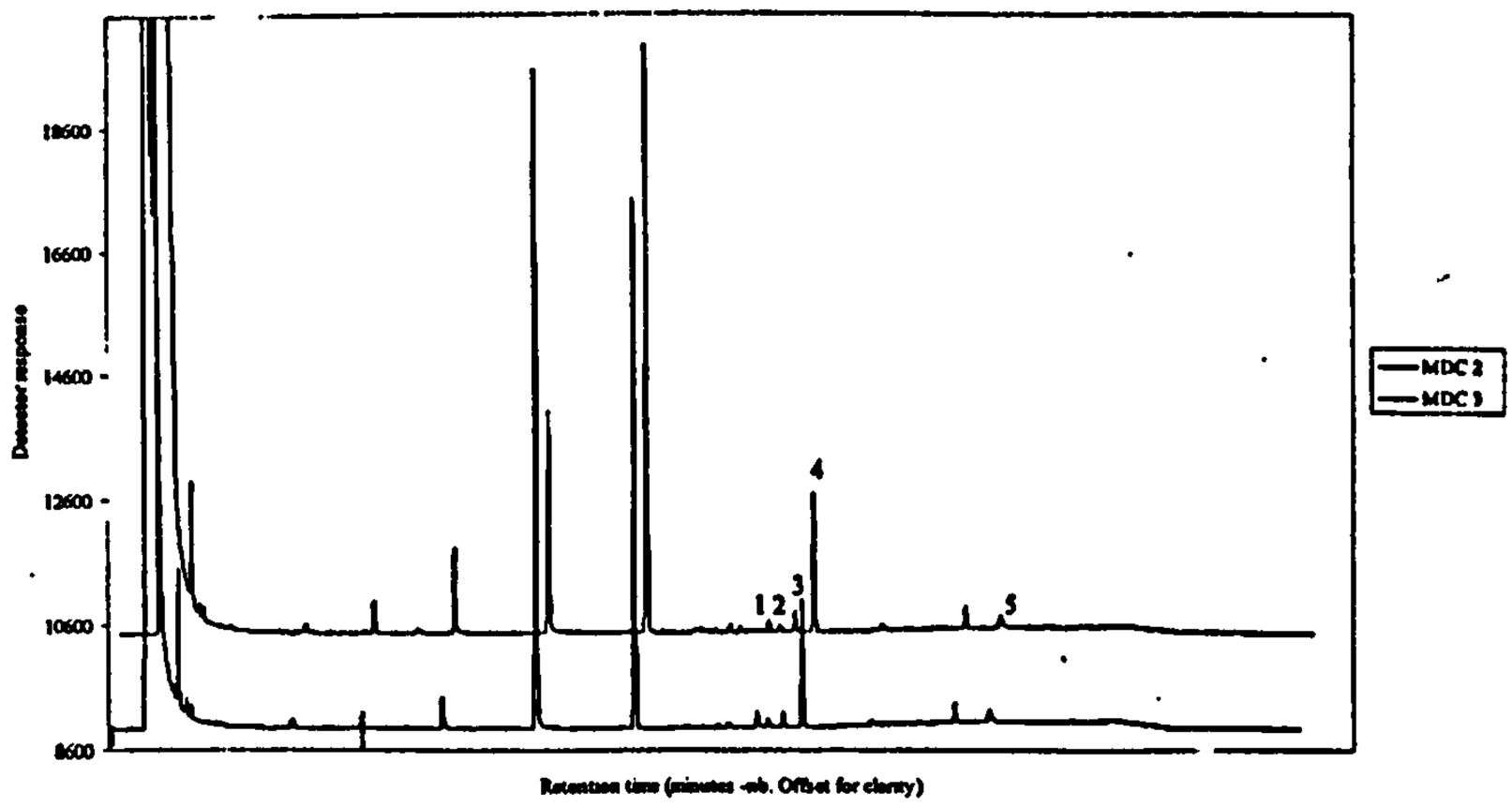

Fig. 2. Gas chromatographic comparison of heroin street samples MDC2 and MDC3. (1) Acetylodeine; (2) morphine; (3) 6-*O*-monoacetylmorphine; (4) *diamorphine; (5) noscapine.*

