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Development of an Analytical Method for the Organic Impurity Profiling of Amphetamine

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This thesis is dedicated to my parents,

Margaret and Michael

and to my sister, Martine.

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Chapter 1

Introduction

1.1. Drugs as a Commodity – Supply and Demand

Illicit drugs form an important sector of the world economy. A report from the United Nations' International Narcotics Control Board (INCB) in 1996 stated that the total revenue generated within the illicit drugs industry was between $300 billion and $500 billion [1]. Estimating that the figure lay around the $400 billion mark, this would represent around 8 % of total international trade - larger than the combined trade in iron, steel and motor vehicles at that time. In 2002, the first ever United Nations Office on Drugs and Crime global survey on ecstasy and amphetamines indicated that at that time the value in amphetamines alone was $65 billion per year [2]. Although the true value is uncertain, somewhere in the region of 10 % of drugs in circulation are intercepted by law enforcement agencies leaving the bulk of drugs available on the street [1]. What is clear, however, is that the export and import of illegal drugs is a multi-billion-dollar business with profits for the producers of between 3000 and 4000 % [2]. The majority of this lucrative business in production and trafficking of drugs is known to be controlled by organised crime groups. Of the 815 UK organised crime groups studied in the strategic intelligence requirement (2000-2001), two-thirds of these groups were reported to be involved in trafficking heroin, cocaine, ecstasy, amphetamine and cannabis [3].

The true extent of the problem of drug abuse in Europe is also difficult to gauge since some countries do not perform extensive drug-use surveys and data may only refer to specific populations, such as those individuals receiving treatment for addiction. However, the standardisation of reporting systems is improving. This has seen progress in the ease of exchange of figures relating to drug-use. One such system compiles and standardises the figures received from across Europe. In 1997, the European Union Centre for Drugs and Drug Addiction (EMCDDA) concluded that between 5 % and 15 % of the European population, over 12 years old, had tried illicit drugs [4]. This particular report also states that at that time, the trend in usage was very much towards synthetic drugs such as amphetamines since they are utilitarian in nature and can theoretically be made to meet consumer demands [5]. Synthetic drugs do not rely on harvests and have the advantage of being produced cheaply in unobtrusive makeshift laboratories, thus avoiding some of the more obvious signs of drug

production, such as acres of poppy fields or large-scale cannabis plantations. Indeed the United Nations global survey concluded that in 2000-2001, 34 million people worldwide abused amphetamine or methamphetamine and 8 million abused ecstasy, which, at that time, exceeded the combined number of cocaine and heroin abusers [2].

1.2. The Present Situation in European Laboratories

On initiation of the project, there was no single, generally accepted method for the profiling of amphetamines in European laboratories. Those laboratories involved in some sort of impurity profiling used procedures developed internally and shown to be suitable for the analysis of amphetamine impurities. While the differences in methods used may have been slight in some cases, the ability to accurately compare analytical data would be improved if the methods were harmonised – even if this meant establishing a compromise between methods already in use. It is clear that while several alternative methods are in use, information gained from analysis of seizures from each of the member countries may not be easily interchangeable. This situation could be perceived as a hindrance to any useful exchange of intelligence information regarding the control of drugs across international borders.

A study previously undertaken on the analysis of amphetamines showed that valuable information could be obtained from an analytical profile of the impurities present in seized samples [6]. This information may then be passed on to law enforcement agencies who could combine this data with intelligence information and suggest links between suspected sources of illicit amphetamine, possibly contribute to their efforts to track down production locations or connect individuals in the trafficking chain [7].

Analysis of seized amphetamine, and subsequent discussion of the findings, has revealed that there are three principal routes of synthesis used across Europe, namely, the Leuckart route (most common), the reductive amination of benzyl methyl ketone (BMK) and synthesis via nitrostyrene [8, 9, 10, 11]. These are illustrated in Figures 1.1, 1.2 and 1.3.

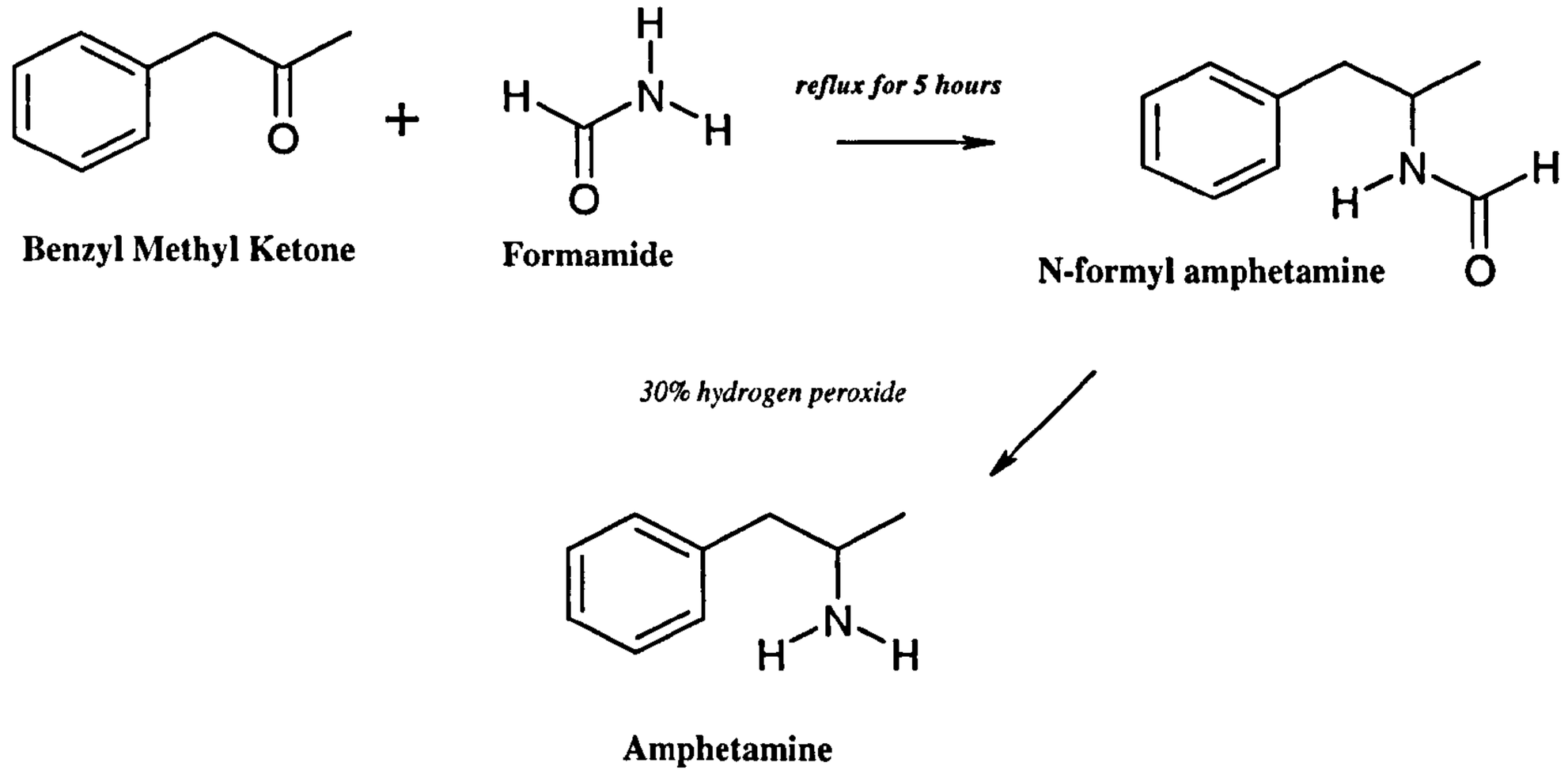


Figure 1.1: Leuckart Route of Amphetamine Synthesis

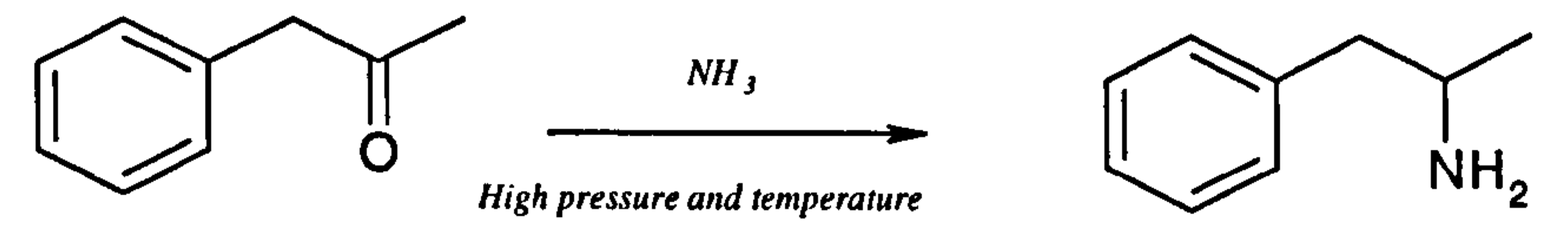


Figure 1.2: Reduction of Benzyl Methyl Ketone to produce Amphetamine

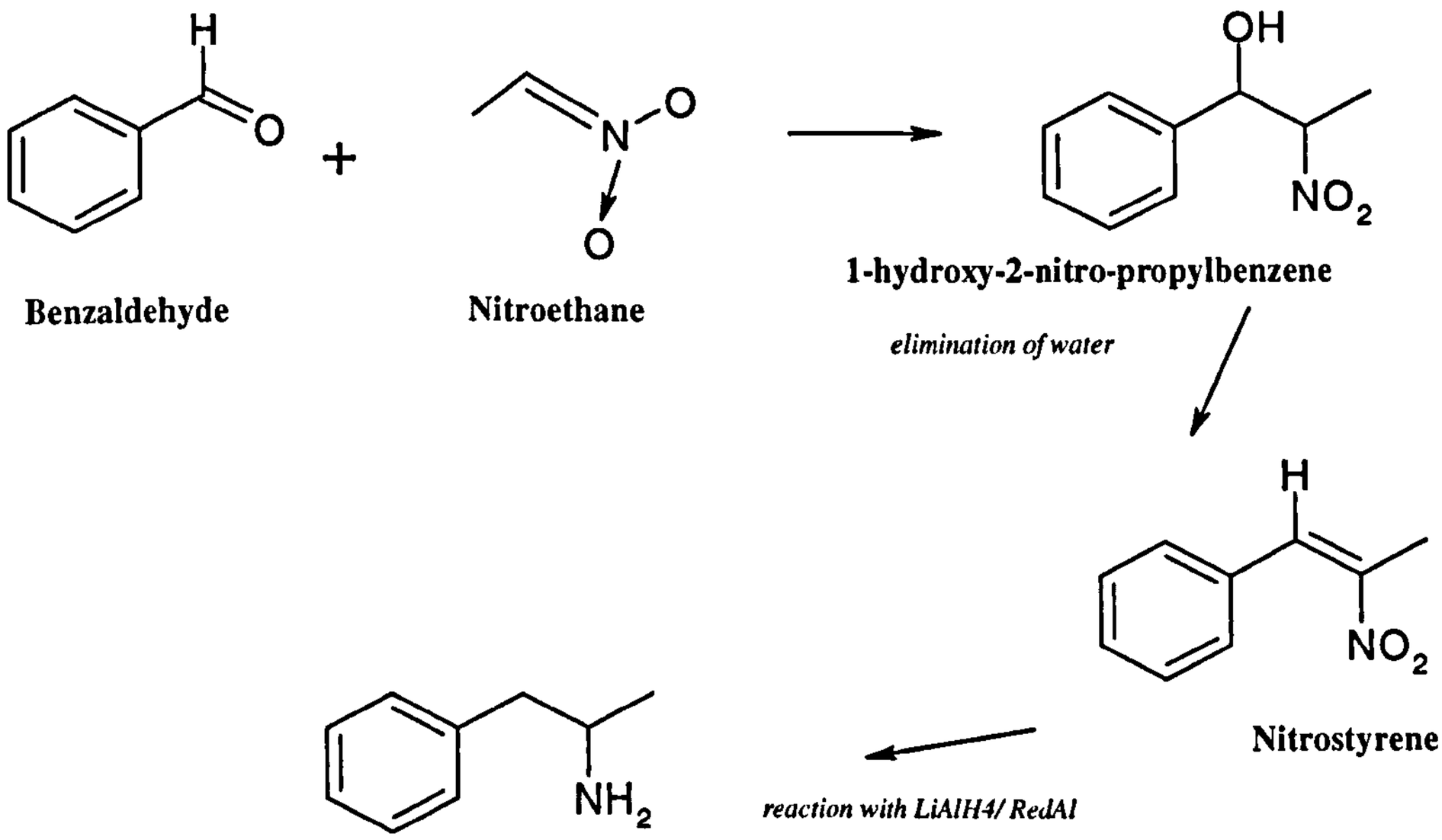


Figure 1.3: Nitrostyrene Route of Amphetamine Synthesis

Other synthesis routes are known but are used infrequently, perhaps as a consequence of more complex or hazardous routes of production [8]. Across Europe and the United States, patterns in the most popular routes of production emerge in specific regions and at different times [11]. For example in 1994 King *et al* reported that seizures of amphetamine in the UK were dominated by samples with high concentrations of DPIA indicating that the majority of samples were being imported from the Netherlands [12]. The Leuckart route was popular in the U.S. and the U.K but the popularity of methamphetamine has resulted in an increase in other methods of production such as iodine-red phosphorous and anhydrous ammonia-lithium metal and in 2001, the Netherlands seen an increase in seizures of amphetamine produced by the reduction of BMK [3]. However, the Leuckart synthesis is still the most frequently encountered route of synthesis for amphetamine across Europe.

Patterns in production are perhaps due to economic factors or the availability of reaction precursors in that region. Controlled precursors are diverted either from licit trade or via formation of 'front' businesses legally using a proportion of the chemicals, the remainder being used in illegal production of drugs. Therefore, at least in theory, when enough intelligence information has been gathered regarding the content of seized batches, the route used for each batch could give some indication of the origin [13].

Each route of production produces a family of impurities, which may be indicative of the synthetic method used, and since illicit amphetamines are generally impure [11], they are commonly seized with numerous impurities present in measurable quantities. A mixture of reaction by-products, impurities in starting materials, un-reacted starting materials and degradation products, these impurities may be specific for one route or more than one route, and so must be interpreted with care. For example, the Leuckart synthesis may be confirmed as the route of production if heterocyclic impurities such as 4-methyl-5-phenylpyrimidine and 4-benzoylpyrimidine are present [11]. The presence of nitrostyrene confirms that the nitrostyrene route has been used as this is the only route-specific impurity in this synthesis. However, impurities such as DPIA are common to both Leuckart synthesis and the reductive amination of BMK since it is formed by the reaction of amphetamine with excess BMK.

Furthermore, each individual synthesis of amphetamine may produce a different number and quantity of impurities, which might be used to link smaller seized samples to each other or to a larger batch [14, 15]. Impurities may also be introduced from the laboratory itself, as 'environmental' contamination. These impurities are of the utmost importance to an analyst when attempting to link batches of seized amphetamine to a clandestine laboratory.

With the lifting of international border controls and with travel across mainland Europe, the U.K. and Scandinavia becoming simpler and more economical than ever before, it is conceivable that a batch of amphetamine may be transported from Scandinavia to southern Europe in a matter of hours. And since it is estimated that almost 90% of drugs seized in the UK have been imported into the country [16], any research which can limit trafficking will decrease the availability of drugs on the street. Trafficking in amphetamine is thought to be predominately regional and intra-European, although there have been incidences of double use of trafficking use – for example trading amphetamines from Europe for heroin from south-east Asia [3].

Because of this ease of trafficking through the continent, one of the focal points for European forensic drug analysts in recent years has been the rationalisation of methods currently in use for the analysis of seized drug samples. In the past, laboratories in different European countries have analysed and linked seized samples using in-house methods. Because these methods of extraction and analysis across Europe are not harmonised, the impurity patterns generated from the same sample may differ, similarities may not be recognised and possible cross-border trafficking routes will not be identified. The 'Comprehensive Actions against Synthetic Drugs in Europe' initiative, CASE has been established to encourage member states to send to Europol, information and intelligence relating to seizures of amphetamine and amphetamine-type stimulants. They would also send samples of seizures to a designated central laboratory for chemical 'fingerprint' analysis to build up a database of profiles which could then be used to link sites of production and seizures across Europe [3].

In order for the harmonisation of analytical protocols to achieve any impact on the production and trafficking of drugs, more frequent scientific communication between analysts from different countries must be established and data exchange methods improved. Only in this way, will the relevant authorities receive meaningful intelligence information provided by drug profiling and be in a better position to stem the flow of illicit amphetamine into, out of and within Europe.

1.3. Amphetamine and the Law

Amphetamine is a substance controlled by law in the U.K. Possession, possession with intent to supply and production of the ß-phenylethylamines is legislated by the Misuse of Drugs Act 1971, and the Misuse of Drugs Act Regulations, 2001 [17]. The Act describes what is *not*

permitted in relation to listed drugs whereas the Regulations describe what *is* permitted in relation to listed drugs and under which conditions. The Act is divided into sections listing which substances are controlled by the Act, how the possession, production, supply or importation of these drugs are controlled and the illegality of allowing premises to be used to produce or take these drugs. The Regulations organises controlled substances into Schedules that balance the drugs' value as medicines while recognising the scope for their abuse. Some drugs listed may be prescribed or used in scientific research under licenced conditions. Amphetamine and its analogues, which include the methylene-dioxyamphetamines and their N-substituted derivatives are listed in Schedule 2 of the Regulations. Only MDMA is a 'Class A' drug, the remainder are 'Class B'. However, while amphetamine, prepared as a salt, is listed as a 'Class B' drug when it is prepared for injection it becomes a 'Class A' drug.

The penalties associated with possession, possession with intent to supply and production of amphetamine as a drug listed in Schedule 2 are found in Section 4 of the Misuse of Drugs Act 1971. Maximum penalties incurred for a class B drug are shown in Table 1.1. The recommended sentence for amphetamine possession in the UK ranges from 2 years for seizures under 500 g to 14 years for seizures over 15 kg.

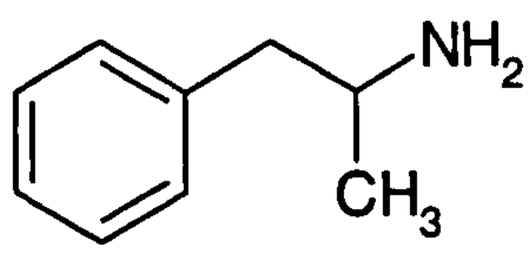
Other drug-related import and export offences such as the trafficking of drugs are also regulated by the Customs and Excise Management Act, 1979 [18] and the Drugs Trafficking Offences Act, 1994. The latter having some significance since its' inception granted the authorities the power to confiscate monies, possessions or property from those convicted of drug offences when these possessions were proven not to have been purchased using legitimately earned income.

Section of the Act	Offence	Penalty (summary)	Penalty (indictment)
S. 4 (2)	Production or concerned in the production of a controlled drug	6 months imprisonment or the prescribed sum or both	14 years imprisonment or a fine or both
S. 4 (3)	Supplying or offering to supply a controlled drug or being concerned in either activity	6 months imprisonment or the prescribed sum or both	14 years imprisonment or a fine or both
S. 5 (2)	Having possession of a controlled drug	3 months imprisonment or £2500 or both	5 years imprisonment or a fine or both
S. 5 (3)	Having possession of a controlled drug with the intent to supply it to another	6 months imprisonment or the prescribed sum or both	14 years imprisonment or a fine or both
S. 8	Being the occupier or concerned in the management, of premised and permitting or suffering certain activities to take place there	6 months imprisonment or the prescribed sum or both	14 years imprisonment or a fine or both

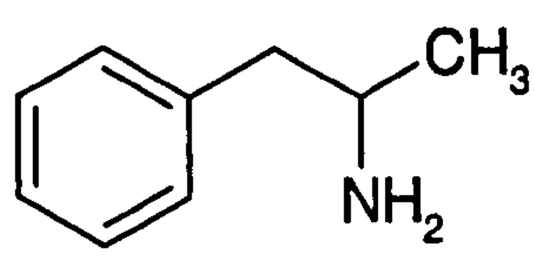
Table 1.1 Maximum penalties relating to 'Class B' drugs from Misuse of Drugs Act 1971

As well as the control of illicit drugs themselves, many precursors used in their production have trade restrictions in place between certain countries and, in some instances; purchase of compounds is quantitatively limited through The Controlled Drugs (Substances Useful for Manufacture)(Intra-community Trade) Regulations 1993 [19, 17]. In other circumstances, a licence may be required to purchase these chemicals. The Criminal Justice (International Co-operation) Act of 1990 (Part II) is aimed at regulating the manufacture and supply of substances with a legitimate use in industry but may also be used in the manufacture of controlled substances [17, 20]. For example, in relation to amphetamine production, the trade of benzyl methyl ketone (BMK) is severely restricted as a Category 1 precursor but does have a legitimate use in chemical manufacture.

In addition to the control of known illicit drugs, the present legislation aims to control any analogues or derivatives of the known drug. This includes, stereoisomeric forms of a drug, (for example, both D and L forms of amphetamine and the racemic mixture), any salt of the substance (amphetamine sulphate or chloride in addition to free-base) and any preparation or product containing the controlled substance (such as a prescribed tablet containing the drug) [17].



D-amphetamine



L-amphetamine

Figure 1.4 D and L forms of amphetamine

Policy-makers and government bodies have the unenviable task of structuring these laws in such a manner that the legislation can keep up with those individuals involved in the illicit production of amphetamine-based drugs. From time to time, new amphetamine analogues are synthesised, and in order to cover the influx of a novel 'designer drug' onto the market, the regulations must be worded carefully so as to include as many chemical derivatives and isomers of illicit drugs as possible. To illustrate this point, problems arose in Italy when 'new' amphetamines, which had not yet been controlled by the Italian drug schedules, were found in seizures [21] and more recently, the Horizontal Drugs Group has requested risk assessments on paramethoxymethamphetamine (PMMA) which is a non-scheduled drug found with paramethoxyamphetamine (PMA) in ecstasy tablets. PMA and PMMA have been associated with 10 deaths in the European Union [3].

Indeed, even when amphetamines are prescribed legitimately, they may be abused by the illegal distribution of the drug. Therefore, the laws must also regulate the possession of prescription drugs, which may be diverted for illicit use. To this end, there have been calls to prohibit long-term prescriptions for amphetamine-based medicines from experts in the field [22]. The extent of the problems which could be encountered from misuse of these drugs is clear when you consider that the authorized sales of amphetamines in the U.S., France, Italy, Germany and the U.K. account for 6 % of the total pharmaceutical markets. These prescription drugs are controlled in the U.K. as separate isomers and additionally through exemption clauses in the Misuse of Drugs Act Regulations, which allow for possession of D-amphetamine with prescription and authorisation for supply when a medic has undergone a registration process.

1.4. Drug Abuse in the U.K.

It is generally accepted that the U.K. and particularly certain sections of the community experiences significant social problems caused by (or instigated by) the abuse of illicit drugs. Opinions on the most effective way in which to tackle problem drug-use in the U.K. generally lie between two polar opposites. At one extreme there are calls for the legalisation or decriminalisation of all drugs with only minimum government regulation (cf. the current age limits on alcohol consumption) [23]. At the opposite extreme, the outright ban on the use of all illicit substances with severe penalties for non-compliance with the law. The current stance taken by the government in its 'ten-year drugs strategy' has the aim of reducing demand for substances through the use of campaigns which highlight the dangers of drug abuse while

imposing financial penalties or custodial sentences for possession, supply and trafficking offences [24]. The intention is also to reduce the levels of these substances on the streets by cutting the supply off at source through targeting the main producers and dealers.

The abuse of drugs in the U.K. has received so much media and political attention that it is almost impossible to break through the public fear, individual prejudice and scare-mongering tactics often employed by the media where illicit drugs are concerned. In order for policy-makers to make informed decisions on how to tackle problem drug-use, they must have access to accurate information on the patterns of drug abuse across the U.K. However, obtaining these figures is not an easy task, with the inherent problem that few people are prepared to volunteer information regarding their own substance abuse. This leaves gaps in the information available to those hoping to target those sections of the community most affected by drugs. Socio-economic factors such as income, education, ethnic origin, marital status, religious beliefs, alcohol consumption and inner-city living have all been shown to have an influence on whether or not an individual is 'likely' to abuse drugs [25]. However, the added psychological and physiological reasons why someone may abuse drugs are rarely explored in general surveys, as these issues cannot be addressed by a quick yes/no response in a questionnaire.

Several different surveys are occasionally conducted in order to obtain a clearer picture of the magnitude and pattern of the use of illicit drugs across the U.K. The primary source of information on the abuse of drugs in the UK, and the statistics on which the government bases drug policy is the British Crime Survey (BCS). In the past, sources such as the Addicts Index, obtained from medical referrals, and the Regional Drugs Misuse Database, using information compiled by the Department of Health, have been used to highlight sections of the community that are most susceptible to abuse and pinpoint where to allocate appropriate resources. There are, however, inherent problems with these databases. The Addicts Index accounts only for opiate and cocaine addiction while the Regional Drugs Misuse Database relies on addicts presenting themselves to a health-worker, which introduces inaccuracies and grossly underestimates the true scale of drug misuse. In essence, this data includes mainly individuals with heroin and cocaine habits who attend a clinic but excludes 'recreational' users of amphetamines and ecstasy, cannabis smokers, glue and solvent abusers, those abusing barbiturates and temazepam and those experimenting with hallucinogens such as LSD or 'magic mushrooms'. Clearly, these make up the bulk of drug abusers in the U.K. and therefore the figure of addicts reported [26] is not a true reflection of the extent of the problem. According to the EMCDDA, amphetamine and amphetamine-like stimulants are the second most common illicit drugs taken in the European Union. It estimates that

amphetamine has been taken by 1-5 % of adults in the European Union but by up to 10 % of the UK population [3]. Indeed, the most recent annual report of the International Narcotic Control Board concluded that Ireland has the highest percentage of amphetamine users in Europe, closely follower by England and Wales [27].

British Crime surveys, which are the government's main source of information on druge misuse figures are carried out every two years [28]. The drugs under examination are split into three classes, two of which relate roughly to the Misuse of Drugs Act classifications, the other being a 'catch-all' class including prescribed tranquillisers, steroids and solvents which may be subject to abuse. However, the information obtained from the questionnaire does not establish whether the drug use is escalating or declining, does not explore the 'stepping stone theory' (the likelihood of a cannabis user escalating to heroin use for example) or make any distinction between chronic abuse and occasional 'recreational' use. The results obtained from this study between 1994 and 2003 are shown in Table 1.2:

	1994 %		2003 %	
	any drug	Class A	any drug	Class A
Ever Used	25.9	9.0	36.0	13
Used in past year	9.4	2.1	12.2	3.3
Used in last month	5.4	0.8	7.4	1.5

Table 1.2 Illicit Drug Use in the UK, Evidence From the BCS, Brit. J. Criminol. 1999 and Findings 2002/2003

These results suggest that at the time the amphetamine profiling project was initiated over a quarter of the population of 18-60 year olds had consumed an illicit drug at some point in their lives and almost one-in-ten has used a 'Class A' drug and that this number was still increasing in 2003.

The Drugs Survey Investigators' Consortium used all relevant U.K. drug surveys to provide a more meaningful statistical analysis of the extent of the problem. Changes in the proportion of 16-24 year olds who reported the use of specific drugs were investigated since it was believed that this age-group were most likely to consume illicit drugs [28].

The results show an increase of at least one-third between '93-'95 and '96 in the reported use of amphetamine and/or ecstasy that year suggesting an escalation in the number of young people using specifically these drugs on a regular basis. It was perhaps results like these that led to work investigating possibilities of profiling amphetamines since these results showed worrying trends towards the use of these drugs.

16-24 year olds	'93-'95 report rate	Increase in report rate in '96
Amphetamine use last year	9.0%	3.4%
Amphetamine use last month	3.8%	0.9%

Table 1.3 Effective Monitoring of Young People's Use of Illegal Drugs, Brit. J. Criminol. 1999 and Findings 2002/2003

These statistics underlined an increase in amphetamine consumption, which had not been highlighted in previous surveys. The BCS (1994, 1996), the Health Education Authority National Campaign Drugs Survey for England (HEA), (1995, 1996) and the Scottish Crime Survey (SCS), (1993, 1996) and the Health Education Monitoring Survey (HEMS), (1996) were used and the data from these surveys is shown in Tables 1.4 and 1.5.

Amphetamine Use in the Last Year

Study	Year	Age Group	% Having Used
BCS	1994	16-24	9.3 %
BCS	1996	16-24	11.3 %
HEA	1995	16-24	8.8 %
HEA	1996	16-24	13.3 %
SCS	1993	16-24	9.0 %
SCS	1996	16-24	12.2 %
HEMS	1996	16-24	14.4 %

Table 1.4 Effective Monitoring of Young People's Use of Illicit Drugs, Brit. J. Criminol. 1999

Amphetamine Use in the Last Month

Study	Year	Age Group	Base Population	Point Estimate
BCS	1994	16-24	1,313	3.9 %
BCS	1996	16-24	1,222	5.3 %
HEA	1995	16-24	1,633	3.7 %
HEA	1996	16-24	1,542	4.2 %
SCS	1993	16-24	667	n/a
SCS	1996	16-24	642	n/a
HEMS	1996	16-24	481	6.7 %

Table 1.5 Effective Monitoring of Young People's Use of Illicit Drugs, Brit. J. Criminol. 1999

From these figures, it may be seen that the abuse of amphetamines among young people in the U.K. in the period before the amphetamine profiling project was initiated was escalating and perhaps explains why these drugs were the focus of research.

However, figures quoted in Europol's situation report on drug production and drug trafficking 2000-2001 [3] suggests that amphetamine use in the UK has decreased from a peak in 1997 where 3296 kg was seized to the year 2000 where only 240 kg was seized. Unfortunately, this may be attributed to the increase in popularity of ecstasy tablets at that time since seizures in 1995 totalled 500,000 tablets and this figure increased to 5,000,000 in 2000.

Home Office sources highlight that amphetamine seizures rose steeply in the period 1990 to 1996 and levelled off in 1997-1998 [29]. Since then the number of seizures has dropped by 28% in 1999 and a further 47% in 2000 with all police forces reporting a fall in the number of cases involving amphetamine. However, in the same year, the number of ecstasy seizures increased by 46% [29].

More recently, the Home Office Findings from the 2002/2003 British Crime Survey reported further decreases in the use of amphetamine as well as a fall in the number of people using ecstasy [30]. The only drug which showed an increase in abuse was cocaine. 4.6% of 20-24 year olds had used amphetamines in the last year compared to 6.4% having used cocaine which would suggest that the trend towards amphetamines is now on a downturn.

In total, 30% of 20-24 year olds had used illicit drugs in the last year which equates to almost 1.5 million individuals. Overall, the BCS claims that 12% of all 16-59 year olds had used an illicit drug in the last year and 3% had used a class A drug which equates to 4 million drug users and 1 million class A drug users in the UK alone.

1.5. Physiological and Psychological Effects of Amphetamine

The abuse of the β-phenethylamines increased steadily in the 1990s to become almost accepted as an integral part of youth culture throughout Europe. The upsurge in the use of amphetamine as a stimulant was initially associated with the 'rave' scene, centred in illegal parties in disused warehouses and fields and, as such, was a relatively 'underground' phenomenon. Today, the 'recreational' use of amphetamine, also known as speed or sulph, is on the downturn but is remains commonplace in many nightclubs and bars.

The popularity of amphetamine and analogues such as methylenedioxy-methamphetamine (MDMA, ecstasy), methamphetamine (ice), methylenedioxy-amphetamine (MDA) and methylenedioxy-ethylamphetamine (MDEA, eve) within 'club culture' stems mainly from their euphoric properties, the sense of well-being they endow (albeit briefly) and the rush of energy they give. Amphetamine stimulates the central nervous system and produces an adrenaline surge, a sense of alertness, and increased self-confidence [31]. This may lower inhibitions and enable users to act without normal social constraints and, for this reason, some amphetamines, especially MDMA have been called 'hug-drugs'.

Amphetamines are widely perceived as a relatively 'safe' drug and, certainly, the actual physical and psychological effects of amphetamine are dependant on the environment, dosage, combination with other substances, method of consumption and the physical condition of the user. Many users will only experience slight side effects such as an elevated temperature, through use. However, as is the case with most illicit preparations, the user is rarely aware of the concentration of drug they have consumed or what diluents or impurities are present. Therefore, although the effects of amphetamine at 'normal' levels are relatively predictable, the consumer never knows how 'normal' their dosage is [31].

In the U.K. Illicit amphetamine is generally obtained as a powder of the sulphate salt in a preparation containing around 4-6 % drug with diluents and bulking agents. However, a study of seizures in Norway obtained amphetamine concentration between 9 % and 99 % of bulk powder and in Denmark between 12 % and 34 % [32, 33]. The average 'wrap' or 'deal' in the U.K. is between 0.5 and 1 g costing £10 [34]. The drug is most commonly swallowed or snorted, the latter producing a more intense and faster effect. The most dangerous method, however, is intravenous injection of an amphetamine solution to produce an almost immediate and intense 'hit'. Here, there is a real chance of overdose if the concentration of the solution is too high, and a heart attack may result [35, 36]. In addition, users fall prey to the inherent risks of drug injection and needle sharing [37, 38] where the risks of HIV infection as well as Hepatitis B and C are the same as those for heroin injection. It is perhaps for these reasons that amphetamine, when prepared for injection is reclassified as a 'Class A' drug under the Misuse of Drugs Act [17]

Amphetamine is, strictly speaking, not physically addictive. However, users may become psychologically dependent on the effects of the drug. Moreover, with prolonged use, the withdrawal period increases in length and intensity and users may become fairly reclusive and irritable during this period [39]. In the case of chronic abuse, after days taking amphetamine to stave off depression and exhaustion, the user can collapse feeling restless and paranoid. In the most serious amphetamine binges this can result in clinical depression or amphetamine psychosis – a medical condition with symptoms akin to mild schizophrenia where the user experiences delusions of persecution and feelings of omnipotence [40, 41, 42]

When combining stimulants with prolonged exercise the body's temperature soars. Consequently, there is a serious risk of heatstroke, initially resulting in a faint feeling but possibly leading to unconsciousness and multiple organ failure [31]. Even drinking water to replenish fluids lost through excessive perspiration can cause serious damage if not consumed

at a sensible rate. Drinking too little results in severe dehydration and too quickly (more than three pints an hour) can result in water poisoning where the brain swells leading to unconsciousness and rapid death [31].

In addition, the combination of stimulants and alcohol is particularly dangerous since the effects of increased body temperature caused by the drug are exacerbated by the dehydrating effect of alcohol. Higher doses of amphetamine, when combined with alcohol, may result in liver and kidney damage when the demands on the body's purification systems become too great. Recreational use of amphetamine and 'ecstasy' has been known to cause hepatitis and acute liver failure [43]. Indeed, a condition known as 'Raver's Haematuria' is described when the combination of strenuous exercise and stimulant abuse causes rhabdomylosis and acute renal failure [44].

The ingestion of any stimulant quickens the pulse and strains the heart when combined with exercise or dancing. However, if dormant coronary problems exist, the effects can be fatal [45]. Acute myocardial infarction complications and cardiomyopathy have been reported after intravenous injection [35, 36] of amphetamine. Increased blood pressure is another potentially fatal consequence of amphetamine abuse. Small blood vessels in the brain may burst leading to paralysis or coma. Cerebral vasculitis and intracranial haemorrhage are well-documented effects of amphetamine abuse [46, 47] and acute pulmonary oedema has been attributed to amphetamine ingestion.

In addition to the effects of amphetamine sulphate when ingested alone, the combination of amphetamine and ecstasy is relatively common [31]. Those who want the euphoric 'hug-drug' properties of MDMA and the energetic feeling provided by amphetamine will take both simultaneously. More recently, ketamine has become popular as a combination drug with amphetamine [3]. These substances, when taken together, may produce an extreme reaction known as 'sledging'. The victim feels cold and shivers violently almost like a fit or seizure. They are unable to speak or move their body voluntarily and may then quickly lapse into a coma [31].

It is for all of the above reasons that amphetamine has been designated as a controlled substance. As underlined previously, the physical and psychological effects of amphetamine are relatively predictable and, therefore, controllable when a small amount is ingested. However, more concentrated doses can cause unexpected adverse reactions even in healthy young adults and fatal complications in users with undiagnosed health problems.

Consequently, the social cost of drug abuse is enormous. Directly or indirectly, addiction to drugs, cigarettes and alcohol is thought to account for a significant proportion of all hospital admissions, premature deaths and serious crimes [48].

1.6. Amphetamine Metabolism

Some other possible problems associated with amphetamine ingestion have been investigated using methods predicting how the drug is metabolised in the human body and the toxicity of the metabolites. Theoretical biochemistry has used the structure of the drug to predict compatible biochemical mechanisms for its uptake in the body. It has been reported that cytochrome P-450 mono-oxygenase enzyme was the only enzyme involved in the amphetamine metabolic pathway [49]. The consequence of mono-oxygenation of amphetamine was shown to be the oxidative deamination resulting in the formation of BMK and ammonia. Ammonia is not toxic in this case unless the glutamic acid, α-oxoglutaric acid and glutamine involved in the body's ammonia-binding mechanism are overwhelmed by excessive amounts of ammonia. However, if this does occur, the free ammonia accumulates in the cerebral tissues and may result in convulsions. BMK is not thought to be toxic and can be further metabolised by an aldoketoreductase to an even less toxic alcohol, which may then be converted to an ether glucoronide which is completely innocuous.

1.7. Medical Use of Amphetamine

Amphetamines are used in many medical preparations for a wide range of purposes. They have been used in the alleviation of depression, treatment of alcoholism, to maintain alertness, combat narcolepsy and as a slimming aid. The fundamental difference between illicit amphetamine and prescribed amphetamine is that the drug prepared in a clandestine lab will contain a racemic mixture of the D and L optical isomers, whereas the pharmaceutically prepared amphetamine is purely the D isomer. The D isomer is known to be 10-20 times more stimulating than the L form and therefore the same effect can be induced by prescribing less than one-tenth of the bulk drug [50].

Amphetamines were regularly administered to servicemen during the Spanish Civil War and the Second World War to keep them alert for longer periods [51]. However, their use was

phased out since their abuse was capable of producing aggression, confusion and panic attacks. The drugs were also used by students revising for exams in the pharmaceutical product form of Drinamyl or 'purple hearts'.

Initially prescribed in cases of obesity, D-amphetamine has anorectic properties and can be abused as such even when obtained from a legitimate source and in the pure enantiomeric form in prescription slimming aids such as Chlorophentermine. Amphetamine-induced anorexia is a well-documented phenomenon [52]. Many cases have been reported when patients lost control of their weight-loss and, as in any case of anorexia, their perception of body-image was further confused by the psychological effects of chronic amphetamine use.

The fact that D-amphetamine can induce a sense of well-being has also been used to treat clinically depressed patients [53]. Ritalin, another medicinal preparation of amphetamine, is controversially used to treat adolescents with Attention Deficit Disorder (ADD) [54]. Patients were thought to benefit from the alertness effects of the drug. Studies have shown that D-amphetamine improves selective attention and decreases the maintenance of attention to irrelevant stimuli thus making it easier for the patient to focus on specific tasks and becoming less distracted by other events around them [55].

Although illicit amphetamine is known to cause serious side effects, the positive attributes of amphetamines cannot be overlooked. It is therefore important to distinguish between amphetamine use and abuse.

1.8. Theories behind Abuse and Addiction

For some time, scientists have put forward the 'dopamine theory' of abuse and addiction which is based on findings that the ability to raise dopamine and noradrenaline levels in the brain is what links all mood-altering drugs. The feelings of pleasure produced by amphetamines are a result of stimulation of the meso(cortico)limbic dopamine function which is believed to be one of the main pathways for pleasure in the brain.

Dopamine is a neurotransmitter, carrying 'pleasure messages' from the storage vesicles in the central nervous system to neurons, or nerve cells, in the brain [56]. The major drugs of abuse can alter the function of neurotransmitters. The neurons which produce dopamine are only present in the order of several tens-of-thousands, from a total of around 100 billion nerve cells

in the brain but affect neurological activity in many regions – most importantly one of the main pleasure centres, the nucleus accumbens.

The level of dopamine in the brain is generally balanced by secretion and re-uptake by neurons. However, insufficient levels of dopamine in specific regions of the brain, causes paralysis and spasms such as those seen in Parkinson's disease. Excess dopamine causes hallucinations and mangled thought processes as observed in schizophrenia. Because of this, new anti-Parkinsonian and anti-Alzheimer's agents act as anti-depressants because of their dopaminergic action which redresses the balance [57].

In the short term, amphetamines activate the sympathic nervous system. Amphetamine blocks the neuronal re-uptake of the neurotransmitters, noradrenaline and dopamine, while similtaneously triggering their synaptic release. This means that more neurons release these neurotransmitters at a greater rate and, since they cannot return to the neurons, as is normally the case, the net result is a great enhancement of dopamine and noradrenaline in the brain [57]. In the long term, however, the knowledge that chronic amphetamine use can lead to depression as the withdrawal period worsens is borne out by the fact the body's natural store of neuronal dopamine in the mesolimbic pleasure centres is depleted by the use of stimulants [56]. Consequently, the user experiences less pleasure from the drug, which may result in an increase in their intake or provoke the user into progressing to notionally 'harder' drugs.

Chapter 2

Analysis of Amphetamine

2.1. Detection and Identification of Amphetamines

In any seizure of an unidentified substance suspected to contain a controlled drug, the analysis must detect and identify any illicit substances present. Before attempting to profile the impurities in a confirmed amphetamine sample, it is fundamental that the analyst identifies which members of the amphetamine family the sample contains. If a drug is sold as 'speed' the only active constituent may be amphetamine sulphate. However, 'street drugs' are seldom pure, and the possibility that a mixture of related drugs, such as other commonly abused phenethylamines are present, cannot be overlooked. In addition, some prescription drugs, which contain amphetamine-based constituents, may be added as bulking agents. Therefore, much research has been undertaken to determine the most successful method to detect, separate, identify and, in some cases, quantify each of the amphetamines present in a seizure [58].

To establish how to proceed with the chemical characterisation or profiling analysis of a seizure, the type of drug (or drugs) the sample contains must be assessed. Colour tests are a common form of preliminary screening for amphetamines although these tests cannot accurately or conclusively identify a specific drug but can be indicative of drug class. Although colour tests work well with concentrated pure drugs any tests performed on illicit seizures can provide only a tentative indication that an amphetamine or a derivative is present [59].

Microcrystal tests may also be used as a preliminary technique for drug identification. These tests have been shown to distinguish the D, L and racemic forms of amphetamine and, as such, may be used to establish if the amphetamine was produced and diverted from a legitimate pharmaceutical laboratory [59].

One of the most widely used techniques employed to differentiate between related amphetamine derivatives is thin layer chromatography (TLC). It is valued as a

complementary identification technique with, for example, HPLC or GC-MS and when colour reagents are employed the technique can be very specific. In 1982, researchers were able to separate and identify 19 amphetamines, a mixture of ring-substituted psychotropic amphetamine derivatives, as well as some over-the-counter drugs. Using retardation factors (Rf) obtained in two different solvent systems and four colour reagents, all of the amphetamines analysed were identified [60]. This technique requires skill and time but is inexpensive and is therefore used as an economical screening technique.

Ultra Violet spectroscopy may also be used but structurally related compounds display very similar absorption characteristics and identification of a specific compound is very difficult. The use of second derivative UV, however, has been able to distinguish between amphetamine, phenethylamine, phentermine, ephedrine and meperidine without the use of complex-forming procedures or dye-pair compounds which were previously used to enhance the UV spectra of amphetamines [61].

Radioimmunoassay techniques have also been proposed as an alternative technique for the detection and identification of amphetamine and its analogues in biological fluids (or in particularly dilute samples) with ^{125}I labelled amphetamine and an amphetamine antiserum [62]. The assay described by Mason *et al.* can detect amphetamine at 10 ng mL^{-1} in blood or urine in small samples (50 µL). However, this technique is unlikely to be used on a wide scale because of the environmental issues associated with radioactive waste.

Gas chromatography is one of the most widely used techniques for the qualitative and quantitative identification of amphetamines although it is generally used after a preliminary technique has detected the presence of an amphetamine. A flame ionisation detector (FID) is used as a universal detector although in some instances, selective detection of compounds containing nitrogen is offered by a nitrogen-phosphorous selective (NPD) detector. In addition, the electron capture detector (ECD) may be used after suitable derivatisation of the amphetamines although this is more commonly used in the detection of amphetamine in body fluids.

Where GC is used in toxicological analysis, the detection and identification of amphetamine may be complicated by the dilute nature of the sample, in which case, sensitivity must be maximised to detect amphetamine in trace amounts. One of the most successful ways to aid the identification and separation of amphetamine analogues (especially in body fluids) is chemical derivatisation of the drug [58,63,64]. This can enhance the chromatographic separation of the components and improve detection. Also, in the case of analysis by Mass Spectroctrometry, some forms of derivatisation can produce a more complex and unique spectra for compounds, which would otherwise produce virtually indistinguishable fragmentation patterns [58].

The formation of the N-Mono-trifluoroacetylated (TFA) derivatives of amphetamines by reaction with N-methyl-bis(trifluoroacetamide) (MBTFA) or trifluoroacetic anhydride (TFAA) is common. N-TFA derivatives are considerably less volatile than the parent amines with improved chromatographic properties [58, 65] and complex predictable fragmentation patterns in both electron impact and chemical ionisation MS. CS_2 derivatisation is commonly employed in working operational laboratories but this practice is being phased out due to the poisonous nature of the chemical.

The use of headspace GC avoids the loss of amphetamine content during the processes of sample preparation or derivatisation in urine analysis. An alkalinised urine sample is transferred to a sealed vial and heated using a water bath. A portion of the gas phase produced is analysed directly using GLC with NPD detection. This procedure is sensitive enough to detect amphetamine at extremely low levels found in urine samples after one single therapeutic dose [66].

In addition, the use of radiolabelled standards in GC-MS can compensate for loss of analyte during analysis. However, while radiolabelled standards are available for amphetamine itself, no standards are available for profiling impurities.

Due to its general acceptance as the most suitable technique for amphetamine profiling and the fact that systems and instrumentation are commercially available, GC-FID and GC-MS were selected as the techniques to be used in this project.

2.2. Amphetamine Profiling

The analytical techniques that have been employed in an attempt to carry out thorough amphetamine profiling, are many and varied. The first step is generally an attempt to separate the impurities from the active drug, normally present in the salt form. In most cases, this is an extraction technique, either liquid-liquid or solid phase extraction. Additionally, the impurities should then be separated from diluents or bulking agents. A further separation and detection technique is then required after the isolation of impurities, to provide data representative of the concentration of each component in the mixture. It may also then be necessary to name each of the components, in which case, an appropriate identification technique is required.

The technique of profiling impurities present in an illicit sample of amphetamine was initially established when the impurities present were found to be of significance in determining which method was used to produce the drug [67]. Research then continued into the most effective ways to tackle profiling with the aim to eventually using the information gained to follow drug trafficking routes [13, 14]. Alternatively, if a completely novel impurity pattern emerges, profiling may highlight the fact that an innovative procedure has been employed in clandestine labs.

Each of the three main routes of amphetamine synthesis produce a family of organic impurities which may be indicative of the synthetic route used and since amphetamines are almost always seized with many impurities still present [10], this class of drug is ideal for impurity profiling. A mixture of reaction by-products, excess starting materials and degradation products, these components may be indicative of the route used or common to several routes. An additional feature of amphetamines that makes them ideal for profiling is that, although the impurity mix may be complex, the components are introduced via the synthesis. Since the chemistry of these routes is well documented, the components are more readily identified than those present due to natural processes.