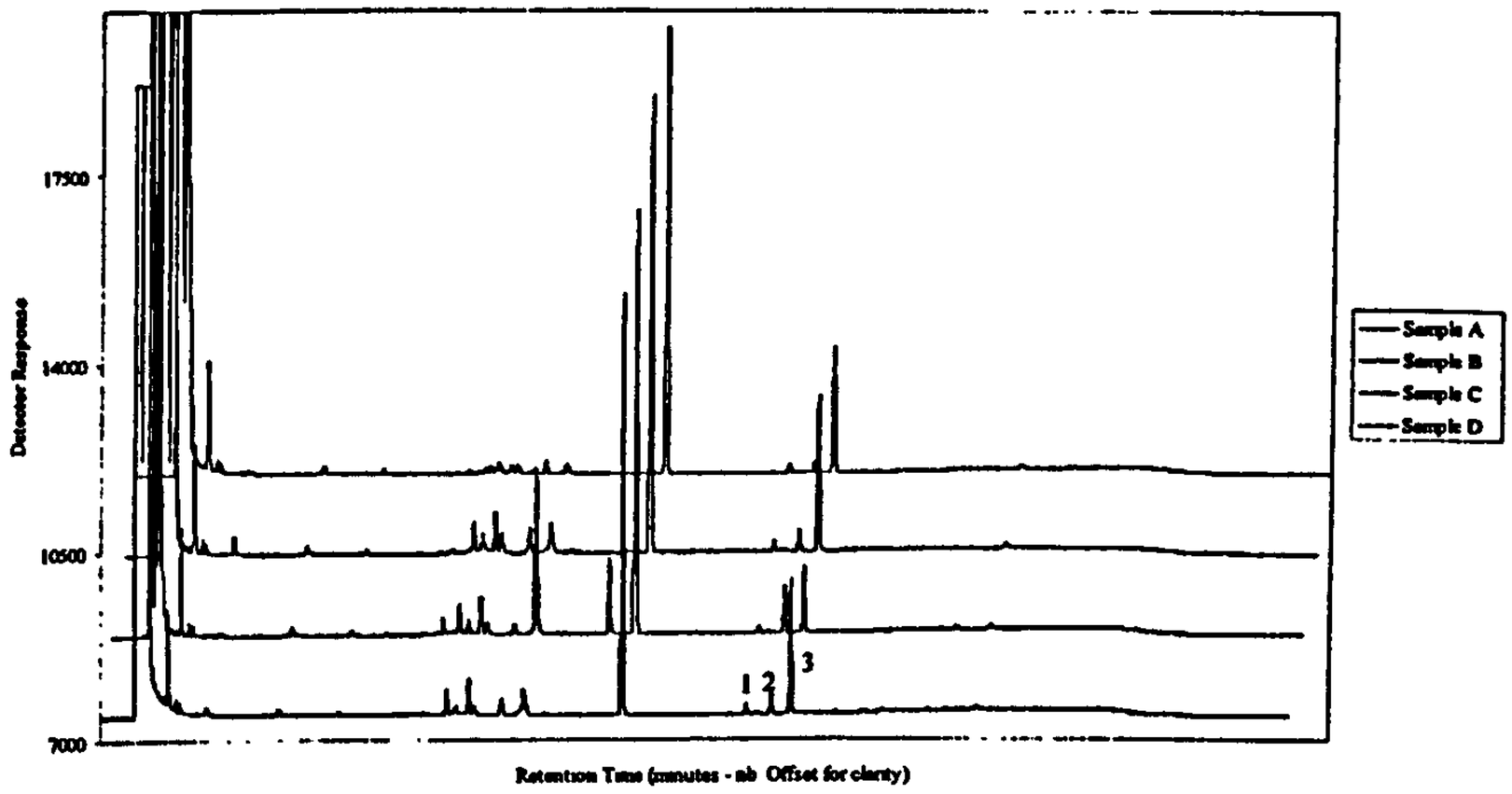

Fig. 3. Gas chromatographic comparison of heroin street samples A, B, C and D. (1) Acetylcodeine; (2) 6-*O*-monoacetylmorphine; (3) diamorphine.

papaverine. Fig. 3 illustrates another set of samples from a multi-sample seizure. In this instance samples A and C could not be discriminated by eye, whilst samples B and D were different from all other samples in the seizure, based on the relative amounts of 6-*O*-monoacetylmorphine and diamorphine present in the samples. The issue raised is whether Fisher's linear discriminant analysis can discriminate between samples MDC 2 and 3 as illustrated in Fig. 2, and samples A and C in Fig. 3, in addition to discriminating the other samples examined in this study.

The data from the chromatographic analysis of the 31 samples was used to generate discriminant coefficients for each of the drugs in each of the samples, respectively. The drug concentrations were then used to test the discriminant models, to determine whether or not the heroin samples in the 'teaching data set' could be correctly assigned to heroin sample. The assignments are given in Table 4. Of the samples analysed in this study, 91.9% of the samples were correctly assigned to their respective street samples. A caveat should be added at this point, in that the use of a teaching set to test the resulting models may result in slightly higher proportion of correct assignments than would be expected in completely blind trials. However, using the models developed, samples which could not be discriminated by eye, for example samples A and C, in addition to samples MDC 2 and 3, were completely discriminated using Fisher's linear discriminant modelling. With further development, such models could potentially provide powerful tools for the discrimination of street samples of heroin.

3.5. Blind trial prediction of batch membership of street samples of heroin

The blind trial involved two heroin samples named MDC 2 and MDC 5. The sample membership was predicted with 100% accuracy when each sample was analysed five

Table 4
Predicted and actual sample membership of the 31 street samples of heroin analysed in this study (*N* = the number of samples in each batch that were analysed)

		Predicted group membership (batch to which the models predict the samples belong)																														
		MDC1	MDC2	MDC3	MDC5	A	B	C	D	NND23	NND24	NND26	NND28	NND30	NND31	3D	3A/1	3A/2	3A/3	3A/4	3A/5	3B/1	3B/2	3B/3	3B/4	4A/1	4A/2	4A/3	4A/4	4A/5	4B/1	4B/2
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31
Actual group membership (Groups to which the samples actually belong)	MDC1 1	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	MDC2 2	0	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	MDC3 3	0	0	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	MDC5 4	0	0	0	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	A 5	0	0	0	0	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	B 6	0	0	0	0	0	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	C 7	0	0	0	0	0	0	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	D 8	0	0	0	0	0	0	0	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	NND23 9	0	0	0	0	0	0	0	0	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	NND24 10	0	0	0	0	0	0	0	0	0	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	NND26 11	0	0	0	0	0	0	0	0	0	0	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	NND28 12	0	0	0	0	0	0	0	0	0	0	0	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	NND30 13	0	0	0	0	0	0	0	0	0	0	0	0	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	NND31 14	0	0	0	0	0	0	0	0	0	0	0	0	0	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	3D 15	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	3A/1 16	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	3A/2 17	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	3A/3 18	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5	0	0	0	0	0	0	0	0	0	0	0	0	0
	3A/4 19	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5	0	0	0	0	0	0	0	0	0	0	0	0
	3A/5 20	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5	0	0	0	0	0	0	0	0	0	0	0
	3B/1 21	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5	0	0	0	0	0	0	0	0	0	0
	3B/2 22	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5	0	0	0	0	0	0	0	0	0
	3B/3 23	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5	0	0	0	0	0	0	0	0
	3B/4 24	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5	0	0	0	0	0	0	0
	4A/1 25	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5	0	0	0	0	0	0
	4A/2 26	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5	0	0	0	0	0
	4A/3 27	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5	0	0	0	0
	4A/4 28	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5	0	0	0
	4A/5 29	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5	0	0
	4B/1 30	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5	0
	4B/2 31	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5
	a	91.9% of original grouped cases correctly classified.																														