Furthermore, each individual batch of synthesised amphetamine may have a different pattern of impurities and these differences may be used to link smaller seized samples to each other or to a larger seized batch [68]. The impurity profile from different batches using the same method should not vary widely in the species present, but the relative concentrations of each of the impurities may differ depending on variation in reaction parameters. The final ratio of impurities is a result of many contributing factors such as: reagent purity, reaction

completeness, purification systems, capabilities of the chemist and so on. Impurities may also be introduced from the laboratory environment itself when a lab is also used for purposes other than the illicit production of amphetamine. These impurities are of utmost importance to an analyst when attempting to link a batch of seized amphetamine to a specific clandestine laboratory.

It must also be noted, however, that storage conditions and handling of the amphetamine after manufacture may also affect the chemical profile through time. The stability, or otherwise, of the impurities will affect the profile with time although it is doubtful that two similar profiles could have resulted from different batches degrading in different ways. It is also practically impossible to determine why two samples, produced by the same method, have slightly different profiles. This could be a consequence of reaction conditions, impurity degradation, storage conditions, purification procedures, addition of adulterants, external contamination or slow degradation of the final amphetamine batch converting back to precursors and perhaps changing the profile over time.

The fact that the number and type of impurities present in a 'street' amphetamine sample is so large and the sample matrix so complex means that any extraction technique or analytical procedure which aims to separate the components, must be robust enough to cope with any batch of amphetamine and its impurities. For example, the procedure must be able to analyse the impurities present in a Leuckart synthesis as well as a nitrostyrene synthesis. For this reason, several techniques have been studied to determine which method of analysis or combinations of procedures are required to successfully harmonise amphetamine profiling.

Many methods of amphetamine analysis, which have been employed, for detection and identification, include sample derivatisation. These procedures can aid analysis in several different ways; improving detection limits, increasing chromatographic separation, concentrating minor constituents and so on. These sample preparation methods are vitally important in the detection of amphetamine in particularly dilute or decomposed samples or body fluid analysis. Many analysts, however, are reluctant to introduce these derivatisation steps in impurity profiling even although the impurities may be present in only minute quantities. This is because profiling relies on an accurate analysis of the composition of the sample at the time of seizure. Some pre-treatments such as fluorescent tagging and radiolabelling which may improve the detection of the drug itself can alter the sample composition and, as such, are unsuitable for profiling. Subsequently, analysts are attempting to develop separation systems which minimise sample pre-treatment and eventually make direct sample analysis possible.

2.2.1. Extraction

In profiling, the impurities must be as fully separated as possible from the bulk amphetamine. The amphetamine is only present at approximately 4% of the bulk sample [69] and these impurities are present at even smaller quantities, they could in theory, be masked by the presence of the drug. One extraction procedure is a liquid-liquid method where the sample is dissolved in a phosphate buffer solution at pH 7.4, shaken with iso-octane for 10 minutes and extracted into the organic phase. [12]. Impurities can then be profiled using a suitable separation and detection technique, in this case GC-FID.

Some of the systems developed for the rapid sampling of impurities in amphetamine samples involve a pre-analysis step as an alternative to the liquid-liquid extraction using a bonded-phase silica sorbent – a solid-liquid extraction. The sample containing amphetamine salt is dissolved in a phosphate buffer, pH 7, and injected onto a C_8 cartridge where the neutral or weakly basic impurities are held initially and then eluted with acetonitrile after the bulk of the amphetamine has been washed away with distilled water. This eluant can then undergo a separation technique such as HPLC or GC [68].

Solid-liquid impurity extraction systems may be coupled to an HPLC. The amphetamine is washed away and the impurities are then injected onto a reverse-phase analytical column for separation. Alternatively, a method has been developed using a column-switching process, which enables the pre-analysis enrichment of impurities to be carried out as part of an automated system [72]. The amphetamine sample is dissolved in an acetonitrile-citrate buffer mixture and injected directly onto a C_8 enrichment column where impurities are retained and concentrated while the major components, (amphetamine and any strongly polar diluents present) are washed out of the system with distilled water. Valves are then opened to allow gradient elution of trace impurities, which are desorbed from the C_8 column and flow directly onto a C_{18} analytical column. Components in the eluant are then detected by UV absorbance at 254 nm and 220 nm. Since the impurity enrichment and extraction procedures are on-line, system automation is simple and, as such, appears to be more convenient for routine screening and comparison of seized samples. In addition, a fully automated analytical system decreases the likelihood of human error in the extraction stage. It also ensures better comparability of impurity profiles than manual extraction by different analysts.

2.2.2. Separation Techniques

Several separation methods have been employed in amphetamine profiling, each with their merits and disadvantages. These include, capillary electrophoresis (CE), gas chromatography (GC) coupled with mass spectrometry (GC-MS) and high performance liquid chromatography (HPLC).

Although capillary electrophoresis has mainly been used to separate and identify amphetamine from adulterants, structural analogues and impurities in drug seizures, CE has been proposed as a complementary technique to GC and HPLC for the analysis of illicit drugs [70, 71]. However, this technique employs non-volatile running buffers that are difficult to remove prior to the MS analysis required to confirm the identity of the components present. The technique outlined by Krogh *et al.* is based on micellar electrokinetic chromatography (MEKC) and can separate neutral and highly charged species in a single run [71]. Since amphetamine and its impurities may cover a range of polarities, this technique could be established as an alternative to other techniques that may require extensive sample preparation. MEKC has good resolving power and is fast, with complete analysis times around 15 minutes. However, the use of MEKC as a technique for the profiling of amphetamines appears to be limited by the fact that the adulterants and amphetamine itself are in such excess that the impurities are masked or not detected.

GC is a high-resolution instrumental technique, which allows separation and analysis of complex mixtures such as those resulting from amphetamine synthesis. However, although the resolving power of the technique is powerful, the possibility of co-elution of components in the mixture still exists. Coupling MS to the GC can allow a more definitive identification of each peak and determine whether co-elution is a problem. In addition, both GC and GC-MS can be used quantitatively provided a suitable internal standard is used. However, there are inherent problems with the techniques. An extraction procedure may be necessary to separate the impurities from the bulk drug since the impurities are generally in such small quantities when compared with the drug itself [68, 32, 10]. The extraction procedure for a Leuckart synthesised amphetamine may not be optimal for use with amphetamine synthesised via nitrostyrene and this hinders harmonisation of the general method. In addition, the high temperatures necessary for vaporisation in GC may prove problematic since primary amines are thought, in some instances to react with other components of the profiling mixture to produce imines. In addition, impurities present in some common solvents may react with the analytes at higher temperatures.

The problem of elevated temperatures and thermal stability of the sample may be overcome by using HPLC [72, 73]. However, analysis times are longer and the technique does not always offer the same resolving power as GC. Co-elution of components and accurate quantification of impurities can become problematic. As a result, statistical analysis, which may be required for profiling is not always possible.

2.2.3. Amphetamine Profiling

One of the first studies into the use of amphetamine profiling to trace possible manufacture sites and chains of illicit drug distribution was carried out in 1975 by Strömberg [67]. In this research, the discovery that analysing the minor components present in cannabis resin could assign different seizures to common sources was extended to other illicit drugs, including amphetamine sulphate. This study used comparative gas chromatography to give a representation of the 'chemical signature' of each sample to determine the extent of 'inter-batch' variation in chemical profile (sampled from different sources) and 'intra-batch variation' (samples removed from a single batch). Experiments were designed to determine how the method of production (electrolytic reduction of nitrostyrene, $LiAlH_4$ reduction of nitrostyrene or the Leuckart synthesis) affected the 'chemical signature'. It was found that the preparation method could be deduced from the presence of recognised trace components originating from the side reactions of the route. A double detector system, utilising both FID and ECD was used for a more definitive recognition of the trace impurities. As well as comparing retention times, the ECD and FID response ratios were used as another parameter for identification.

In addition, within this experiment, a series of Leuckart amphetamine syntheses were carried out, with slight alterations in the experimental conditions each time. These showed a strong influence of reaction conditions on the 'chemical signature'. However, when conditions were kept constant, reagents were obtained from the same manufactured batch and the reaction procedure was followed exactly, the patterns produced were extremely similar. These findings provided a very strong argument for the use of profiling of smaller seizures in order to link them together to a larger batch.

Many of the studies carried out to date, have focused on impurities found in the Leuckart synthesis as the production method most frequently employed in Western Europe and the US [14, 9]. A study of the seized amphetamine in Norway between 1975 and 1982 found that, of the 28 amphetamine samples analysed, 22 contained Leuckart specific impurities suggesting that an estimated 79 % of the total amphetamine seized is Leuckart amphetamine [32]. This

percentage obviously varies between countries and over time. However, many of the findings on Leuckart synthesised amphetamine are applicable to the analysis of alternative routes. Research by Lambrechts *et al.* produced amphetamine sulphate by the Leuckart route while altering various purification conditions after synthesis in order to assess the occurrence of specific impurities and reproducibility under each set of conditions [68]. Ether extraction, steam distillation or recrystallisation of a Leuckart product, was carried out and replicated six times. The amphetamine sulphate produced was analysed by GLC-FID and GLC-MS. It was found that the level of impurities present varied and their relative proportions were significantly altered by the purification method. As expected, similar impurity patterns were obtained from batches produced using the same experimental procedure but every single batch had an impurity profile distinct from the others.

Perhaps the first definitive research published on the profiling of amphetamines was undertaken by Verweij where a pre-analysis process was used to extract and concentrate impurities present in an illicit amphetamine sample [11]. Initially, the sample was dissolved in water and tartaric acid was added to obtain a weakly acidic solution. Next, the solution was extracted with ether and subsequently extracted with 4 N hydrochloric acid. A portion of the hydrochloric acid solution was made alkaline and extracted with chloroform to give a fraction containing weak bases. A portion of the ether layer was evaporated to give a fraction containing neutral species. The tartaric acid solution was made alkaline and extracted with chloroform to give a fraction containing strong bases. Preparative TLC was then used to isolate each component of the fractions. Mass spectrometry, ^{1}H NMR and ^{13}C NMR were then used to confirm the identity of each of the species. The data published gives an account of each successfully identified impurity, for each of the common routes of synthesis along with their MS data. From the findings, it is concluded that the analysis of seized amphetamine by HPLC can be used to determine if amphetamine preparations originate from the same batch. However, the argument is also made that the Leuckart synthesised amphetamine is more suitable for impurity profiling than the other routes of synthesis since, as a result of the condensation reaction, there are many more reaction pathways and therefore more variables in the impurities in the profile [11].

In 1984, a study to determine how the GC chemical profile of amphetamine batches differed between syntheses, carried out by the same chemist using different sets of standard conditions, was undertaken based on experimental notes found in a clandestine laboratory. Reagent concentration, reaction time, temperature, amphetamine base extraction method and treatment of precipitated amphetamine sulphate were studied and a standard procedure established which was then followed rigidly by five chemists acting independently, each

producing two batches of amphetamine. The batches produced were then analysed by gas chromatography with FID detection, producing complex multi-peak chromatograms. The research showed that, although the reaction conditions for each synthesis were reproduced very closely and the chemical profiles produced were similar, different patterns were obtained for each of the ten syntheses. In conclusion, variation in batches occurred between batches produced by different chemists using the same procedure and by a single chemist using the same method to produce more than one batch [14]. In most cases, the same impurities were identified although at different concentrations. Nonetheless, in some syntheses even the number and type of impurities varied. This study therefore provides strong support for the evidential value of gas chromatographic profiles of amphetamine seizures to link two samples having originated from a single batch. It does, however, raise questions about the evidential value of impurity profiles linking two samples made by the same chemist on different occasions following exactly the same procedure.

However, this does highlight some crucial points. In a real clandestine laboratory, would conditions be followed extremely closely for every batch? Would the same source be used for precursors? Would every batch be kept separately or would the batches be combined after synthesis in one mixed bulk sample? If a professional chemist prepared batches which had similar, but not identical, profiles what results would an untrained individual obtain? These points all limit the evidential value of amphetamine profiling but are outwith the control of the authorities and are questions that cannot be answered in this thesis.

What this project set out to do was establish an analytical method which can be used to extract and analyse samples of amphetamine for profiling purposes. It will test whether extracts from a single source will generate the same profile consistently. If samples from a single synthetic batch are divided and distributed to different individuals and these samples arrive separately in the laboratory for testing will the extraction and analysis of these samples generate identical profiles?

2.2.4. Data Processing

While the methods employed in the analysis of impurities are of importance, the way in which the data obtained is processed is also fundamental. A visual comparison of chromatograms with those of known standards will give a good indication of which synthesis method was used. A visual comparison of chromatograms obtained from two samples will highlight similarities in the profile. However, some statistical analysis of the chromatographic data may be required to obtain a meaningful comparison of samples [74, 75]. Data presented

statistically and graphically also eases the exchange of analytical information between relevant authorities. It is perhaps preferable to have 'numbers' to present in criminal proceedings rather than present two chromatograms and simply point out similarities.

However, due to time constraints and limited experience in statistical analysis, I have focused on visual examination of profiles while employing basic statistics to determine the variation between samples where appropriate. I recognise that numerical classification and statistical analysis may be appropriate in some case and this could be explained in further work with this original data.

2.3. GC-MS Introduction

In choosing the most suitable method of analysis for amphetamine profiling, there are many factors to consider. As well as providing a means of successfully and consistently separating and analysing complex mixtures, the system chosen should be commercially available, and be relatively robust to minimise the amount of maintenance required. It should be completely automated to reduce man hours spent on analysis and be capable of high throughput analysis for use in a busy laboratory.

2.3.1. Gas Chromatography Basics

In gas chromatography, a mobile phase continually sweeps a sample mixture in the gaseous phase through a column containing a stationary phase which is coated onto the inner wall of the column.

The whole concept of gas chromatography relies upon the fact that the extent of separation of components is relative to the amount of time that molecules of different components are associated to the stationary phase. This period is determined by the attraction of the solutes to the stationary phase and the comparative vapour pressures of components.

As a separation technique, GC is restricted to those compounds that are volatile but thermally stable. However, molecules of limited volatility and stability may be chemically derivatised as outlined in section 2.1, changing their molecular structure to improve their suitability for the technique.

2.3.2. Partitioning Processes

When injected onto the GC column, sample molecules partition between the gaseous and stationary phase. The extent of this partition is governed, predominantly, by their attraction to the stationary phase chemistry and the vapour pressures of the sample components.

At its simplest level, the path of a component through the column may be considered a stepwise process. Solute molecules in the gaseous phase pass through the column at the same velocity as the carrier gas. As those molecules are exposed to the stationary phase, they become associated to it and are retained depending on how strong their interaction with the stationary phase is. They then re-vaporise, enter the mobile phase and travel along the column until they encounter fresh stationary phase and re-associate. The process is more complex than isolated vaporisation and resolution steps and at any given time, solute molecules are present in both stationary and mobile phases of the system.

To improve partition the solutes should be subjected to the maximum possible dissolving and vaporising steps along the column to separate solutes with similar vapour pressures. If the relative polarity and attraction of two components for the stationary phase is very similar, the order in which solutes are eluted and the degree of solute separation may be predicted on the basis of solute vapour pressures. If the vapour pressure of a species is too high, the species will remain in the mobile phase, spend less time associated to the stationary phase and no true partition between the phases will occur. If the vapour pressure is too low, the species will remain in the stationary phase for too long, analysis may take longer and may broaden the solute band and the chromatographic peak. To combat this, increasing the temperature as the sample is swept through the column has the effect of gradually increasing the vapour pressure of the solute encouraging it into the mobile phase. In addition, since the vapour pressure of a solute is exponentially related to temperature, a small change in the column temperature will bring about a significant change in its vapour pressure. This enables separation of components with very similar vapour pressure using temperature gradients.

2.3.3. Separation and Resolution of Peaks

Ideally the vaporised sample will enter the column as a sharp band of mixed molecules, bands of different molecules separate from each other along the column and molecules of each separate component will remain in close proximity until they elute and are detected as sharp discrete peaks. The time spent in the stationary phase for all molecules of one species should be identical so that a tight band of solute molecules emerges from the column as a sharp peak.

A system's ability to resolve peaks may, however be affected by peak-broadening processes along the column that may result in co-elution of solutes. A following peak's front may elute simultaneously with the tailing edge of a previous peak resulting in incomplete separation of components. Although the theoretical difference in retention time of two components may be sufficient effect a separation, significant peak broadening may cause co-elution. Resolution is a nominal measure of the degree to which two closely eluting components are separated and relates to the difference in the retention times of two components in relation to their combined width at half peak height.

2.4. Components of a GC System

The main components of a gas chromatographic system are briefly discussed in the following sections.

2.4.1. Carrier Gas

The choice of carrier gas in a GC system is limited but fundamental. The gas should not interact in any way with the sample. The gas is used for the sole purpose of transporting the vaporised components from the injector to the detector. Carrier gases must be entirely oxygen and moisture-free since both will quickly degrade columns and are incompatible with mass spectrometers. The gases should also be free of other contaminants that may be detected and significantly increase the baseline response. Since capillary GC benefits from using the carrier gas with the smallest molecular weight possible, *He* and H_2 are the most suitable gases. However the inherent risk of explosion in using H_2 has resulted in *He* being used most frequently and is employed in this study.

2.4.2. Injection Port

The injection block in GC can take several forms depending on the preferred method of injection, the state of the sample and the volume to be injected. The inlet guides the syringe through a self-sealing septum into the glass liner of the vaporising chamber and onto the column. The block is held at a specified pressure at the column head to ensure that the carrier gas flow is constant. More sophisticated injection techniques such as cool-on-column may be required to place a very small quantity of sample onto the column without compromising separating efficiency.

In splitless mode, a heating block surrounds the vaporising chamber into which the sample is injected. The vaporising chamber volume must be sufficient for the expansion of gas from the injections. While the split valve is closed the vapour formed in the vaporising chamber is swept onto the column. This valve is then opened and the remaining vapours flushed out of the system through a purge vent. Short purge times decrease solvent tailing and enable the remaining solute to reach the column quickly. The injector temperature must be sufficiently hot to vaporise all of the components within the sample but not degrade the sample or cause thermal shock in the molecules.

In split mode, the vaporised sample gas is split through three routes. A portion of the vapour is allowed onto the column and a specific volume escapes through the split exit valve. The split ratio used will depend on the injection volume and the loading capacity of the column. A small amount of vapour is flushed through the septum purge outlet to prevent bleeding of organic components from the septum through the column.

2.4.3. Retention Gaps

A retention gap is a length of deactivated, fused silica column without stationary phases or free silanol groups. Retention gap columns are fitted to the injector port in the same way as a normal capillary column. One end of the analytical column is then attached to the end of the retention gap and the other to the detector. The retention gap in GC can be a pre-concentration step for large volume injections, for example, when a splitless injection is used. At injection, the sample vapours spread over the retention gap surface and condense in a thin film but do not adhere to the surface. There is an abrupt change in the stationary phase entering the column causing the mixed band to form a plug focusing the band before any separation. The solvent begins to evaporate at the inlet side of the column, concentrating the molecules present in the condensate while carrying the entire mixture forward and when all solvent has evaporated the analytes are carried through the column as a concentrated band of vaporised sample molecules. Therefore, on reaching the analytical column, the sample will be held by the stationary phase in a narrow band while the partitioning process between mobile and stationary phase begins.

Where retention gaps are used and flow is split between two columns, column connectors and y-shaped splitters are necessary. Glass press-fit connectors are light and do not stress the columns. The gas-tight seal is achieved by pressing the polyimide layer on the outside of the column to the inner glass layer of the connector. These are essentially one-use connectors

since the polyimide sticks to the connector on heating. If re-used these polyimides may act as a site of activation for solutes.

Both retention gaps and y-shaped splitters are used in this study to connect one column to two detectors and one retention gap to two columns.

2.4.4. Ovens

The control of column temperature is vital in isothermal and programmed runs as used in this project. The column temperature is controlled independently of the injection port and the detector and must accurately and rapidly respond to computer control to ensure that temperature programmed runs are comparable every time.

2.4.5. The Column Stationary Phase

Three main types of interaction between the solute molecules and column stationary phase may occur: dispersion, a relatively weak association, stronger dipole-dipole interactions and strong acid-base bonds.

Dispersion

These attractions form the basis of most interactions between solutes and stationary phase molecules. If dipoles within a molecule are created by oscillations of the molecule's electrons through different positions, then at any instant the molecule will have a specific instantaneous dipole depending on the asymmetry of the electrons. This dipole will, in turn, result in polarisation of nearby molecules causing attraction between those molecules. If weaker dispersion interactions are the dominant force between stationary phase and molecules, the relative volatility and vapour pressure of the components becomes a greater influence in determining retention time.

Dipole

This form of interaction is less common than dispersion but is fundamental in separations of more polar compounds. If both solute and stationary phases contain permanent dipoles, the two may be aligned causing a strong attraction between them. If only one possesses a strong permanent dipole, an instantaneous dipole may be induced in the other. These interactions are normally a result of individual functional groups of the components.

Acid-Base

If solutes contain a hydroxyl group, the possibility of hydrogen bonding with the stationary phase is an important factor in separation as these stronger interactions will significantly increase the retention times of a component.

As a general rule, a stationary phase which has more interaction with the solute molecules is likely to achieve better separation of these components. Alternatively, if solutes with high boiling points or a very strong interaction with the stationary phase are present, this could result in a very long analysis time and it is then more suitable to choose a stationary phase with less retentive properties.

Polysiloxane stationary phases are now the most widely used column type. 100% polydimethylsiloxane columns are low polarity stationary phases with a broad range of applications. The polysiloxane molecule exists normally as a coiled helical structure which shields the siloxane bonds on the inside projecting the alkyl groups outwards to interact with the solute molecules.

The dimethylsiloxane phases may be cross-linked and covalently bonded to the support surface or column wall. These gums form films with uniform thickness and produce high efficiency columns. The main problem with these columns is that they have no real functionality and interaction of the stationary phase with solutes is limited to dispersive forces. Therefore, the elution of solutes is based almost solely on vapour pressures and solutes with identical boiling points may co-elute. Therefore, in order to separate these components, a more selective phase may be required.

Substitution of a proportion of the methyl groups for other functional groups imparts greater selectivity to the column. However, this disrupts the helical structure of the molecules and properties, such as the thermal stability of the phase, are compromised. Substitution of 5 % of the methyl groups for phenyl groups in the stationary phase has given rise to one of the most widely used column types available. The 5 % phenylmethylsiloxane columns have slightly increased polarity without compromising the thermal stability of the column surface. As the percentage diphenylsiloxane content increases, the polarity increases. As this approaches the 35-50 % range, the phases are of mid-polarity (such as HP-50 or DB-35). Substitution of more polar functional groups increases the likelihood of dipole interactions and possibly acid-base interactions between the solute and stationary phase making them more selective to specific components in a mixture. For example, phenylmethylsiloxane columns can share

electrons with aromatic compounds and display greater selectivity towards them. Nevertheless, with more substitutions, these columns become unstable and although they have high selectivity for specific types of solute they may display little or no selectivity for other solutes and are consequently less useful for general analysis.

2.4.6. Peak Tailing/Fronting

Peak tailing or fronting is caused by band broadening processes occurring during analysis. Some injection methods can increase the possibility of band broadening even before the sample reaches the column. The use of a splitless injection increases the volume of sample taking longer for the total contents of the heated chamber to enter the column resulting in the sample spreading over the inlet-side of the column. Very broad peaks may result if the separating power of the column is not sufficient to fully resolve discrete bands of analyte.

The situation may be improved by on-column focusing through temperature programming but the column must initially be at least 80 °C below the boiling point of the first compound of interest to completely focus the band. Since the column is then colder than the injection port, the sample enters the column as a small plug, either by attraction to the stationary phase or by the sample condensing on the column almost immediately.

Another option is to use a split technique with a high split ratio resulting in the rapid removal of most of the vaporised sample material from the injection port. This means that the small volume of sample left can be rapidly swept onto the column. Split methods give a narrower bandwidth and sharper peaks but with a loss of system sensitivity because most of the analyte does not enter the column.

As the solutes pass through the column, more broadening processes come into play. Diffusion in the mobile and stationary phases may result in broader peaks. Longitudinal diffusion causes band broadening, tailing and overlapping peaks – diffusion of molecules from a region of high concentration to a region of low concentration occurs due to thermal processes.

Peak tailing in hydrocarbon molecules may be attributed to flashback in the injection port (where the sample volume exceeds the capacity of the vaporising chamber) or insufficient gas flow. Peak tailing may also be a consequence of active sites in the system such as free silanol groups or polyimide residues in column connectors. These have additional attraction for more polar solute molecules and may cause a proportion of these molecules to be held for a period of time in addition to that spent associated with the stationary phase. This increases the

retention time of those molecules relative to the bulk solute band leading to a skewed distribution of the solute. Similar band broadening processes may also be attributed to non-volatile residues that have been trapped in the injection port liner. If the liner is not completely free of contamination, polar solutes in the injected mixture may be held by polar residues in the liner.

Peak fronting may be caused by column overload. If the amount of solute associated to the stationary phase is large, the solute in that region then acts like an additional stationary phase. Therefore, solute molecules in the mobile phase may be affected differently depending on which region of stationary phase they become associated to. Those at the front of the band pass over fresh stationary phase and as such have a 'normal' path through the column. Other molecules in the band may experience the region with 'additional stationary phase' and progress more slowly through the column. On a chromatogram this appears as a gradually increasing concentration of molecules following a 'normal' path through the column followed by molecules which have been associated to the additional phase region and a sharp decrease as the last molecules emerge from the column. This can be corrected by decreasing the sample size, increasing the temperature of the column, using a thicker column film or using a more effective stationary phase to give better separation for the analytes of interest.

Peak tailing or fronting may be given a numerical value by calculation of the peak tailing factor as determined by chromatographic software (Chemstation Enhanced Data Analysis) as follows.

Peak tailing = BC/AB.
Peak Height = DE, 10 % Peak Height = BD, Peak Width at 10 % Peak = AC, Apex = E

2.4.7. Flame Ionisation Detector

Detectors for GC systems are located in a heated zone at the outlet of the column. In some systems, more than one detector is employed for different purposes. The effluent from a single column may be split to the detectors or the injected sample may be split to two columns with separate detectors. However, the universal detector for GC is the flame ionisation detector (FID) and even in systems with multiple detectors an FID is normally present.

The popularity of the FID as a detector in combination with a GC is mainly due to its high sensitivity to virtually all organic compounds. It has little or no response to water, CO_2, *He or*

N_2. Providing that the carrier gas is contaminant-free, the FID produces stable baselines and, in terms of detector response, the FID shows good linearity over a wide working range.

The analytical column feeds straight into the detector. Just above the column tip as the solutes emerge they enter an *H_2/air* flame. In the flame, organic molecules undergo a series of reactions causing thermal fragmentation, chemi-ionisation, ion-molecule and free-radical reactions. They undergo thermal cracking and stripping of protons and terminal groups.

Pure H_2 and air contains H•, O• and OH• radicals but no charged species. However, when organic molecules are introduced into the flame, ionisation occurs which is proportional to the number of carbon atoms present.

$$C + O \rightarrow CHO^+ + e\text{-}$$

The ions formed travel to the collector electrode, which is maintained at a negative potential with respect to the flame so that the electrical current created is proportional to the concentration of charged species in the flame.

2.5. Mass Spectrometers

Mass spectrometers separate and measure ions formed from molecules on the basis of their mass-to-charge ratio, m/z. The pattern of m/z ion abundances is a characteristic of the original molecule that allows for tentative identification. Although most ions are formed with a single positive charge in which case, m/z is equal to m, the mass of the ions, doubly or triply charged ions are achieved in some instruments to allow for a wider range of molecular mass to be studied.

2.5.1. The Formation of Ionic Species from Neutral Molecules

As a general rule, mass spectrometers function by the creation, detection and quantification of charged particles. The nominal mass and relative abundances of these ions are representative of the original molecule of the compound of interest. Therefore, in order to identify 'neutral' compounds using mass spectrometry, the formation of ions from non-ionic molecules is fundamentally important.

If necessary molecules may be converted to a charged state either by the removal or addition of an electron resulting in positively or negatively charged species. The most common means

of introducing charge to a neutral molecule (and the method used in this study) is electron impact ionisation (EI) which involves the removal of an electron to form a positively charged ion species. These processes do not cause a significant change in the overall mass of the molecule so the ionised molecules have the same nominal mass as the original molecule. Alternatively, the molecule may be ionised by addition or subtraction of charged species with a mass significantly large to produce ions having a different nominal mass to the original molecule as in chemical ionisation (CI).

2.5.2. The Fragmentation Process

If a molecule is ionised solely by the ejection of an electron, the ion formed, is said to be the molecular ion, or in some cases the parent ion. The parent ion will have the same nominal mass as the original molecule since the mass of an electron is so small as to be negligible.

Ions having no excess energy would be stable enough to prevent subsequent fragmentations and would generate information only on the molecular mass providing very little structural information. In cases where this molecular ion contains excess energy, fragmentation or cracking can occur. Specific bonds in the molecule are broken, and in the process, new neutral fragments and fragment ions are created. If a new fragment ion also has excess energy, it too may fragment in the same way and further decomposition will occur until species are formed which do not have enough excess energy to fragment further.

This whole decomposition process from molecular ion to smaller, more stable ions is a fragmentation pathway. However, one molecular ion may travel along several different fragmentation pathways and therefore a set of identical molecular ions may create alternative sets of fragments depending on which fragmentation route they follow. The combined total of the molecular and fragment ions from all of these routes creates the fragmentation pattern. The complete fragmentation pattern is displayed in the spectrum and it is this complete image that is characteristic of the original molecule.

All of these processes and changes in the composition of the molecular ion are what determine the eventual m/z distribution of the molecular and fragment ions and eventually make up the m/z spectrum. The mass spectrum is normally recorded as a percentage of total ion current (%TIC). That is, the current achieved in the detector, a product of the abundances of all ions of significant size (normally above m/z of 40) combined to give the total ion current. Each m/z abundance is reported as a percentage of this total value. It should be noted that the mass spectrum is not a well-defined and easily transferable property of a molecule.

The spectrum generated is dependant on many more factors than the molecule's structure and there is no guarantee that the spectrum obtained from one instrument will be identical to that obtained from another, even when using the same instrument type under what are though to be identical conditions. Ideally, a spectral library obtained on the same instrument should be used for comparisons.

2.6. Mass Spectrometer Instrument Design

2.6.1. Sample Inlet

In terms of mass spectrometers used in conjunction with gas chromatographic systems, the effluent from the gas chromatograph normally consists of carrier gas along with separated compounds. Although the flow rate through most capillary GC systems are around 0.5 to 2 mL min^{-1}, a pumping system will allow the mass spectrometer to maintain an extremely low pressure even when the total stream of effluent enters the system to maintain the mass spectrometer vacuum.

2.6.2. Ion Source

The ion source is the region of the instrument in which ionisation occurs. This normally comprises a small chamber in which the ions are produced and are propelled out, drift out or are trapped in the source depending on which type of analyser is used. In the quadrupole mass spectrometer (as used in this study) the ions drift out of the source under the influence of a small electrostatic field. Although neutral species are formed after fragmentation of the molecular ion, their movement is not influenced by the electrostatic field and these species are not detected by the instrument. In positive ion mode, any negative ions formed by the capture of an electron will be discharged at the positively charged repeller and be ejected.

2.6.3. Electron Ionisation

Inside the ion source, a hot metal filament produces energetic electrons with energy equivalent to eV where e is the electron charge. The mass spectra may be obtained at any voltage provided that it exceeds the required ionisation energy of the molecules in the chamber. The voltage applied can be adjusted but most mass spectra are obtained at or around 70 V since this voltage yields the maximum number of ions in a reproducible manner. Most

spectral libraries are obtained at 70 V and therefore for ease of comparison, this has been used throughout this study. The electrons formed are accelerated through the ion chamber past gaseous molecules of the sample and if the accelerated electron passes close to a gaseous molecule, electron waves and the electric field of the molecule interact and interfere with one another. Some of the electron waves have the correct energy to interact with the electrons of the molecule and an electron may be propelled from the neutral molecule creating a positive overall charge. Since electron ionisation relies on the electron coming in close enough proximity to the molecule to effect any change in the overall charge less than 1 % of the sample molecules are actually converted to molecular ions.

Being in a vacuum, the molecular ion cannot lose any more energy from collisions and so if the excess energy is sufficient to cause the molecular ion to be unstable, it will fragment to create further ions of smaller mass. Using a higher voltage will increase the extent of fragmentation of the molecular ion, which is useful in some cases where the abundance of the molecular ion is very high and few fragments are formed making it difficult to ascertain any information other than the compound mass. In some cases, a molecule may fragment too rapidly resulting in very small fragments with no real structural information to be gleaned from the spectra. In this case, a low voltage is advantageous to control the extent of fragmentation.

2.6.4. Analysers

Here, ions are separated according to their m/z ratios. This is accomplished mainly by the use of electric or magnetic field.

In the quadrupole analyser, electric fields are created to allow ions having different m/z ratios to sequentially pass through the analyser to the detector. Ions from the source are propelled towards the analyser by a small applied voltage. Four parallel rods are arranged in a tube, with opposite rods connected and a DC voltage applied to them. A radiofrequency potential is also applied, superimposed onto the DC voltage.

The combination of electric fields forces the ions to follow oscillating paths through the quadrupoles. If the oscillations have a defined path with finite amplitude, they will pass through the poles to the detector. If the ion trajectory is not defined then they move through the poles with an unpredictable path, collide with the poles and do not reach the detector. At specific values of DC voltage and radio frequency amplitude, some ions will have stable oscillations and pass to the detector whereas others will not. By carefully varying the DC

voltage and the amplitude of the radiofrequency potential, ions with a specific m/z ratio will be sequentially allowed through the quadrupole to the detector. In reality, the DC/RF voltage ratio is fixed but the voltages increased maintaining the ratio and bringing ions with different m/z ratios into their 'stable' region. At any point in a scan across the voltages, only ions with one m/z ratio will pass to the detector and the rest will be excluded.

In modern instruments, the entire m/z range under investigation can be scanned very quickly, obtaining many spectra per second. This speed is vital if compounds eluting from a chromatographic system emerge in very narrow bands passing through the system in a fraction of a second.

2.6.5. Ion Detection

Ions emerging from the analyser section are detected producing a signal proportional to their ion abundance. In the case of the quadrupole analyser, the ions that have been sequentially separated from each other are directed to a single channel detector. In most cases, the detector is an electron multiplier, which consists of a series of electrodes, and when a rapidly moving ion hits the first electrode, a cascade of electrons is created. This electron cascade is accelerated towards the next electrode causing another cascade and so on. This series of cascades continues through around twenty electrodes and each time causes an increase in the electric current from the electrons. In total, a gain in current of 10^8 can be achieved. The amplified signals from a single channel detector can be transferred directly to a computer which converts the electronic signals for each individual scan of the m/z range to a mass spectrum.

2.7. Project Information

Experimental work on the 'Development of a Harmonised Method for Profiling of Amphetamines' began in February 1999 with funding provided by the Standards and Methods Testing (SMT) department of the European Union, DGXII. The overall aim of the project was to study, establish and report on a harmonised, collaboratively tested method for the analysis of seized amphetamine sulphate and free-base to be used in forensics laboratories throughout participating European countries. This was mainly an attempt to increase the distribution of meaningful analytical data among relevant institutions since information exchange was difficult at that time.

Four partner laboratories in Scotland (Strathclyde University), Finland (National Bureau of Investigation, NBI) , Sweden (SKL) and Switzerland (University of Lausanne) were responsible for practical and experimental work throughout the project. An additional three laboratories in Denmark, the Netherlands and Portugal were involved on a consultancy basis, providing support and guidance to the project based on their knowledge and particular experiences in amphetamine casework.

Essentially, the project may be divided into six phases, which, on completion should enable the group to establish and make recommendations on the most suitable method of analysis of amphetamine seizures for the purposes of chemical characterisation. The phases were as follows:

Section 1. Synthesis of standards (known impurities) for use throughout the project:
Section 2. Study of the stability of the impurities as mixtures in solution:
Section 3. Optimisation of gas chromatographic and detector systems:
Section 4. Optimisation of impurity extraction protocols:
Section 5. Determination of stability and variability of extraction and analysis protocols:
Section 6. Investigation of the numerical classification schemes for amphetamine.

My involvement in the project began part way though Section 1 and continued though Sections 1-5 when my period of study finished. This thesis follows the order of these sections to some degree.

The project management was such that each section was headed by one of the four partner laboratories, and the design of each section and control of experiments was the responsibility of that particular partner. For example Strathclyde University set up Section 1 and divided the task of synthesising and identifying impurities within this section between participating laboratories as described in Chapter 3.

When experimental work in each section was complete, a compromise between what each partner had concluded was established and agreed. It was this compromised method that was then taken forward to the next section of work. This will become apparent as each section of work in this thesis will have a conclusion which will apply to the work which I had undertaken (ie. relating to the nitrostyrene synthesis route). However, this may not necessarily have been in agreement with the combined partner laboratories' findings and therefore the next section of work will have its basis as compromised agreed method.

Chapter 3

Synthesis and Analysis of Standards

3.1. Aims

Initially, the focus of the SMT project was a combined attempt by the four partner laboratories to synthesise standards, in gram quantities, of some of the most common impurities encountered in amphetamine seizures in Europe. These 'analytical' standards were then used in the course of the project as a library for the identification of those impurities in synthesised batches of amphetamine sulphate. The impurities were individually synthesised and purified as much as possible. Analytical data was obtained from the purified compounds using methods such as Infra-Red Spectrometry (IR), Ultra Violet-Visible Spectrometry (UV-VIS), GC, MS and Nuclear Magnetic Resonance (NMR). In addition, a representative batch of amphetamine sulphate produced was synthesised via each route and the impurities known to result from this synthesis characterised. This batch was then used as a test sample with which to optimise the extraction and analysis of reaction impurities. The responsibility of synthesising the impurities and obtaining all the necessary analytical data was split between methods and laboratories as detailed below. As the Leuckart synthesis yields more identified impurities than the other routes, the synthesis of these standards was split between two laboratories.

Impurities produced in the Leuckart route (part A) – SKL, Linköping, Sweden

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

4-Methyl-5-phenylpyrimidine

4-Benzylpyrimidine

Impurities produced in the Leuckart route (part B) – IPSC, Lausanne, Switzerland

N-Formylamphetamine

N,N-di(ß-phenylisopropyl)-amine (DPIA)

N,N-di(ß-phenylisopropyl)methylamine (DPIMA)

N,N-di(ß-phenylisopropyl)formamide

Impurities produced via reductive amination of benzyl methyl ketone – NBI, Vantaa, Finland

The most significant of the impurities produced by this route are the imines, resulting from the reaction of amphetamine (primary amine) with the starting material (ketone).

Impurities produced via the nitrostyrene route – Strathclyde, Scotland

Nitrostyrene

Benzyl methyl ketoxime

2-Benzyl aziridine

2-Methyl-3-phenyl aziridine

N-(β-phenylisopropyl) benzaldiimine

Since Strathclyde had the task of producing some impurities from the nitrostyrene route, only these syntheses will be discussed.

3.2. Nitrostyrene Route of Amphetamine Synthesis

The synthesis of nitrostyrene from benzaldehyde and nitroethane and the subsequent reduction of nitrostyrene to amphetamine using lithium aluminium hydride ($LiAlH_4$) or in some cases sodium dihydro-bis(methoxyethoxy)aluminosilicate (RedAl) is not a particularly common route in the UK but has been used incidentally in Sweden. Common impurities encountered in this synthesis are as follows:

1. ***benzyl methyl ketoxime*** – from the partial reduction of nitrostyrene
2. ***N-(β-phenylisopropyl)benzaldiimine*** – the Schiffs base formed from amphetamine and excess benzaldehyde
3. ***2-benzyl aziridine and 2-methyl-3-phenyl aziridine*** – from the reduction of the ketoxime or dehydration of nitrostyrene
4. ***nitrostyrene*** which has not been consumed in the reaction
5. traces of ***benzaldehyde*** remaining in the nitrostyrene crystals

The reaction pathways for formation of amphetamine and selected impurities are shown in Figure 3.1.

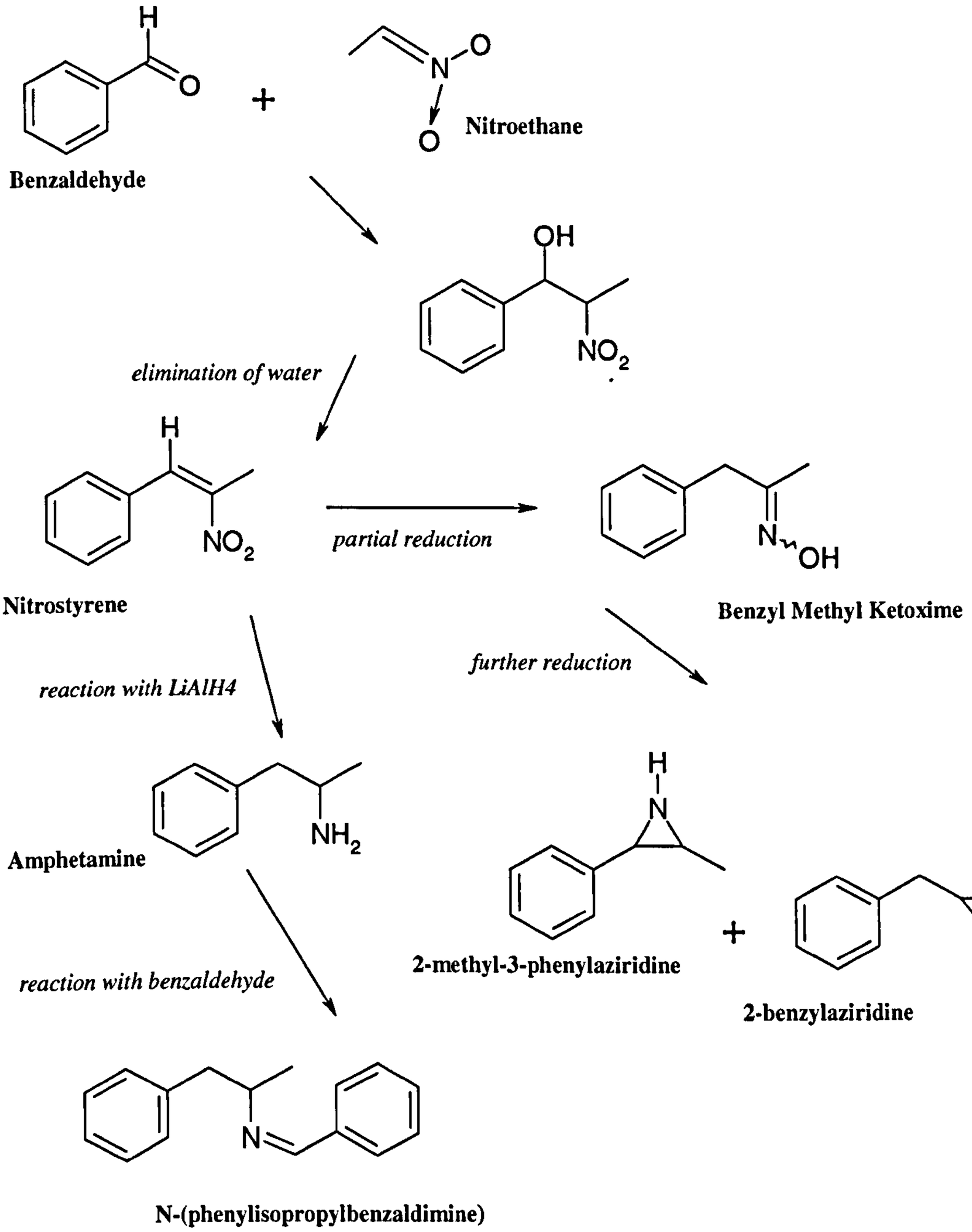


Figure 3.1 Nitrostyrene Route of Amphetamine Synthesis – Impurity Formation

3.3. Experimental Procedures

3.3.1. Treatment of Glassware

All glassware to be used in each of the syntheses was cleaned using an eluotropic series of solvents in order to reduce any impurities from the laboratory environment. These comprise impurities not directly resulting from the synthesis itself and, since, in the case of nitrostyrene, the product would subsequently be used to prepare amphetamine, these would obviously affect the impurity profile but would not necessarily be reproducible or easily attributed to specific stages of the synthesis.

Solvents were used in the following eluotropic series: n-hexane, ethyl acetate, ethanol, distilled water then ethanol, ethyl acetate and n-hexane. This procedure was subsequently carried out with all glassware to be used in the synthesis and purification of all standards.

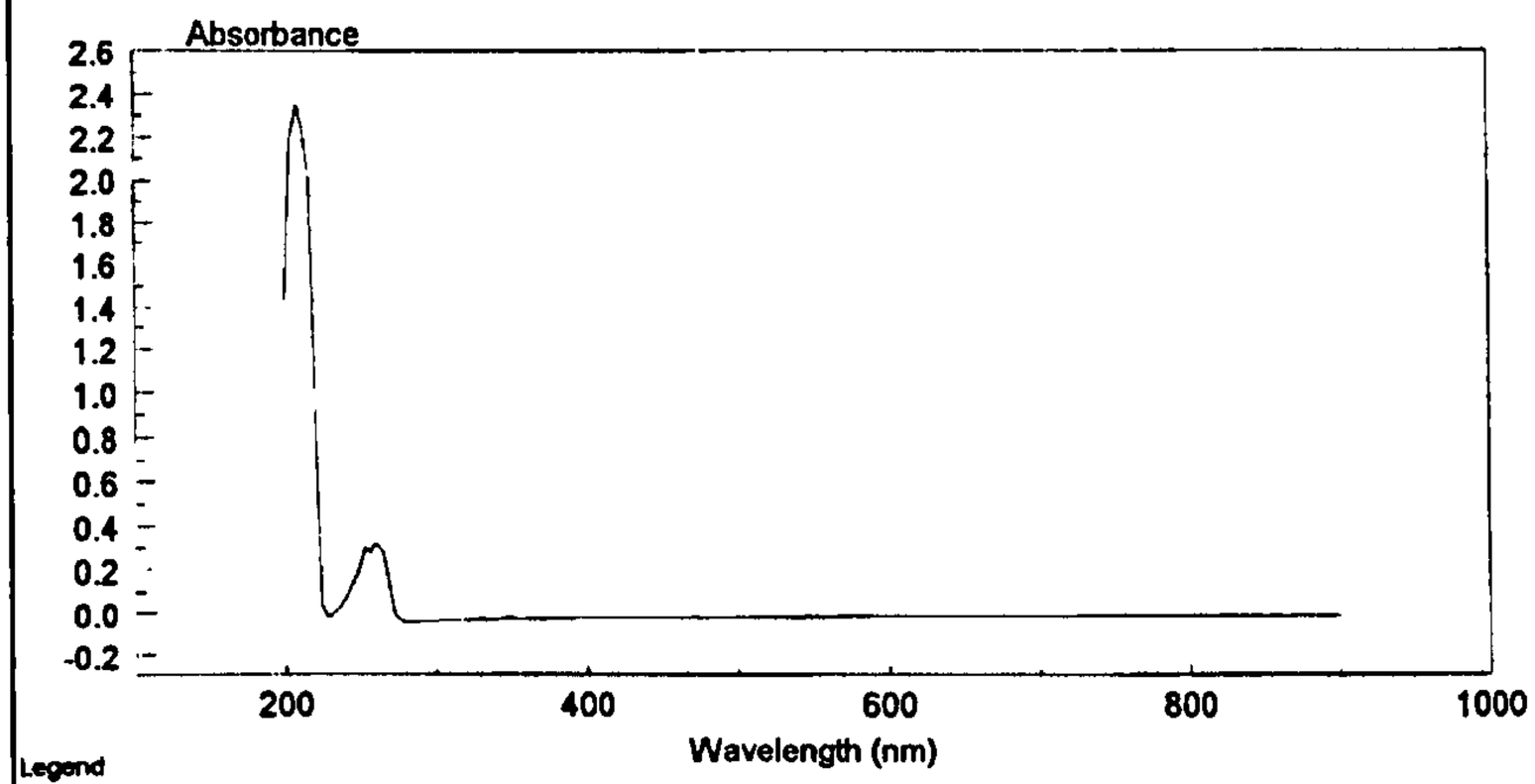
3.3.2. Ultra Violet-Visible Spectroscopy

3.3.2.1 UV-VIS Basics

Radiation in the ultraviolet/visible range is passed through a molecule and light of specific wavelength is absorbed. This absorption wavelength corresponds to the energy required to promote an electron in the molecule from a lower to a higher energy level. When an electronic absorption spectrum is generated a series of absorption bands results, each corresponding to a different electronic transition. The data of interest is usually the λmax (nm) – the wavelength at which the greatest amount of light is absorbed. An exemplar UV-VIS absorption spectra of amphetamine in methanol is shown in Figure 3.2

UNICAM UV-VISIBLE SPECTROMETRY ~ VISION SOFTWARE V3.40

Operator Name	(None Entered)		
Department	(None Entered)	Date of Report	13/04/99
Organisation	(None Entered)	Time of Report	16:23:01
Information	(None Entered)		



scan008,Sample004,Cycle01

Description	amphetamine		
Date Collected	13/04/99	Time Collected	16:14:54
Operator Name		Instrument ID	061602

Manipulations

Manipulation 1	Convert to Absorbance		
Date Performed	13/04/99	Time Performed	16:14:54

Scan Method - (Untitled)

Scan Speed	Intelliscan	Data Interval	Zip
Lamp Change	325.0 nm	Bandwidth	2.0 nm

Figure 3.2 Exemplar UV-VIS Spectra

3.3.2.2 Experimental Detail

For each of the impurities, UV-VIS absorption spectra were obtained in methanol, dilute acid (5 % H_2SO_4 in methanol) and dilute base (5 % NaOH in methanol) using matched quartz cuvettes and a UNICAM UV2 instrument with Vision software version V3.40. Absorbance values were measured from 200 to 900 nm in all cases.

3.3.3. Fourier Transform Infra-Red Spectroscopy

3.3.3.1 FTIR Basics

When infrared light is passed through an organic compound, light of specific frequency is absorbed while other frequencies are transmitted through the sample. A plot of absorbance or transmittance against frequency results in an infrared spectrum. A molecule will only absorb infrared light of a particular frequency if there is an energy transition within the molecule corresponding to that frequency. The transitions involved in infrared spectroscopy are associated with vibrational changes within the molecule, bonds either stretching or bending. Different bonds in a molecule have different vibrational frequencies and we can detect the presence of these bonds by their characteristic frequencies as an absorption band in the infrared spectrum.

An exemplar FTIR spectra of styrene is shown in Figure 3.3.

3.3.3.2 Experimental Detail

Infrared spectra were obtained, in the case of crystals, by grinding a few crystals with dry KBr powder and pressing a disk. Analysis was performed using an ATI Mattson Genesis 1 instrument with Winfirst software. Blanks were obtained from a freshly prepared KBr disk, of the same dimensions as the sample disk, immediately before analysis of the sample. 16 scans were obtained in each case and resolution was set to 4 with signal gain at 1. Transmittance values were recorded from 4000 to 400 cm^{-1}.

In the case of liquid samples, a blank KBr disk was prepared and 'background' spectrum obtained. The KBr blank disk was then re-ground into powder and a small amount of the sample, dissolved in chloroform was added. The chloroform was allowed to evaporate off and the KBr now containing the sample was pressed into a disk ready for a background-subtracted analysis.

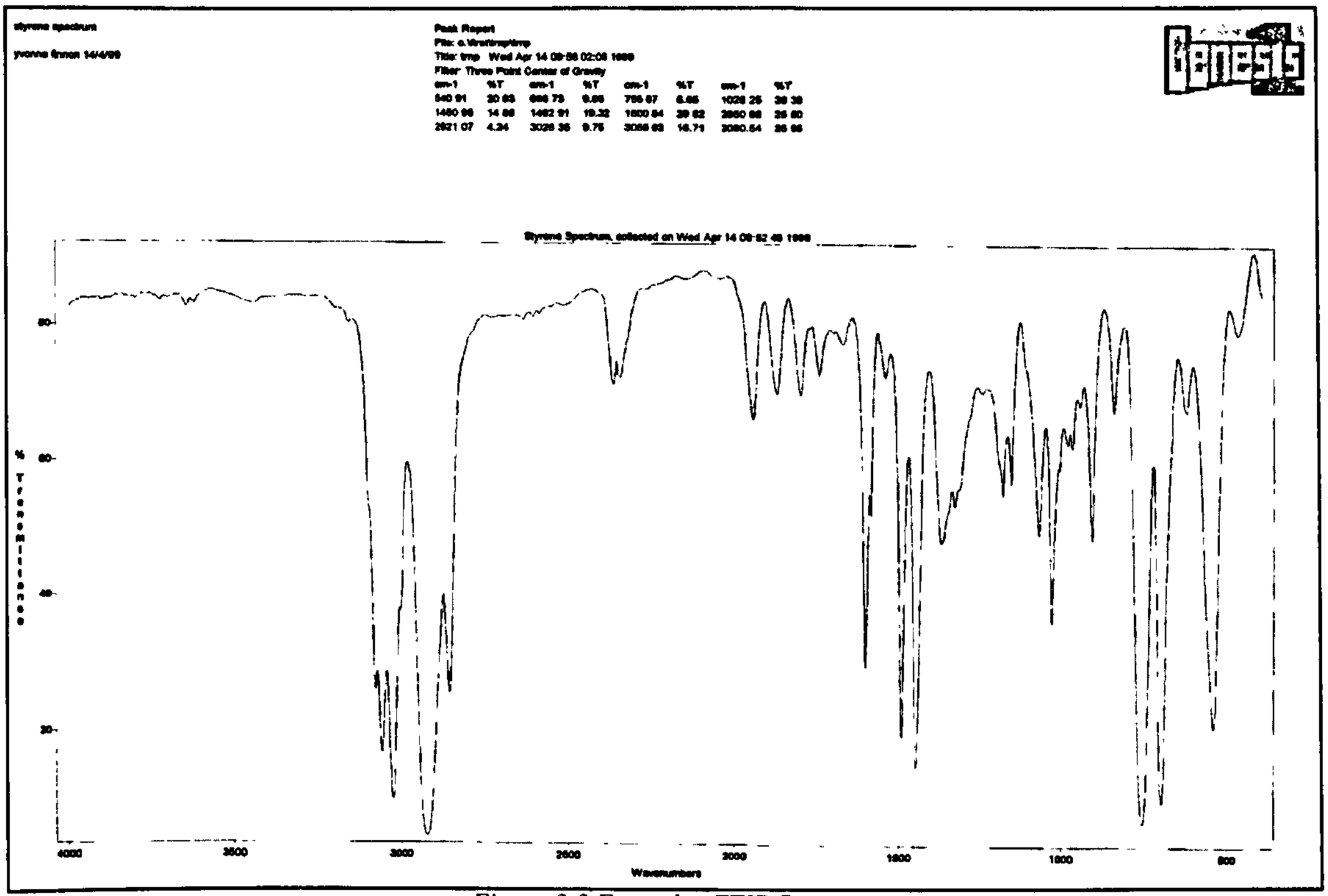


Figure 3.3 Exemplar FTIR Spectra

3.3.4. Gas Chromatography-Mass Spectrometry

3.3.4.1 GC Basics

The basis on which chromatographic separation is achieved and mass spectra formed is detailed in Chapter 2. Exemplar chromatograms and mass spectra can be seen throughout this thesis.

3.3.4.2 Experimental Detail

Unless otherwise stated, the GC conditions for this stage were based in part, on published methods and were a compromise based on experimentation by the project group previously.

Instrument: HP 6890 gas chromatograph, HP 5973 mass selective detector

Software: HP MS Chemstation rev. B.01.00

Columns: methyl silicone (HP Ultra 1) 25 m, 0.2 mm internal diameter, 0.33 µm d_f

5 % phenyl methyl silicone (HP 5MS) 30 m, 0.25 mm internal diameter, 0.25 µm d_f

Carrier Gas: Helium, 25 cm s^{-1} at 60 °C, constant flow

Sample Introduction: 1 µL splitless, 30cm min^{-1} total flow after 1 min

gas saver 20 mL min^{-1} after 1.5 min, splitless liner with volume 250 µL

Temperatures: injection port – 260 °C

oven temperature program 60 °C (1 min), 10 °C min^{-1}, 300 °C (10 min)

GC-MS interface – 305 °C

MS Information: solvent delay – 3.5 – 6 min depending on solvent

mass range – 30 – 550 amu

MS quad temp – 150 °C

MS source – 230 °C

3.3.5. Nuclear Magnetic Resonance

3.3.5.1 NMR Basics

NMR involves the study of the magnetic properties of certain atomic nuclei, most commonly the nucleus of the hydrogen atom (^{1}H) and that of the carbon-13 atom (^{13}C). Examining a molecule by NMR is essentially looking at the magnetic properties of the magnetic nuclei present and predicting where these nuclei are positioned within the molecule. By placing a sample of a compound in a magnetic field, each nuclei experiences this imposed magnetic field but is also affected by the additional magnetic field generated by the magnetic nuclei around it. In this way, each atom in an organic compound experiences a unique magnetic environment. NMR may establish how many different magnetic environments there are in the molecule and how many atoms are within those environments and from this information the molecular structure may be deduced.

3.3.5.2 Experimental Detail

NMR data was obtained by dissolving sample material (crystals or oils) in $CDCl_3$ in Willimad 50 NMR tubes at approximately 10 mg mL^{-1}. For ^{1}H data, a 250 MHz Bruker instrument was used. ^{13}C data was obtained at 90 MHz. For ^{1}H spectra, 64 scans were obtained and for ^{13}C spectra, 2000 scans were obtained.

3.3.6. Quality Assurance

In order to check the comparability of results obtained, a simple quality assurance procedure was established. A Grob sample was pre-prepared in the Finnish laboratory and distributed amongst the partner laboratories. Before analysis of samples took place, a Grob sample was analysed using the agreed GC method (see section 3.3.4 for details). This Grob sample was also included as a part of any sequence which was set up to run over several days.

The Grob sample consisted of a mixture of 16 compounds and was prepared in n-hexane. The chromatographic profile of the sample was monitored as an indicator of how well the instrument was performing. Any obvious changes in the Grob profile highlighted possible problems within the system and a gradual change in the profile can be used as a gauge on the ageing of parts of the system, such as the liners and septa. Therefore, the Grob sample was regarded as an early 'warning' device for the instrument and a pointer as to when routine maintenance may be required.

The Grob sample in use in this project contained the following compounds prepared in n-hexane: n-decane, n-octanol, 2,6-dimethylphenol, 2-ethylhexanoic acid, 2,6-dimethyl aniline, n-dodecane, n-tridecane, docanoic acid methylester, tetradecane, undecanoic methylester, dicyclohexylamine, n-pentadecane, dodecanoic acid methylester, n-hexadecane, n-heptadecane, n-octadecane. The acid and the amine were studied most closely as markers of changes in instrument performance.

For each sample, measurements were taken of the selectivity (as compared to dodecane) and inertness tailing for each of the compounds (see Chapter 2 section 2.4.6 for an explanation of the peak tailing/fronting calculations). These values were plotted on a Shewart chart which had 'warning levels' for the compounds based on the deviation from the mean of the first 20 values obtained after a new column was been installed. A deviation of "plus or minus 2 standard deviations from the mean" was regarded as the 'warning' level. A deviation of "plus or minus 3 standard deviations from the mean" was the 'action' level at which the instrument problems were investigated and resolved.

An example of a GC profile of a Grob sample and an exemplar Shewart chart for 2,6-dimethylaniline is shown in Figures 3.4, 3.5 and 3.6.

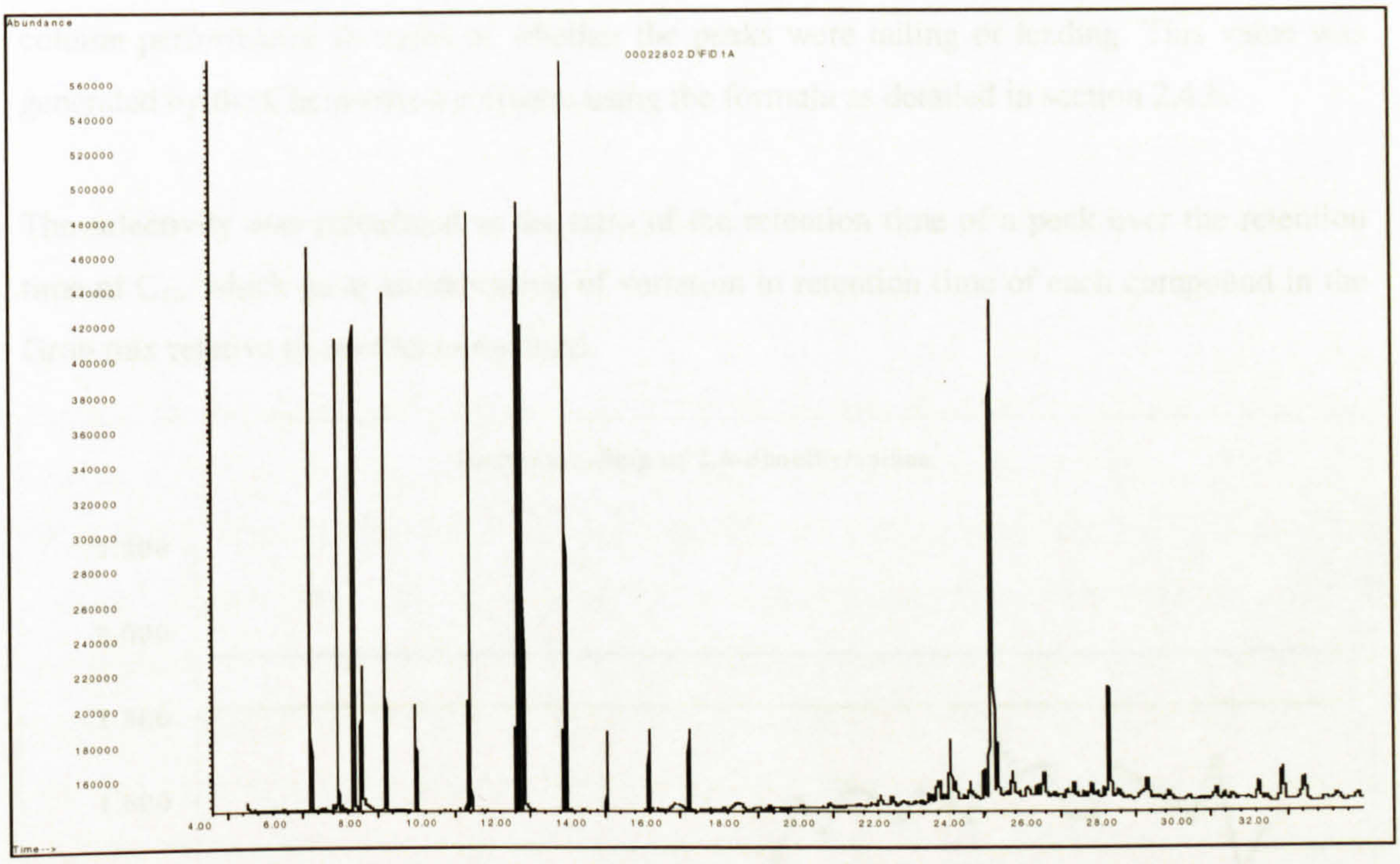


Figure 3.4 Representative Chromatogram of a Grob Mixture

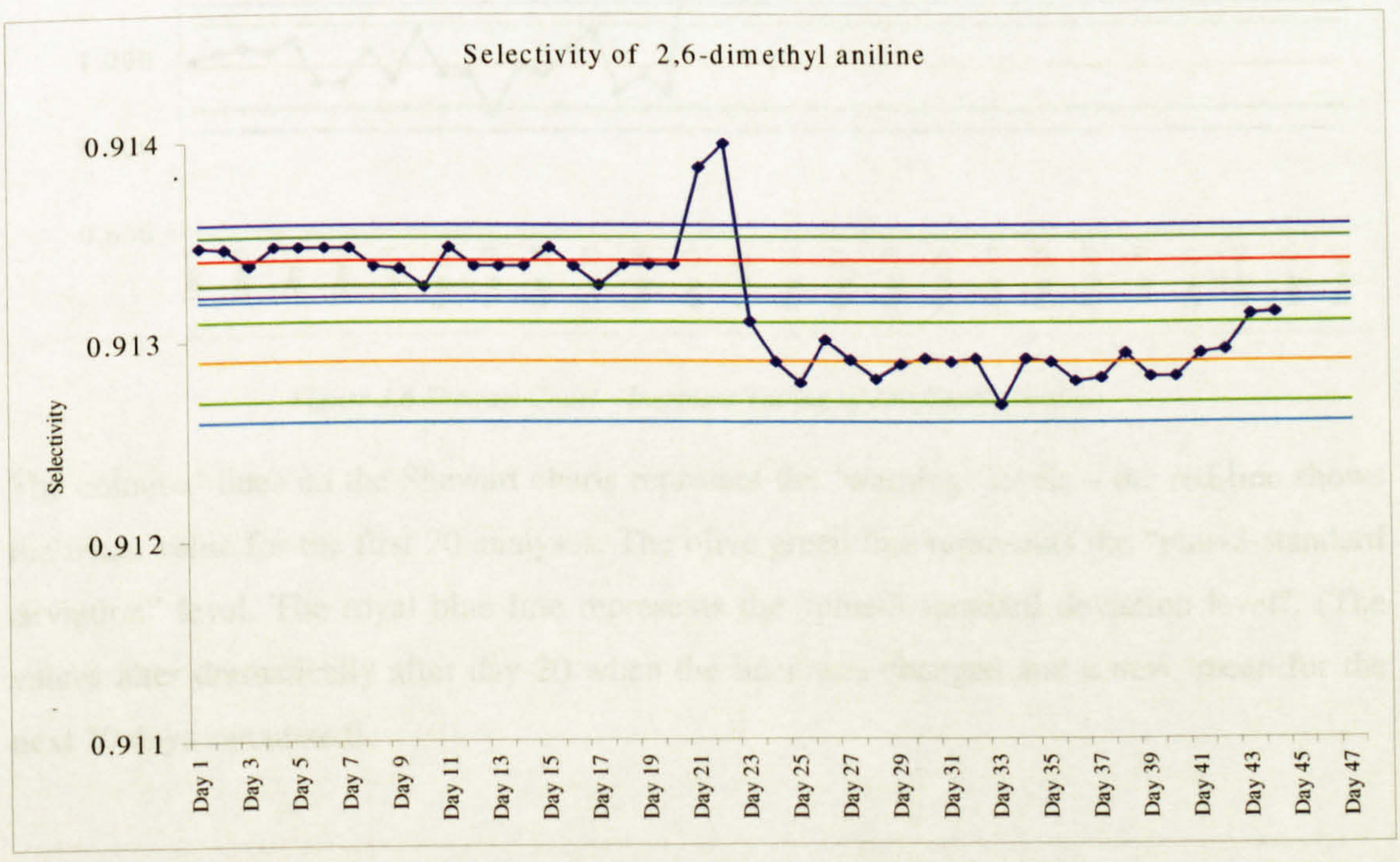


Figure 3.5 Shewart Chart – Selectivity of 2,6-dimethylaniline

The inertness tailing value (as discussed in Chapter 2. section 2.4.6) gives an indication of column performance in terms of whether the peaks were tailing or leading. This value was generated by the Chemstation software using the formula as detailed in section 2.4.6.

The selectivity was calculated as the ratio of the retention time of a peak over the retention time of C_{12}, which gave an indication of variation in retention time of each compound in the Grob mix relative to another compound.

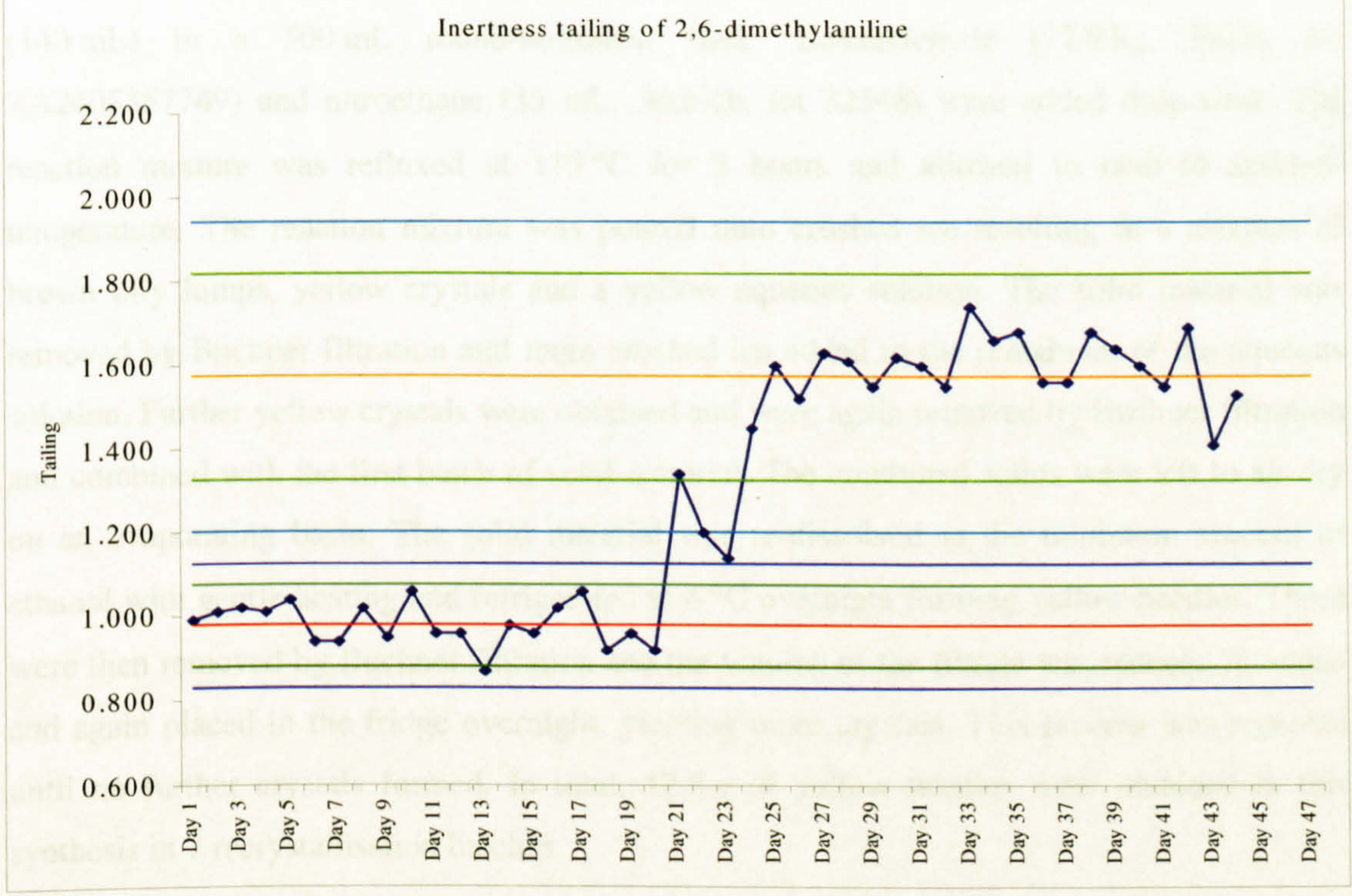


Figure 3.6 Shewart Chart – Inertness Tailing of 2,6-dimethylaniline

The coloured lines on the Shewart charts represent the 'warning' levels – the red line shows the mean value for the first 20 analyses. The olive green line represents the "plus-2-standard deviation" level. The royal blue line represents the "plus-3-standard deviation level". (The values alter dramatically after day 20 when the liner was changed and a new 'mean for the next 20 days calculated).

3.4. Synthesis and Analytical Data - Nitrostyrene

3.4.1. Synthesis of Nitrostyrene

Nitrostyrene is formed as a reaction intermediate in the synthesis of amphetamine as detailed in Figure 3.1.

Ammonium acetate (14.46 g BDH, lot A109068834) was dissolved in glacial acetic acid (140 mL) in a 500 mL round-bottomed flask. Benzaldehyde (17.93 g, BDH, lot ZA2405357749) and nitroethane (35 mL, Aldrich, lot 32548) were added drop-wise. The reaction mixture was refluxed at 110 °C for 3 hours and allowed to cool to ambient temperature. The reaction mixture was poured onto crushed ice resulting in a mixture of brown oily lumps, yellow crystals and a yellow aqueous solution. The solid material was removed by Buchner filtration and more crushed ice added to the remainder of the aqueous solution. Further yellow crystals were obtained and were again removed by Buchner filtration and combined with the first batch of solid material. The combined solids were left to air-dry on an evaporating basin. The solid material was re-dissolved in the minimum amount of ethanol with gentle heating and refrigerated at 4 °C overnight forming yellow needles. These were then removed by Buchner filtration and the volume of the filtrate was reduced *in-vacuo* and again placed in the fridge overnight, yielding more crystals. This process was repeated until no further crystals formed. In total, 12.8 g of yellow needles were obtained in this synthesis in 7 recrystallisation batches.

Yield

100 % yield based on 17.93 g of benzaldehyde would give 22.21 g of nitrostyrene.

12.8 g of nitrostyrene represents a 57.6 % yield for this exemplar synthesis.

3.4.2. Gas Chromatographic Data

The reaction was followed by GC analysis after removing a sample drop from the reaction at one-hour intervals, after crystallisation over ice and after recrystallisation from ethanol. 10mg was also removed from each batch of crystals, and dissolved to 1.5 mL in ethanol and analysed by GC-MS. The GC traces from the original reaction mix and after recrystallisation are shown in Figures 3.7 and 3.8. The purity of nitrostyrene, based on peak integration, varied between batches, approaching 100 % in some samples.

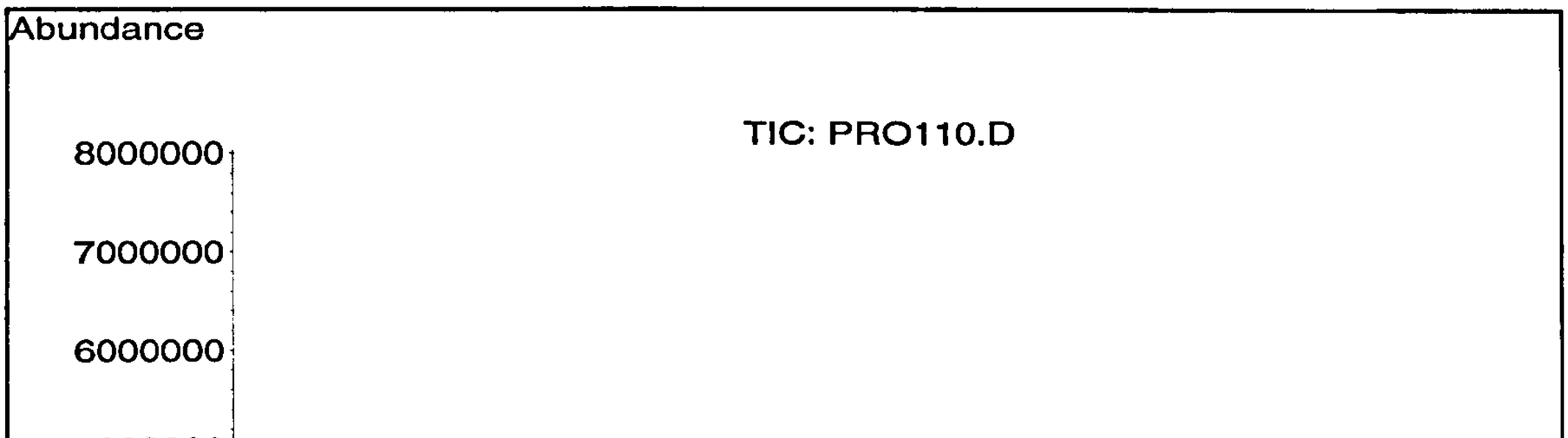


Figure 3.7 Reaction Mixture in Ethanol Before Recrystallisation

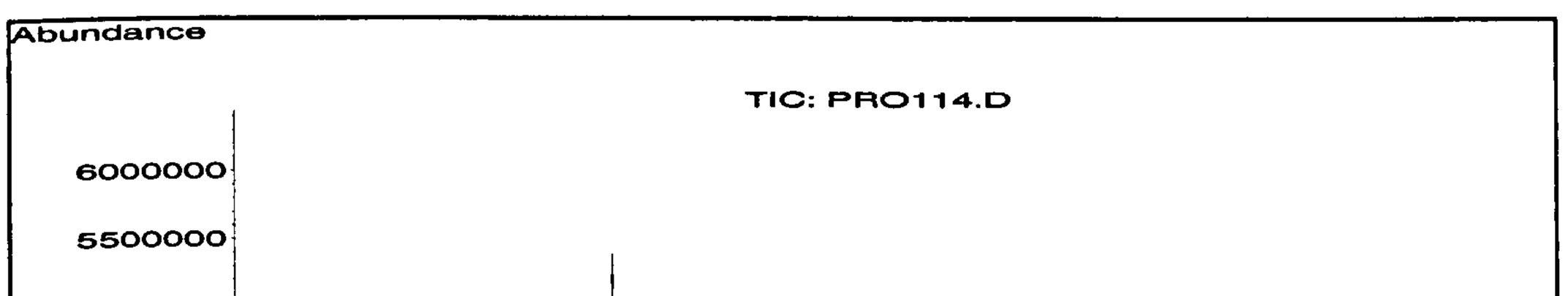


Figure 3.8 Reaction Mixture in Ethanol After Recrystallisation

3.4.3. UV-VIS Absorption Spectra

The numerical values obtained are as follows:

UV λmax (nm): [MeOH] 224

[0.1M NaOH] 212, 220

[0.1M H_2SO_4] 196, 216, 224 (shoulder)

The only literature value available for comparison is suggested by Currie *et al.* at 305.5 mμ obtained in methanol [76] which does not agree with our findings.

3.4.4. Fourier Transform Infrared Spectrometry

The numerical values obtained are as follows:

IR γmax (cm^{-1}): 690, 708, 763, 941, 979, 1295, 1322 (principal peak), 1488, 1517 (principal peak), 1651 (principal peak), 2837, 3020 (broad).

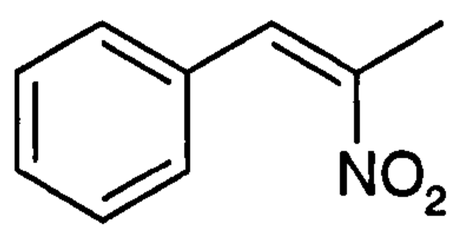
The predicted IR spectra should display two peaks at 770-730 cm^{-1} and 720-680 cm^{-1} due to the presence of five adjacent aromatic protons. The methyl group present should give rise to two or three bands at 2960-2580 cm^{-1}. The presence of the nitro group would produce two peaks in the region 1570-1540 cm^{-1} and 1390-1340 cm^{-1}. C=C should result in multiple peaks at 3040-3010 cm^{-1}.

When compared to literature values the results obtained show relatively good correlation although the presence of many more peaks than would be expected may be attributed to impurities in the sample.

Ranu and Chakraborty [77] report IR values for nitrostyrene as obtained in $CHCl_3$ with principal peaks at 1380 and 1550 cm^{-1} which does not agree with our findings. However, the most appropriate comparison is found in a paper by Currie *et al.* [76] where the spectra are obtained as KBr disks as in our experimental procedure. The principal peaks obtained were at 1520 cm^{-1} (our data – 1517 cm^{-1}) corresponding to νC-N, 1658 cm^{-1} (our data –1651 cm^{-1}) corresponding to νC=C, 1324 cm^{-1} (our data – 1322 cm^{-1}) corresponding to NO_2.

3.4.5. Nuclear Magnetic Resonance Spectrometry

The numerical data for ^{1}H is as follows:



^{1}H NMR: δ = 2.46 (3H, s, Me), 7.46 (5H, m, phenyl-H), 8.10 (1H, s, H-1)

These results compare well with published data. Benhaoua *et al.* [78], using the same sample preparation methods, report values of δ = 2.40, 7.41 and 8.03 respectively for the same protons. However, the results published are for the E stereoisomer only and since we have not isolated each stereoisomer we would expect our results to differ slightly.

The numerical data for ^{13}C is as follows:

^{13}C NMR: δ = 136.54 (C), 157 (C), 13.5 (C), 126-129 (Ph)

The results obtained may be compared with those obtained by Bailey and Legault in 1981 [79]. They assigned the carbon atoms as follows:

C_1 = 132.82, C_2 = 130.27, C_3 = 129.24 C_4 = 130.27, C5 = 129.24, C_6 = 130.27, C_α = 133.79, C_β = 148.25, C_γ = 14.03

3.4.6. Mass Spectrometry

An exemplar spectra is shown in Figure 3.10.

The numerical results and m/z assignments are as follows:

m/z = 115 $[M\text{-}H_2\text{-}NO_2]^+$, 105 $[C_6H_5CH_2CH_2]^+$, 91 $[C_7H_7$, tropyllium ion$]^+$, 130 $[M\text{-}H\text{-}O_2]^+$, 163 $[M]^+$, 146 $[M\text{-}O]^+$

These results may be compared with data obtained by Verweij [11], who obtained, in order of decreasing intensity, m/z = 163, 105, 115, 91, 77, 116, 106, 51 and although the order of intensity does not agree with our findings the principal fragments including the molecular ion are present.

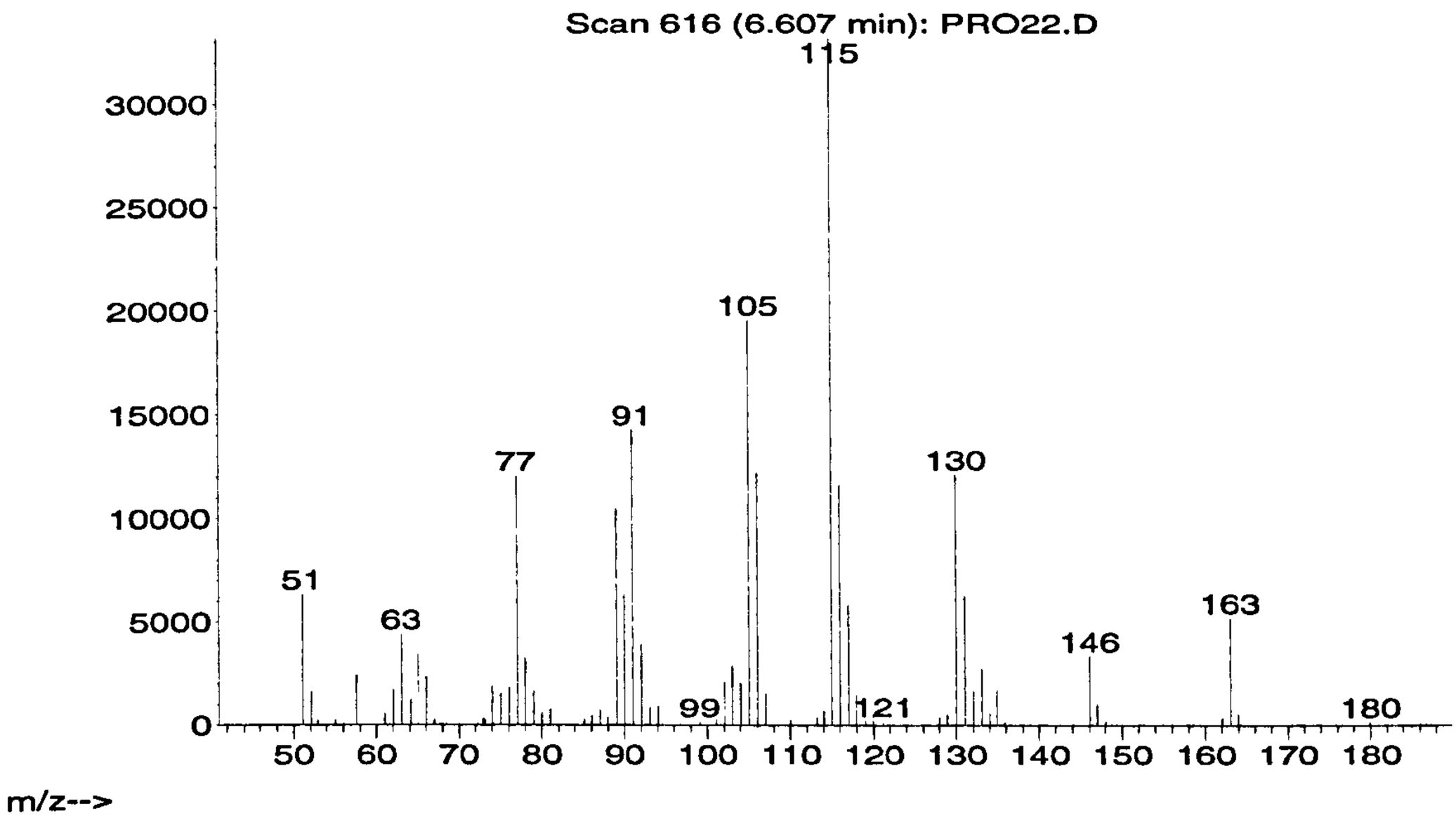


Figure 3.9 Nitrostyrene Mass Spectra

3.5. Synthesis and Analytical Data - Benzyl Methyl Ketoxime

3.5.1. Synthesis of Benzyl Methyl Ketoxime

Benzyl methyl ketoxime is formed by the partial reduction of nitrostyrene in the synthesis of amphetamine as detailed in Figure 3.1.

Hydroxylamine hydrochloride (12.00 g, Sigma lot 88H0433), sodium acetate (catalytic amount, Fisons lot 50034) and benzyl methyl ketone (BMK), (12.07 g, Fisher lot 9768216 477 - controlled reagent) were dissolved in 80 mL of distilled water in a 500 mL round-bottomed flask. Approximately 50 mL of ethanol was added and the reaction refluxed for 3 hours at 80 °C. The reaction mix was allowed to come to ambient temperature and poured onto chilled water. This resulted in a suspension of red oil globules in an aqueous solution. 50 mL of sodium-dried diethyl ether was added to extract the organic products. This extraction process was repeated twice and the organic layers combined. The solvent was removed *in-vacuo* resulting in the final product of 9.36 g of viscous red oil.

Purification

Following GC-MS analysis of the red oil, it was clear that, in order to use these samples as measured standards for analysis, it would be necessary to further purify the ketoxime to obtain a level of purity around 95 %.

Experiments were performed to determine the most feasible method of purification for the ketoxime. Attempts at crystallising the product by refrigeration and freezing failed, as did attempts at vacuum distillation.

Preliminary Thin Layer Chromatography (TLC) trials were then run. The reaction mixture was run against BMK using a 1:1 (hexane, ethyl acetate) solvent system. It was visualised using iodoplatinate and the ketoxime isomers appeared to be separable from residual BMK but were not resolved from each other. Indeed, GC did not fully separate the isomers. This was not a major concern since the project task did not stipulate separation of optical isomers, since it is assumed that they would both be present in an illicit amphetamine sample. From this point, both preparative TLC and column chromatography were considered as possible purification methods.

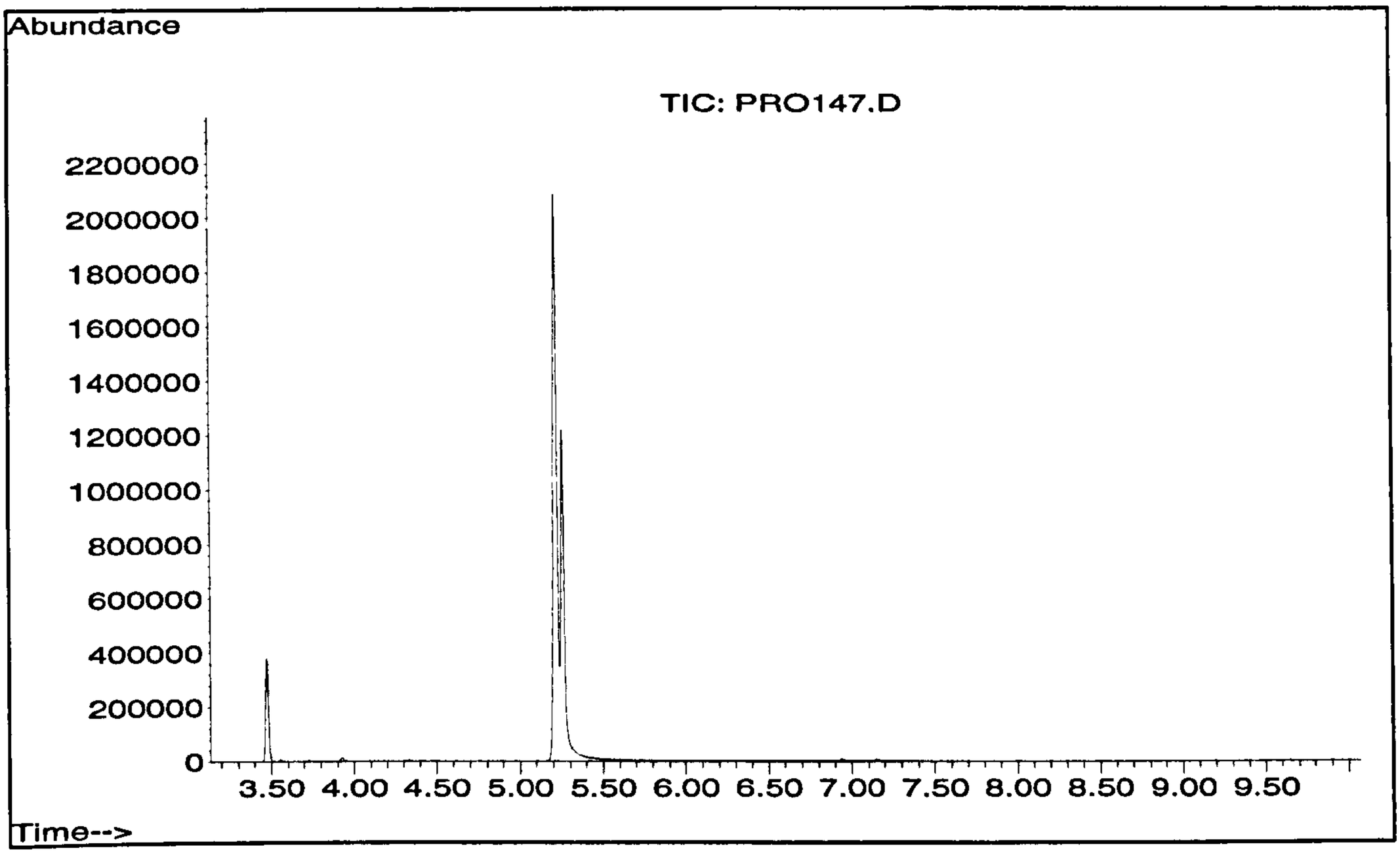


Figure 3.10 Ketoxime Product Before Purification

Column chromatography was considered a more practical method of purification. A 50mL burette with internal diameter of 10mm was adapted to a chromatography column using a plug of silanised wool at the base and packing with wetted silica gel (chromatography grade Prolabo 60, 35-75 µm particle size) to the 20 mL mark. The impure ketoxime sample was combined with some silica gel to create a 1 mL sample plug at the top of the column. The solvent system used was 5:4 (hexane, ethyl acetate) and 5 column volumes of 10 mL fractions were collected. Each fraction was analysed by TLC initially and those which showed the possible presence of ketoxime were analysed by GC-MS. Those fractions, which were considered 'pure' (at least 95 % as determined by peak integration) were combined to provide a sample of pure benzyl methyl ketoxime.

Yield

A 100 % yield based on 12.07 g of BMK would produce 13.35 g of ketoxime. 9.36 g produced represents a 69.2 % yield for this exemplar synthesis.

3.5.2. Gas Chromatographic Data

The reaction was followed by removing a sample drop from the reaction at one-hour intervals during reflux, after formation of the oily suspension and after extraction with ether. Each drop was dissolved in 1.5 mL ethanol and analysed by GC-MSD. The GC traces before and after

purification are in Figures 3.10 and 3.11. Note that the two unresolved peaks are the optical isomers of the ketoximes.

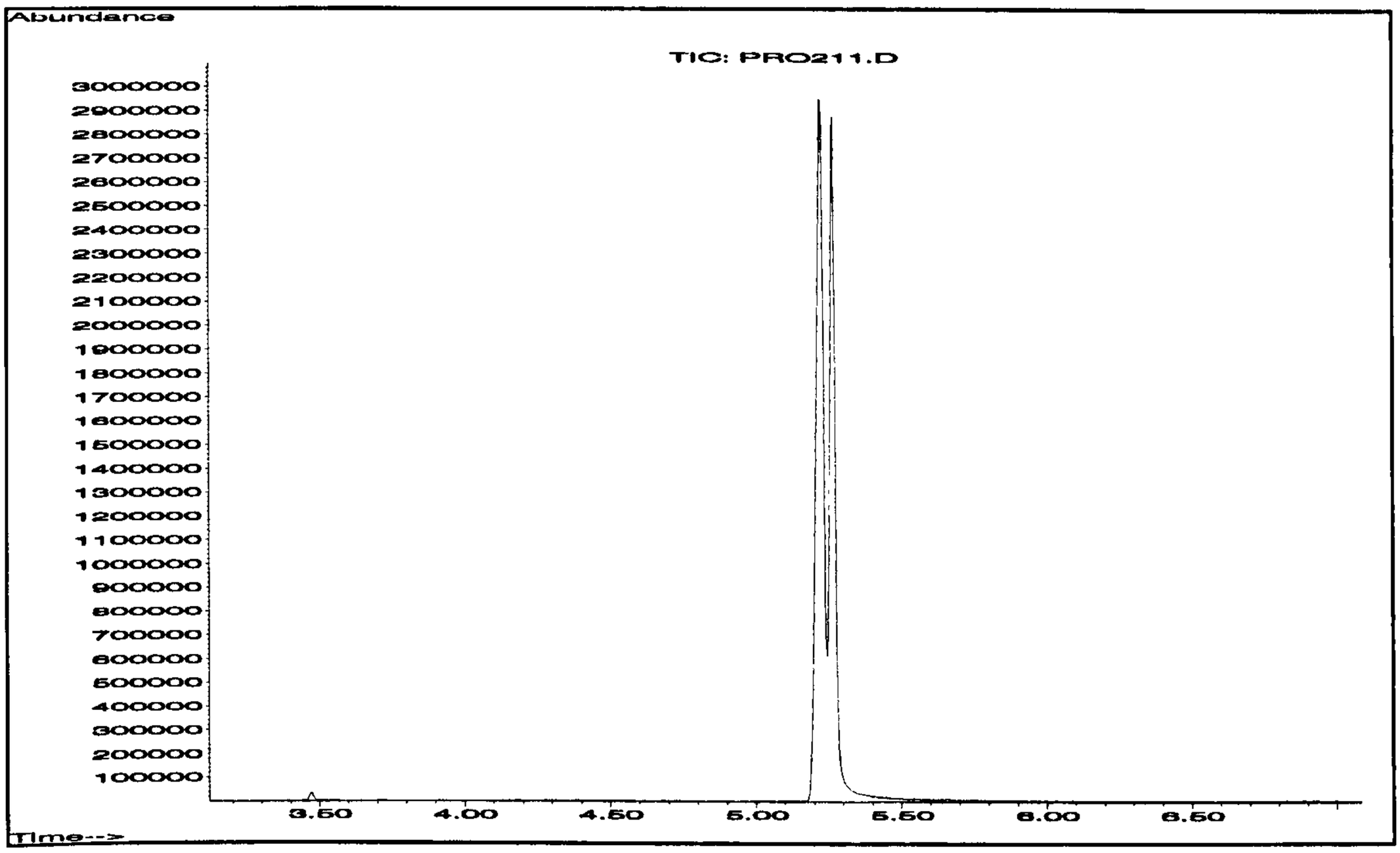


Figure 3.11 Ketoxime Product After Purification

3.5.3. UV-VIS Spectrometry

The numerical values obtained are as follows:

UV λmax (nm): [MeOH] 212, 220, 260, 284 (sh)

[0.1M NaOH] 208, 224, 260

[0.1M H_2SO_4] 196, 216, 224 (sh)

Literature values were obtained from work completed by Warren *et al.* [80] who studied the concept of homoconjugation which can occur where there are π orbitals β to the C or the N of the C=N bond. They found the absorption maxima to be 225.5 nm (n→π* band) and 259 nm (π→π* band) in a methanolic solvent which shows relatively good correlation with the data obtained. No data was available for comparison with the acidic and basic conditions.