times and the data placed in the discriminant models generated using the 'teaching data set'. Whilst the authors recognise the limitations of the small sample size and trial number, the data achieved to date indicates that this type of modelling may represent a powerful tool for street sample discrimination, especially when it is remembered that sample MDC 2 was very close, by eye, to sample MDC 3. This modelling technique allowed complete discrimination of these samples.

4. Conclusions

Analysis of this set of street heroin samples from Glasgow, UK, indicates that a number of batches of heroin may be present in street samples at any one time. This presents a problem to an analyst who may be faced with matching one or more samples to others, often involving large and complex data set. The use of unweighted Euclidean distances and cluster analyses is precluded when the analytes being measured are strongly correlated, as in this study. Use of Fisher's linear discriminant modelling may allow this problem to be overcome and may offer a greater degree of discrimination than may be achieved by simply examining the chromatograms by eye.

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Development of a harmonised pan-European method for the profiling of amphetamines

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Introduction

There is now a documented increase in the number of drug trafficking and related offences in the European Union and on a world-wide basis [1,2]. A number of different routes of trade have been identified for different drug classes, amongst which are routes for the trafficking of amphetamine and related drugs. Given the reduction in border controls between some EU member states the problems of drug control are still further exacerbated.

There is now considerable interest in the possibility of harmonised methods for the comparative analysis of drugs of abuse. Such interest is at the national and international levels, and discussions have already taken place within the European Network of Forensic Science Institutes (ENFSI) and at the Police Co-operation Working Group (PCWG), which meets in Brussels. Such harmonised methods would allow the exchange of data and intelligence information which, with the correct use, could potentially assist in the policing and control of trafficking offences. One study, to develop a harmonised method for the profiling of heroin, has already been undertaken [3].

In the early days of such discussions and with these potential aims in mind, a seven nation consortium developed a proposal for the development of a harmonised pan-European method for the profiling of amphetamines. The ultimate goal of the project is to develop a method for the profiling of amphetamines which can be used on samples synthesised by any of the commonplace routes of amphetamine synthesis, namely the Leuckart synthesis, the nitrostyrene route of synthesis and the reductive amination of benzyl methyl ketone. This paper describes the development of the proposal, the process which was undertaken to endeavour to secure funding, the development and execution of the project to date and the successes and difficulties that have been encountered during the project.

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Key Words: Forensic science, amphetamine, chemical impurity profiling, method development.

The participating laboratories

At the outset of the project, four laboratories (Forensic Science Unit at the University of Strathclyde, Glasgow, UK; The National Bureau of Investigation, Vantaa, Finland; The SKL National Laboratory of Forensic Science, Linköping, Sweden; The Institut de Police Scientifique et de Criminologie, University of Lausanne, Switzerland) initiated the experimental design and application process. As the project developed, three further laboratories (The Netherlands Forensic Institute, Rijswijk, The Netherlands; The Department of Forensic Medicine, University of Aarhus, Denmark; and The Policia Judiciaria, Lisbon, Portugal) joined the project. It was ultimately felt that this represented a good geographic balance of European laboratories, in addition to including laboratories with a great deal of drug profiling and/or research experience. Within the context of the project, each could also bring a variety of skills that would enable a rounded approach to its progression. Further, the group includes academic members, those involved in casework and those involved in both activities. It was again thought that this would bring a rounded view to the group. Finally, one laboratory was nominated as the co-ordinating laboratory, which acts as the link between the European Commission and the partners in the group. This is not an inconsiderable task and requires that the laboratory chosen has the necessary administrative infrastructure to deal with the various types of paperwork involved. In this project it has been found that the required infrastructure includes: financial and accountancy offices; a legal department (the contract with the European Commission is a legally binding document); and a purchasing department (especially for large equipment purchases for more than one laboratory). Further, in each laboratory it is necessary to have the required technical support.

The development of the project

The project developed out of an initial meeting between the four original laboratories following study of the documents issued by DG XII of the European Commission. In these documents a section was identified under which research related to forensic science might be supported. The consortium identified the need to study amphetamine profiling and wrote the proposal around this subject area.

In terms of development of the project, the subject was broken down into a number of discrete phases. These were:

(i) The synthesis, identification and documentation of the relevant impurities likely to be found in amphetamine synthesised by different routes;

(ii) A study of the stability of impurities in different solvents;

(iii) Optimisation of the GC and detector system;

(iv) Optimisation of the extraction process;

(v) Study of the variability of the results;

(vi) Investigation of numerical classification schemes;

(vii) The writing up phase.

Within the context of the development of the project, it was necessary to have the phases attached to a timeline. The durations of the phases and the intended timeline are shown in the Gantt chart (Figure 1).

Each phase was required for a specific reason and had a defined outcome. The synthesis, identification and documentation of the impurities was necessary because it was clear that whilst many impurities are known, standards needed to be available for method development. At the time of the first management report, 21 impurities had been synthesised and ^{1}H and ^{13}C NMR data, mass spectroscopic, infra-red and ultraviolet spectroscopic data collected. This is the largest single data set for this group of compounds that the authors are aware of. An example of the data collected is shown in Table 1.

In order that the system be valid, it is necessary to know whether or not the impurities are stable, or otherwise, in the solvent in which they are presented to the GC system. A summary of the data collected is shown in Table 2. Once a solvent in which the impurities are stable has been identified, it is then possible to attribute any other variation to experimental parameters which have subsequently been changed.

The third stage of the work was to develop a GC system in which the impurities were stable and which elicited the greatest response, with least variation, to the compounds from the detectors. This in turn would minimise the random and systematic errors associated with such analyses. Both GC-FID and GC-MSD were used. Parameters which, at the completion of the project, will have been studied include (i) injection volume, (ii) injection port temperature, (iii) split/splitless injection, (iv) column type, (v) detector type and conditions. This data is currently being fully evaluated.

Currently under investigation is the optimisation of the extraction conditions. This includes the use of both liquid–liquid extraction and solid phase extraction systems. Parameters that will have been investigated by the end of the project include (i) the buffers employed at different pH and ionic strengths, (ii) the organic solvents used to extract the impurities from the buffers, (iii) solid phase extraction (SPE) stationary phases and (iv) the eluting solvent for SPE extractions, and (v) different volumes of buffers and solvent for the different processes.

Having determined the best operating parameters, finally the systems for impurity extraction and profiling will be decided upon. The project will then move on to the synthesis of large batches of amphetamine by each of the three routes. These batches will then be divided and circulated to participating laboratories to determine whether it would be