3.5.4. Fourier Transform Infrared Spectrometry

The numerical values obtained are as follows:

IR γmax (cm^{-1}): 701, 744, 1031, 1078, 1371, 1454, 1494, 1670 (principal peak), 3300 (broad)

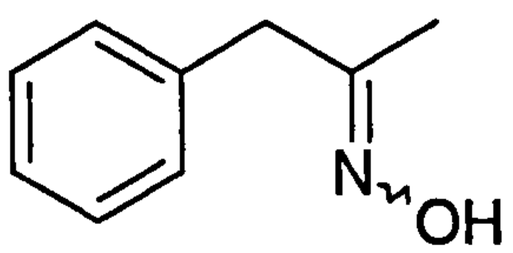
The predicted spectra of the ketoxime would show peaks at 770-730 cm^{-1} and 720-680 cm^{-1} due to the presence of five adjacent aromatic protons. The methyl groups would result in two or three bands at 2960-2580 cm^{-1}. The presence of an N-OH group should show broad absorbance at around 3300 cm^{-1} and a C=N bond would produce a characteristic band at 1600 cm^{-1}.

Although the principal peaks at 3500-3100 cm^{-1} and 1670 cm^{-1} are in agreement with the literature values, there are many more absorption peaks than would be predicted in the spectra and these additional peaks may be attributed to impurities in the samples.

Warren *et al.* report the presence of a peak corresponding to νC=N at 1660 cm^{-1} (1670 cm^{-1} obtained) [80]. Wessling and Schäfer, in their study of the electrochemical reduction of nitroalkanes to oximes, report values of ν= 3500-3100 cm^{-1} (OH) which can be seen as a very broad peak in the spectra obtained, and a sharp peak at 1660 cm^{-1} (C=N) as reported previously [81]. Kotera *et al.* [82] report peaks at 3603 and 3290 cm^{-1} (OH), 1670 cm^{-1} (C=N) whereas Ranu and Chakraborty published values of 3280 cm^{-1} broad (OH), 1665 cm^{-1} (C=N) [77].

3.5.5. Nuclear Magnetic Resonance Spectrometry

The numerical data for ^1H is as follows:



Z-isomer

^1H NMR: δ = 1.85 (3H, s, Me), 3.77 (2H, s, H-1), 7.29 (5H, m, phenyl-H)

E-isomer

^1H NMR: δ = 1.83 (3H, s, Me), 3.53 (2H, s, H-1), 7.29 (5H, m, phenyl-H)

Lustig, in his paper on syn-anti isomerism in ketoximes, states that the presence of isomerism in a sample is indicated by the presence of two resonance lines for the protons on C atoms adjacent to the C=N-OH or C=N-OR group [83]. Separation of the resonance lines is generally only observed in aromatic solvents. However, the effect of the phenyl group in benzyl methyl ketoxime exerts a sufficient effect to achieve a measurable separation. The oxime molecule is thought to experience the additional magnetic field of an aromatic compound which is cylindrical and, since the -C=N-OH is not linear, the various parts of the molecule lie in different regions of the field. It is this slight difference in local field which is thought to be responsible for the observed separations. The sequence of intensities of the peaks should also be in the ratio of 1:3 for CH_2 and 3:1 for CH_3.

Wessling and Schäfer report values of δ = 1.80 E, 1.82 Z (3H, 2s, Me), 3.50 E, 3.75 Z (2H, 2s, CH_2), 7.2-7.4 (5H, m, phenyl-H) which correspond almost exactly to our observed values [81].

In addition, the results obtained by Varma *et al.* [84] agree with our observed values, δ = 1.82 Z, 1.83 E (3H, 2s, Me), 3.51 E, 3.76 Z (2H, 2s, CH_2). Ranu and Chakraborty, however, do not report peak separation of the methyl peaks in their paper on the reduction of disubstituted nitroalkenes to oximes [77]. The results they obtained were as follows: δ = 1.77 (3H, 2s, Me), 3.43, 3.66 (2H, 2s, CH_2), 7.13 (5H, s, phenyl-H).

The numerical data for ^{13}C is as follows:

Z-isomer

^{13}C NMR: δ = 19.00 (CH_3), 35.00 (CH_2), 126.7-129.1 (Ph), 157.00 (C=NOH), 136.65 (Ph quaternary C)

E-isomer

^{13}C NMR: δ = 13.22 (CH_3), 40.03 (CH_2), 126.7-129.1 (Ph), 157.62 (C=NOH), 136.65 (Ph quaternary C)

The results obtained may be compared with data obtained by Fox and Reboulet [85]. They assigned the carbon atoms as follows:

Z-isomer

^{13}C NMR: δ = 19.80 (CH_3), 34.09 (CH_2), 126.9-129.0 (Ph), 157.1 (C=NOH), 136.00 (Ph quaternary C)

E-isomer

^{13}C NMR: δ = 13.30 (CH_3), 42.20 (CH_2), 126.9-129.0 (Ph), 157.7 (C=NOH),

136.00 (Ph quaternary C)

These results are comparable with our own which allowed us to use the ketoxime in the next stage of the synthesis to produce aziridines.

3.5.6. Mass Spectrometry

An exemplar spectrum for both isomers is shown in Figures 3.12 and 3.13. Although GC did not fully resolve the isomers, MS data could be obtained separately for both.

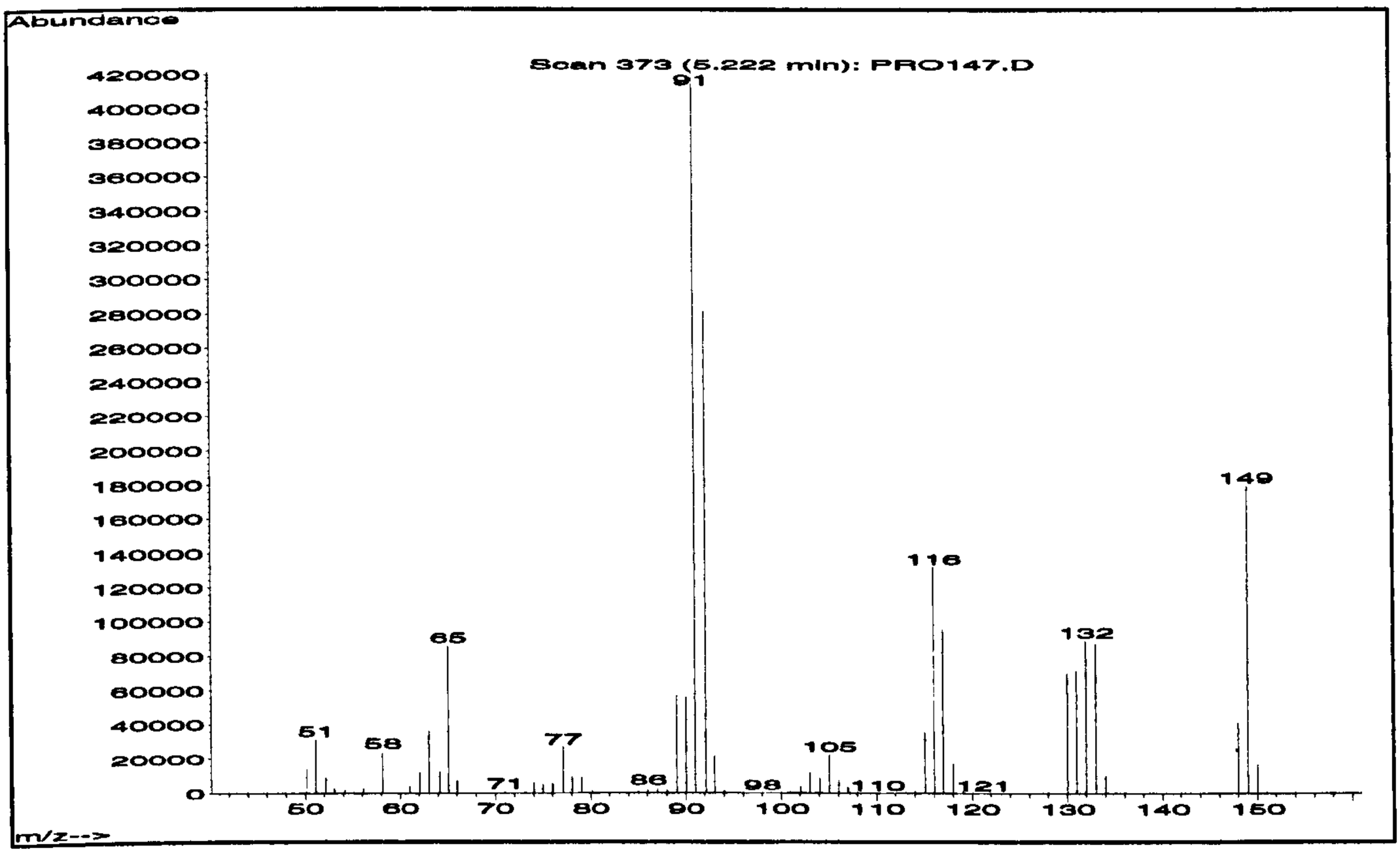


Figure 3.12 Ketoxime Isomer 1 Mass Spectra (5.22min)

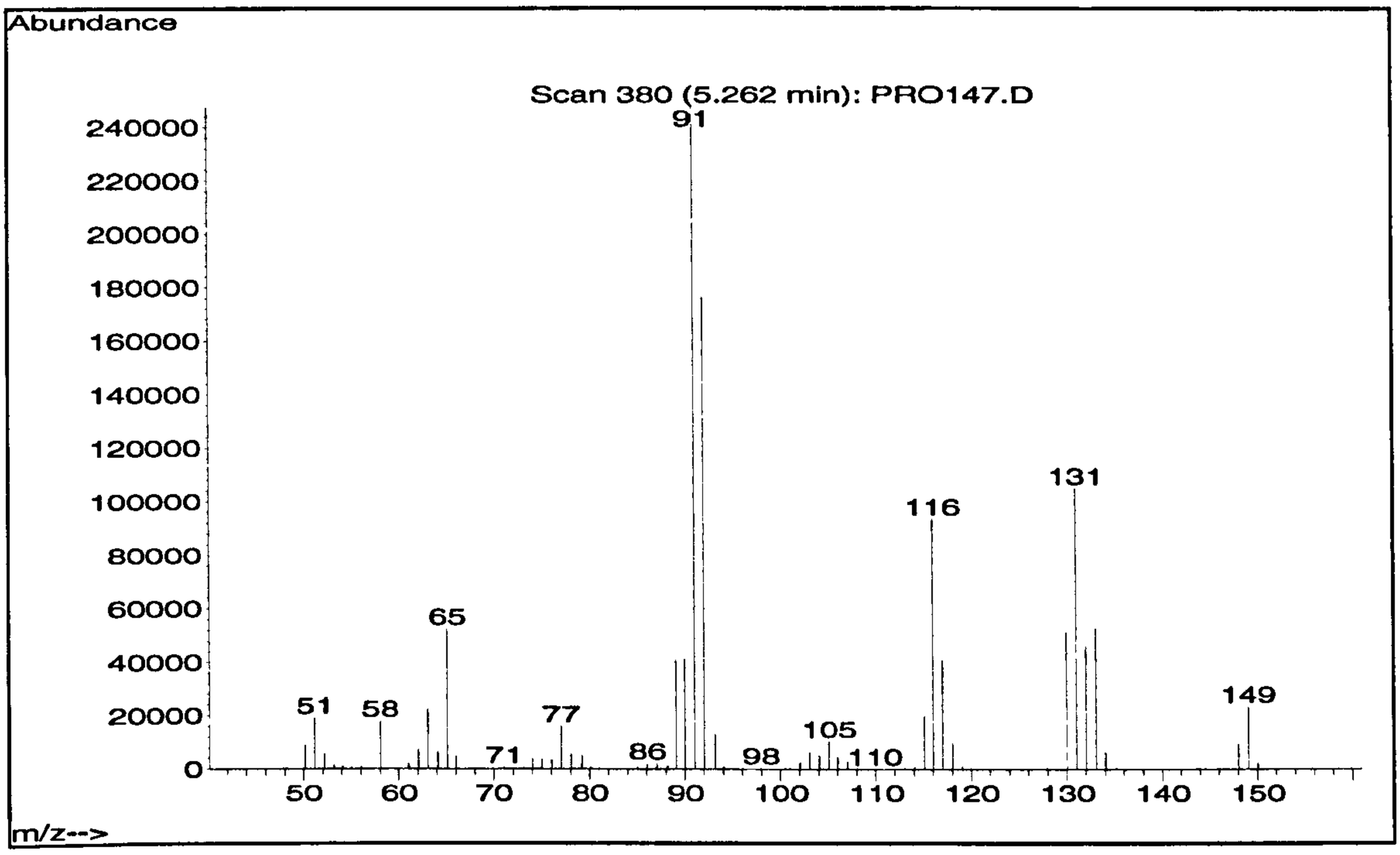


Figure 3.13 Ketoxime Isomer 2 Mass Spectra (5.26min)

The numerical results and m/z assignments are as follows;

Isomer 1

m/z = 91 $[C_7H_7]^+$, 92 $[C_7H_7]^+$, 149 $[M]^+$, 116 [M-2H-NOH]$^+$, 133 [M-O]$^+$,
117 [M H-NOH]$^+$, 132 [M-OH]$^+$, 131 [M-H_2O]$^+$, 130 [M-H-H_2O]$^+$

Isomer 2

m/z = 91 $[C_7H_7]^+$, 92 $[C_7H_7]^+$, 131 [M-H_2O]$^+$, 116 [M-2H-NOH]$^+$, 133 [M-O]$^+$,
132 [M OH]$^+$, 130 [M-H-H_2O]$^+$, 117 [M-H-NOH]$^+$, 149 $[M]^+$,

Data published by Wessling and Shäfer may be used as a comparison [81]. The data is available for the racemic mixture only, since the E and Z isomers were not completely separated in this study.

m/z = 91 $[C_7H_7]^+$, 149 $[M]^+$, 116 [M-2H-NOH]$^+$, 117 [M-H-NOH]$^+$, 131 [M-H_2O]$^+$,

The fragments formed are identical to those reported by Wessling and Shäfer although we detected some additional fragments which may be a due to incomplete separation of the isomers by the GC. The ability to show the presence of the molecular ion was essential to confirm that the ketoxime was synthesised. However, most importantly, it has been shown that the fragmentation pattern for each isomer is distinct and recognisable.

3.6. Synthesis and Analytical Data - N-(β-phenylisopropyl)benzaldiimine

3.6.1. Synthesis of N-(β-phenylisopropyl)benzaldiimine

N-(β-phenylisopropyl)benzaldiimine is formed as a by-product of the nitrostyrene route when excess benzaldehyde present in the reaction vessel reacts with amphetamine as it forms as detailed in Figure 3.1.

Amphetamine sulphate (+)-α, (13.71 g Sigma) was dissolved in 200 mL of distilled water. 200 mL of 0.1 M NaOH (BDH, lot B152048) was added to the amphetamine sulphate solution resulting in formation of the free-base form of amphetamine. The free-base was then extracted into 4x100 mL aliquots of sodium-dried diethyl ether and the combined volume dried over anhydrous sodium sulphate. A further 100 mL of ether was then added.

The amphetamine free-base in ether was transferred to a 500 mL round-bottomed flask with 15 g of molecular sieve 4A as a dehydrating agent. Benzaldehyde (5.45 g, BDH lot ZA2405357 747 GPR) was added and the reaction stirred overnight.

After 20 hours of stirring, a drop of the reaction mix was removed and dissolved in 1.5 mL of ether for GC-MSD analysis to determine if the reaction had gone to completion. The reaction was left stirring for a further 5 hours and sampled again. The reaction mix was then filtered to remove the molecular sieve and the residue washed with ether. The solvent was removed *in-vacuo* and then under nitrogen leaving viscous, yellow oil.

Purification

Since GC-MSD analysis showed traces of unreacted starting materials, a column chromatography system was set up in the same way as for the ketoxime reaction following attempts at distillation and preparative TLC which failed to produce sufficient material for analysis (see section 3.5.1). A 50 mL burette with internal diameter of 10 mm was adapted to a chromatography column using a plug of silanised wool at the base and packing with wetted silica gel (chromatography grade Prolabo 60, 35-75 μm particle size) to the 20 mL mark. The impure benzaldimine sample was combined with some silica gel to create a 1 mL sample plug at the top of the column. The mobile phase used was 5:4 (hexane, ethyl acetate) and seven column-volumes of 10 mL fractions were collected. Fractions were analysed by TLC and then, if benzaldimine was determined to be present, analysed by GC-MSD. Those fractions considered to be pure enough after chromatographic peak integration (fraction 1-6) were

combined and the solvent removed under nitrogen. This process yielded 6.71 g of 'pure' product and 0.331 g of crude product where the product could not be separated from the impurities.

Yield

A 100 % yield based on 5.45 g of benzaldehyde would give 11.4 g of benzaldiimine.

6.71 g of benzaldiimine represents a 58.9 % yield for this exemplar synthesis.

3.6.2. Gas Chromatographic Data

The reaction was followed by GC-MSD after removing a sample drop from the reaction after 20 and 25 hours of stirring. An example of the chromatogram of the purified product is shown in Figure 3.12. The purity, based on peak integration, was determined as 100 % in some fractions and in the combined sample.

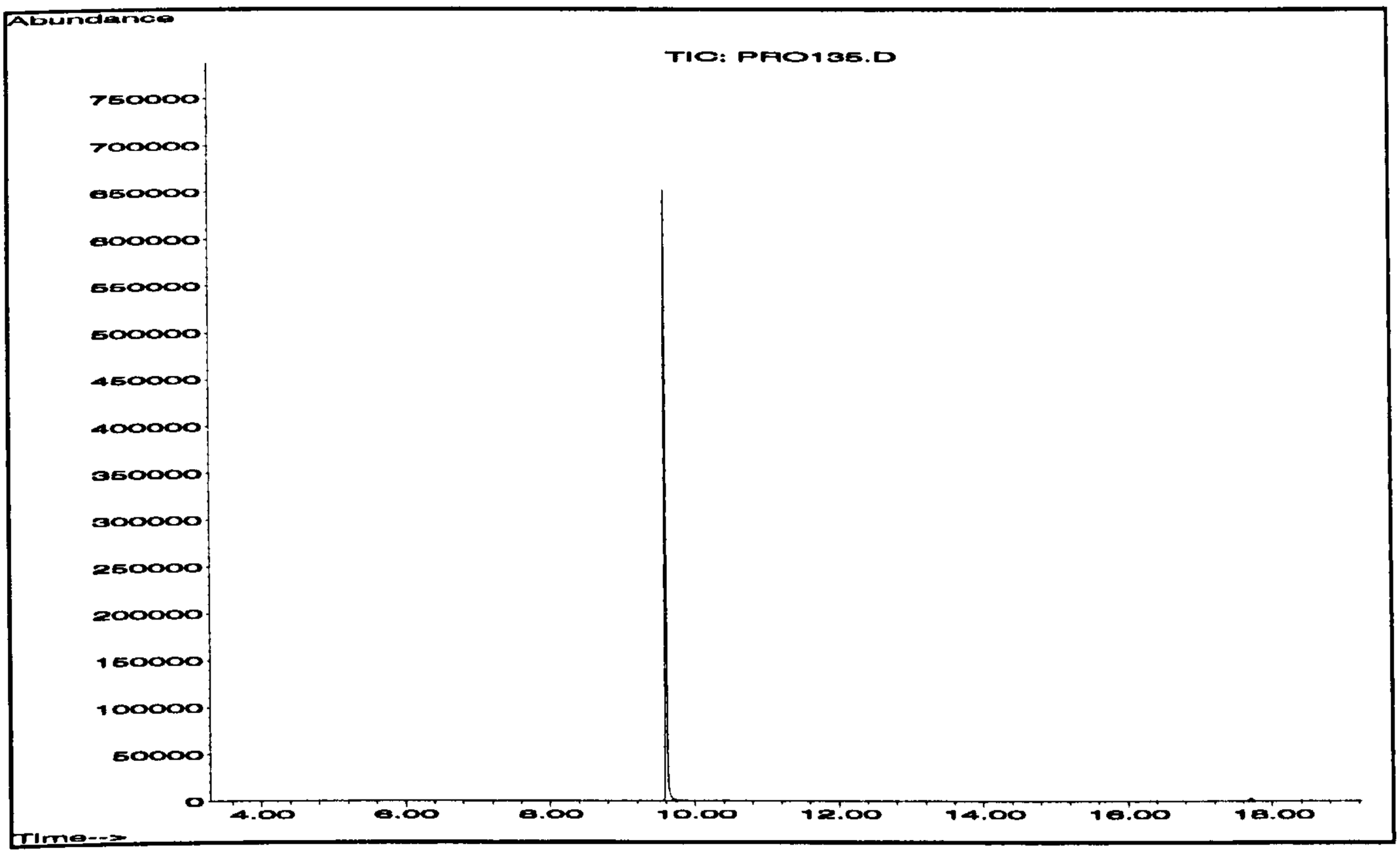


Figure 3.14 Benzaldimine Product After Purification

3.6.3. UV-VIS Absorption Spectra

The numerical values obtained are as follows:

UV λmax (nm): [MeOH] 240

[0.1M NaOH] 204, 212, 220

[0.1M H_2SO_4] 212, 220, 240

The only literature values available for comparison were obtained in ethanol by Sunagawa and Yoshida who report λmax = 246 nm [86].

3.6.4. Fourier Transform Infrared Spectrometry

The numerical values obtained are as follows:

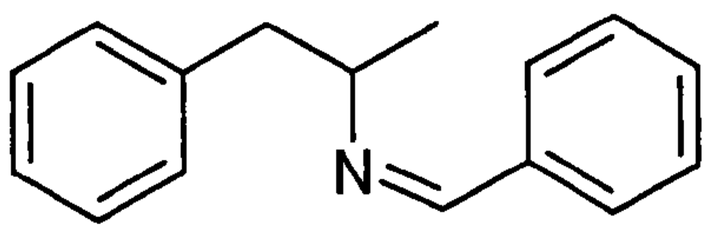
IR γmax (cm^{-1}): 696, 746, 968, 1454, 1495, 1641 (principal peak), 2353, 2362, 2848, 2973.

The predicted spectra for benzaldiimine would have characteristic peaks at 770-730 cm^{-1} (746 cm^{-1} found) and 720-680 cm^{-1} (696 cm^{-1} found) due to the presence of five adjacent aromatic protons. The presence of a methyl group should result in two or three bands at 2960-2580 cm^{-1} (2793 cm^{-1} found) A C=N bond would show characteristic absorbance at 1650-1700 cm^{-1} (1641 cm^{-1} found) and imines would produce multiple peaks at 3500-3300 cm^{-1}.

When compared to literature values the results obtained show relatively good correlation. Sunagawa and Yoshida report a peak corresponding to νC=N at 1640 cm^{-1} (1641 cm^{-1} obtained) tested as a liquid film [86]. Rogalska and Belzecki found a peak corresponding to the CN group with exactly the same value [87].

3.6.5. Nuclear Magnetic Resonance Spectrometry

The numerical data for ^{1}H is as follows:



^{1}H NMR: δ = 1.36 (3H, m, CHCH_3), 2.97 (2H, m, PhCH_2), 3.59-3.62 (1H, m, NCH), 8.07 (1H, s, NC*H*Ph) and 7.21-7.75 (10H, m, Ph).

These results compare well with published data. Rogalska and Belzecki report values of δ (ppm) = 1.2 (3H, d, CHCH_3), 2.78 (2H, d, PhCH_2), and 3.40 (1H, dq, NCH) [87]. Kreig's paper on the stability of amphetamines and benzylamphetamines, the results obtained are very similar. He reports δ (ppm) = 1.25 (3H, d, CHCH_3), 2.77 (2H, d, PhCH_2), 3.20-3.60 (1H, m, NCH), 7.85 (1H, s, NC*H*Ph) and 6.8-7.7 (10H, m, Ph) which again agree with our findings [88].

The numerical values for the ^{13}C data are as follows:

^{13}C NMR: δ = 22.24 (CH_3), 44.59 (CH_2), 68.20 (CH), 125.97 (C^4), 126.24 (C^{10}), 128.04 ($C^{3,5}$), 128.48 ($C^{9,11}$), 129.25 ($C^{2,6}$), 129.69 ($C^{8,12}$), 136.37 (C^1), 139.35 (C^7), 159.30 (N=CH)

No data was available in the literature for comparison and confirmation of the identity of the prepared compound.

3.6.6. Mass Spectrometry

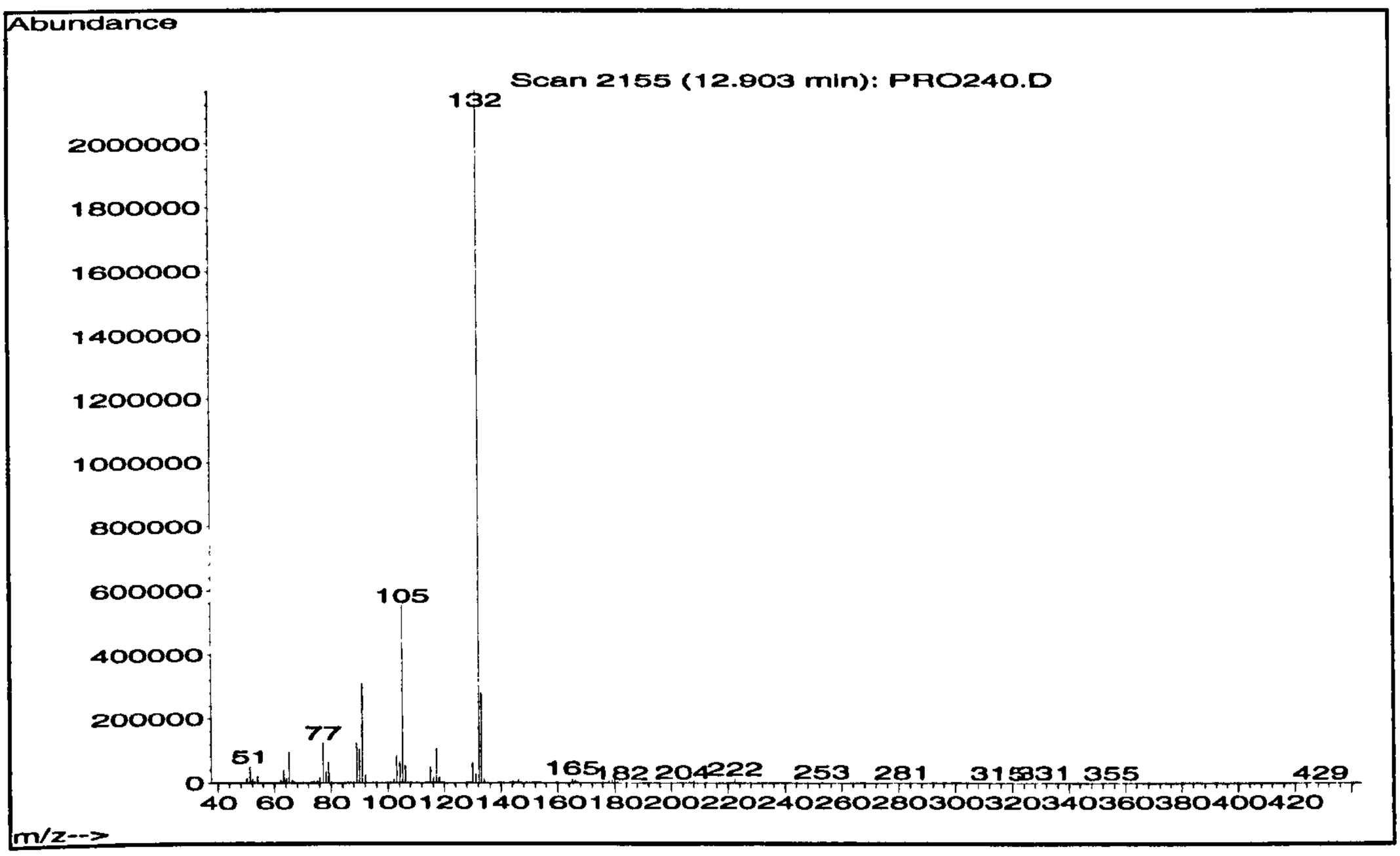


Figure 3.15 N-(β-isopropyl)benzaldiimine

The numerical results and mass to charge assignments for the spectra shown in Figure 3.15 are as follows:

m/z = 132 $[M\text{-}C_7H_7]^+$, 105 $[C_7H_7N]^+$, 91 $[C_7H_7]^+$, 133 $[M\text{-}C_7H_6]^+$, 77 $[C_6H_5]^+$, 117 $[M\ C_7H_8N]^+$, 222 $[M\text{-}H]^+$

This may be compared with the results obtained by Hanus *et al.* in their study of the fragmentation patterns of some aliphatic amines [89]. The m/z values found were, in decreasing order of abundance, 132, 105, 91, 77, 223, 117, 179, 165 and 208. Although the fragments at 223, 179, 165 and 208 are not detected in our spectra, this may be due to a tuning issue, the principal fragments are found in the correct order of abundance.

3.7. Synthesis and Analytical Data - 2-Benzyl Aziridine & 2-Methyl-3-Phenylaziridine

3.7.1. Synthesis of 2-Benzyl Aziridine & 2-Methyl-3-Phenylaziridine

Both aziridines are formed by two alternative ring closures when benzyl methyl ketoxime formed by the partial reduction of nitrostyrene is further reduced as detailed in Figure 3.1.

Benzyl methyl ketoxime (0.74 g, racemic mixture obtained from previous synthesis) was dissolved in 20 mL of distilled tetrahydrofuran (THF). The vessel was evacuated and the solution stored under a blanket of Argon. $LiAlH_4$ (0.5 g from laboratory store) was weighed into a Schlenk tube in a dry box. The $LiAlH_4$ was suspended in THF and cooled to –78 °C while the tube was attached to a Schlenk line and Argon bubbled through the $LiAlH_4$ suspension. The ketoxime solution was added drop-wise to the suspension via a syringe, while the solution was stirred and kept cool using a dry-ice/acetone bath. The reaction mix was allowed to rise to ambient temperature after approximately 1 hour of cooling and stirred at room temperature for 3 hours. The reaction mix was then refluxed for 4 hours and stirred overnight to ensure it had gone to completion. The reaction was then quenched using 5% NaOH (aqueous) until no more gas evolved and a white precipitate formed.

The reaction mix was transferred to a separating funnel and the organic products were extracted with 3x20 mL sodium-dried diethyl ether. The ether was then removed *in-vacuo* to leave a yellow oil, a drop of which was removed and dissolved in 1.5 mL of ethanol for GC-MSD analysis.

Purification

From this GC data, it was apparent that some ketoxime remained in the sample along with two aziridine structural isomers. In order to separate the aziridine isomers, preliminary TLC was performed using a 1:1 (hexane, ethyl acetate) mobile phase and triple development. The plates were scraped and each spot extracted with ethyl acetate and the resulting sample analysed by GC-MSD.

Since the TLC was successful in separating the isomers, a preparative TLC system was set up using the same mobile phase. This procedure was also successful in separating the aziridine isomers but did not yield enough of each isomer to obtain any meaningful analytical data. A column chromatography system was then set up using the same procedure as the

benzaldiimine purification system. A 50 mL burette with internal diameter of 10 mm was adapted to a chromatography column using a plug of silanised wool at the base and packing with wetted silica gel (chromatography grade Prolabo 60, 35-75 μm particle size) to the 20 mL mark. The impure aziridine sample was combined with some silica gel to create a 1 mL sample plug at the top of the column. The mobile phase used was 5:4 (hexane, ethyl acetate) and 24 column-volumes of 10 mL fractions were collected. In this case, all fractions were retained and analysed by GC-MSD for the presence of an aziridine and those containing only one isomer were combined and the solvent removed *in-vacuo* to yield enough of each compound to obtain FTIR, UV-VIS data and so on. Fractions 5-11 contained aziridine 2. Fractions 22-24 contained aziridine 1.

This whole procedure was been repeated several times to yield enough of each aziridine to continue with the project. However, it was found that benzyl aziridine was unstable and could not be retained on the bench as it appeared to degrade. Therefore it had to be synthesised as soon as practical before analysis. In addition, this was thought to present problems in the analysis of amphetamine samples which may have come from a single batch but were then split and stored in different environments thus introducing the possibility of the impurity profile changing over time.

3.7.2. Gas Chromatographic Data

The reaction was followed by GC-MSD after extraction of the organic product into ether. In addition, each fraction was analysed after column chromatography. The GC traces for the crude reaction mix and the two purified aziridines are shown in Figures 3.16, 3.17 and 3.18 with the mass spectra for each peak shown in Figures 3.19 and 3.20. Purity, based on peak integration, was shown to be 98 % in some samples.

3.7.3. UV-VIS Absorption Spectra

The UV-VIS data for the aziridines was not obtained due to the fact that very little of these compounds was available after purification and it was thought that it would be more analytically useful if used for FTIR and GC-MS analysis since these analytical techniques were more likely to yield useful information than UV-VIS.

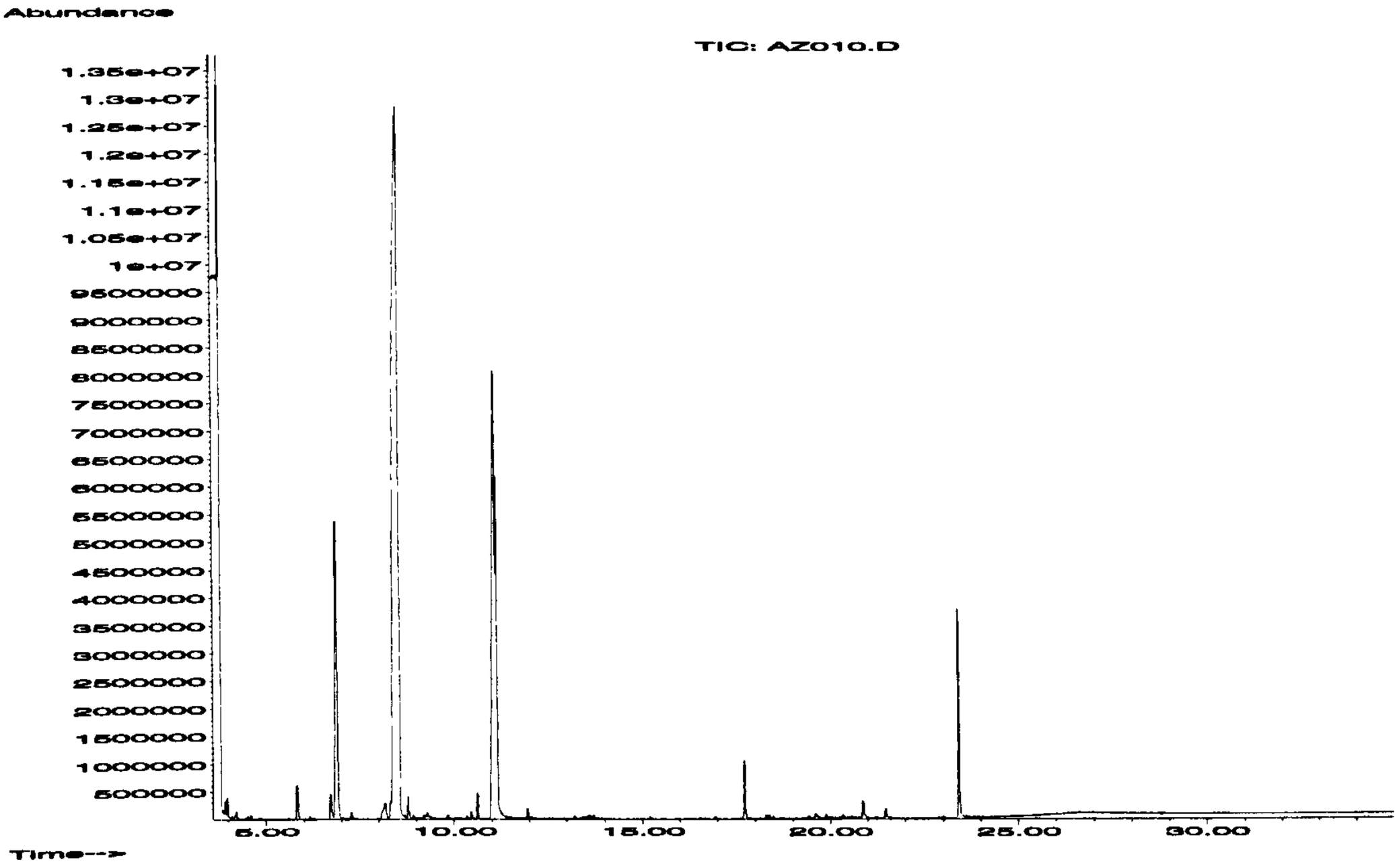


Figure 3.16 Crude Reaction Product Containing Excess Ketoxime

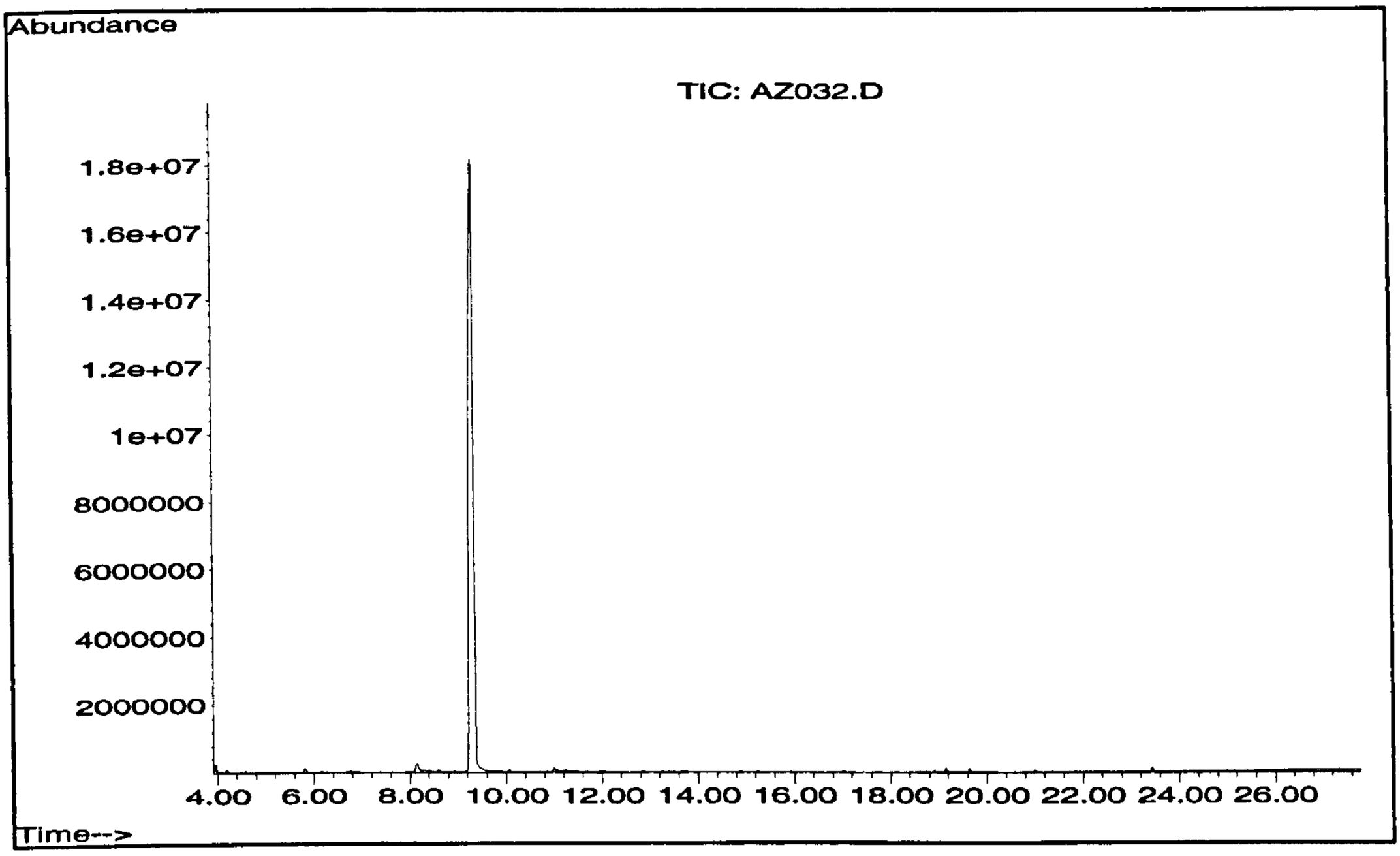


Figure 3.17 Aziridine Isomer 1

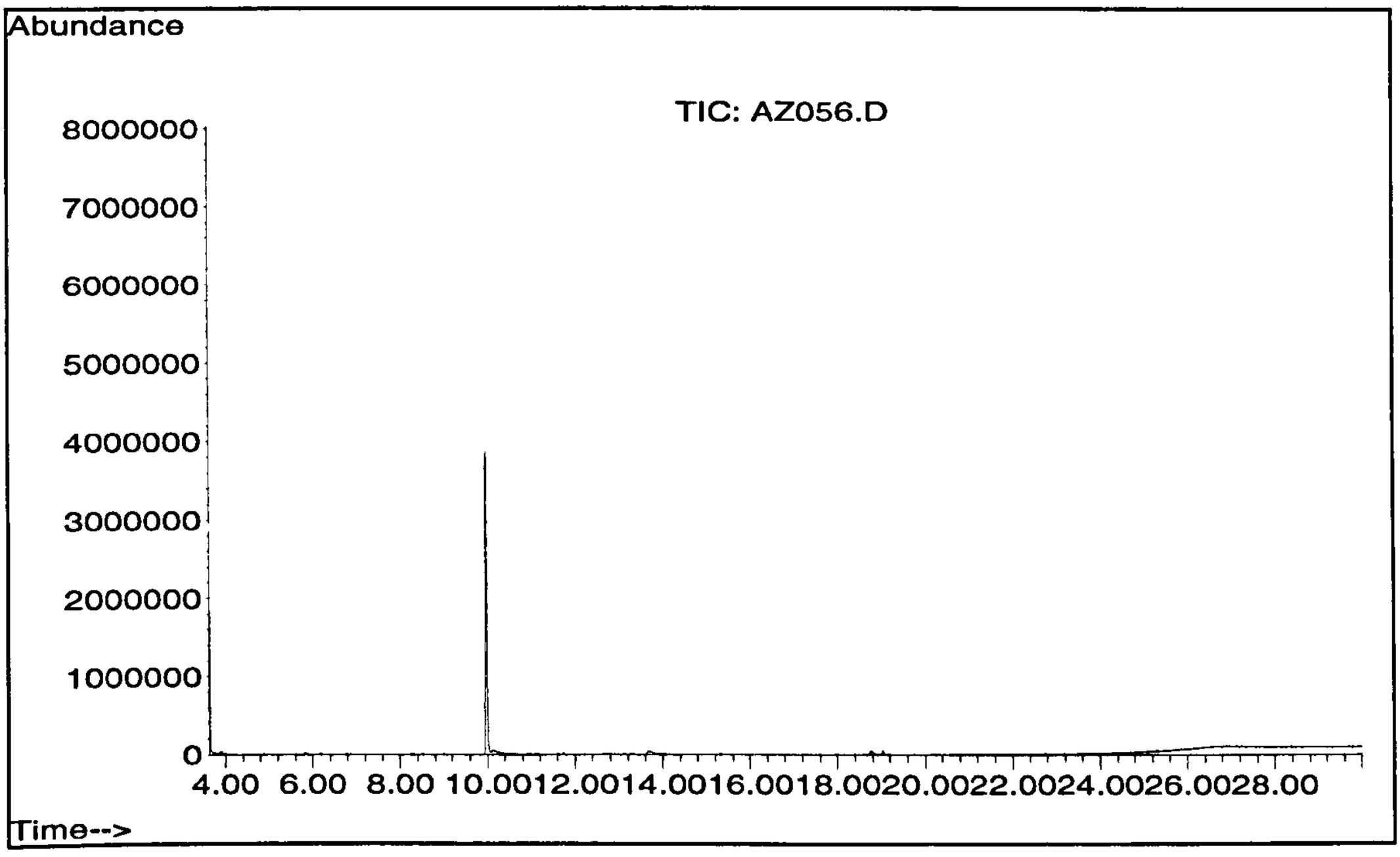


Figure 3.18 Aziridine Isomer 2

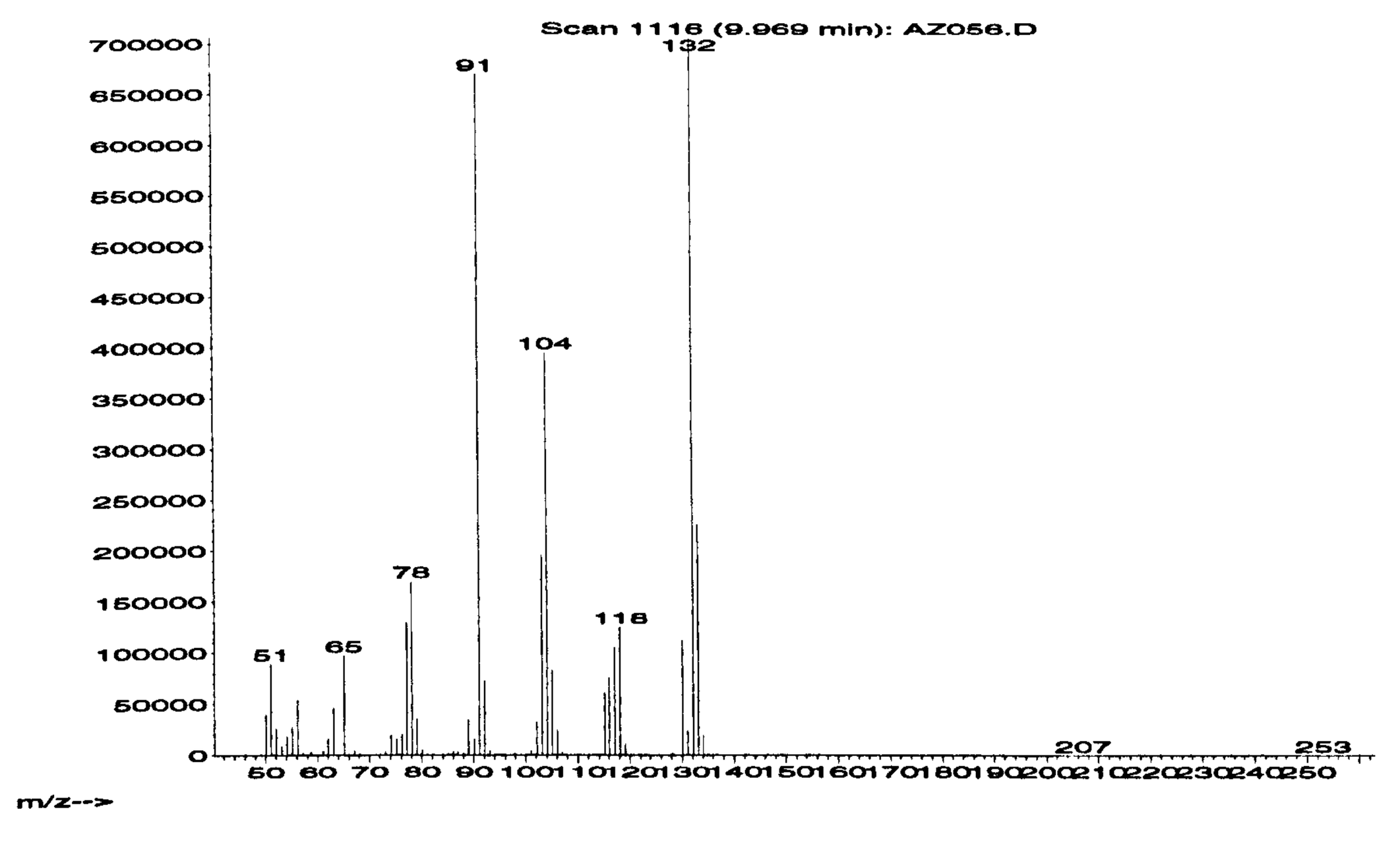


Figure 3.19 Mass Spectrum of Aziridine 2 (9.7min)

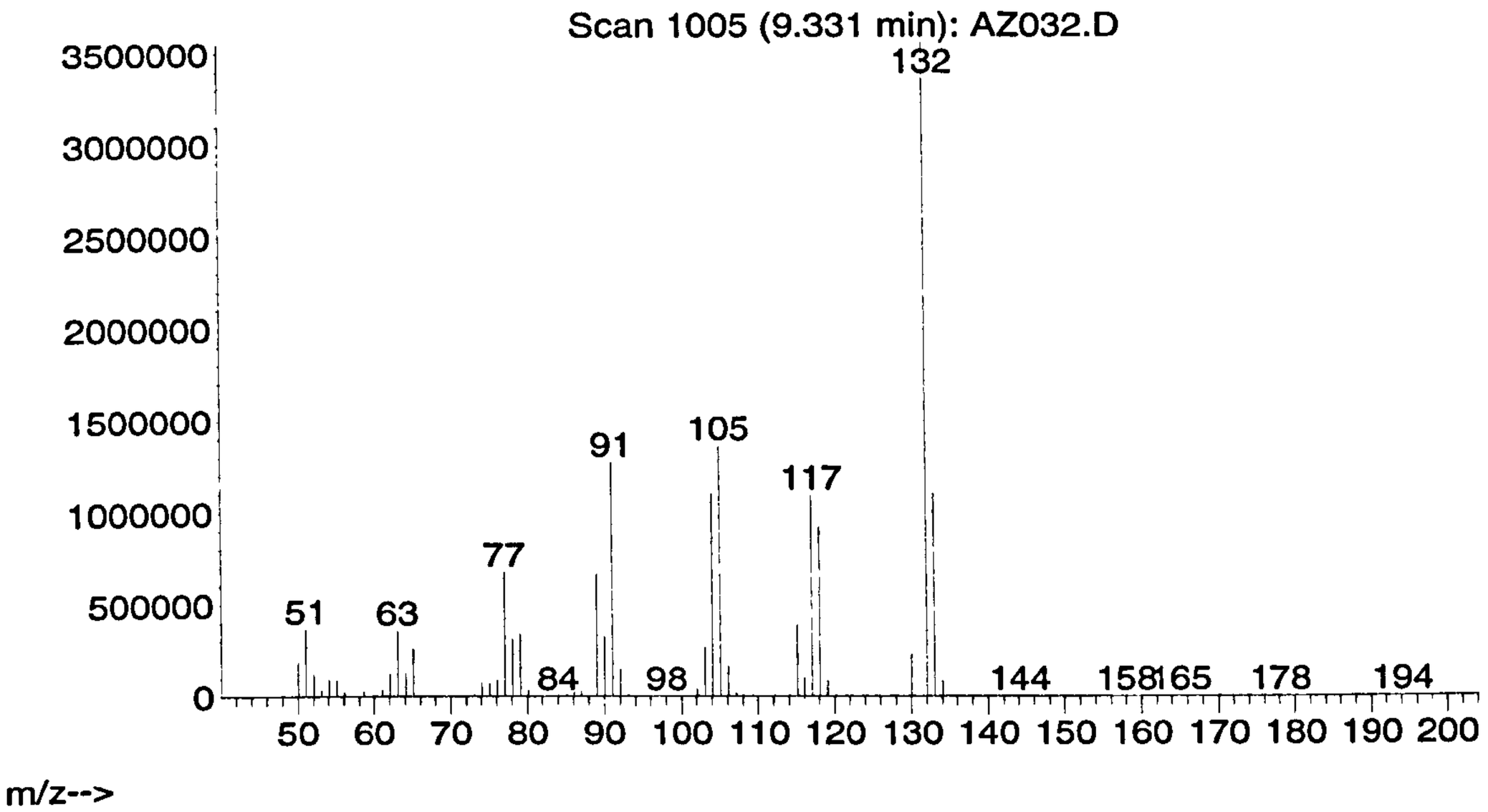


Figure 3.20 Mass Spectrum of Aziridine 1 (9.3min)

3.7.4. Fourier Transform Infrared Spectrometry

The numerical values obtained are as follows:

2-methyl-3-phenyl aziridine

IR γ_{max} (cm^{-1}): 701, 736, 803, 842, 1027, 1054, 1097, 1262, 1450, 2963, 2992

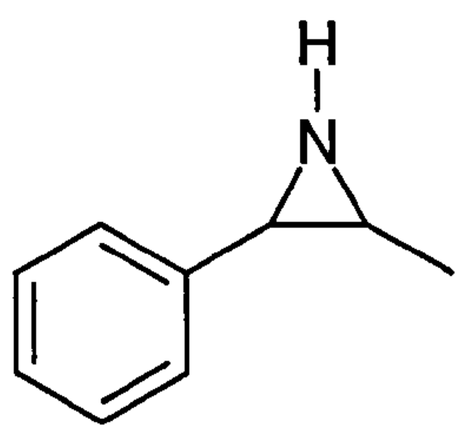
2-benzyl aziridine

IR γ_{max} (cm^{-1}): 702, 803, 815, 1026, 1047, 1097, 1262, 1456, 1668, 2968

The predicted IR spectra of these compounds would have characteristic peaks at 770-730cm^{-1} and 720-680cm^{-1} due to the five adjacent aromatic protons on the benzene ring. The presence of a methyl group can distinguish between the two isomers – two or three bands at 2960-2580cm^{-1} would denote a methyl group. This does not agree with the findings above however this may be due to impurities in the samples.

3.7.5. Nuclear Magnetic Resonance Spectrometry

The numerical values for the ^{1}H data are listed below.



2-methyl-3-phenyl aziridine

^{1}H NMR: $\delta = 0.87$ (1H, s, CH_3), 2.31 (1H, s, CH_α), 3.14 (1H, s, CH_β), 7.2-7.3 (5H, m, Ph)

These data values were then compared with those found by Galindo *et al.* [90] in their paper on the synthesis of 2-methyl-3-phenyl aziridine. This paper reports values of ^{1}H: $\delta = 0.9$ (d, 3H, J = 7.6), 2.38 (m, J = 7.6 and 8.8, 1H), 3.22 (d, J=6.2, 1H), 3.76 (s, 1H, NH), 7.2-7.3 (m, 5H, Ph).

The numerical values for the ^{13}C data is as follows:

^{13}C NMR: $\delta = 137.39$ (C^1), 127.67 (C^2), 127.68 (C^3), 126.4 (C^4), 36.93 (C^α), 31.96 (C^β), 13.38 (CH_3)

^{13}C NMR: $\delta = 137.6$ (C^1), 127.9 (C^2), 127.8 (C^3), 126.6 (C^4), 37.1 (C^α), 32.1 (C^β), 13.6 (CH_3) were the reported values of Galindo *et al.* which compare well with observations [90].

Benzyl aziridine

No suitable carbon data for this compound was obtained.

3.7.6. Mass Spectrometry

Exemplar spectra are shown in Figures 3.21 and 3.22. The numerical results and m/z assignments are as follows;

2-methyl-3-phenyl aziridine

m/z = 132 $[M-H]^+$, 117 $[M-NH_2]^+$, 91 $[C_7H_7]^+$, 105 $[C_8H_9]^+$, 104 $[C_8H_8]^+$, 118 $[M-NH]^+$, 133 $[M]^+$, 77 $[C_6H_5]^+$

2-benzyl aziridine

m/z = 132 $[M-H]^+$, 105 $[C_8H_9]^+$, 91 $[C_7H_7]^+$, 133 $[M]^+$, 117 $[M-NH_2]^+$, 104 $[C_8H_8]^+$, 118 $[M-NH]^+$,77 $[C_6H_5]^+$

This may be compared to the results obtained by Porter and Spear [91] which list the m/z values in order of abundance for 2-methyl-3-phenyl aziridine as:

m/z = 91, 133, 104, 78, 51, 39, 28, 77, 118, 105

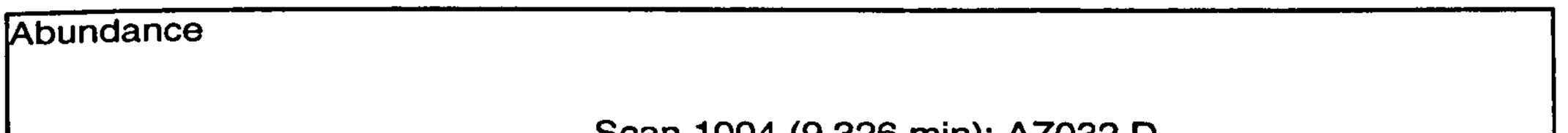


Figure 3.21 2-methyl-3-phenylaziridine Mass Spectra

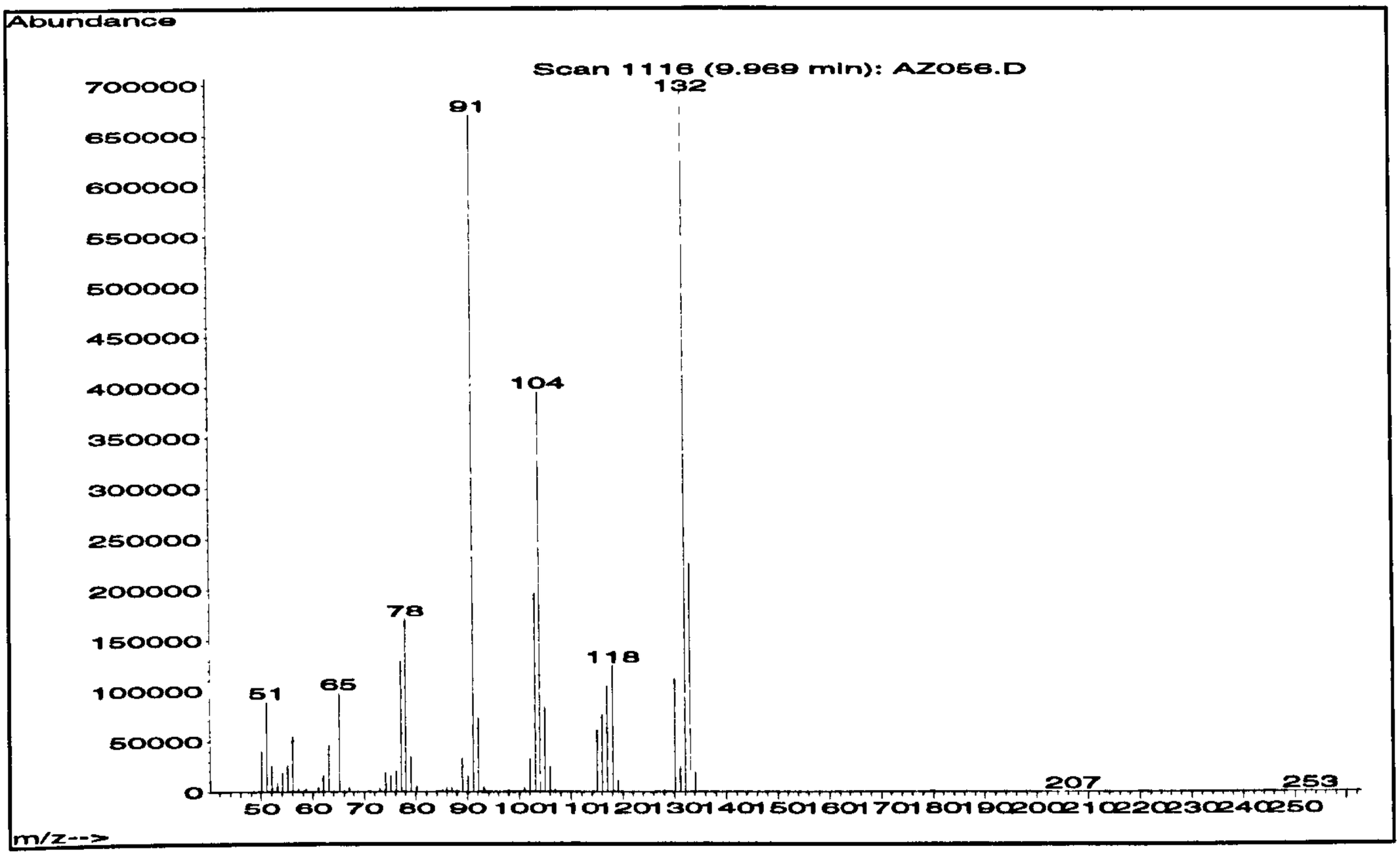


Figure 3.22 2-Benzylaziridine Mass Spectra

3.8. Synthesis of Amphetamine Batches

3.8.1. Synthesis of Amphetamine – $LiAlH_4$ Reduction

The Nitrostyrene route of synthesis for amphetamine production is detailed in Figure 3.1.

Nitrostyrene was synthesised as described in section 3.4.1. 5 g nitrostyrene was obtained by combining several recrystallisation batches and was dissolved in 20 mL anhydrous THF.

2.2 g of $LiAlH_4$ was weighed out in a dry-box and transferred to a Schlenk tube, which had previously been oven-dried, evacuated and filled with Argon. The tube was then attached to the Schlenk line and Argon bubbled through while 20 mL of anhydrous THF was added to form a $LiAlH_4$ suspension. To this, the nitrostyrene solution was added via a cannular while the reaction vessel was cooled to –78 °C using a dry-ice/acetone bath. The reaction was maintained at this temperature for an hour with stirring. The temperature was then allowed to slowly rise to ambient while stirring for several hours. The reaction vessel was placed in an oil bath at 80 °C and refluxed for 24 hours. The reaction was allowed to cool to ambient and 10 % aqueous sodium tartrate solution was added to quench any remaining unreacted $LiAlH_4$ (until no gas was evolved). A further 20 mL of distilled water was added to convert all the aluminium salts to a grey solid which was removed by Buchner filtration. The filtered solids were then washed with 20 mL of sodium-dried diethyl ether. The remaining filtrate was extracted with 3x10 mL sodium-dried diethyl ether to obtain amphetamine free-base and any organic impurities present. The solvents present were removed *in-vacuo* and the sample at this stage is our 'crude' amphetamine.

This sample was then split in two and one part retained as a 'crude' sample. The second part underwent a purification stage as suggested by Shulgin in his synthesis of methoxy-amphetamine [92]. The residue was dissolved in 25 mL of dilute aqueous H_2SO_4. The solution was then washed with 3x25 mL of CH_2Cl_2, which removed much of the orange/pink colour. The aqueous phase was made basic with dilute NaOH and re-extracted using 3x30 mL CH_2Cl_2. The solvent was removed *in-vacuo* leaving a yellow oil which was dissolved in 20 mL of iso-propyl alcohol which was neutralised with concentrated HCl and diluted to 60 mL with sodium-dried diethyl ether. White crystals formed which were removed by filtration and washed with ether and air-dried to produce the 'clean' amphetamine sample.

3.8.2. Synthesis of Amphetamine – RedAl Reduction

75 mL toluene was placed in a round-bottomed flask and 25 mL RedAl was added via a condenser to minimise heat evolution. 1.25 g nitrostyrene (from synthesis 3.4.1) was dissolved in 25 mL of toluene and added to the RedAl solution dropwise. The reaction was refluxed for 6 hours and the solution allowed to cool to ambient temperature.

25 mL of a THF:water (20:1) mix was added with stirring. The solution was allowed to stir for 1 hour. 25 mL of distilled water was added with stirring. After 1 hour, 50 mL of THF containing 3 NaOH pellets was added. The solvents present were rotary-evaporated, almost to dryness. 100 mL water was added with stirring and the solution made acidic by the addition of concentrated HCl. This mix was covered and stirred overnight.

The following day, the acidic mix was extracted with dichloromethane (2x50 mL). The aqueous phase was retained and basified with aqueous NaOH. The basic mix was then extracted with dichloromethane (2x50 mL). The organic phase was retained and the solvent removed *in-vacuo* to leave 0.629 g of amphetamine free-base.

Yield

A 100 % yield based on 1.25 g of nitrostyrene would give 1.03 g of amphetamine.
0.629 g of amphetamine represents a 61 % yield for this exemplar synthesis.

Before optimisation of the extraction procedures could proceed, it was necessary to synthesise at least 50 g of amphetamine sulphate using the nitrostyrene route. This would ensure that sufficient sample material was available to experiment with several different extraction and analysis methods. The processes that had been used previously to prepare amphetamine samples had been small-scale $LiAlH_4$ reduction reactions. Since the use of $LiAlH_4$ on a larger scale was not permitted due to its explosive nature, an alternative method was used. This method was modified from one found on an internet-site where synthetic chemists working in clandestine labs post instructions on how to increase yields etc and was through to reflect the ways in which 'street' amphetamine could be prepared.

The new modified method involved the use of sodium dihydro–bis(methoxyethoxy) aluminosilicate (RedAl), which is less explosive than $LiAlH_4$ but does not give as high yields. Therefore, the production of 50 g of amphetamine was a time-consuming and laborious process.

In addition to synthesising this 'home-made' amphetamine, its purity was assessed by HPLC and, since production of a single batch was not possible, the combination of all synthesised batches was necessary to provide a single sample from which many aliquots could be removed, analysed and compared. The reasoning behind this was to ensure that each of the batches of amphetamine sulphate prepared was pure enough that the combined sample would be properly representative of a 'pure' illicit sample. This sample of 'pure' drug would be used to mimic an uncut amphetamine such as may be found at a site of production.

It was also vital that the sample prepared from mixed batches would be homogeneous, since inhomogeneous samples would cause significant difficulties in the profiling of any drug. Any comparison of extracts must be carried out with the certainty that the aliquot taken from the bulk sample is representative of the whole sample otherwise intra-batch variation could lead to linked samples not being picked up during data analysis and chromatographic comparison.

3.8.3. Synthesis of Amphetamine Sulphate - Modified Version

The Nitrostyrene route of synthesis for amphetamine production is detailed in Figure 3.1 on p.61 with the substitution of RedAl in place of $LiAlH_4$.

Nitrostyrene (8 g), prepared as described in section 3.4, was dissolved in toluene (20 mL). RedAl (50 mL) purchased pre-dissolved in toluene, was combined with an additional volume of toluene (50 mL).

The nitrostyrene solution was added drop-wise to the RedAl solution via a condenser into a 500 mL round bottom flask. Heat was evolved and the solution was then allowed to reflux with stirring for a further 30-45 minutes.

5 % NaOH in H_2O (75 mL) was added drop-wise, forming a white suspension in the organic mixture. This suspension was then stirred for 30 minutes to ensure any active RedAl remaining had been consumed.

The aqueous layer was removed and the organic layer washed with distilled water (2x100 mL). The organic layer was acidified with 5 % HCl solution and stirred for 20 mins. The organic layer was then removed and the aqueous layer retained.

The aqueous solution was then basified using a concentrated NaOH solution until amphetamine free-base came out of solution as oil. Ether was then added to extract the

amphetamine free-base. This ether was then removed by rotary evaporation, leaving free-base oil which was then dissolved in ethanol.

H_2SO_4 (in ethanol) was added drop-wise to free-base (also in ethanol) with stirring. A white precipitate formed which was then removed by vacuum filtration. H_2SO_4 (in ethanol) was added again to the free-base solution to form more crystals which were again filtered off. These steps were repeated until no crystals formed. The batches of amphetamine sulphate crystals were then combined, washed with ether and left to dry in a desicator.

HPLC, GC-MS, FTIR and TLC analyses were carried out to confirm the presence of amphetamine sulphate crystals. This data was compared with that obtained previously from the smaller batches prepared via the $LiAlH_4$ method.

3.8.4. HPLC Analysis of Amphetamine Prepared Via Nitrostyrene

In all, 15 batches of amphetamine sulphate were synthesised using the procedure as described in section 3.8.3. The total weight of amphetamine sulphate obtained before homogenising the batches was 53.4 g.

Individual samples of each separate batch for HPLC analysis were prepared at a concentration of approximately 1mg/mL in methanolic HCl, the exact concentration being recorded for each sample. The HPLC calibration for amphetamine sulphate was carried out using SIGMA D+ amphetamine sulphate standard.

Quantification of the amphetamine purity levels in each individual batch was determined as well as the percentage purity of the sample prepared as a combination of all batches.

HPLC System

Gilson 308 and 306 (slave) pumps were used to maintain a flow of 1 mL min^{-1} through a 0.5 µM ID octadecylsilicate (ODS) column. UV-VIS detection was at 254 nm and integrator used was Shimadzu C-R3A with attenuation set at 4.

HPLC eluant used was as follows: MeOH : HCl : NH_3 (2000:5.8:18.4) v/v.

Samples were prepared in methanolic HCl (175 µL HCl in 100 mL methanol) and calibration was carried out using SIGMA Amphetamine (D+) batch number 46H1366 at concentrations of 0.1, 0.25, 0.5, 1, 1.5 mg mL^{-1}.

Each sample was injected 5 times to give replicate analyses. Methanolic HCl blanks were included between each run.

The calibration curve for the HPLC analysis of SIGMA D+ amphetamine is shown in Fig 3.23. The R^2 value is 0.9979. The linear regression equation generated was then used to determine the percentage purity of each of the 15 batches of 'home-made' amphetamine.

Table 3.1 shows the batch number and the percentage purity as determined by the HPLC analysis. Although some of the batches have been determined to be more than 100 % pure, the error in analysis is ±15 % and therefore these values are acceptable. In addition, some batches are particularly impure (Batch 6 and 15) but these batches were included to provide impurities at a higher level.

From this data as well as the GC analysis of each batch of amphetamine sulphate, which showed a low level of impurities, the bulk amphetamine sulphate prepared via the nitrostyrene route was thought to be pure enough to be used in the optimisation of the extraction system.

Batch Number	% purity
Batch 1	97.3
Batch 2	103.5
Batch 3	96.7
Batch 4	90.8
Batch 5	90.2
Batch 6	48.3
Batch 7	109.8
Batch 8	98.3
Batch 9	114.2
Batch 10	102.7
Batch 11	107.0
Batch 12	114.1
Batch 13	86.3
Batch 14	101.1
Batch 15	68.8

Table 3.1 Percentage Purity of Amphetamine Batches

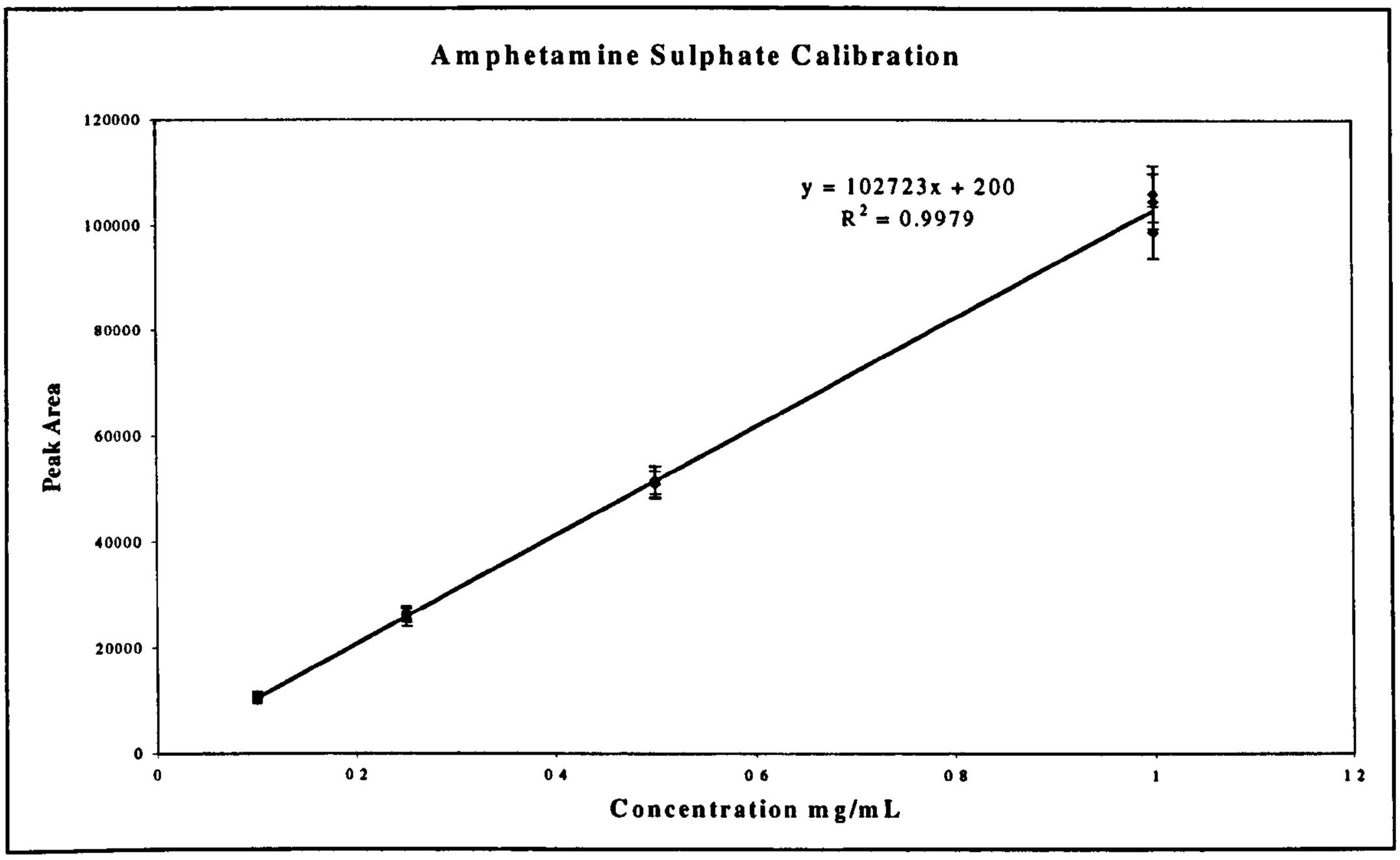


Figure 3.23 Peak Area of Amphetamine Against Concentration

Chapter 4

Linearity of Detector Response, Stability and Homogeneity Studies

4.1. Linearity of Response

The objective of this task was to study the linearity of response of the MSD as a detector, with respect to an increase in concentration of each of the individual standards. If the MSD showed a linear range, this would allow quantification of compounds as well as identification based on both on retention time and spectra. This particular stage of the study was carried out in parallel with the synthesis of compounds and since, at this point, the aziridine mixture had not yet been separated; these were not included in this study.

If the experiments show that the MSD and FID cannot provide a linear response to an increase in the concentration of each of the impurities, then their use as detectors for accurate quantification and therefore amphetamine profiling would be severely limited. Since the process relies on an accurate comparison of the ratio of impurity concentrations in each seizure to an internal standard, determination of the linearity of detector response is fundamental.

4.1.1. Procedure

Samples of each impurity were prepared individually in iso-octane at concentrations of 0.005, 0.01, 0.025, 0.05, 0.1, 0.25 and 0.5 mg mL^{-1}. Internal standard, $C_{24}H_{50}$ (tetracosane) was included at 0.1 mg mL^{-1} in each case.

A bulk sample at each concentration was divided into five aliquots in separate GC vials for separate analysis. Iso-octane blanks were included between each sample. The GC temperature programme used for this study was previously agreed between the partner laboratories to ensure results were comparable and is as described in section 3.3.4.

4.1.2. Results & Discussion

Since the benzyl methyl ketoxime isomers were incompletely resolved, integration, to obtain the area under each peak, was performed manually to achieve the best approximation for the total area under both peaks. The results of the change in impurity peak area (relative to the internal standard) in response to concentration, is presented in Table 4.1 for each compound showing the line of best fit and the r-squared value calculated. The R^2 values calculated for each impurity are presented for the 0.005-0.1 mg mL^{-1} range and the 0.005-0.5 mg mL^{-1} range.

From the results shown graphically below for the benzaldimine impurity, it can be seen that, for this compound the detector response is linear up to 0.1 mg mL^{-1} concentration in iso-octane. Above this concentration, the detector response is enhanced and the peak areas are disproportionately large, relative to the change in concentration.

It was thought that the actual concentrations of the impurities were unlikely to be used routinely to profile the impurities since this would necessitate a calibration curve to be established for each impurity before each batch of samples are run. This would impose some time constraints on the routine analysis of samples. At this stage, it was thought that since impurities are generally found at very low levels in amphetamine samples, the established range of linearity was sufficient for our purposes. However, in hindsight it would be useful to provide calibration lines and quality control samples to run alongside samples to accurately quantify impurities since this is undertaken routinely in the pharmaceutical industry in the multicomponent analysis of drugs and their metabolites. Establishing separate calibration lines based on a mixed stock of standard compounds before each run would provide easy numerical comparisons between batches. This would, however, rely on the standards being commercially available and certified which is not currently the case.

Compound	R^2 Value 0.005–0.1 mg mL^{-1}	R^2 Value 0.005–0.5 mg mL^{-1}
Nitrostyrene	0.9901	0.9739
2-(β-phenylisopropyl) benzaldiimine	0.9993	0.9601
Benzyl Methyl Ketoxime	0.9995	0.9853
Benzaldehyde	0.9974	0.9886

Table 4.1 r^2 Values for Impurities in Iso-Octane Using MSD

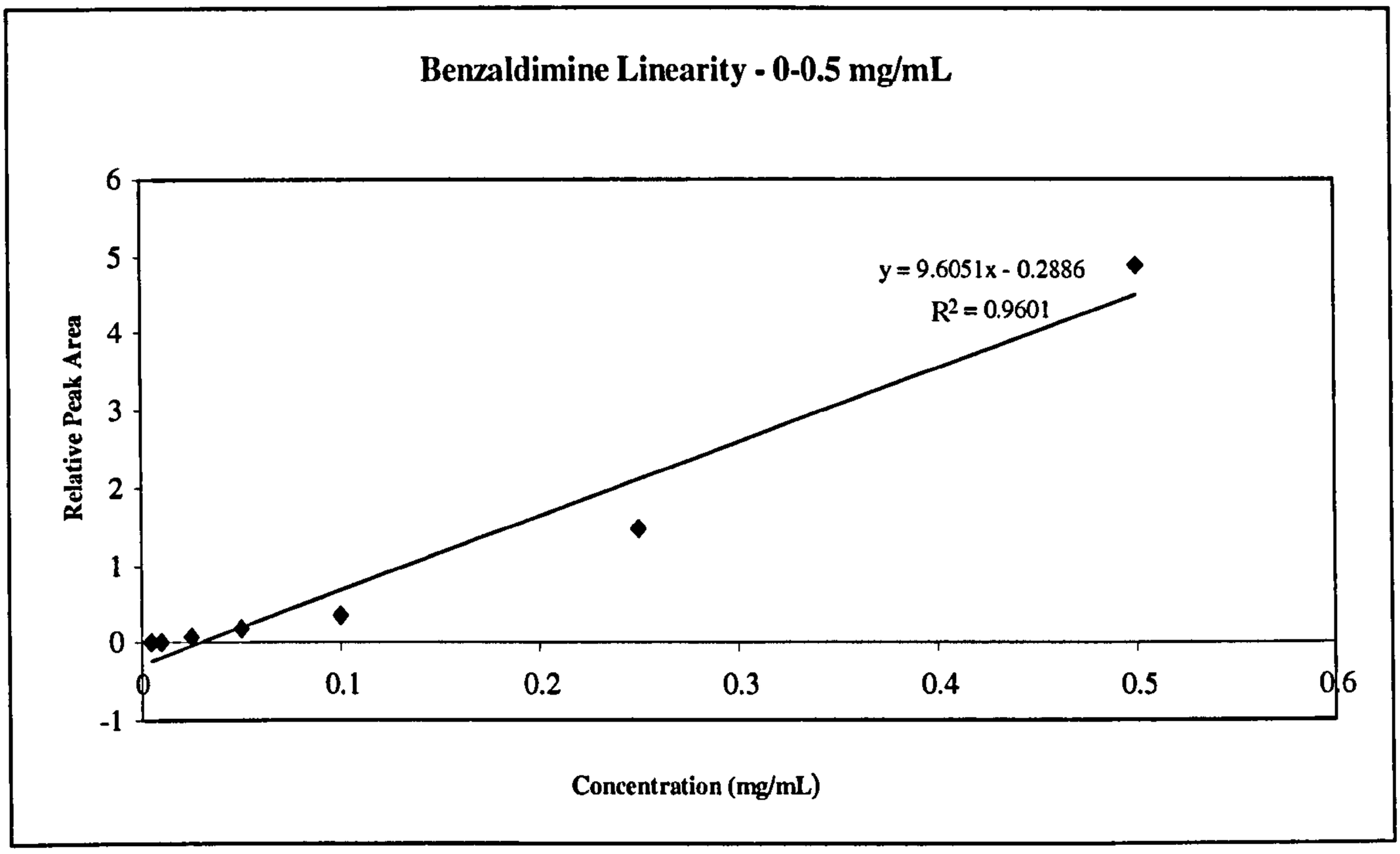


Figure 4.1 Linearity of Response for Benzaldimine in Iso-Octane (0-0.5mg/mL)

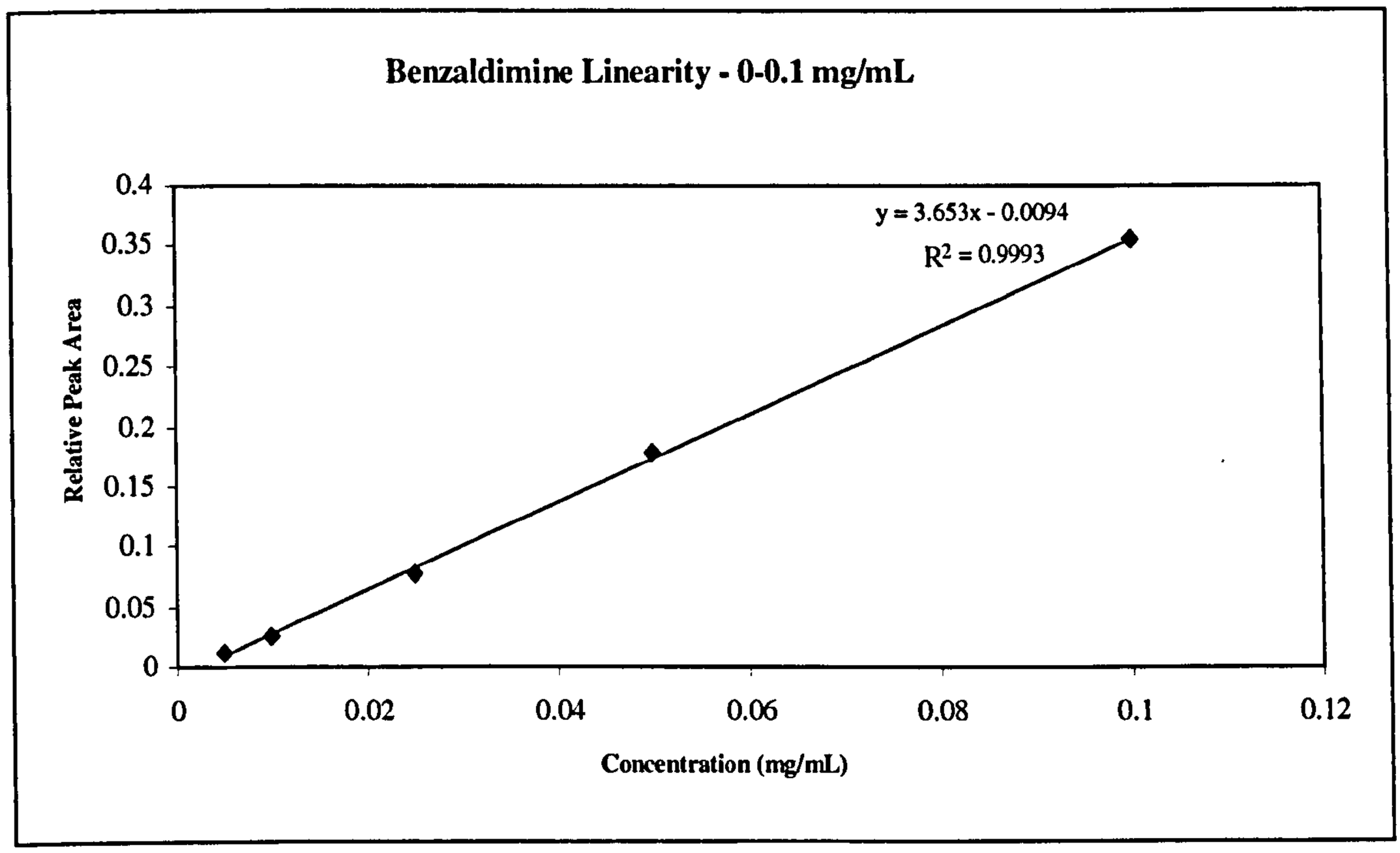


Figure 4.2 Linearity of Response for Benzaldimine in Iso-Octane (0-0.1mg/mL)

It should be noted that the inability to separate the ketoxime isomers and integrate them separately was, at this stage in the project, not a great problem since the data analysis was undertaken by each laboratory individually (see Figure 4.3). In order to completely automate extraction and analysis and perform data manipulation which has no element of subjectivity, manual integration of peaks (such as the ketoxime isomers) should be avoided. The fact that humans are subjective in determining the area under a peak could result in identical batches of amphetamine being integrated in a slightly different way. However, this integration could be checked and countersigned by two individuals for quality purposes to avoid this possibility.

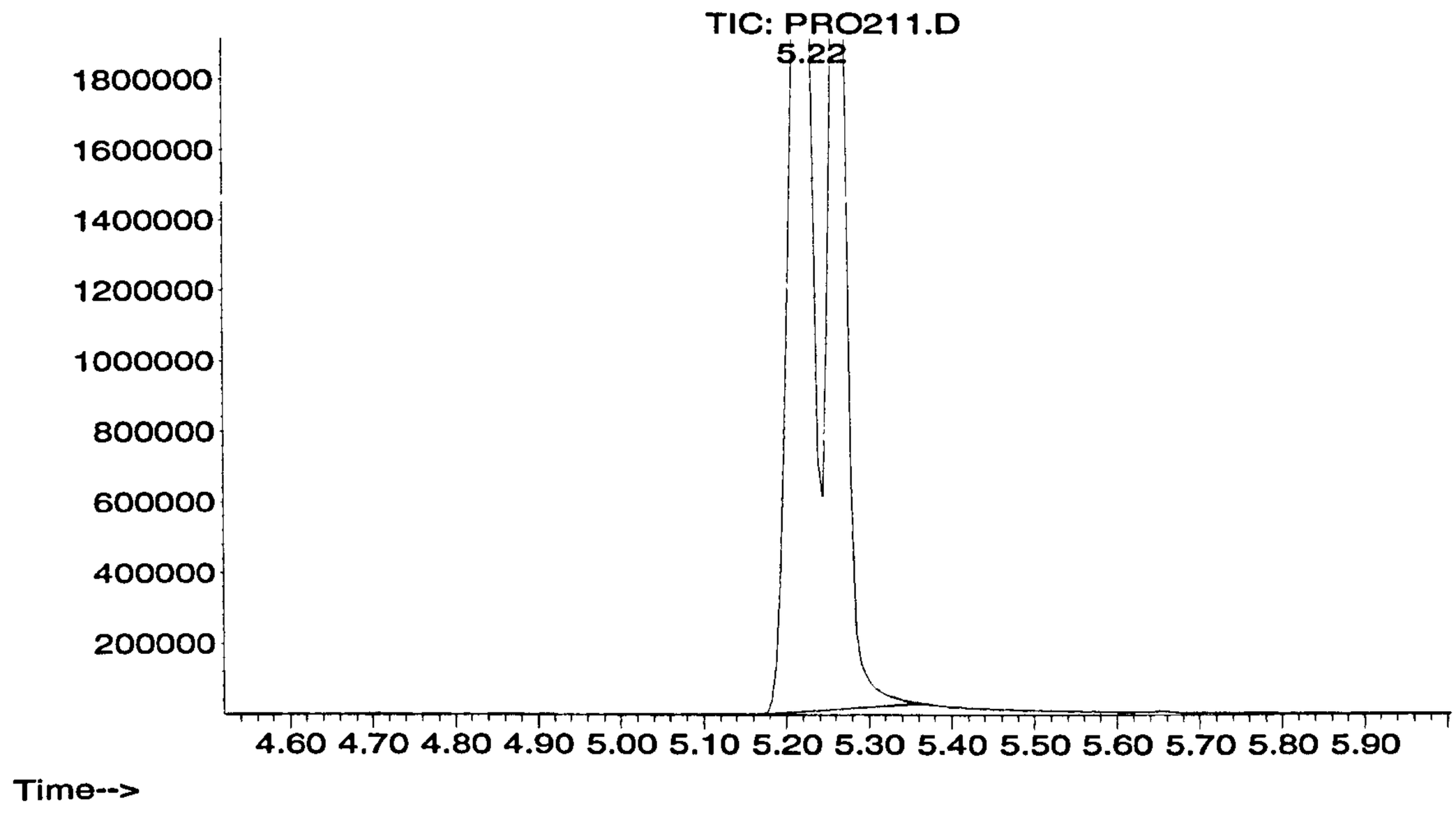


Figure 4.3 Ketoxime Isomer Co-elution and Integration

4.2. Stability Study

4.2.1. Aims

The stability of the impurities in solution is also an important consideration in choosing the most suitable solvent for extraction and profiling. Their solution stability becomes of major importance for profiling purposes when the proportions of each impurity with respect to one another are assessed. These experiments set out to explore the premise that the concentration of each impurity may alter if the sample is left for a lengthy period in specific solvents. Since the impurities are by-products or intermediates in the same reaction, the possibility that species may react together cannot be overlooked. Alternatively, they may degrade only in specific solvents at higher temperatures.

The stability study focuses on the chromatographic response showing either the presence of artefacts which were not in the original sample, or a diminished response to analytes over time. This possible problem of degradation of, or reaction between analytes, becomes especially significant in working laboratories where, due to high case-throughput, impurities may be extracted into a solvent and the samples left in a queue on an autosampler carousel at room temperature for an extended period. Therefore, these tests should determine if the impurity profile obtained from a sample immediately after extraction would be significantly different to that obtained after a given time on an autosampler carousel.

Experiments were designed to mimic the possible post-extraction waiting time on an autosampler. Each impurity was studied as part of a mixture in several different solvents, which could be used as extraction solvents in profiling to selectively separate the impurities from the bulk drug.

The purpose was to determine the length of time and temperature at which the analytes are sufficiently stable for analysis to be performed from a full autosampler. In the 'worst-case scenario', the available autosampler held 100 samples and the average run time of our temperature programme is 45 minutes. Therefore, the analysis of 100 samples (and 100 wash samples or solvent blanks to prove that the system is free of contamination) would take approximately six days.

4.2.2. Procedure

This study was carried out using mixtures of the synthesised impurities each at 10 $\mu g\ mL^{-1}$ concentration dissolved in each of the solvents under consideration. Internal standard $C_{24}H_{50}$ (tetracosane) was spiked at 100 $\mu g\ mL^{-1}$ in all mixtures. It was also thought that the presence of amphetamine itself may have an effect on the stability of the impurities, therefore, the study was repeated with impurity mixtures also containing amphetamine base at 10 $\mu g\ mL^{-1}$.

Solutions were sampled immediately after preparation and placed on an autosampler carousel, which was maintained at a constant 25 °C using a water-bath and pump constantly circulating warm water around the carousel. Analysis was carried out in duplicate with appropriate blank samples of solvent between each stability sample to prevent analyte carry-over and to prove that the system itself was free of latent impurities.Solutions were sampled from separate vials after 4-5, 12-13, 24-25, 36-37, 48-49, 72-73 and 96-97 hours left on the carousel.

If changes in profile were observed at 25 °C, the experiment was repeated at 8 °C to determine if degradation could be prevented by chilling the samples to inhibit any possible reactions.

The solvents chosen for this experiment were thought to be representative of possible solvent types used for amphetamine extractions. A branched hydrocarbon (iso-octane), an alcohol (ethanol), an ether (sodium-dried diethyl ether), an aromatic solvent (toluene), a chlorinated solvent (dichloromethane), and an ester (ethyl acetate).

It is unlikely, however, that ethanol or ethyl acetate would be selected as an extraction solvent from an aqueous buffer because of the inherent problem that they would be partially miscible. However, these solvents could be useful as solvents in solid phase extractions and were therefore included in this study. In addition if these solvents were to take up any of the aqueous phase this could result in severe problems for the GC in terms of column deterioration and damage to the source and therefore should be avoided if at all possible. Environmental concerns over the use of chlorinated solvents also makes them less attractive as an option in extraction, however, a case could be made if dichloromethane proved to be far superior to the alternatives. Problems associated with evaporation of diethyl ether also

limit its use as an extraction solvent but with a chilled autosampler these can be overcome if this solvent gives improved extraction stability.

In addition, previous to impurity samples being analysed, the instrument was checked by running a Grob mixture, hexane blank and iso-octane blank to ensure that the instrument was free of contamination and that the detector response was comparable with the previous batch.

The timetable and GC programme for this experiment were set up, making use of the time delay facility of the instrument, to sample each of the solutions at the appropriate times throughout the day and night.

Peak integration was carried out manually on each of the chromatograms and the ratio of impurity peak area relative to the internal standard, was obtained. It was thought that the internal standard in this experiment ($C_{24}H_{50}$) would compensate for the differences in injection volumes, changes caused by solvent evaporation and variation in the MS detector response. The mean value of the duplicate injections was obtained and the relative peak areas plotted against the number of hours spent on the autosampler carousel for each of the solvents.

After the full complement of results had been obtained at 25 °C, only ethanol, ethyl acetate, dichloromethane and ether were studied at 8 °C.

4.2.3. Results and Discussion

Exemplar chromatograms of the changes in impurity profile obtained with dichloromethane with respect to time are shown in Figures 4.4 and 4.5.