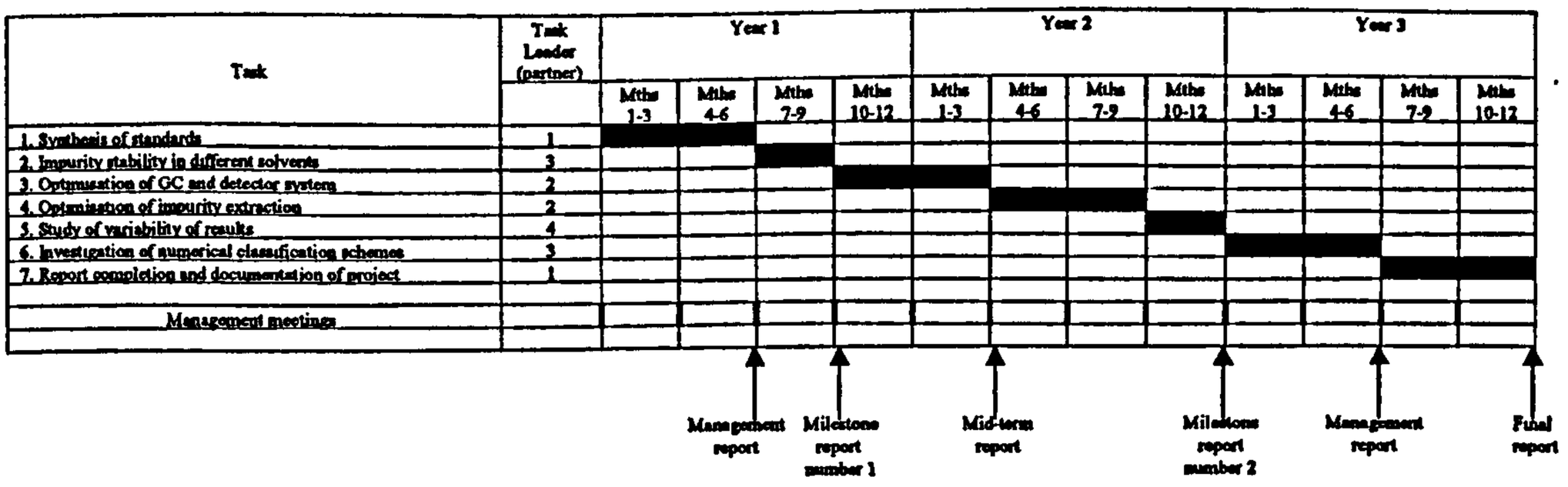
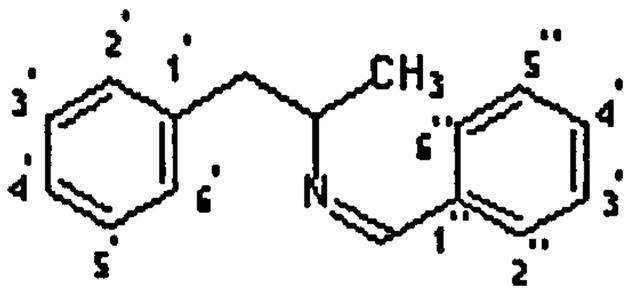


FIGURE 1 Timeline established for the management of the project. The Gannt chart illustrates the timescales involved for each of the tasks described in this project and the points at which management meetings will be held and reports prepared.

TABLE 1 Illustrative data for N-(β-phenylisopropyl)benzaldimine indicating the spectroscopic information collected for each of the impurities.



UV data	λmax (nm) [MeOH] 240; [0.1M NaOH] 204, 212, 224; [0.1M H_2SO_4] 212, 220, 240.
MS data	m/z = 222 $[M-H]^+$ 0.7%, 133 $[M-C_7H_6]^+$ 14%, 132 $[M-C_7H_7]^+$ 100%, 117 $[M-C_7H_8N]^+$ 5%, 105 $[C_7H_7N]^+$ 29%, 91 $[C_7H_7]^+$ 15%, 77 $[C_6H_5]^+$ 6%.
IR data	γ_{max} (cm^{-1}): 696, 746, 1641, 2973, 2848, 1494, 1454, 968, 487.
^1H NMR	δ = 1.37 (3H, m, Me), 2.97 (2H, m, H-2), 3.60 (1H, m, H-1), 8.07 (1H, s, C-H proton), 7.00 – 7.80 (10H, m, phenyl-H).
^{13}C NMR	δ = 21.7 (methyl), 44.0 (methylene), 67.6 (methine), 125 – 129 (phenyl), 135.7 and 138.9 (quaternary), 158.7 (imine).

possible to obtain the same profiles using the designated method. Variations in the data within laboratories and between laboratories will be investigated.

In time, once a method has been identified as being suitable for amphetamine profiling, a large body of data will be developed. In order that related samples can be identified, it is necessary that numerical classification schemes be developed which can identify and predict sample relationships. The penultimate stage of the project will include an investigation of the methods available for the comparison of samples and prediction of the relationships between different samples. If necessary, new models will be developed to enable such relationships to be established.