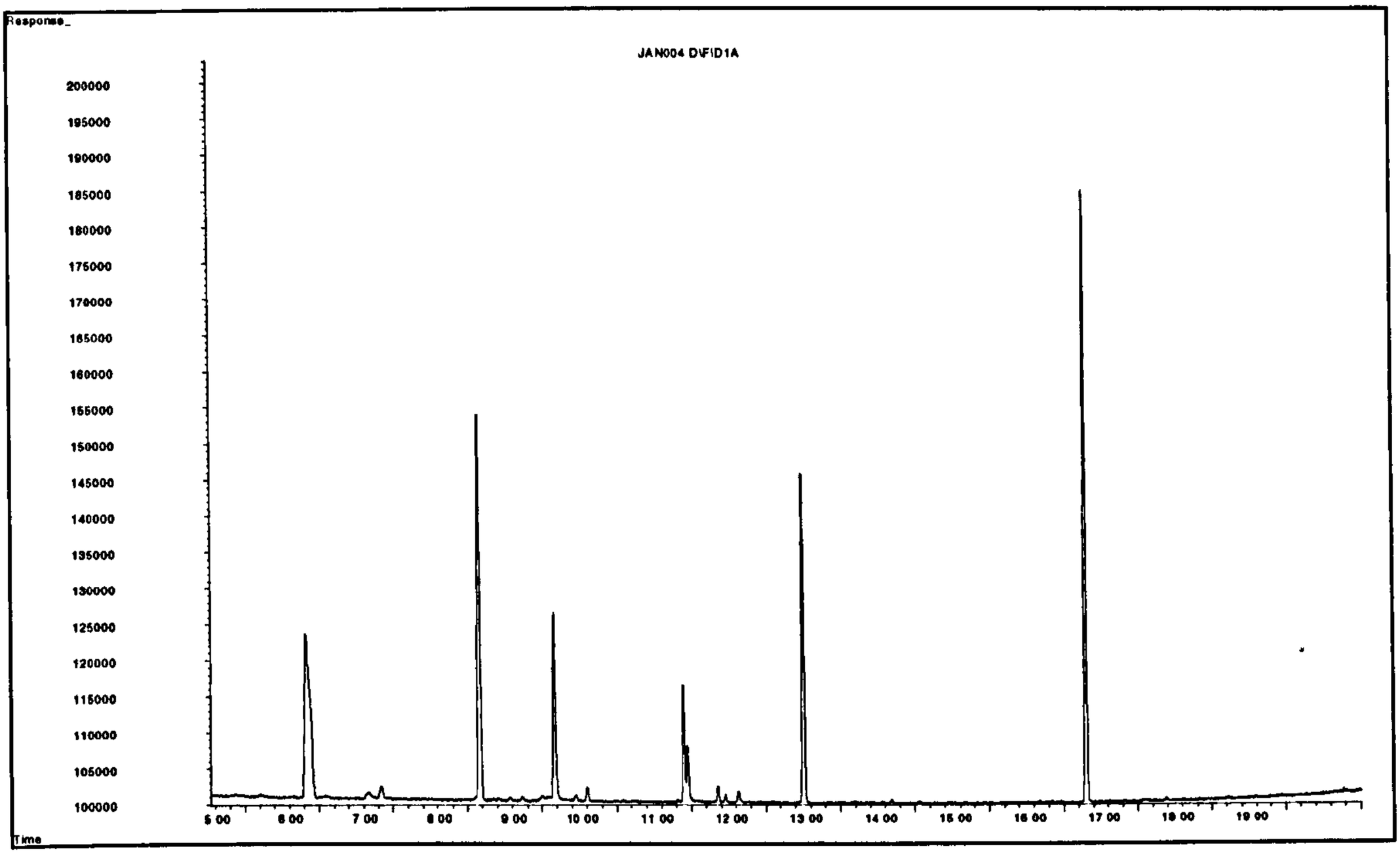


Figure 4.4 Mixture of Impurities in Dichloromethane Immediately Following Preparation

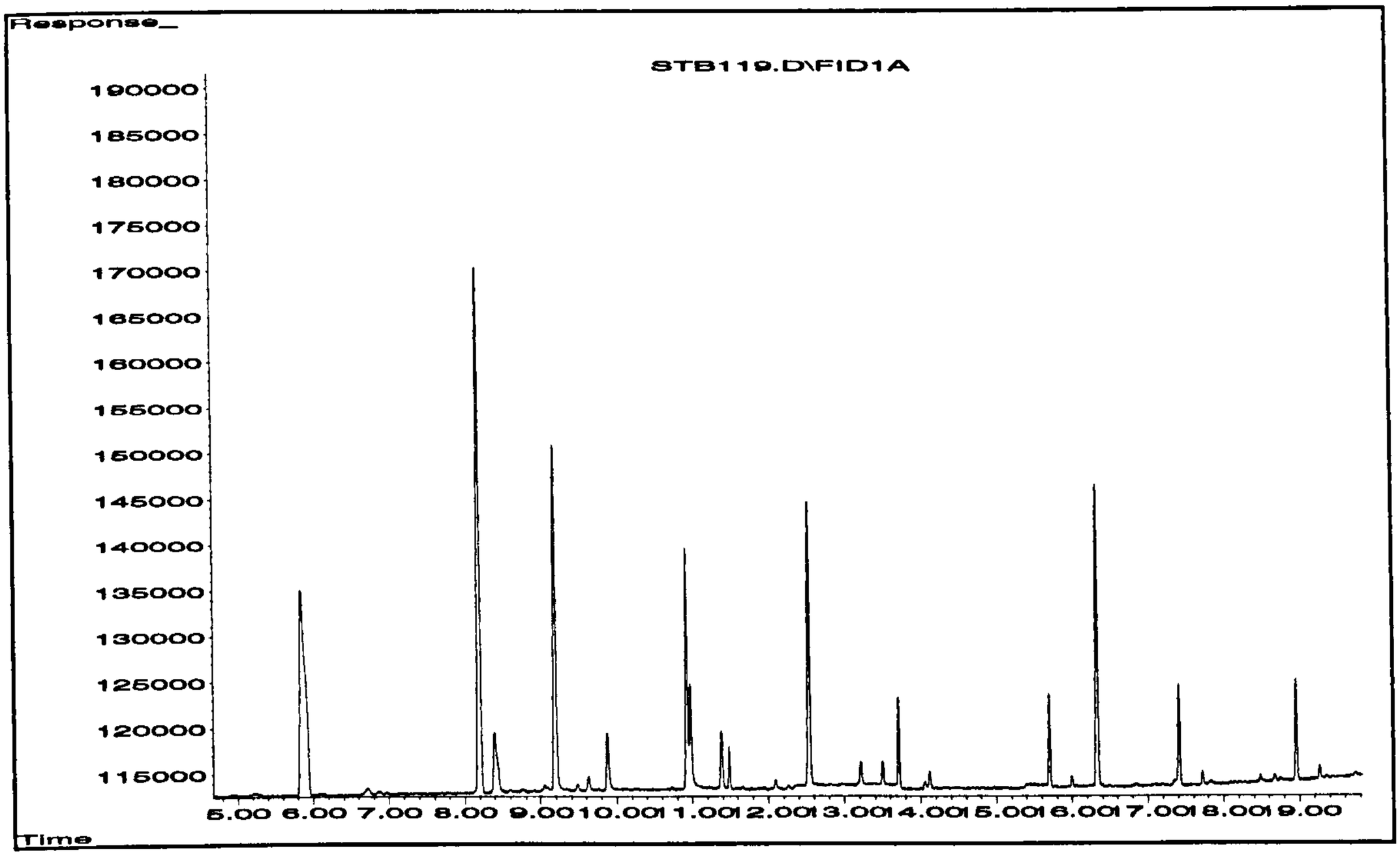


Figure 4.5 Mixture of Impurities in Dichloromethane After 96 Hours

From the results obtained, it was shown that the profiles of the impurity mixtures changed significantly over time in some of the solvents in question. In the next stages of the optimisation process, the fact that the impurities are not stable in specific solvents is, in some ways a hindrance because it renders solvents, which may otherwise be excellent extraction solvents, impractical. On the other hand, it does narrow down the choice of solvent and reduces the number of experiments necessary to determine the solvent which gives the most efficient extraction.

Iso-octane

This solvent was used only in the 25 °C study since there was no visibly appreciable level of degradation over time. The levels of all monitored impurities remained relatively constant over the experimental period of 96 hours. Therefore, iso-octane was proposed as a solvent to be included in the next experimental stages. The results are displayed graphically as relative peak area for each detected impurity against time in Figure 4.6 and 4.7.

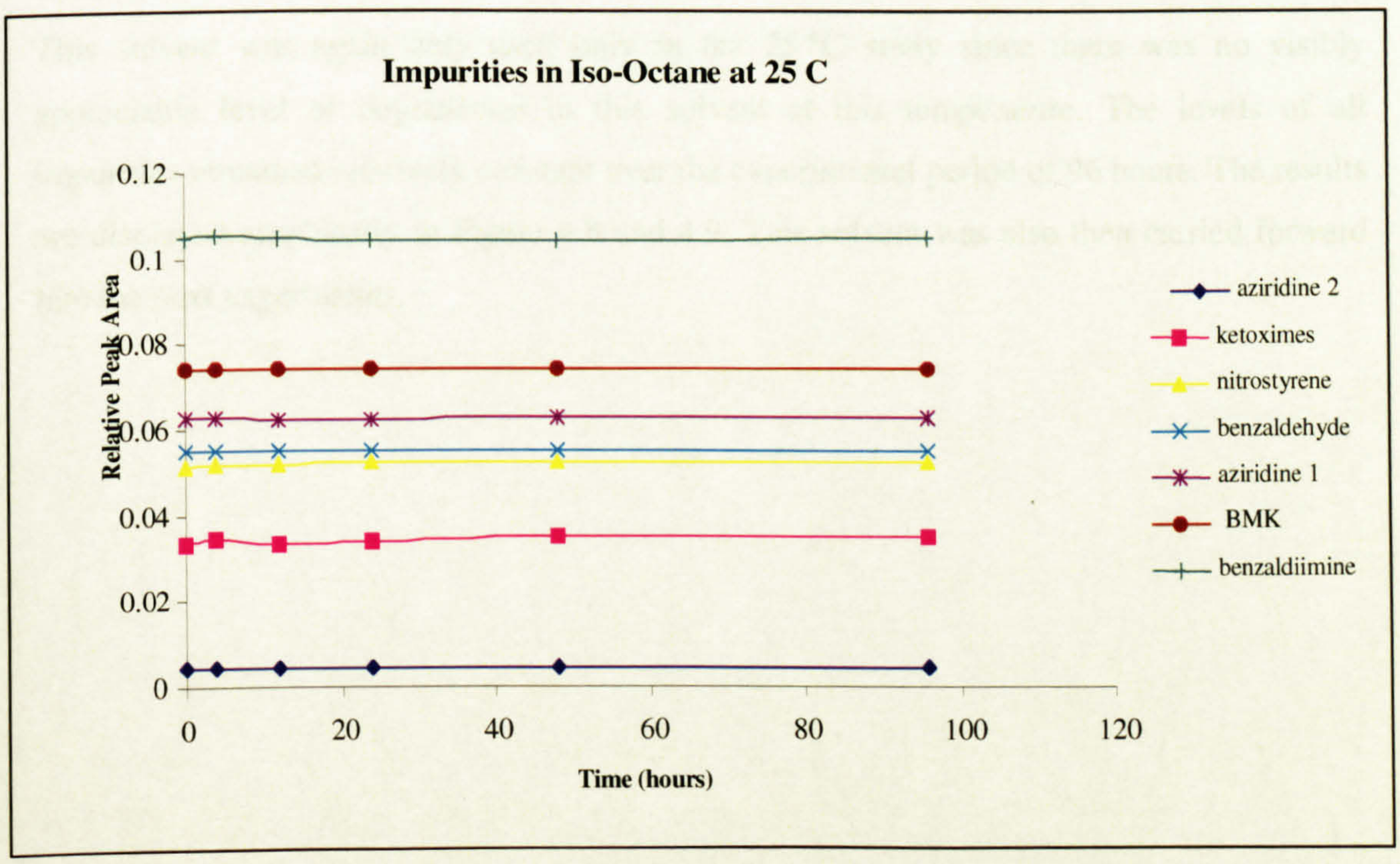


Figure 4.6 Stability of Impurities in Iso-Octane at 25°C

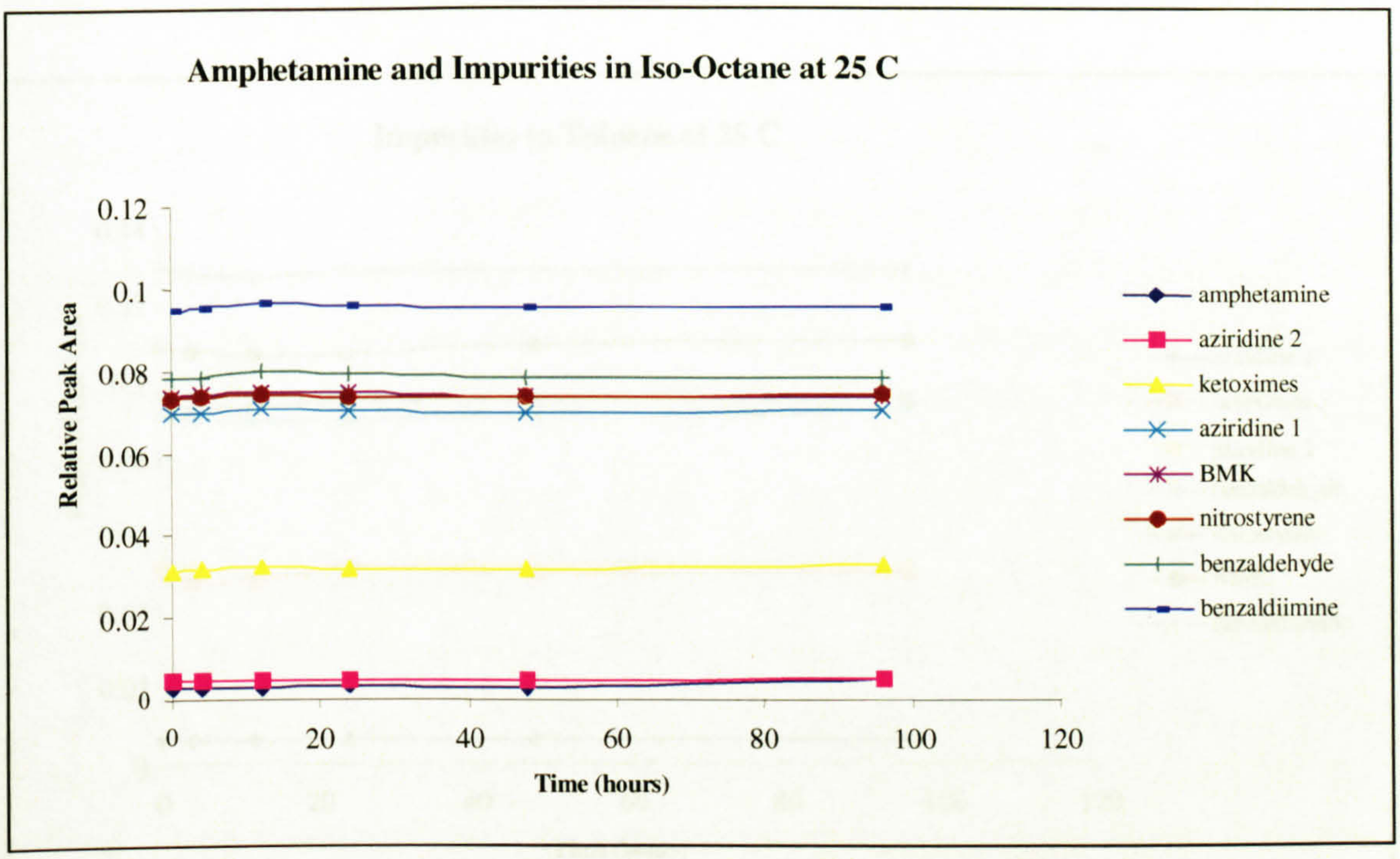


Figure 4.7 Stability of Impurities With Amphetamine in Iso-Octane at 25°C

Toluene

This solvent was again only used only in the 25 °C study since there was no visibly appreciable level of degradation in this solvent at this temperature. The levels of all impurities remained relatively constant over the experimental period of 96 hours. The results are displayed graphically in Figure 4.8 and 4.9. This solvent was also then carried forward into the next experiments.

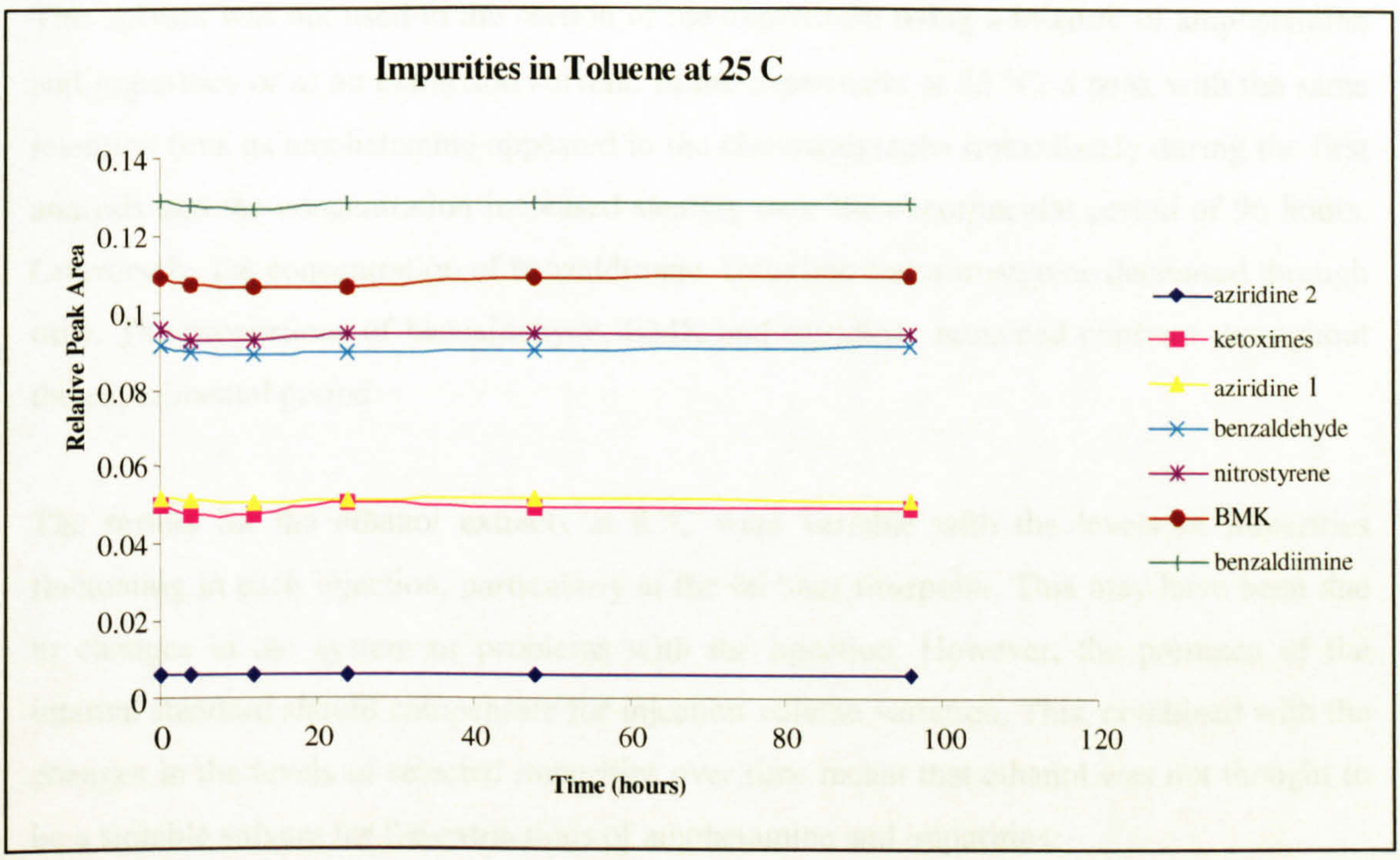


Figure 4.8 Stability of Impurities in Toluene at 25°C

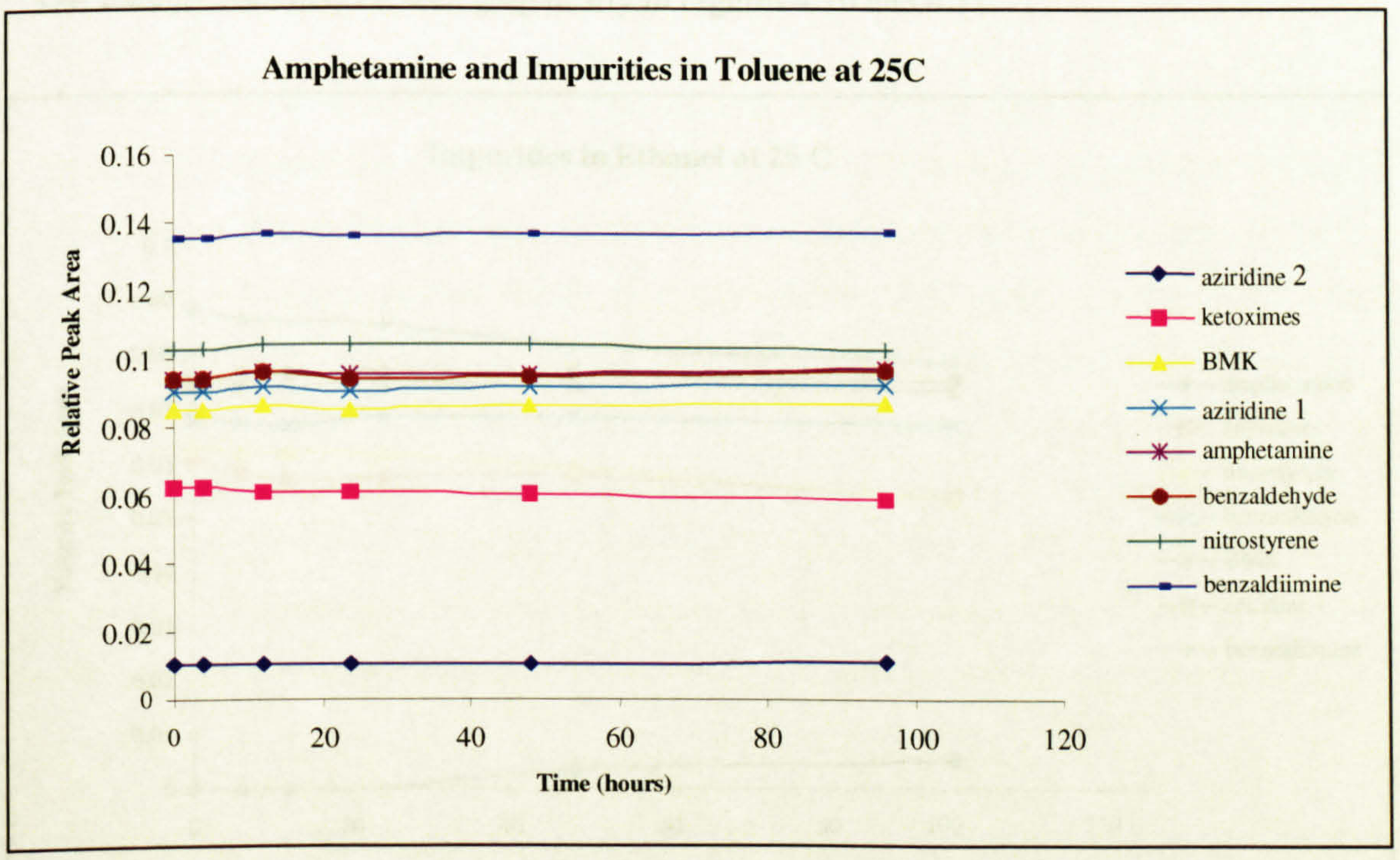


Figure 4.9 Stability of Impurities With Amphetamine in Toluene at 25°C

Ethanol

This solvent was not used in the section of the experiment using a mixture of amphetamine and impurities or as an extraction solvent. In the experiment at 25 °C, a peak with the same retention time as amphetamine appeared in the chromatographs immediately during the first analysis and the concentration increased steadily over the experimental period of 96 hours. Conversely, the concentration of benzaldimine, ketoxime and nitrostyrene decreased through time. The proportions of benzaldehyde, BMK and aziridines remained constant throughout the experimental period.

The results for the ethanol extracts at 8 °C were variable with the levels of impurities fluctuating in each injection, particularly at the 60 hour timepoint. This may have been due to changes in the system or problems with the injection. However, the presence of the internal standard should compensate for injection volume variation. This, combined with the changes in the levels of selected impurities over time meant that ethanol was not thought to be a suitable solvent for the extractions of amphetamine and impurities.

The stability data may be seen graphically in Figures 4.10 and 4.11.

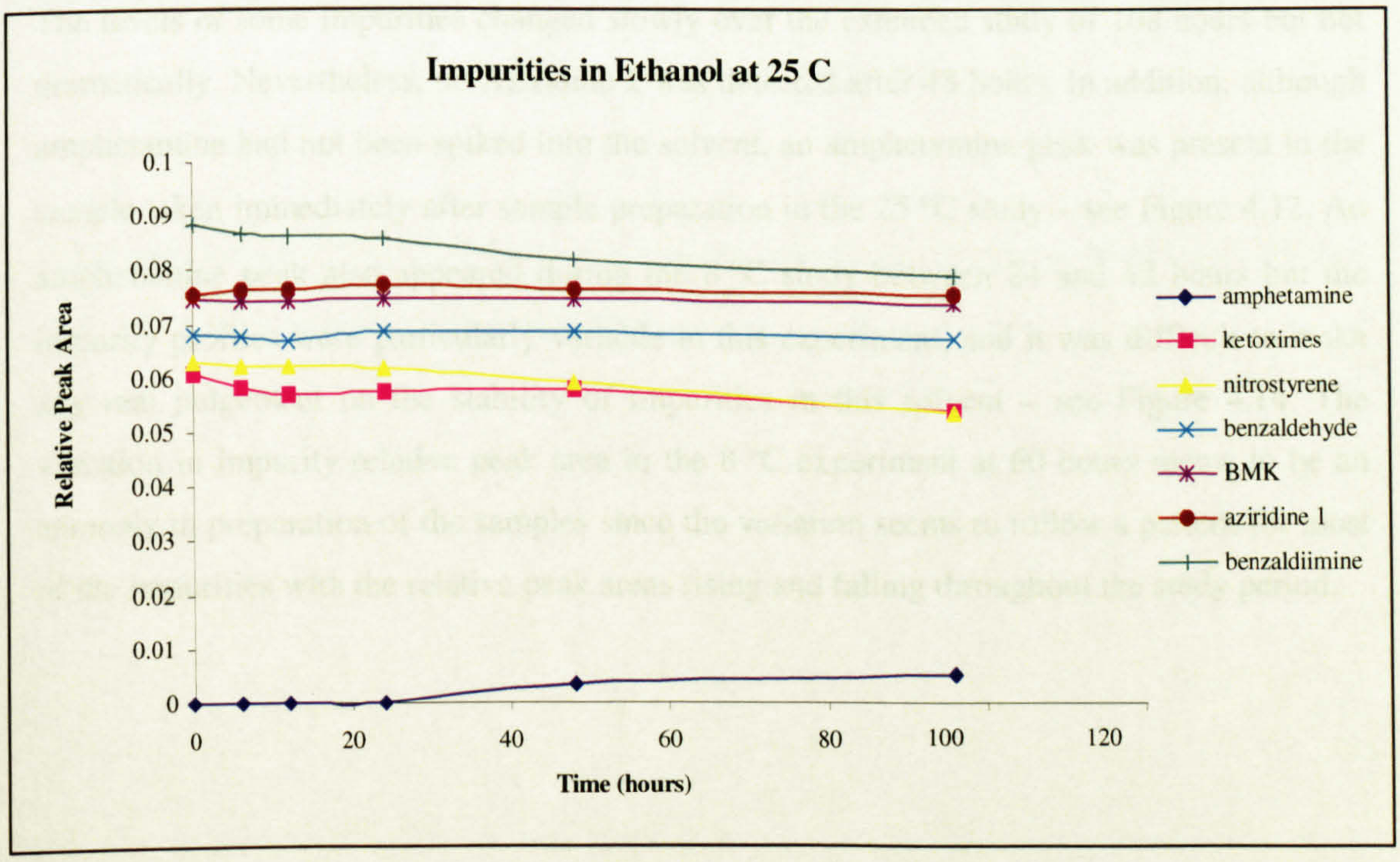


Figure 4.10 Stability of Impurities in Ethanol at 25°C

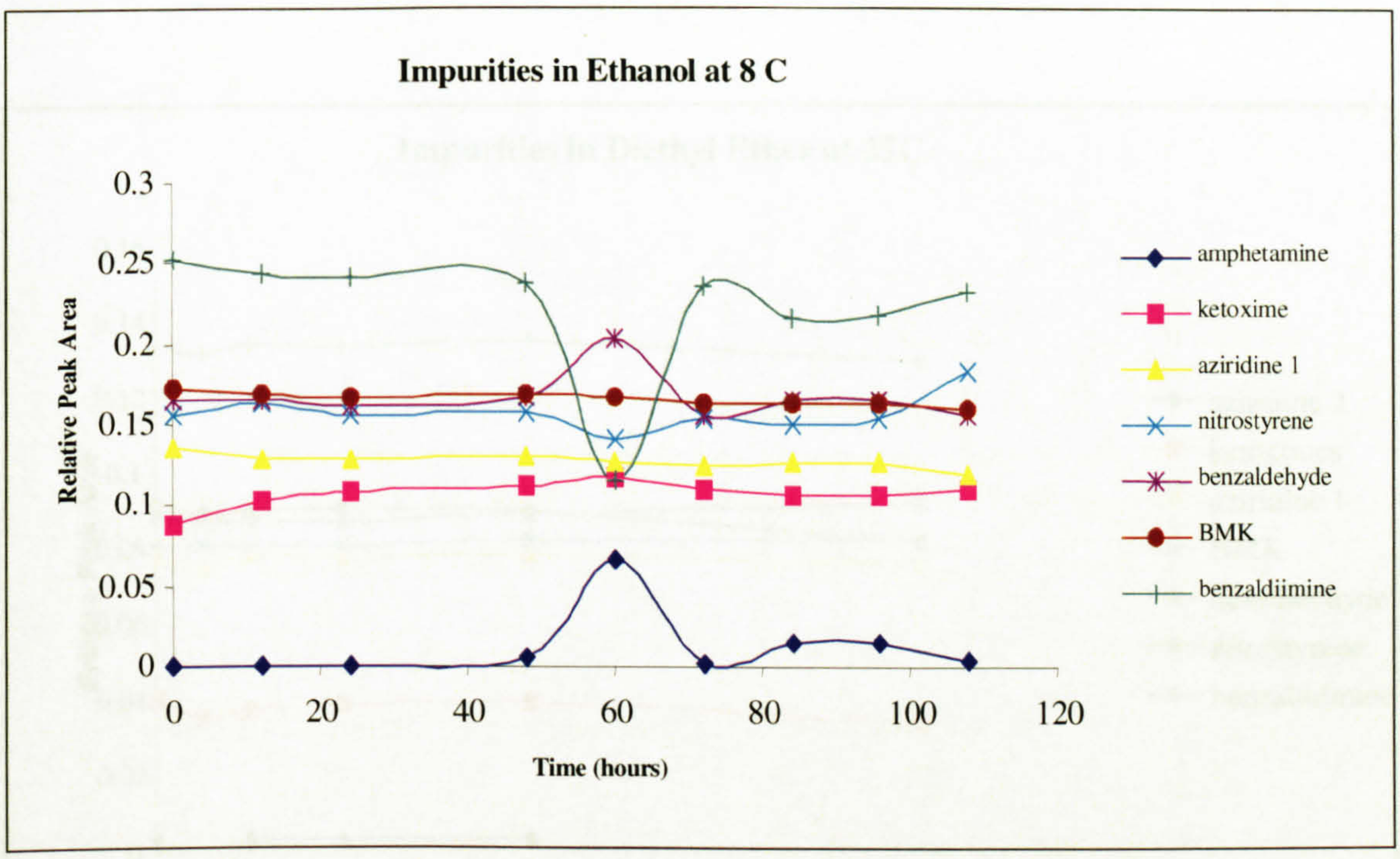


Figure 4.11. Stability of Impurities in Ethanol at 8°C

Diethyl Ether

The levels of some impurities changed slowly over the extended study of 108 hours but not dramatically. Nevertheless, no Aziridine 2 was detected after 48 hours. In addition, although amphetamine had not been spiked into the solvent, an amphetamine peak was present in the sample taken immediately after sample preparation in the 25 °C study – see Figure 4.12. An amphetamine peak also appeared during the 8 °C study between 24 and 48 hours but the impurity profiles were particularly variable in this experiment, and it was difficult to make any real judgement on the stability of impurities in this solvent – see Figure 4.14. The variation in impurity relative peak area in the 8 °C experiment at 60 hours seems to be an anomaly in preparation of the samples since the variation seems to follow a pattern for most of the impurities with the relative peak areas rising and falling throughout the study period.

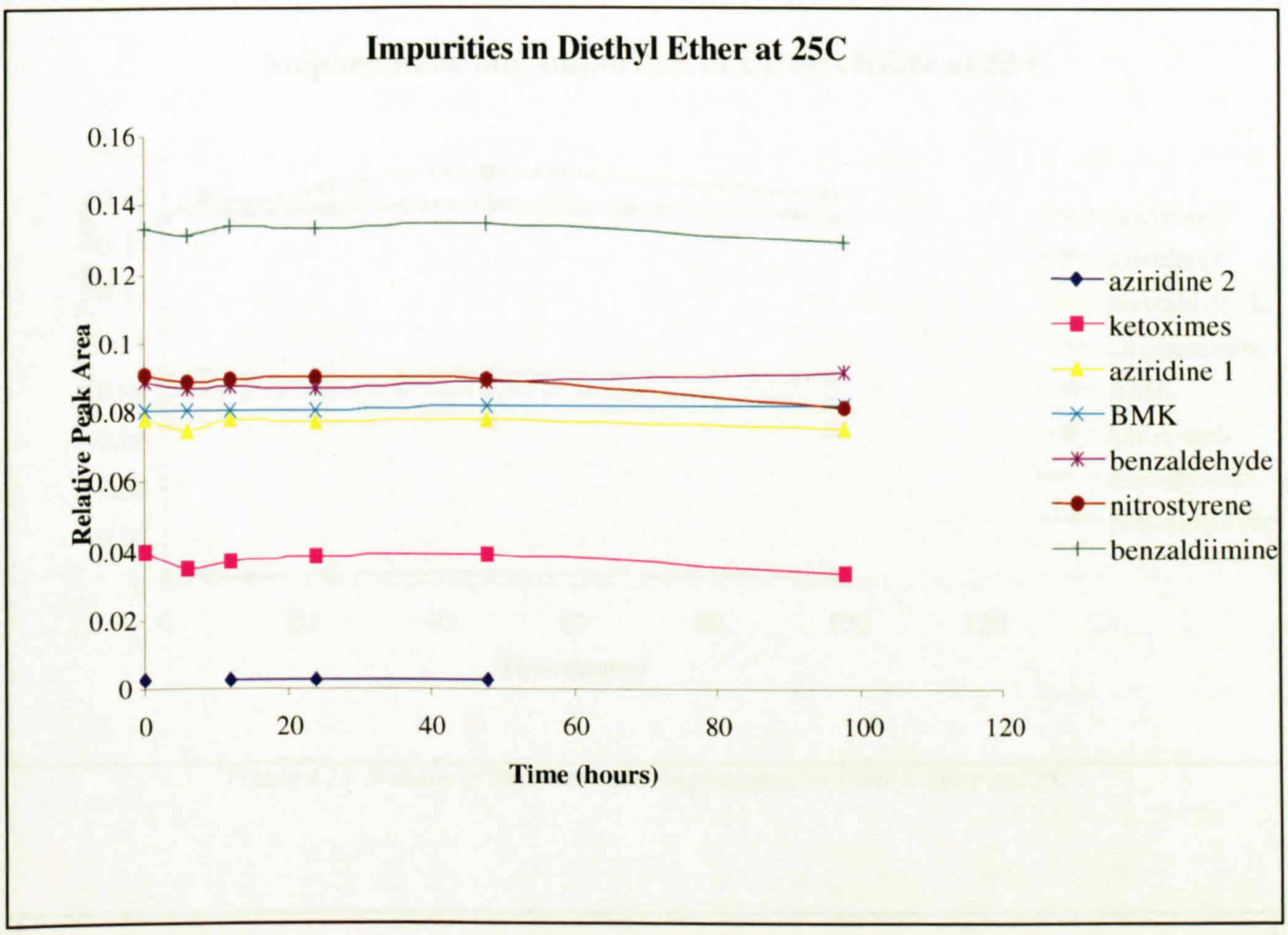


Figure 4.12 Stability of Impurities in Diethyl Ether at 25°C

When amphetamine was included in the original sample, the sample composition remained relatively stable throughout the experiment.

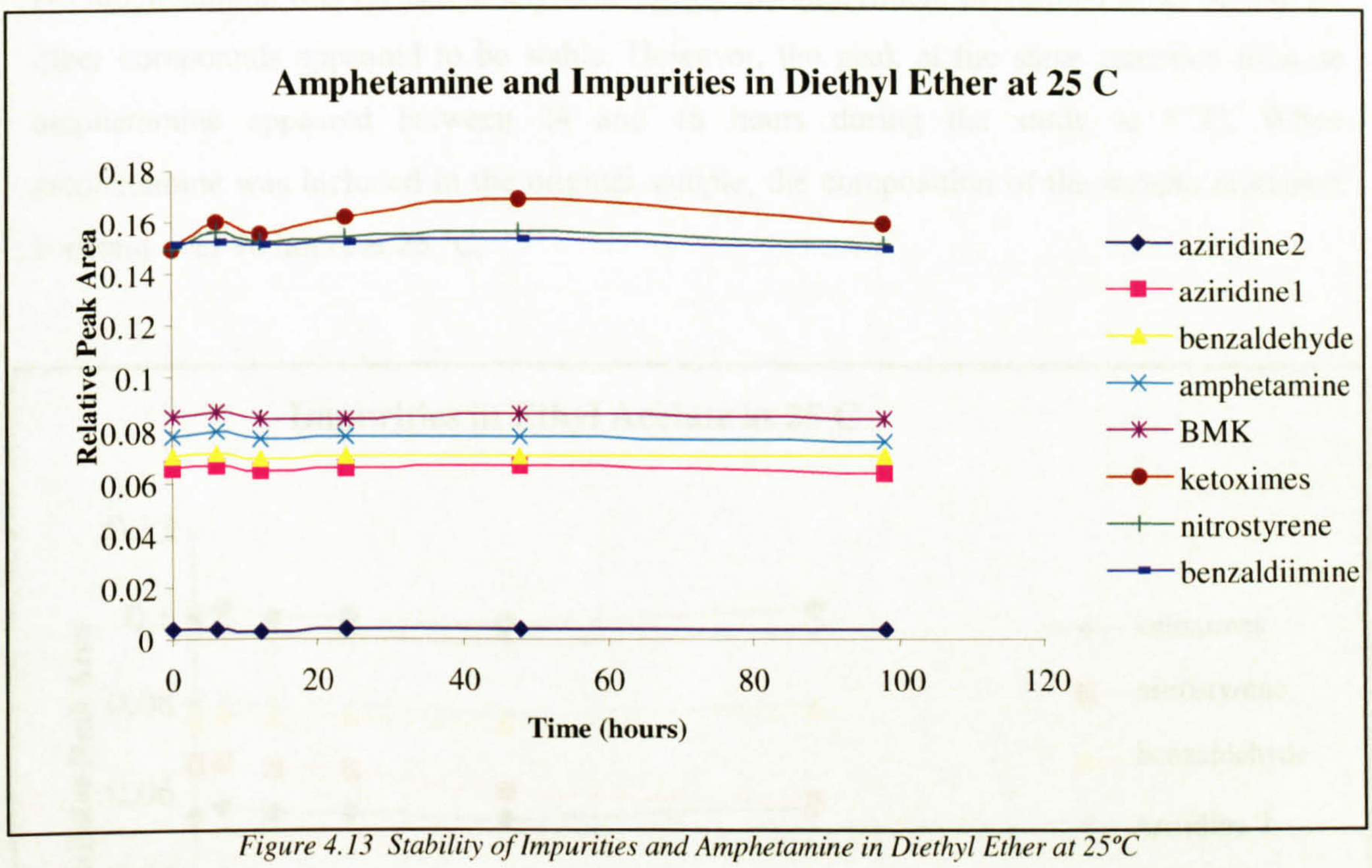


Figure 4.13 Stability of Impurities and Amphetamine in Diethyl Ether at 25°C

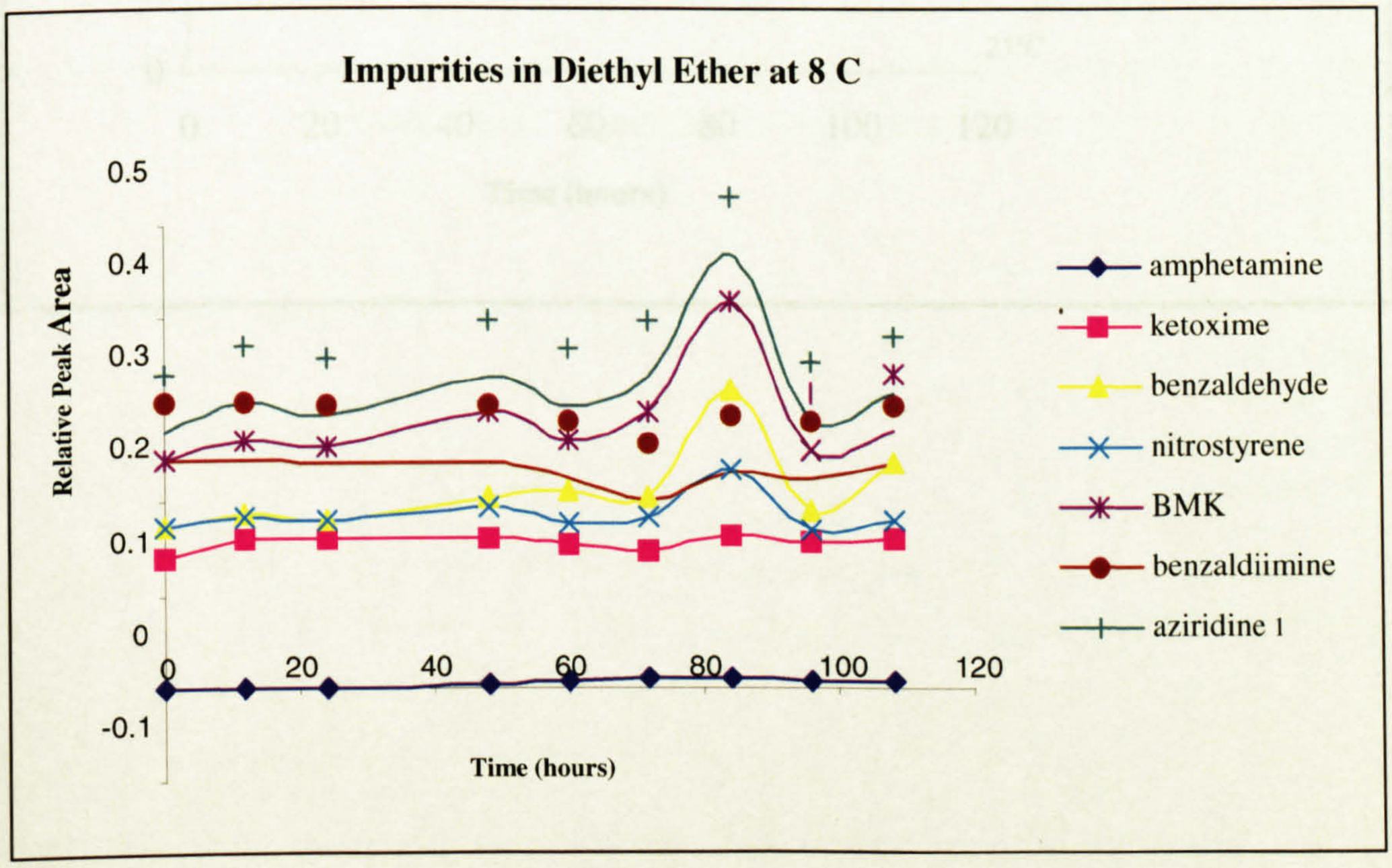
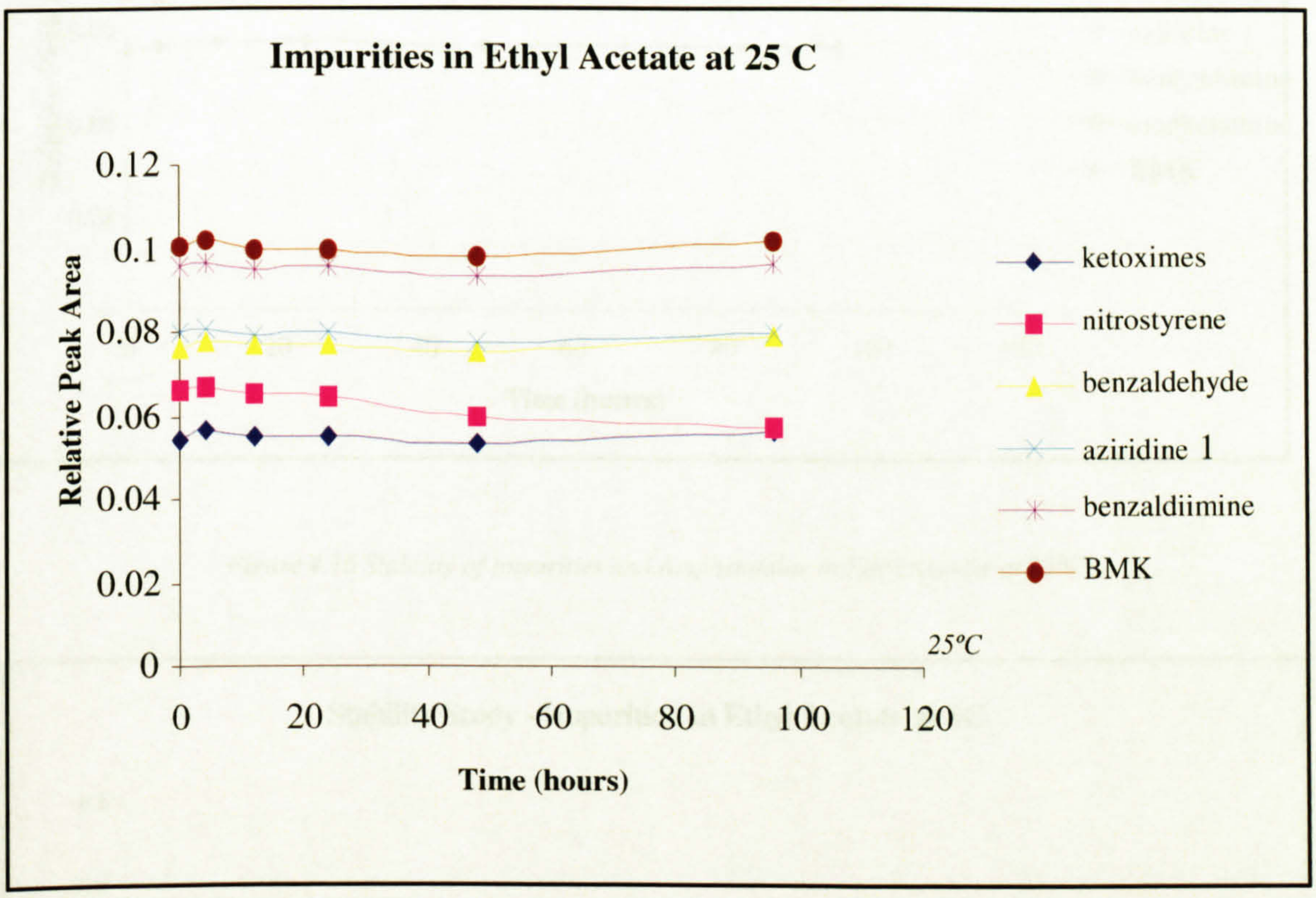


Figure 4.14 Stability of Impurities in Diethyl Ether at 8°

Ethyl Acetate

No amphetamine was formed at any time during the experiment performed at 25 °C and all other compounds appeared to be stable. However, the peak at the same retention time as amphetamine appeared between 24 and 48 hours during the study at 8 °C. When amphetamine was included in the original sample, the composition of the sample remained constant over 96 hours at 25 °C.