The final phase of the project will involve the writing up and distribution of information arising from the project. Dissemination will be achieved through report preparation for the European Union, poster presentations, conference presentations and publication of peer-reviewed papers. All of the publications which arise from this project require clearance from DG XII of the European Commission and each of the partners involved.

In respect of report preparation for the European Union, reporting deadlines have also been identified. These have been mapped onto critical phases of the project (Figure 1). In addition, release of funds for the work are tied to the submission of the necessary reports. One final piece of paperwork is completed according to a scheduled timetable – the project quality indicator (PQI) document. This provides an opinion, from both the participants and the Commission on the value and effectiveness of the project.

The process of securing funding

Once authored, the project and related paperwork were submitted to the relevant organisation within DG XII of the European Commission, through the project co-ordinator. At this point, a reasonable wait was anticipated whilst the project was reviewed. The project was assessed on a number of criteria, including scientific necessity and merit, satisfaction of the subject matter and selection criteria identified in the calls for projects, the pan-European nature of the project and the added value that would be achieved for the European Union. Once assessed, judgements are made on whether the project will receive funding.

In the case of this project, discussion with DG XII of the European Commission was entered into and final negotiated sums arrived at. At this point the contractual

TABLE 2 Summary of results arising from the determination of the stability of the impurities in different solvents. X indicates that at least one impurity was observed to decompose. * No decomposition was observed for the impurities studied by this research group.

Solvent	*Lab*			
	Strathclyde (nitrostyrene)	*NBI, Finland (reductive amination)*	*SKL, Sweden (Leuckart part 1)*	*IPSC, Switzerland* (Leuckart part 2)*
Iso-octane				
Toluene				
Dichloromethane	X		X	
Ethyl acetate		X	X	
Diethylether		X	X	
Ethanol	X			

arrangements were negotiated and the documentation completed by legal representatives from each of the participating institutions. Once the money was secured, the project began.

Development and execution of project to date

The project commenced with what is described as a "Kick off' meeting at the Commission, with the Scientific Officer of the Commission who was assigned to oversee the project. Equipment was delivered in order to allow the project to proceed. Once this was completed the project commenced. The work programme was undertaken according to the time schedule and the reports written and circulated. The internet has proven to be an invaluable tool in rapid communication within the project and between the project and the Commission. Without it, the project would be considerably slowed down. However, one drawback is that there can, on occasion, be a flood of information that is difficult to deal with. It is interesting to note that in the new framework programme, there is provision for the salary of an administrative officer to deal with this volume of paperwork.

The monthly reports and management meetings have progressed as scheduled. One of the major difficulties is collating the information generated in a common format for the milestone reports. However, with the co-operation of the participants, this has proven possible so far. The provision of the administrative officer (above) in future programmes should ease these difficulties. The PQI document is easily completed. The project has, so far, generated one poster and a number of papers are currently being authored. Again, one laboratory, usually the task leader for which the paper is being written, takes on the responsibility for authoring these materials prior to their circulation. Again, e-mail and the internet is invaluable in circulating these materials.

Whilst painting a picture of success, there are a number of difficulties that have been encountered. The principal difficulties include staff turnover and equipment failure. Neither can be predicted, and as a consequence a degree of flexibility needs to be built into the work programme in anticipation of these difficulties. In general, however, this particular project has developed amicably, according to the work programme and it is anticipated that it will be completed within the designated time frame.

Conclusion

Clearly, the project is not yet complete. However, certain conclusions about the project can be drawn at this early stage. In order for an application and subsequently funded project to be successful, there are a number of criteria which must be met. These include

(i) a well structured project, which can realistically be completed within the envisaged time frame, in which the participants have a sense of ownership;

(ii) a good working relationship with the European Commission;

(iii) a willingness of the participants to assist each other;

(iv) a good communication system;

(v) a spirit of partnership.

If these criteria can be met, there is a good chance that the project will be successful in its outcome.

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