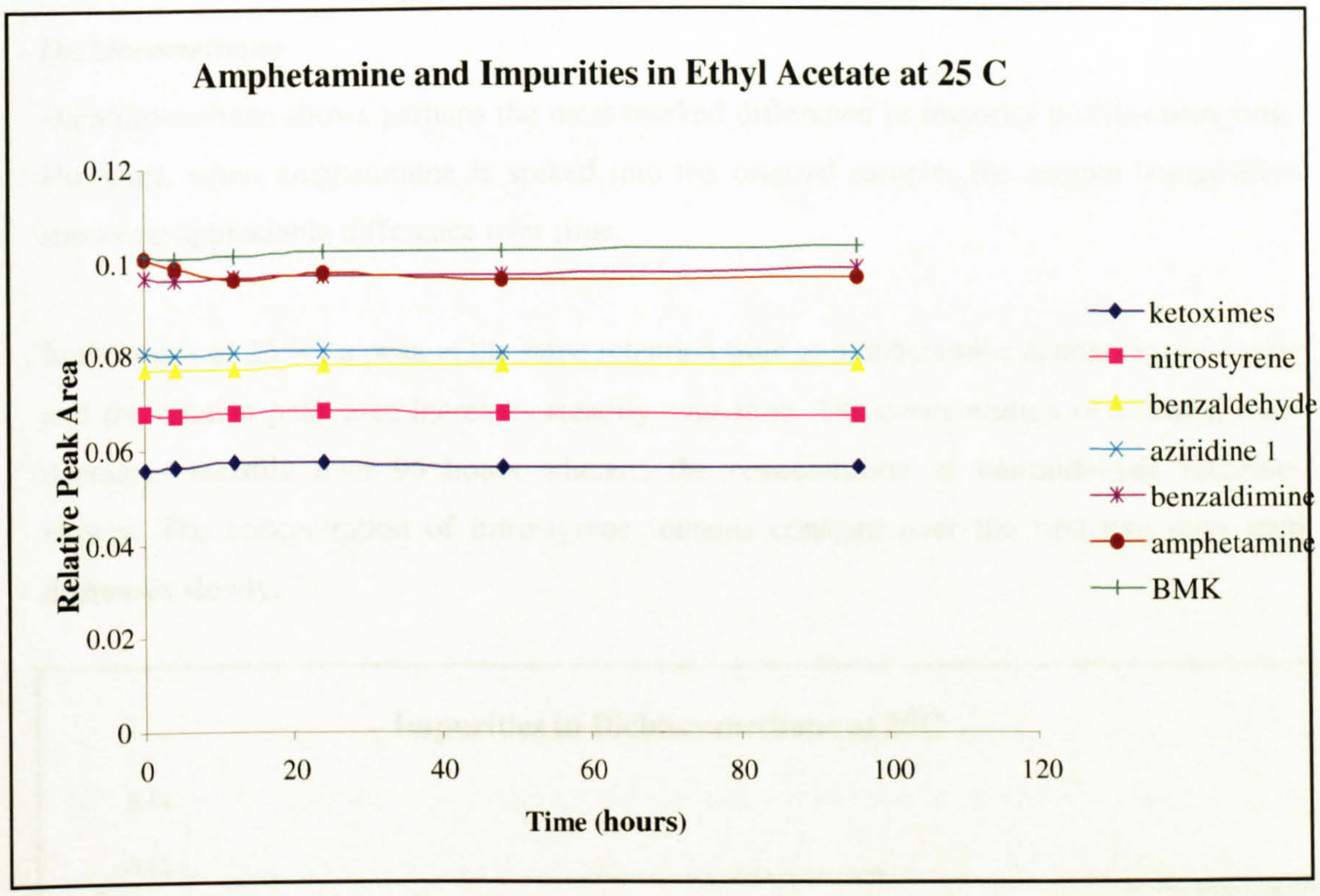


Figure 4.16 Stability of Impurities and Amphetamine in Ethyl Acetate at 25°C

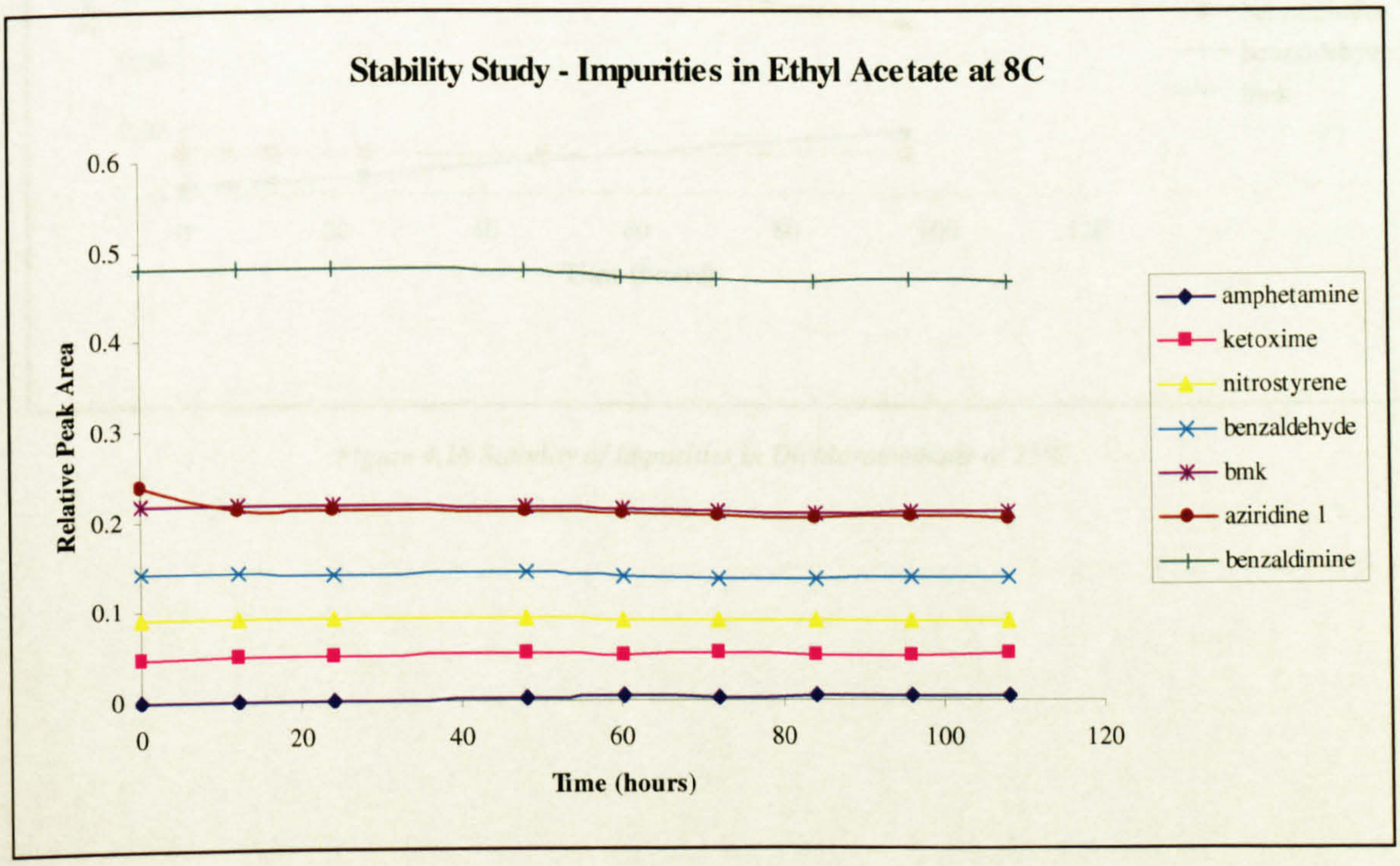


Figure 4.17 Stability of Impurities in Ethyl Acetate at 8°C

Dichloromethane

Dichloromethane shows perhaps the most marked difference in impurity profiles over time. However, when amphetamine is spiked into the original sample, the sample composition shows no appreciable difference over time.

In the study at 25 °C, a peak at the same retention time as amphetamine appears immediately and the relative peak area increases steadily over time. The concentration of benzaldiimine decreases steadily over 96 hours whereas the concentration of benzaldehyde increases slowly. The concentration of nitrostyrene remains constant over the first two days then decreases slowly.

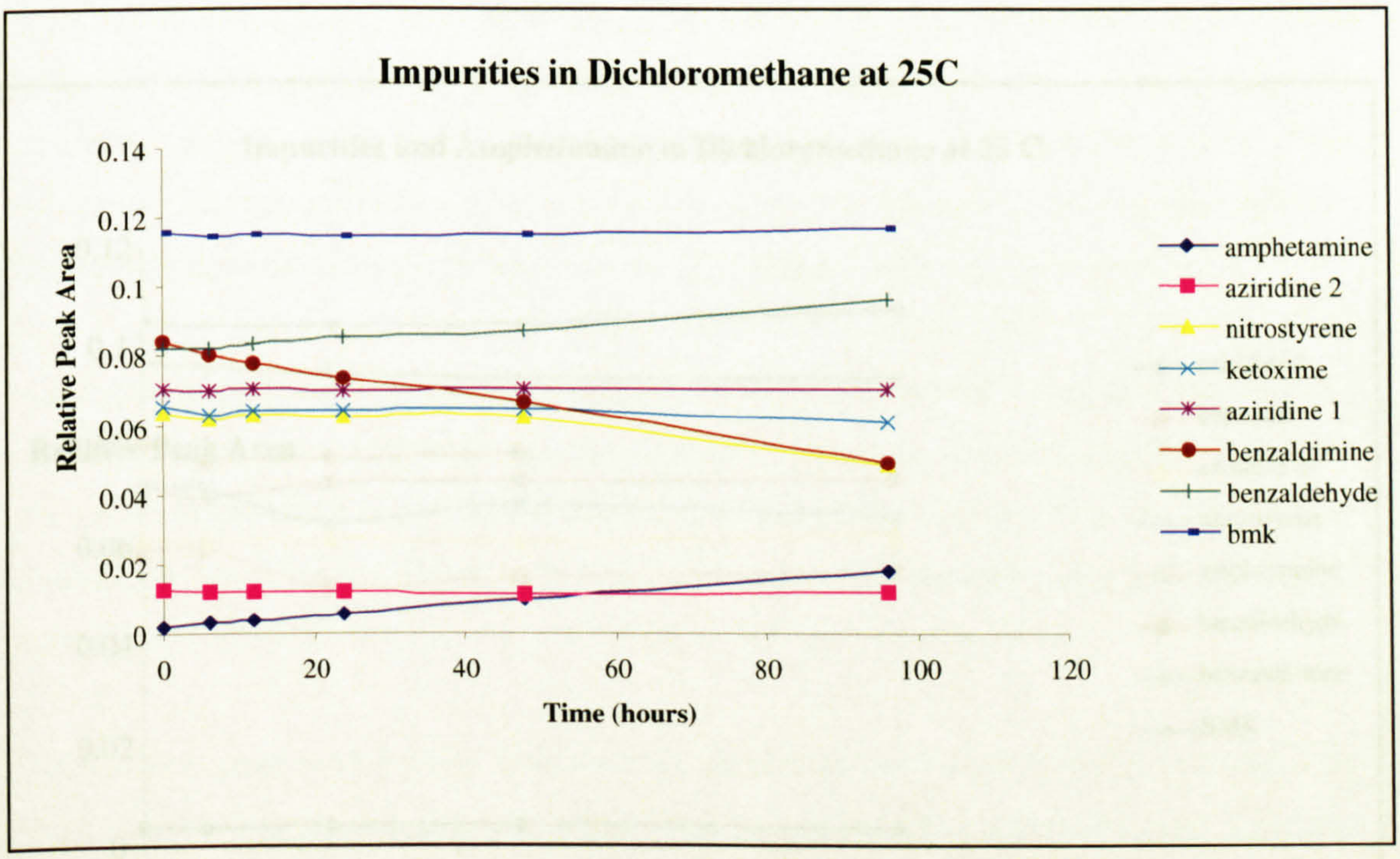


Figure 4.18 Stability of Impurities in Dichloromethane at 25°C

At 8 °C, the concentration of benzaldiimine decreases rapidly within 24 hours. A peak with the same retention time as amphetamine appears to form within 4 hours. Initially, nitrostyrene decreases and ketoximes increase but their concentrations level out over time.

It was thought that since the benzaldiimine was originally formed by the reaction between amphetamine and benzaldehyde (in excess from the starting materials used in the nitrostyrene route), there was a possibility that in the presence of the chlorinated solvent, the benzaldiimine breaks down into amphetamine and benzaldehyde. If this is the case, the composition of the impurity profile of a dichloromethane extract would change significantly over time.

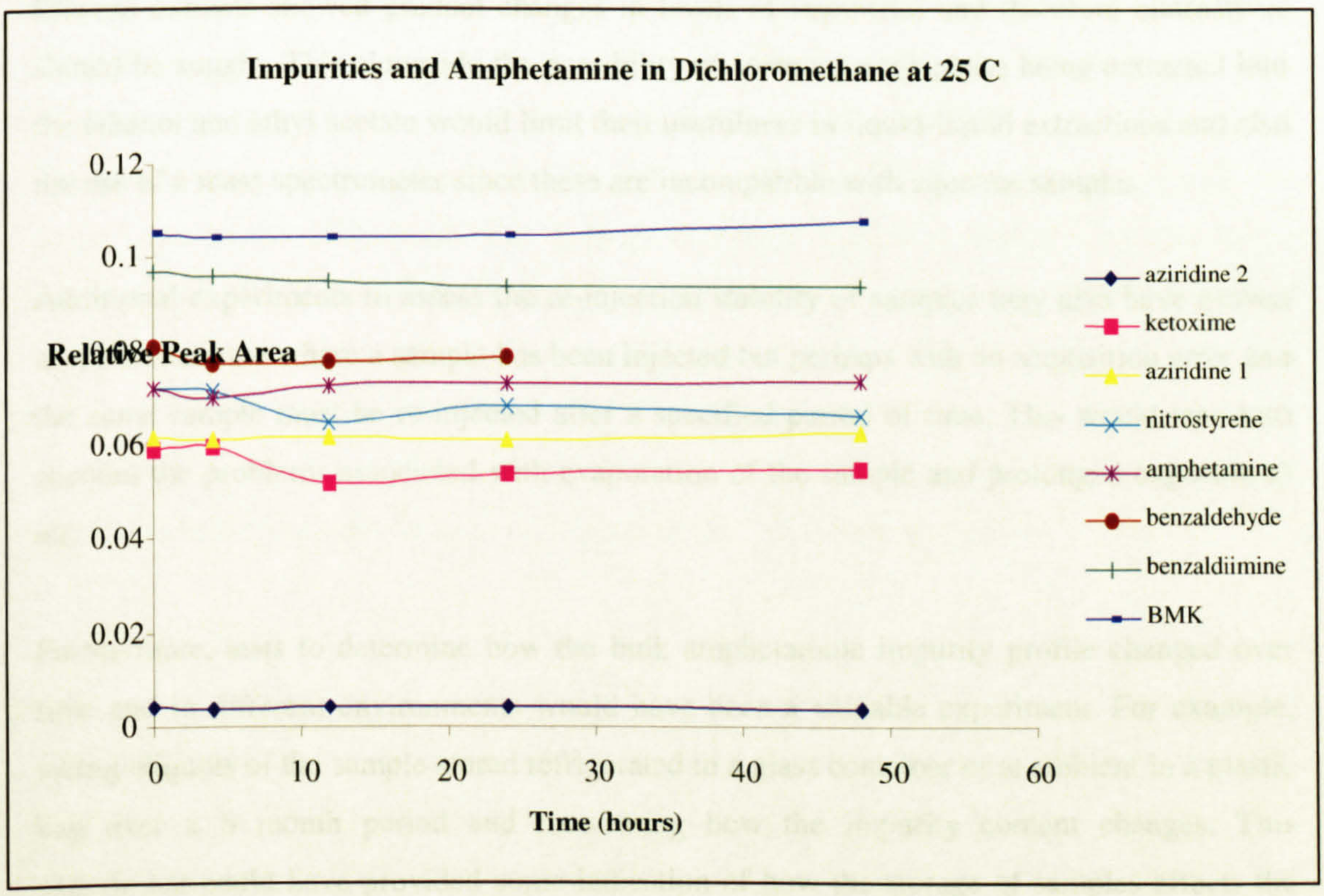


Figure 4.19 Stability of Impurities and Amphetamine in Dichloromethane at 25°C

4.2.3. Conclusions

From the stability study data, if extraction is relatively efficient for solvents such as toluene, iso-octane and ethyl acetate, impurity profiling should be more successful. This would remove any of the doubt as to the possible alteration in the impurity profile caused by a lag time between sample extraction and analysis.

If possible, it would be preferable to avoid the use of dichloromethane entirely as it has shown to change the profile dramatically over time as well as the possibility of restrictions in the use of chlorinated solvents limiting its use in the near future. The use of diethyl ether brings practical problems such as the possibility of sample evaporation and concentration. Ethanol extracts showed gradual changes in levels of impurities and therefore alternatives should be sought. This alongside the possibility of some aqueous phase being extracted into the ethanol and ethyl acetate would limit their usefulness in liquid-liquid extractions and also the use of a mass spectrometer since these are incompatible with aqueous samples.

Additional experiments to assess the re-injection stability of samples may also have proved useful in the case where a sample has been injected but perhaps with an acquisition error and the same sample must be re-injected after a specified period of time. This would take into account the problems associated with evaporation of the sample and prolonged exposure to air.

Furthermore, tests to determine how the bulk amphetamine impurity profile changed over time and in different environments would have been a valuable experiment. For example, taking aliquots of the sample stored refrigerated in a glass container or at ambient in a plastic bag over a 6 month period and monitoring how the impurity content changes. This experiment could have provided some indication of how the storage of samples affects the profile and if changes were significant enough to limit the scope of impurity profiling. However, time constraints did not allow for these additional phases of work.

It must be noted here that these results are for the nitrostyrene amphetamine only and the partner laboratories had to come to a conclusion regarding which solvent would be suitable for all synthesis routes studied in the project.

4.3. Homogeneity Study

4.3.1. Aims

Since the sample to be used in extraction method development had been prepared from 15 separate batches of amphetamine free-base which were to be combined to form a single batch, it was necessary to determine if the entire combined sample was homogeneous. It was thought that if the sample was not sufficiently homogenised, the amphetamine content and impurity profile of aliquots taken from different areas of the sample would be significantly different and therefore any dissimilarity in profiles would not necessarily be attributable to the extraction method.

4.3.2. Homogenising the Sample

The 15 samples prepared via the nitrostyrene route were ground thoroughly in a mortar and pestle and made into a thick slurry by addition of a small amount of diethyl ether. This slurry was then placed in a rotameter for 30 minutes to mix the sample thoroughly. The ether was then allowed to evaporate off and the sample re-ground.

The initial experiment consisted of extraction of aliquots of the combined sample followed by chromatographic analysis and comparison of the five solvent extracts.

4.3.3. Experiment 1

Five 200 mg aliquots were taken randomly from the bulk sample and dissolved in 1 M, pH 7 phosphate buffer, 2 mL. The vial was mechanically shaken for 30 minutes to ensure that the sample was as thoroughly mixed as possible.

Iso-octane, 200 µL (solvent chosen by group since this had been historically used in profiling), containing internal standard $C_{34}H_{70}$ (since this was thought to elute after all analytes of interest) at 10 µg mL^{-1} was added and the combined buffer and solvent were mixed for a further 30 minutes. The top organic phase was removed and placed in a GC vial with glass insert. The extracts were then analysed by GC-MS and GC-FID using the method agreed between the partner laboratories previously.

Results

Problems were encountered using $C_{34}H_{70}$ as the internal standard for the GC analysis of the extracts. This long chain hydrocarbon was found to be unsuitable when using this particular sample matrix. An unidentified impurity eluted at the same retention time as the internal standard making it difficult to integrate using the TIC trace and impossible to identify properly using FID. The decision was then made to prepare a fresh batch of solvent using $C_{20}H_{42}$ (eicosane) as internal standard at the same concentration. However, in order that some data could be gleaned from this initial experiment, the GC-FID data was analysed without comparison relative to the internal standard and the impurity patterns of the chromatograms generated looked very comparable.

In all, 78 integrated peaks were present in all five samples (not that in each of the following experiments only those peaks present in all five sample extracts were included in the statistics and the graphics) and the mean values and relative standard deviation for these peak areas were obtained. Of these, 50 peaks present had an RSD less than 10 % and 30 peaks had RSD less than 5 % based on absolute peak area. Note that no internal standard was used so these results are not compensated for differences in injection volume or detector response.

The mean peak area, standard deviation and RSD for identified peaks are shown in Table 4.2. Again, the ketoxime peak areas were combined to give the total peak area for both isomers.

	Peak Area (mean)	**Standard Deviation**	**RSD**
Amphetamine	22882517	3460623	**15.1**
Aziridine 1	7171661	638329	**8.9**
Ketoxime	8323682	330650	**4.0**
Aziridine 2	332476	32126	**9.7**
Benzaldiimine	7580883	409649	**5.4**
Reduced form of Benzaldiimine	15843523	472377	**2.3**

Table 4.2 Analytical Data: Homogeneity Study Experiment 1

As may be seen from these results, even without the reference to an internal standard and taking into account the possible variation due to the extraction procedure and analysis which was not known at this point, all of the recognised impurity peaks (although not amphetamine) had an RSD less than 15 %. In addition, 30 peaks were present in the extract with an RSD less than 5 % and therefore had no significant difference between their values

across the five aliquots. A tentative conclusion would be that the sample is indeed homogeneous although further experiments with a more suitable internal standard were necessary to confirm this. The areas of peaks present in all five replicates were plotted to give an overall view of the variation of the impurity pattern of the samples. The plot can be seen below. Since the profiles are similar and are not clearly visible when superimposed, the profiles have each been shifted slightly up the y-axis.

As may be seen from Figure 4.20, Sample 5 impurity profile deviates quite significantly from the pattern followed by the others in the latter part of the chromatogram. Excepting this anomaly, the impurity profiles follow a very similar pattern.

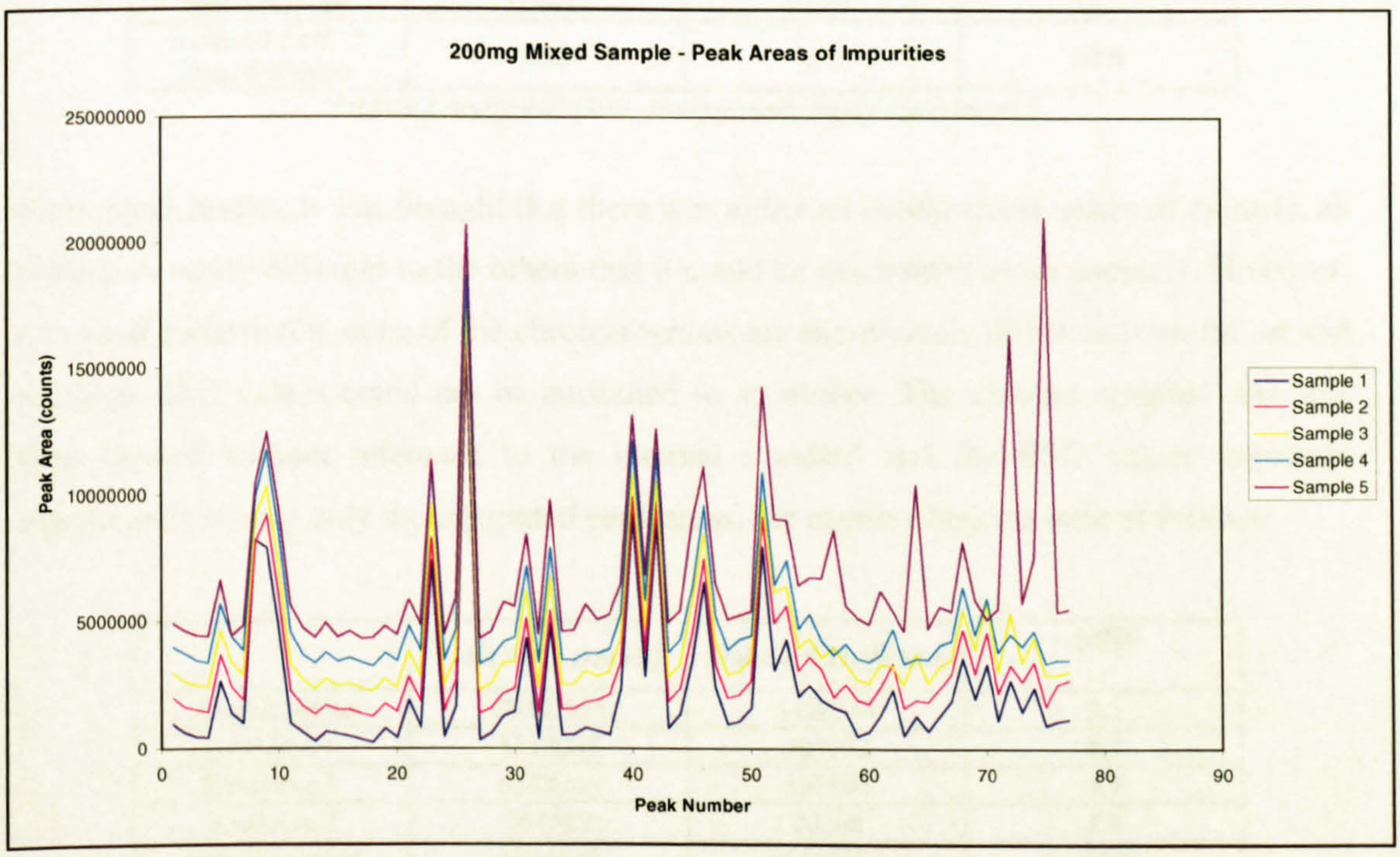


Figure 4.20 Peak Areas of Impurities versus Peak Number: Homogeneity Study Experiment 1

4.3.4. Experiment 2

The same extraction process as in Experiment 1 was repeated on a further five 200 mg aliquots using $C_{20}H_{42}$ as the new internal standard at the same concentration as previously.

Results

$C_{20}H_{42}$ proved to be a better internal standard, eluting in an area of the chromatogram otherwise free of impurity peaks. On an initial visual analysis of the chromatograms, the pattern of impurity peaks appears to be very similar and so, the relative peak areas for the identified impurities in each of the five chromatograms was studied and the results are shown in Table 4.3.

	Relative Area (mean)	**Standard Deviation**	**RSD**
Amphetamine	7.46	4.61	**61.8**
Aziridine 1	0.963	0.639	**66.3**
Ketoxime 1	0.626	0.376	**60.1**
Aziridine 2	0.0926	0.0557	**60.2**
Benzaldiimine	0.442	0.321	**72.7**
Reduced form of Benzaldiimine	3.62	2.19	**60.6**

Table 4.3 Analytical Data: Homogeneity Study Experiment 2

From these results, it was thought that there was either an outlier in the series of extracts, an extract so vastly different to the others that it could be discounted as an anomaly. However, on visual comparison, none of the chromatograms are significantly different from the set and the high RSD values could not be attributed to an outlier. The chromatographic data was then viewed without reference to the internal standard and the RSD values improved significantly. Using only the integrated peak areas, the results obtained were as follows:

	Peak Area (mean)	**Standard Deviation**	**RSD**
Amphetamine	28007555	1458534	**5.2**
Aziridine 1	3513097	304962	**8.7**
Ketoxime 1	6578986	307880	**4.7**
Aziridine 2	351582	20268	**5.8**
Benzaldiimine	1524849	516868	**33.9**
Reduced form of Benzaldiimine	13406564	732441	**5.5**

Table 4.4 Analytical Data: Homogeneity Study Experiment 2

The RSD values obtained for the impurities and amphetamine content were now much more comparable with the results from experiment 1 with all of the identified impurities having an RSD less than 15 % except for benzaldiimine which had already been found in the stability experiment to give particularly variable results. Furthermore, looking at the unidentified impurities in the profile, the overall profile contains 55 peaks present in all five replicates. Of these 10 with an RSD less than 5 %.

The integration data was also treated in the same way as for experiment 1 and a plot of peak area versus peak number was obtained and is shown in Figure 4.21.

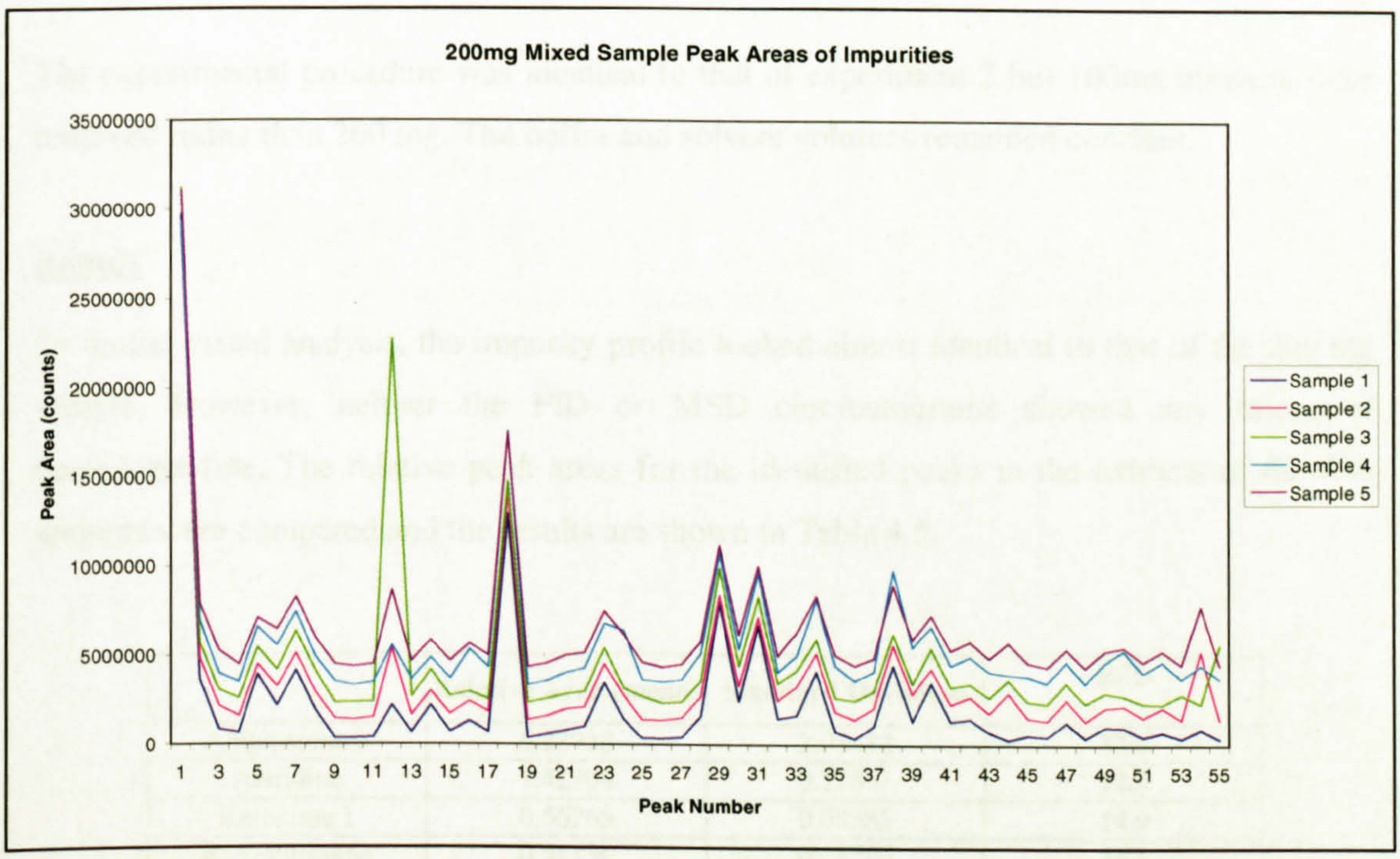


Figure 4.21 Peak Areas of Impurities versus Peak Number: Homogeneity Study Experiment 2

Again, in this plot we see that the five replicate extractions give a very similar impurity profile although Sample 3 profile has an outlying peak 12 which is has a higher response than the rest of the set.

4.3.5. Experiment 3

During both experiments 1 and 2, it was considered that perhaps the variation in extractions could be a consequence of sample solubility problems we were experiencing with the sample in the buffer. Even with 30 minutes of mixing, some of the sample was not completely dissolved. Problems had already been experienced in preparation of the 1M phosphate buffer where the solution was so saturated that crystals precipitated out of solution overnight. Trying to dissolve 200 mg of sample in 2 mL solvent caused a similar problem.

This experiment set out to determine whether reducing the sample weight would improve the reproducibility and therefore allow us to determine whether the small variation in the sample profiles was due to sample inhomogeneity or because of the solubility of the sample in the buffer.

The experimental procedure was identical to that of experiment 2 but 100mg aliquots were removed rather than 200 mg. The buffer and solvent volumes remained constant.

Results

On initial visual analysis, the impurity profile looked almost identical to that of the 200 mg sample. However, neither the FID or MSD chromatograms showed any traces of benzylaziridine. The relative peak areas for the identified peaks in the extracts of the five aliquots were compared and the results are shown in Table 4.5.

	Relative Area (mean)	**Standard Deviation**	**RSD**
Amphetamine	6.22938	2.05442	**33.0**
Aziridine	1.42709	0.17567	**12.3**
Ketoxime 1	0.56265	0.08385	**14.9**
Benzaldiimine	0.51136	0.14599	**28.6**
Reduced form of Benzaldiimine	3.22135	0.09731	**3.0**

Table 4.5 Analytical Data: Homogeneity Study Experiment 3

As may be seen from these results, the RSD values for these impurities are higher than for the 200 mg sample and there is a great deal of variation in the amphetamine and benzaldiimine content. Again, the data was reassessed using only the peak area without calculating the area relative to the internal standard and the results are shown in Table 4.6. Again, the amphetamine and benzaldiimine content is highly variable. The variation in amphetamine content may be explained by the fact that iso-octane was first selected as a solvent because it does not extract amphetamine well and should give a cleaner extract. Since it does not extract amphetamine successfully it may not extract it consistently either.

	Peak Area (mean)	Standard Deviation	RSD
Amphetamine	10217577	3621343	**35.4**
Aziridine	2318250	253742	**11.0**
Ketoxime 1	2092469	187552	**9.0**
Benzaldiimine	838019	288972	**34.5**
Reduced form of Benzaldimine	5248713	327112	**6.2**

Table 4.6 Analytical Data: Homogeneity Study Experiment 3

Plotting the areas of the peaks present in all five replicates, however, the pattern is relatively similar as can be seen in Figure 4.22.

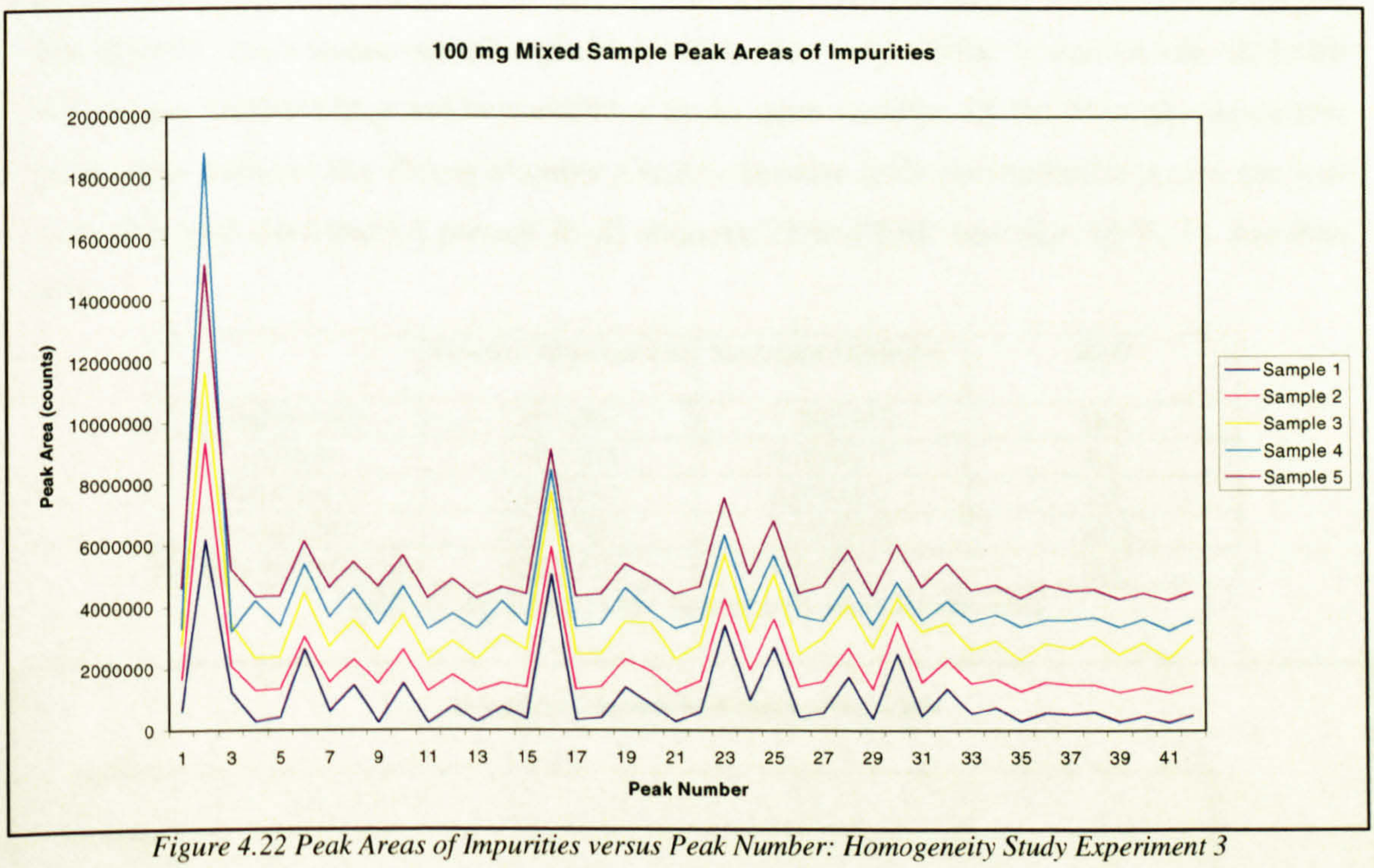


Figure 4.22 Peak Areas of Impurities versus Peak Number: Homogeneity Study Experiment 3

Without using the internal standard, of the 42 peaks present (less peaks present in all sample extracts possibly because some peaks are not too small to be detected consistently) in all aliquots, 14 have RSD less than 10 % but none less than 5 %. Relative to the internal standard, 16 peaks have an RSD less than 10 % with 7 having an RSD less than 5 %.

Considering that the impurity levels are more variable and the benzylaziridine had disappeared, it was thought that the 200 mg sample would still be more appropriate than the 100 mg aliquot. However, from this data it was difficult so say whether the sample was homogeneous or not.

4.3.6. Experiment 4

Although the 100 mg sample did not seem to give a definitive conclusion as to the homogeneity of the sample, 50 mg samples were then tried to determine if a smaller sample might give a more reproducible result.

Results

In this study, one of the samples appeared to have no impurities present so this was rejected as an outlier (possibly a poor extraction or a problem with the injection). The remainder of the aliquots gave chromatographic profiles which were very similar as can be seen in Table 4.7. Again, amphetamine and benzaldimine peaks were variable. Of the 54 peaks (again less peaks than found in the 200mg aliquots possibly because their concentration is now too low to be detected consistently) present in all aliquots, 25 had RSD less than 10 %, 11 less than 5 %.

	Relative Area (mean)	**Standard Deviation**	**RSD**
Amphetamine	7.8156364	1.2922446	**16.5**
Aziridine	1.5866841	0.1008453	**6.4**
Ketoxime 1	2.1442912	0.0534431	**2.5**
Benzaldimine	0.8311686	0.2109412	**25.4**
Reduced benzaldimine	5.2633515	0.1645575	**3.1**

Table 4.7 Analytical Data: Homogeneity Study Experiment 4

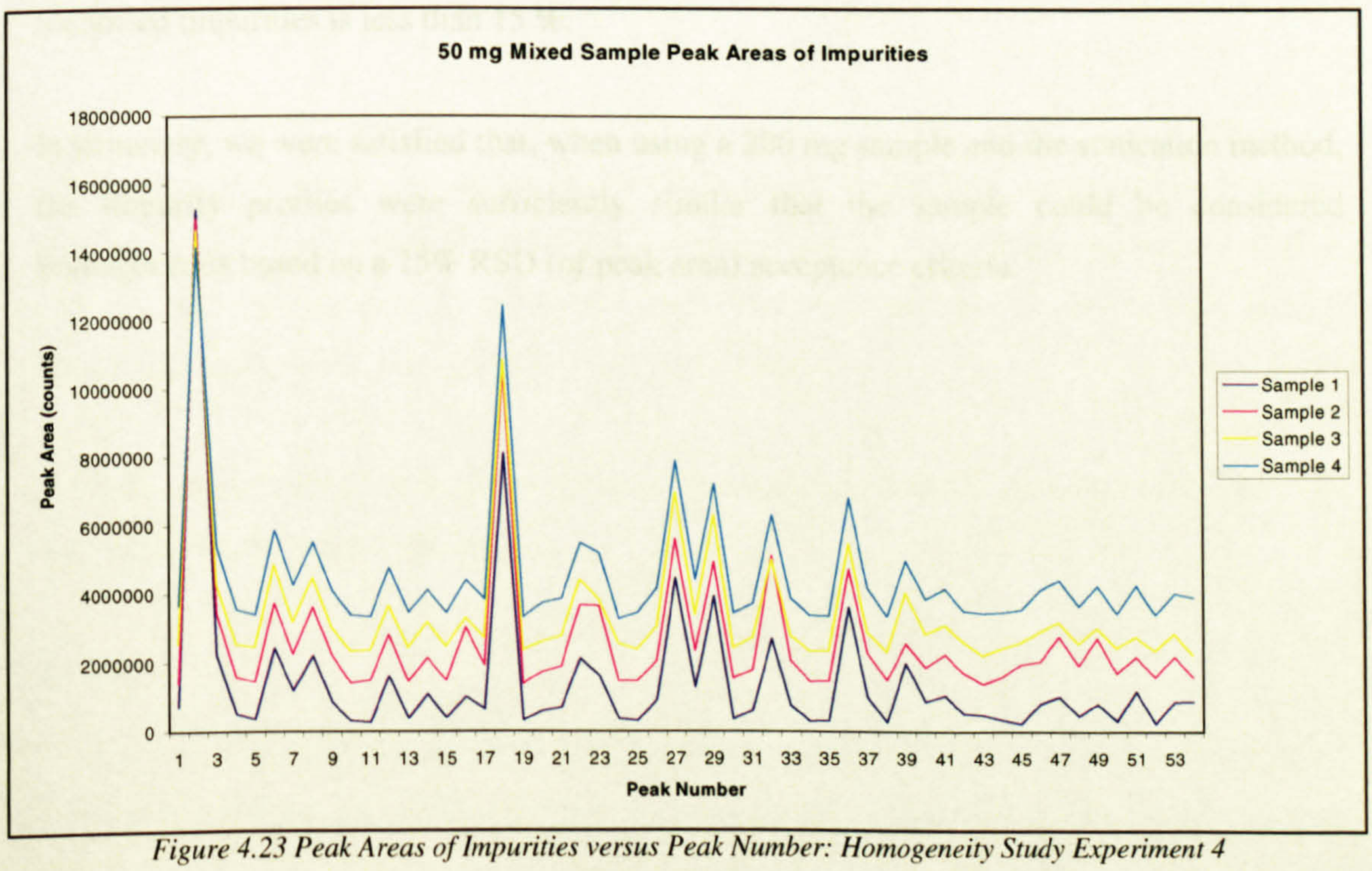


Figure 4.23 Peak Areas of Impurities versus Peak Number: Homogeneity Study Experiment 4

4.3.7. Experiment 5

Instead of shaking the sample vials, the sample was placed in a sonic bath for 30 minutes before and after the organic solvent was added. Five 200 mg aliquots were again used in this experiment.

Results

Of the 103 peaks present in all aliquots (increase in peaks present in all extracts possibly due to the sonic bath giving more consistent mixing and extraction), 33 have an RSD less than 10 %, 8 peaks with RSD less than 5 %.

	Peak Area (mean)	**Standard Deviation**	**RSD**
Amphetamine	25565314	957270	**3.7**
Aziridine	18285762	534653	**2.9**
Ketoxime	6036341	247164	**4.1**
Benzaldiimine	15281001	1815795	**11.9**
Reduced form of Benzaldiimine	19873898	836275	**4.2**

Table 4.8 Analytical Data: Homogeneity Study Experiment 5

From the above results it can be seen that, compared to the mixed sample, the sonicated sample had less variation in the amphetamine and benzaldiimine content and the RSD of all identified impurities is less than 15 %.

In summary, we were satisfied that, when using a 200 mg sample and the sonication method, the impurity profiles were sufficiently similar that the sample could be considered homogeneous based on a 15% RSD (of peak area) acceptance criteria.

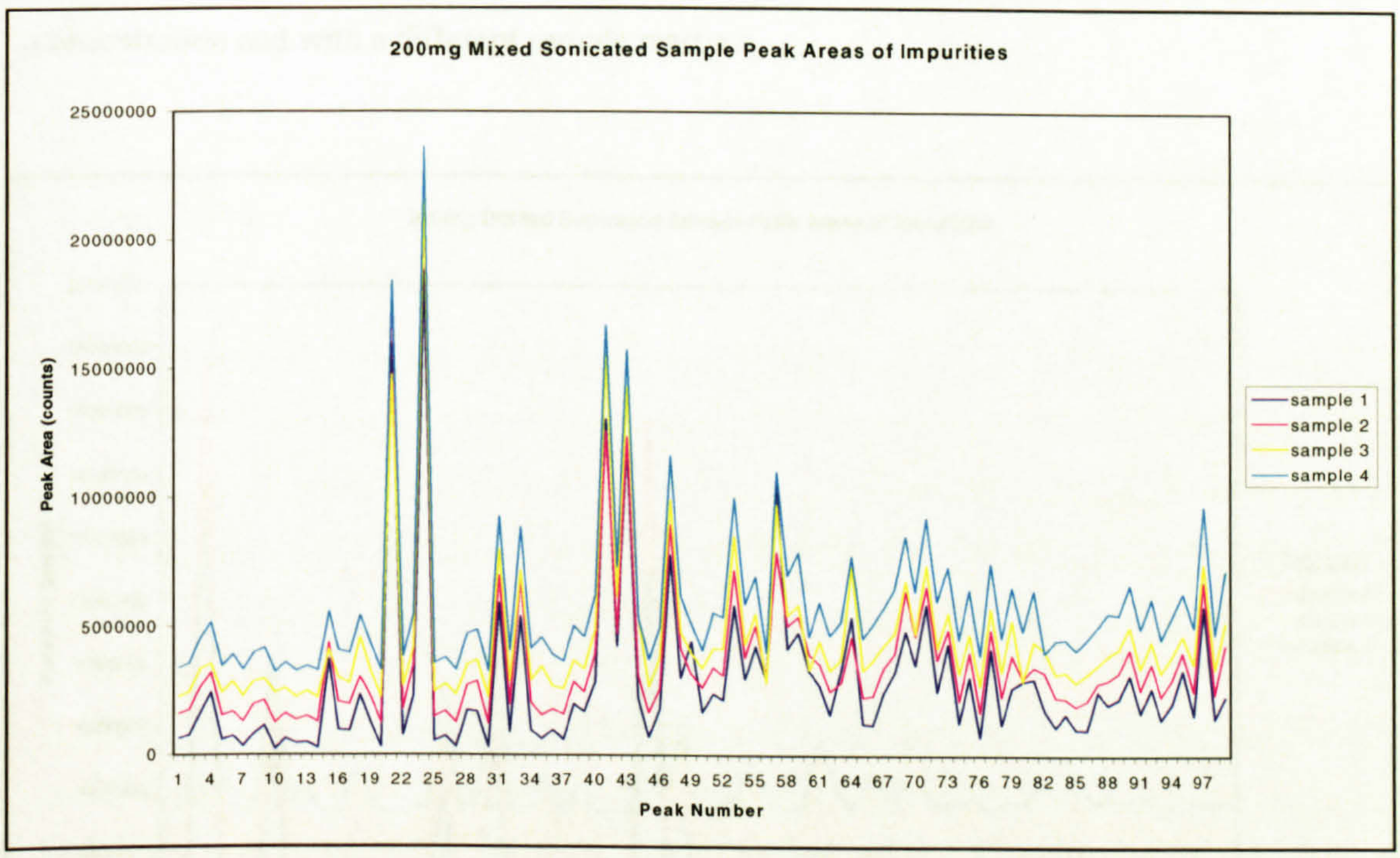


Figure 4.24 Peak Areas of Impurities versus Peak Number: Homogeneity Study Experiment 5

4.3.8. Experiment 6

When we were satisfied that the combined amphetamine sample was homogeneous, a sample of this was taken and a bulked matrix created containing 20% combined amphetamine, 40% caffeine and 40% lactose to mimic a 'street' sample. The diluted sample was ground thoroughly to try to homogenise it as much as possible.

Five 200 mg aliquots of this diluted sample were removed and the same procedure as in the previous experiments was followed.

Results

From the relative peak areas of the identified impurities it may be seen that the sample, even at 1/5 of the concentration still appears to be homogeneous. The RSD values for the impurity peaks are all below 15%. Of the 63 impurity peaks present (less peaks detected possibly due to the lower concentration of impurities in the extract – some not detected consisteltly at

20% of previous concentration) in all replicates, 33 peaks have an RSD less than 10% and 18 peaks with RSD less than 5%. Again, looking at the results graphically, it is clear that the impurity profile for each of the replicate aliquots is very similar, even at this lower concentration and with a different sample matrix.

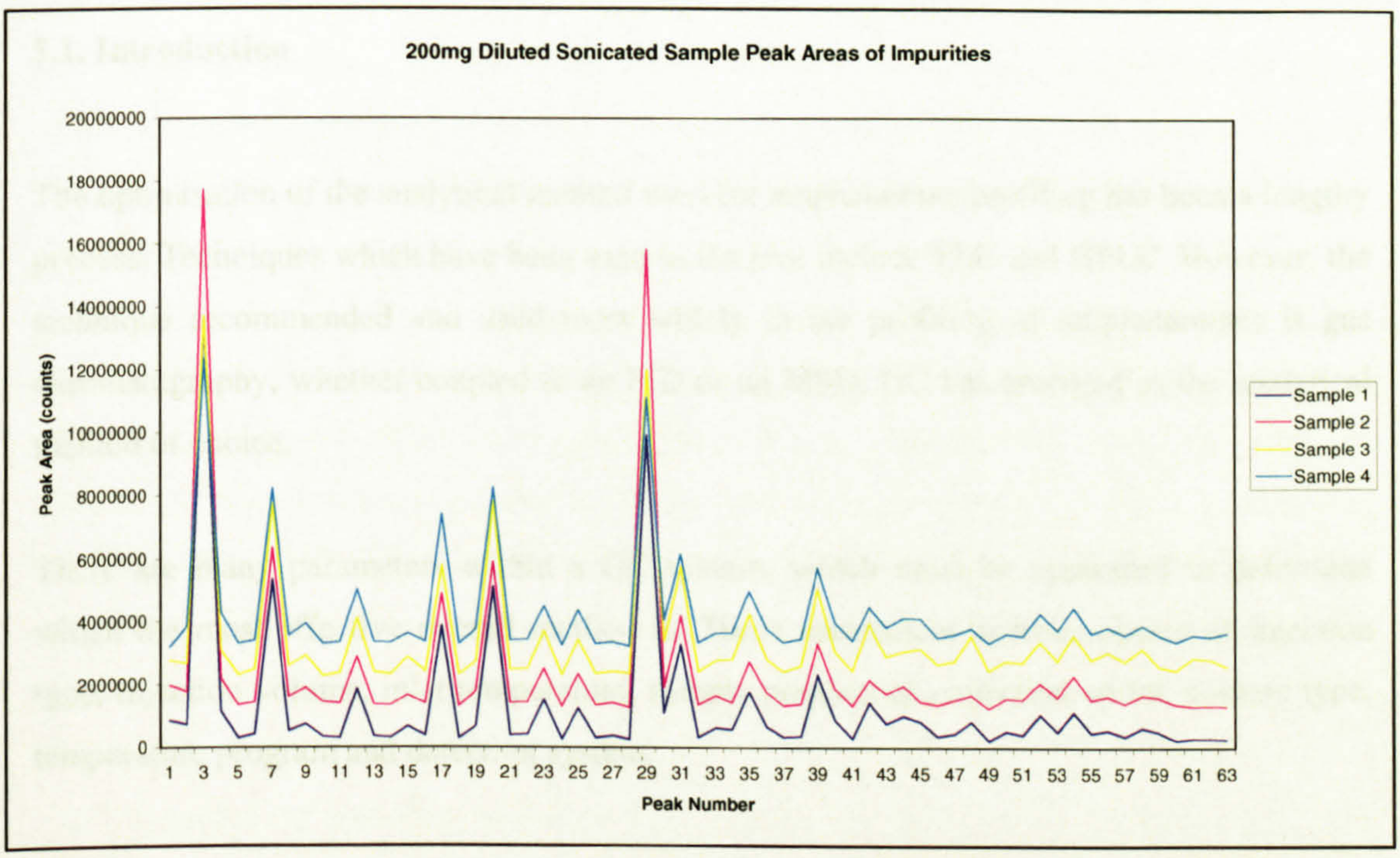


Figure 4.25 Peak Areas of Impurities versus Peak Number: Homogeneity Study Experiment 6

	Relative Area (mean)	**Standard Deviation**	**RSD**
Amphetamine	6.6482	1.9167	**28.8**
Aziridine	2.7654	0.1286	**4.7**
Ketoxime	0.2844	0.0184	**6.5**
Benzaldimine	2.0331	0.1641	**8.1**
Reduced Benzaldimine	2.6894	0.0886	**3.3**

Table 4.9 Analytical Data: Homogeneity Study Experiment 6

Summary

From the combined results of all the mini-experiments, it was shown that the combined sample was homogeneous and that this sample was suitable to continue to use both in the concentrated and dilute form in the method development of the extraction procedure

Chapter 5

GC-MS System Optimisation

5.1. Introduction

The optimisation of the analytical method used for amphetamine profiling has been a lengthy process. Techniques which have been used in the past include TLC and HPLC. However, the technique recommended and used most widely in the profiling of amphetamines is gas chromatography, whether coupled to an FID or an MSD, GC has emerged as the analytical method of choice.

There are many parameters within a GC system, which must be optimised to determine which the most effective overall method is. These parameters include, choice of injection type, injection volume, inlet temperature, sample preparation, injection speed, column type, temperature program and detection system.

The GC system must be optimised for a specific sample type since different analytes and different sample compositions would be more effectively analysed using different operating parameters. Therefore, in order to determine the best system for amphetamine profiling, the samples tested must be representative of the type of sample one would expect to see in a 'street' sample.

To this end, the samples tested in this particular experimental section were prepared in such a way as to represent a typical amphetamine impurity extraction. Synthesised impurities were dissolved in an extraction solvent at levels that might be expected in a 'street' sample. However, since samples prepared via the nitrostyrene route of synthesis are rarely encountered in the UK, no 'street' samples were available to us.

5.1.1. Injection Port Temperature

The choice of injection port temperature is of fundamental importance in GC analysis since the analysis relies on successful rapid volatilisation of analytes so that no loss of efficiency results from the injection technique. However, the temperature must be low enough to ensure that no sample decomposition occurs and no chemical reactions are induced in the port. As a rule, the injection port is generally kept at approximately 50°C higher than the boiling points of the analytes. In profiling, however, the analyte composition is initially unknown so the optimisation of the injection port temperature is, at this stage, an estimation of the most effective setting.

The analytes in these experiments were a synthetic mixture of amphetamine and impurities that may be present in an illicit sample of nitrostyrene-synthesised amphetamine. The solvent used to prepare the sample was iso-octane, which was currently in use in operational profiling laboratories in Scandinavia. Synthetic impurities were dissolved, along with amphetamine free-base at a concentration of approximately 10µg/mL and after preparation were stored at 4°C until analysis.

The variability of injection technique was studied for peak areas obtained using split injection and splitless injections at 220, 240, 260 and 280°C. An otherwise identical oven temperature programme was used in each case. Three replicate injections were performed at each setting. From this, the relative standard deviation (RSD) was also obtained for each impurity using each injection technique at each of the inlet temperatures. If a large RSD value was obtained, this would suggest a change in sample composition within the three replicates at this injection temperature. Obviously, any change in the profile of the sample due to the injection technique is unacceptable and therefore, this particular injection temperature and method would have to be omitted from further experiments.

5.1.2. Columns

The choice of column type and temperature program in combination is perhaps the most fundamentally important choice of all in the optimisation of a GC system.

In this study, the volatility and polarity of the compounds in our 'extract' were already established since our matrix was a synthetic one and our target compounds known. This

would allow us, in principle, to choose the best system with respect to those target compounds. However, the analytes to be studied in each of the three routes in the study cover a wide range of possible polarity and volatility. Therefore, the columns chosen for inclusion in the optimisation procedure reflect this and include columns suitable for a wide range of organic impurities. The evaluation of all columns available on the market would obviously be more comprehensive but not realistically practical.

5.1.3. Temperature Programs

The use of a temperature program instead of an isothermal method of separation was decided early in this part of the project since the use of programmed temperature gas chromatography (PTGC) allows the separation of analytes with a wider range of boiling points. Since the components present in an illicit amphetamine sample are, for the most part, unknown to the analyst beforehand, the use of an isothermal system would cause substantial difficulties in determining which temperature is best for every possible combination of analytes.

Increasing the temperature throughout the chromatographic analysis has the effect of decreasing the partition coefficients of analytes which are still on the column which in turn enables them to move faster through the column allowing a shorter run time. As the temperature increases, the vapour pressure of the compound increases logarithmically. This results in an increase in the relative amount of the compound in the vapour phase moving through the column. The retention time decrease and separation is achieved in a faster time.

In addition, for a homologous series, the retention times of compounds in the series are logarithmic whereas in PTGC they are almost linear. This allows better separation of compounds with lower vapour pressures and allows a faster separation of compounds with higher vapour pressures, which would otherwise remain in the stationary phase. Also, since peak widths rarely increase in PTGC, the later eluting compounds will have less peak broadening, higher peak height above 'noise' and therefore a lower detection limit. The peak widths for each component in a single analysis are, in general, equal simply because of the fact that they spend almost the same amount of time actually partitioning down the column.

However, the choice of temperature program in GC is always going to be a compromise between resolution, good peak shape and purity and practical considerations. Although, for example, an extremely slow ramp rate might give better resolution, the practicalities of a 3

hour GC analysis time cannot be overlooked. In a busy operational lab with a high sample throughput, the analysis time should be kept to a minimum while losing the minimum separation power of the system.

5.2. Experimental Design

5.2.1. Optimisation of Injection Port Temperature

Two sample types were used in this section of the optimisation of the GC system. One sample type was a standard mixture of synthetic impurities (ketoximes, benzaldiimine, aziridines, benzaldehyde and nitrostyrene) at 10µg/mL in iso-octane. The second sample type was a 'mock extract' of these impurities that may be found in a batch of amphetamine synthesised by the nitrostyrene route dissolved in iso-octane. This sample type also includes amphetamine free-base and the impurities at a higher concentration level than in the standard mixture.

Injection port temperatures were set at 220, 240, 260 or 280°C.

Injection was either split or splitless. A 4µL injection was split 1:20 and a split liner (containing deactivated glass wool) was used. In splitless injection mode, a 2µL injection volume was used with a splitless liner.

The temperature program was initialised at 60°C, held for 1 minute and then ramped at 10°C per minute to 300°C then held for 15 minutes at this final temperature. The total run time was 40 minutes.

The column in use was a 25m long Hewlett Packard Ultra 1, 0.2µm ID column with FID and MSD detection.

The chromatograms obtained from the FID detector were analysed using Chemstation software. The FID data was used in preference to the MSD data since, historically FID is known to have a larger linear response range. Relative peak areas were obtained using this

software but integration was also checked manually in the case of ketoxime isomers which the software has a tendency to integrate incorrectly.

For each peak, in the case of the standard mixture of impurities, the peak area was measured relative to an internal standard and the relative standard deviation (RSD) for the triplicate injections was obtained. In the case of the 'extract' sample, the RSD values for the largest peaks (by area) were also obtained.

5.2.2 Choice of Capillary Column and Oven Temperature Program

The columns chosen were commercially available at the time of the initiation of the project and were purchased in 50m lengths. The columns were then cut in half and rewound around a second cage. This ensured that each 'pair' of columns had exactly the same specifications since inevitably, the manufacture of columns results in slight variations in each batch.

In addition, a 2.5m retention gap was fitted to the inlet of the system and the columns attached via a y-shaped splitter. The inclusion of the retention gap was a practical consideration to increase the lifetime of the columns as much as possible.

A dual column system was put in place for all three types of column. A 2.5m, 0.32mm id retention gap was used from the injection port, connected to a Y-shaped glass splitter with identical columns in place for FID and MSD detection. It is noted that a single column could have been used with the flow from this column split to two detectors using the Y-shaped splitter. This would eliminate problems encountered when the MS and FID detect peaks at different times because of variation in the lengths of their respective analytical problems (after trimming column ends etc.). However, the decision of the group was that the dual column system would be preferable.

The columns chosen are as follows:

Ultra 1 – 100% dimethyl silicone – most suitable for non-polar analytes

Ultra 2 – 5% phenyldimethyl silicone – better for slightly more polar analytes

HP-50+ - 50% phenyldimethyl silicone – best for medium polarity analytes

The temperature range of the analysis remained the same for each of the columns since each of the columns may prove more effective at a different ramp rate. The temperature programs

range from a rapid linear temperature gradient of 12°C increase per minute, to a very slow gradient, 2°C per minute. All temperature programs started at 60°C with a 1 minute hold time to allow the oven temperature to stabilise and end at 300°C with increments of 2, 4, 6, 8, 10, 12°C/min followed by a 15 minute hold-time when the maximum temperature was reached. This was to ensure that all components remaining on the column, even at 300°C would be removed before the next analysis.

Each of the column specifications state that the columns are stable up to 310°C and therefore, 300°C seemed a reasonable maximum temperature limit. Each new column was also baked overnight at this temperature in the oven after having been fitted to the GC.

Injections, in all cases, were by 4μL fast syringe injection with a split ratio of 1:20 to a split liner containing deactivated glass wool.

Separation power, inertness and resolution of target compounds were evaluated for each of the columns and each of the temperature programs. The column and oven temperature program, which gave the highest separation power, good enough resolution and inertness would then be chosen for the next stage of the task.

The initial and final oven temperatures were fine-tuned at a later stage by taking into account the sample introduction technique, maximum allowable column temperature of the chosen column and the influence of those diluents such as sugars, starches and caffeine, which may be present in an extraction. This fine-tuning, although not an integral part of the actual analysis, should ensure that all residual compounds are removed from the column before the next injection.

A batch of 'mock extract' (solvent spiked with amphetamine and impurities) was used and contained internal standard (eicosane – C_{20}) at 10μg/mL. One large batch of synthetic 'extract' was prepared and divided into 50μL aliquots in GC vial with glass inserts. Between analyses, the samples were maintained at 4°C and brought to room temperature one hour before analysis.

The Ultra 1 column was analysed in the first instance, followed by Ultra 2 and lastly, HP-50+. The temperature programmes used for each column were randomised and three

replicate injections were obtained in each case. Table 5.1 shows the time taken for each of the temperature programs used in the experiment.

Column	Temperature Program	Total Analysis Time
Ultra 1	2°C/min 4°C/min 6°C/min 8°C/min 10°C/min 12°C/min	2 hours 16 min 1 hour 16 min 56 min 46 min 40 min 36 min
Ultra 2	2°C/min 4°C/min 6°C/min 8°C/min 10°C/min 12°C/min	2 hours 16 min 1 hour 16 min 56 min 46 min 40 min 36 min
HP 50+	2°C/min 4°C/min 6°C/min 8°C/min 10°C/min 12°C/min	2 hours 16 min 1 hour 16 min 56 min 46 min 40 min 36 min

Table 5.1 Experimental design for column and temperature study

5.3. Results and Discussion

5.3.1. Optimisation of Injection Port Temperature

Exemplar chromatograms (Fig 5.1 and 5.2) are shown below of the data obtained from the split and splitless injections of the standard solution using an injection port temperature of 280°C.

Table 5.2 contains the integrated peak area for each of the standards measured relative to the internal standard for each of the three replicate sample injections. Also shown are the average values for each peak and the RSD values for the triplicate injection using both split and splitless injection at 280°C.

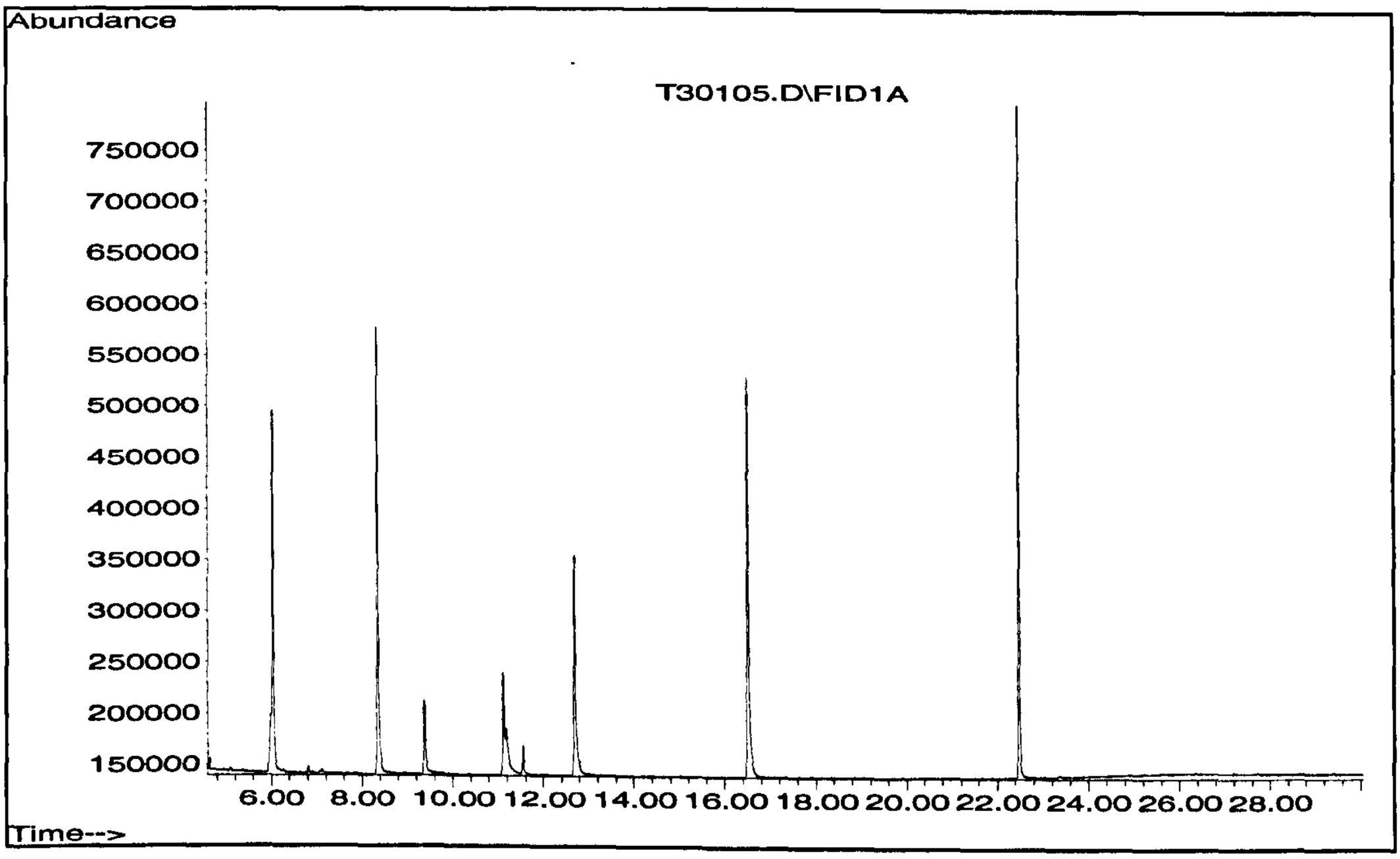


Figure 5.1 Standard impurities run using splitless method at 280 ℃

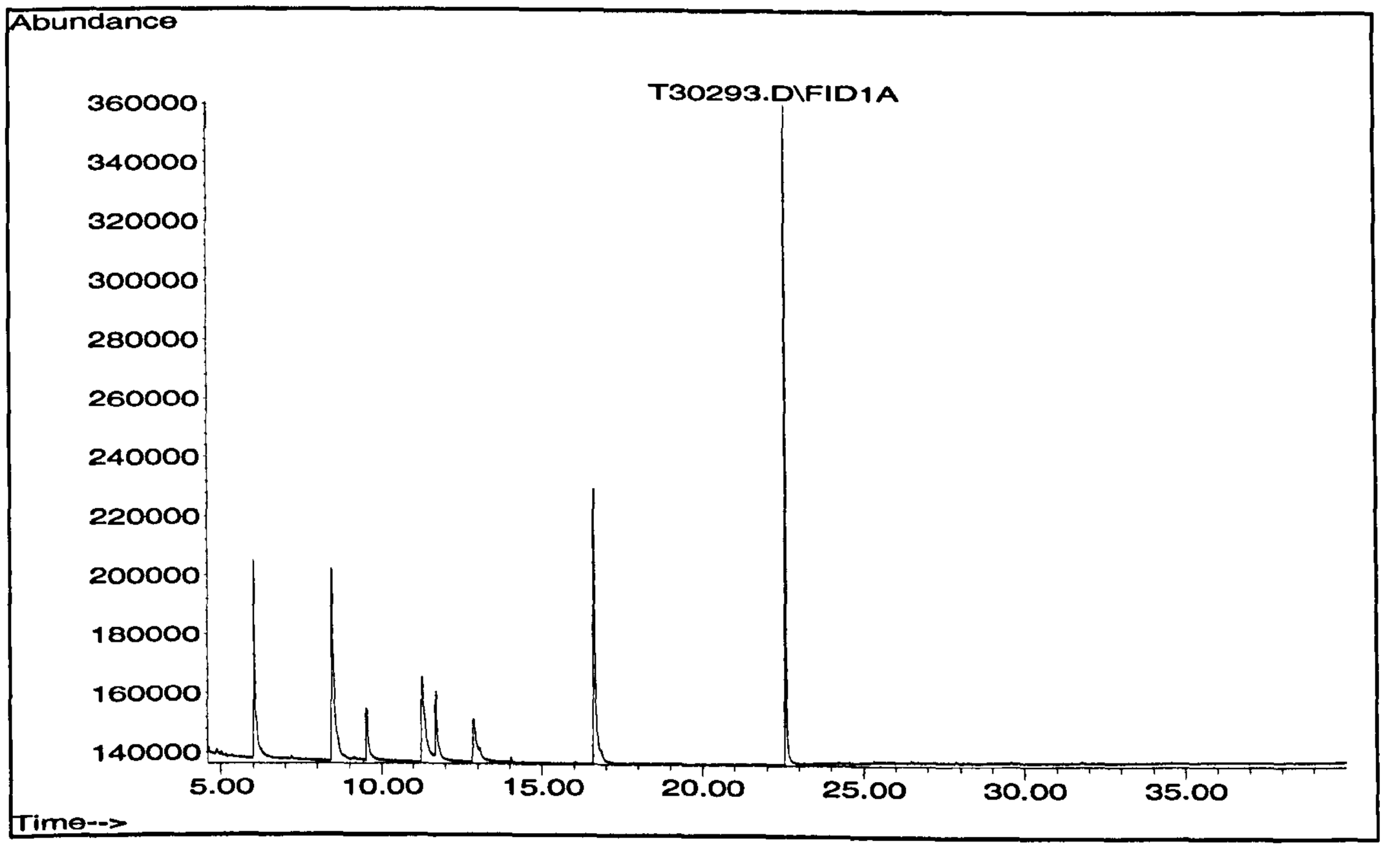


Figure 5.2 Standard impurities run using split method at 280 ℃

Splitless data 280°C						Split data 280°C					
RT	1	2	3	**mean**	**RSD**	**RT**	1	2	3	**mean**	**RSD**
5.97	0.13	0.14	0.14	0.14	**1.42**	-	-	-	-	-	-
6.01	0.65	0.66	0.65	0.65	**1.01**	6.03	0.50	0.49	0.50	0.49	**0.51**
8.33	0.98	0.98	0.97	0.98	**0.31**	8.45	0.71	0.71	0.72	0.72	**0.88**
9.39	0.17	0.17	0.15	0.16	**5.59**	9.543	0.15	0.15	0.16	0.15	**1.37**
11.12	0.21	0.20	0.18	0.20	**6.94**	11.27	0.38	0.39	0.40	0.39	**2.31**
11.18	0.19	0.18	0.16	0.18	**6.12**	11.69	0.17	0.17	0.17	0.17	**1.13**
11.54	0.05	0.05	0.05	0.05	**1.70**	-	-	-	-	-	-
12.68	0.54	0.54	0.54	0.54	**0.24**	12.87	0.14	0.14	0.15	0.14	**2.93**
16.49	0.95	0.95	0.94	0.95	**0.43**	16.63	0.70	0.77	0.75	0.74	**3.93**

Table 5.2 RSD values for standard peaks using split or splitless injections at 280 ℃

From this data it may be deduced that with the inlet temperature set at 280°C, split injection gives the best overall reproducibility of results and with an average RSD value of 1.86, there appears to be no major statistical difference in the profile of standard impurities using this injection temperature.

Table 5.3 shows the data obtained from the identified impurities in the 'mock' sample prepared to resemble an extraction sample. The RSD values are shown for each impurity at each temperature for both split and splitless injection. The overall average RSD value is also shown. These values may be compared with the values obtained for a cool on column (COC) injection of the same sample with the same column and temperature program. This data was supplied by the Finnish Nation al Bureau of Investigation (NBI) since we did not have the correct equipment to carry out this experiment. Note also that the data reported here includes the results for acetylamphetamine and formylamphetamine which were being studied by the Finnish lab as impurities in the Leuckart synthesis but may also be found as impurities formed via the nitrostyrene route.

	COC	**Splitless**				**Split**			
Impurity		**220**	**240**	**260**	**280**	**220**	**240**	**260**	**280**
BMK	2.87	2.64	6.14	16.48	1.01	1.61	1.72	1.32	1.14
Phenyl-2-propanol	2.89	2.62	5.49	14.62	1.46	2.59	1.26	1.94	1.99
Phenyl-1propanone	3.10	1.45	5.95	15.19	2.49	1.71	1.61	1.29	0.41
Ketoxime (1)	2.86	1.35	4.98	11.00	0.82	2.12	6.37	2.36	2.77
Ketoxime (2)	2.97	2.47	2.89	8.57	0.51	10.2	1.10	1.57	3.05
Formylamphetamine	2.38	2.15	7.29	7.66	3.38	3.48	3.2	0.43	21.78
Acetylamphetamine	2.67	4.26	22.55	16.61	17.59	9.50	10.29	6.25	1.82
Benzaldimine	3.09	4.53	24.03	7.73	9.42	2.06	1.85	2.26	0.35
Average	**2.85**	**2.68**	**9.91**	**12.23**	**4.58**	**4.16**	**3.42**	**2.18**	**4.16**

Table 5.3 Relative Standard Deviation Values for Peak Areas of Impurities

It may be concluded that the optimum injection techniques, in terms of reproducibility of results for the impurities derived from the nitrostyrene route (assuming that the other impurities which were, at this stage unidentified behave in the same way as those studied in this part of the experiment) are splitless injection at 220°C and split injection at 260°C.

260°C was chosen as the injection port temperature since it was sufficiently hot to allow complete vaporisation of the sample and the experimental data showed that the RSD values at this temperature were comparable with COC injections and therefore would be sufficiently reproducible for profiling.

The split injection technique was chosen based on these results since overall, the average RSD values for any of the injection temperatures studied was lower using the split method. None of the temperatures studied using split injection resulted in an RSD value above 5%. Split injection was also a popular introduction technique for many practical reasons since it has the advantage of working well with concentrated samples. It also allows the operator to adjust the split ratio to decrease the amount of sample passing onto the column. In addition, using split injection, the massive solvent fronts, which are common in splitless injections, are no longer a problem. However, it has also been shown that splitless injections may be used almost as successfully as split injections if only a small volume of solvent is available (for example if the solvent accidentally is allowed to evaporate during the extraction procedure).

In addition to running the standard mixture of impurities and the synthetic 'mock extract', a second set of experiments was set up using a solvent extract of amphetamine which had been

freshly prepared via the nitrostyrene route. These samples were treated in exactly the same way as the standard mixture of impurities, each sample being analysed at 220, 240, 260 and 280°C injection port temperature. These samples were also analysed split and splitless in triplicate. The largest 11 peaks were analysed, although these were not necessarily the 'target' impurities, nor were they all identified.

The RSD values for each of these peaks is shown below in Table 5.4 along with the total and average RSD values for each technique.

	Split					Splitless			
RT	**220**	**240**	**260**	**280**	**RT**	**220**	**240**	**260**	**280**
5.98	0.92	0.92	1.70	0.04	6.16	1.28	4.52	4.19	2.62
8.43	3.32	1.45	1.62	1.18	8.57	6.50	2.81	10.08	2.79
11.24	3.99	2.18	2.99	0.31	9.07	7.75	3.42	11.85	3.82
13.34	8.18	1.76	6.26	2.76	9.41	7.26	6.90	9.54	3.90
19.5	4.67	1.41	2.29	0.49	11.23	9.24	5.01	17.80	4.34
20.76	4.31	1.51	2.03	0.34	13.17	9.44	4.92	18.60	4.36
20.93	4.45	2.64	3.41	1.24	18.15	11.91	12.21	25.42	8.94
21.07	4.34	2.40	3.19	1.10	19.5	9.62	7.02	21.27	3.13
21.13	4.54	4.76	5.45	4.63	20.76	9.40	6.81	21.73	3.16
22.57	4.86	1.63	1.54	0.94	20.89	10.10	8.21	24.27	4.16
23.45	3.97	1.47	2.05	0.68	20.96	8.98	7.05	21.51	3.25
					21.08	9.38	7.27	22.83	3.07
					22.56	8.55	8.22	23.37	3.47
Total	47.57	22.13	32.50	13.70	total	109.43	84.36	232.46	50.99
Average	**4.32**	**2.01**	**2.95**	**1.25**	**Average**	**8.42**	**6.49**	**17.88**	**3.92**

Table 5.4 Relative Standard Deviation Values for the Largest Peaks in Extracts

These results would tend to suggest that the 'real' extract which has a more complex sample matrix than either the standard solution or the mock extract and is best analysed using a higher injection port temperature of 280°C. This would make much more sense since the higher temperature would help to volatilise what is essentially a far more concentrated sample.

This experiment was repeated using a different 'extract' in order to determine whether the extract used in this section was particularly dirty or concentrated and to verify these results with the final column chosen since this had not been decided at this stage – see section 5.5 for these results. Also, changes in the sample matrix or diluents added in high concentration may affect the chromatographic performance in that, the temperature may need to be increased to completely volatilise the sample so these findings may need to be reassessed at a later date.

5.3.3. Choice of Capillary Column and Oven Temperature Program

The most suitable temperature program and column choice was also decided by determining the tailing and resolution of the system.

The tailing or inertness of peaks in a chromatogram is also important since a tailing or fronting peak could potentially mask another smaller peak. This may be picked up by monitoring the mass spectra of every peak but this is a very time-consuming process and in a high throughput situation is undesirable. Also when using software integration, the computer program has a tendency to split peaks, which are either tailing or fronting. This inability to deal with different peak shapes would result in a necessity for manual integration which, in addition to being time consuming, also introduces subjectivity in integration between different operators. In profiling, this source of possible 'error' could result in possible 'matches' in profiles being missed.

Another fundamentally important aspect of the chromatography is the ability to resolve two peaks with very similar retention times. The peak resolution is particularly important in profiling where the chemical structures of many of the impurities are very similar and could be expected to have very similar interactions with the column and therefore elute very close together.

The inertness or tailing values for each of the target compounds were then obtained for the standard mixture. In the 'extract', however, 6 peaks which appeared in every chromatogram were evaluated for tailing and the total 'value' for the tailing or fronting calculated and compared to the ideal values for inertness which lie between 0.9 and 1.2 with a higher value indicating a greater degree of tailing. These values can be seen in the tables below.

Tables 5.5, 5.6 and 5.9 show the tailing/fronting values for each column and temperature program using the standard mix. Acceptable tailing values are highlighted in red.

Ultra 1	2°C	4°C	6°C	8°C	10°C	12°C
Peak 1	1.15	2.51	3.18	3.09	2.36	2.79
Peak 2	4.81	2.71	7.30	8.04	5.16	5.29
Peak 3	6.14	8.63	9.08	12.25	6.34	6.41
Peak 4	2.38	4.75	4.61	4.73	3.85	3.58
Peak 5	0.00	2.74	2.85	3.04	2.39	2.55
Peak 6	3.51	1.20	1.38	1.36	1.17	1.17
average	**3.60**	**3.76**	**4.73**	**5.42**	**3.55**	**3.63**

Table 5.5 Tailing values for standard impurities using Ultra 1 column

Ultra 2	2°C	4°C	6°C	8°C	10°C	12°C
Peak 1	1.13	1.06	0.99	1.10	1.0	1.02
Peak 2	1.70	1.43	1.32	1.42	1.42	1.31
Peak 3	3.90	3.61	3.55	3.95	3.95	3.47
Peak 4	0.80	1.06	0.81	1.01	1.01	0.89
Peak 5	N/a	0.87	0.96	0.90	0.90	0.89
Peak 6	N/a	1.10	0.68	1.04	1.04	0.68
average		**1.10**	**0.95**	**1.10**	**1.07**	**0.96**

Table 5.6 Tailing values for standard impurities using Ultra 2 column

HP 50+	2°C	4°C	6°C	8°C	10°C	12°C
Peak 1	1.26	1.28	1.23	1.10	1.28	1.18
Peak 2	3.15	2.62	2.57	2.04	2.36	2.19
Peak 3			5.73	5.05	5.33	4.50
Peak 4	1.09	1.06	1.05	1.10	1.05	1.06
Peak 5	1.21	1.07	1.00	1.02	1.01	1.00
Peak 6	1.49	1.33	1.24	1.15	1.15	1.01
average	**1.63**	**1.47**	**1.42**	**1.28**	**1.37**	**1.25**

Table 5.7 Tailing values for standard impurities using HP-50+ column

As may be seen from the tables above, the general inertness of the Ultra 1 column is far lower than either the Ultra 2 or the HP 50+ columns. The peak shape using the Ultra 1 column was particularly bad considering that, at this stage, the sample was a standard solution of known compounds at a reasonable concentration. It was thought that this column simply would not perform well enough in a profiling situation with a complex mixture of unknown compounds in a complex matrix of diluents etc.

The chromatograms below (Fig 5.3, 5.4 and 5.5) show the standard mixture of impurities run on each of the columns at 10°C/minute ramp rate. Simply by looking at the chromatograms, it is clear that the peak shape obtained using the Ultra 1 column is poorer than the other columns with tailing clearly evident.

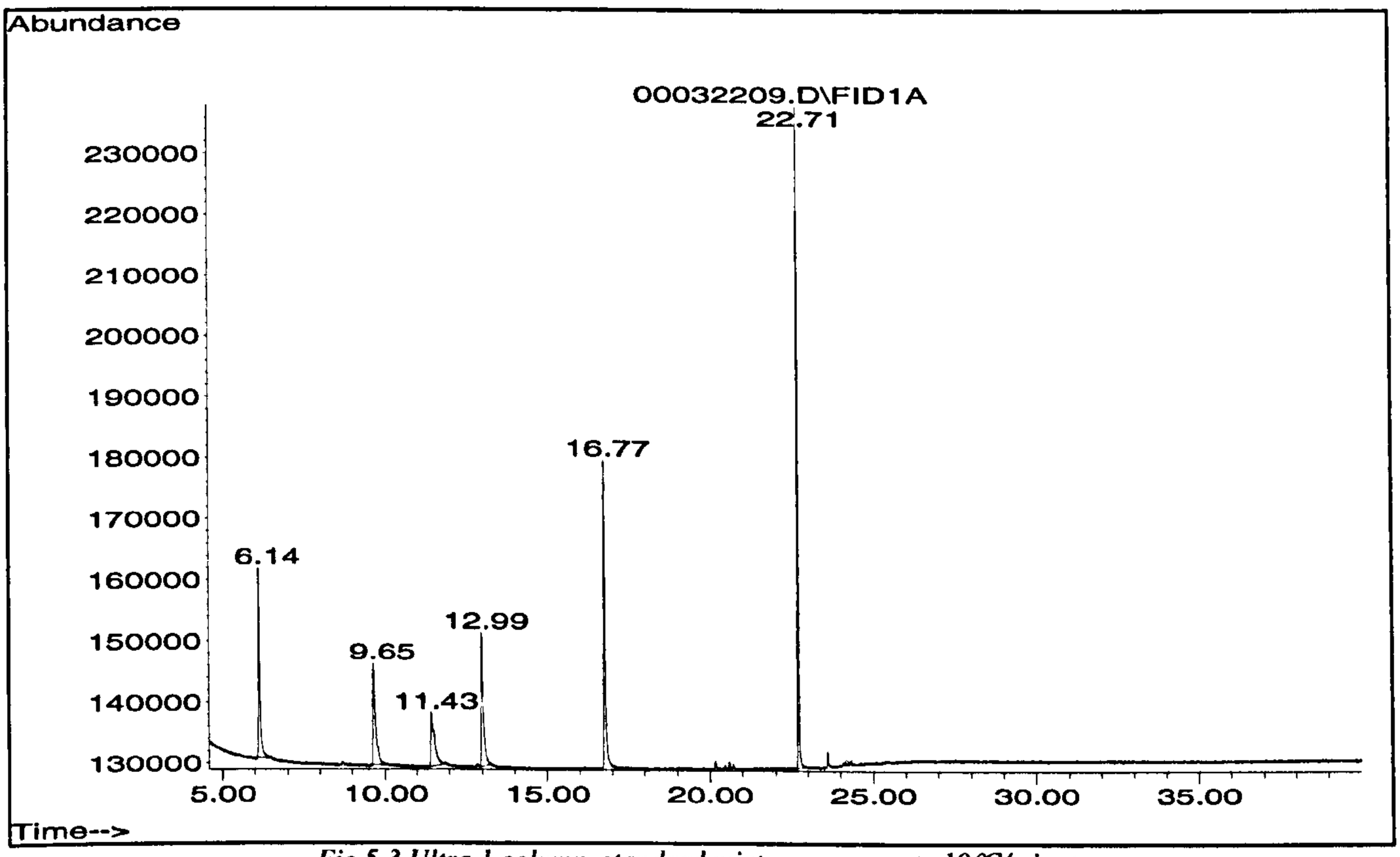


Fig 5.3 Ultra 1 column, standard mixture, ramp rate 10°C/minute

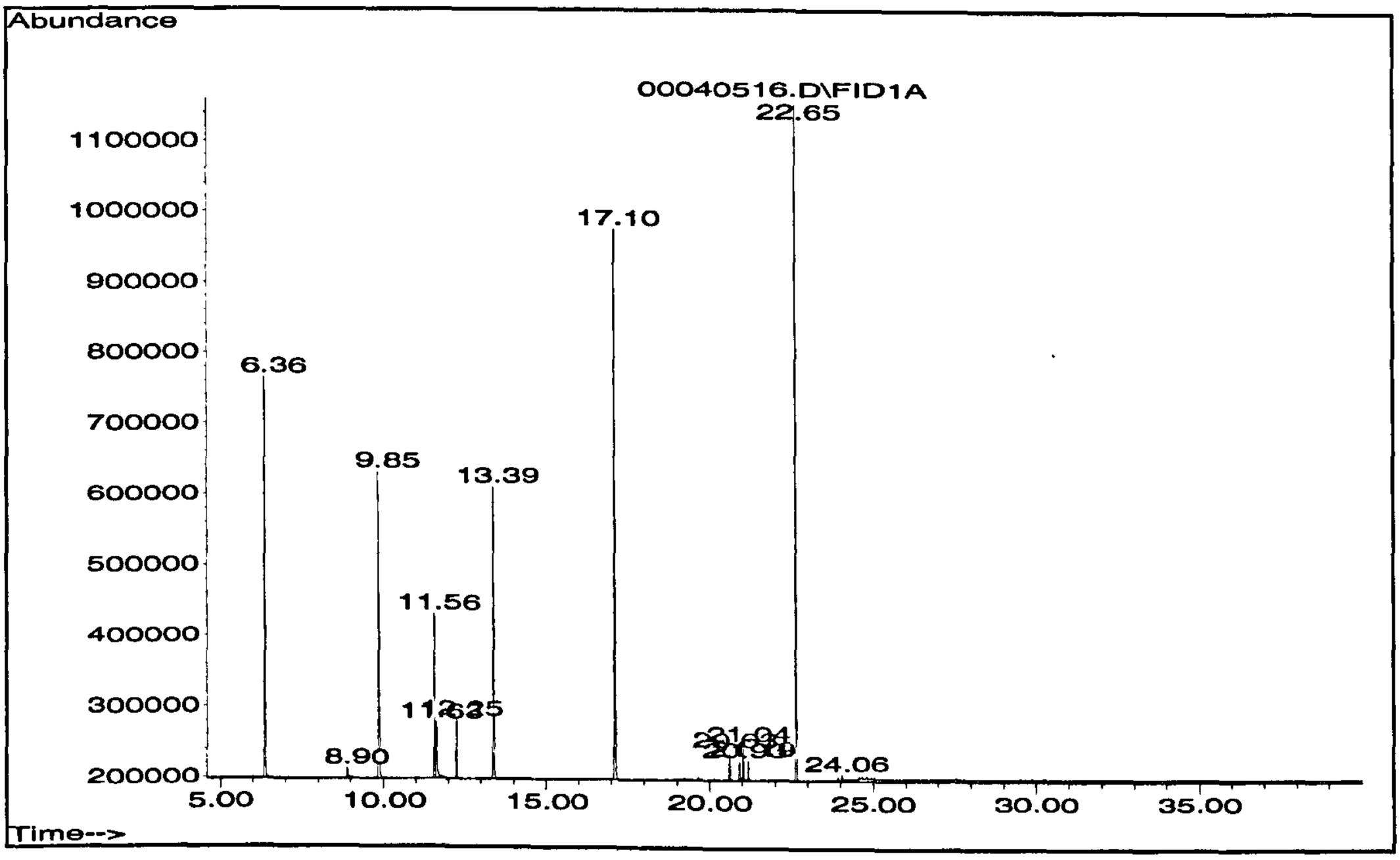


Fig 5.4 Ultra 2 column, standard mixture, ramp rate 10℃/minute

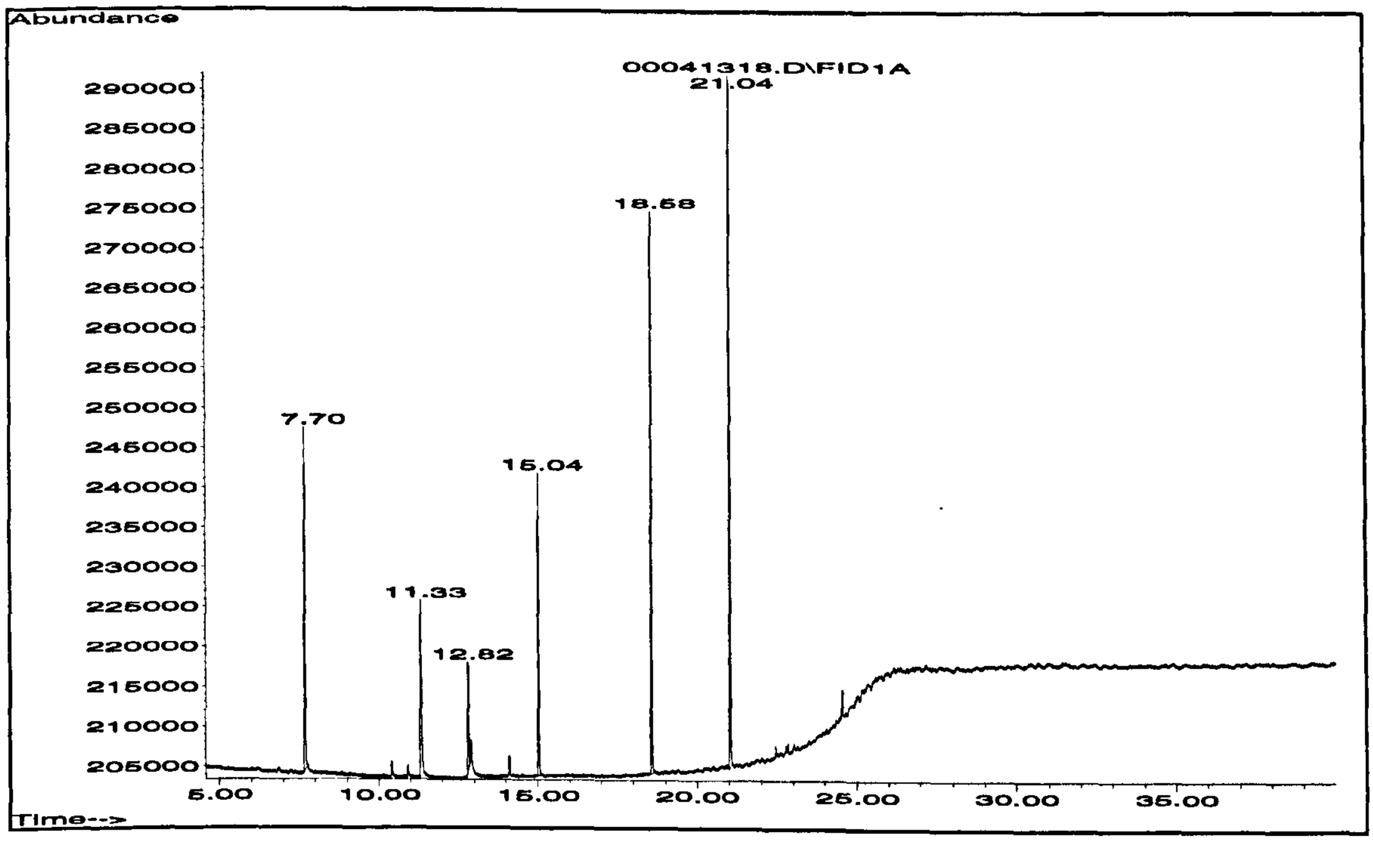


Fig 5.5 HP-50+ column, standard mixture, ramp rate 10℃/minute

The tailing values for the columns were then evaluated using a nitrostyrene amphetamine extract sample which had a far more complex mixture of compounds as well as a high concentration of amphetamine present. The chromatograms below show the extract sample run at 8°C ramp rate using each of the three columns.

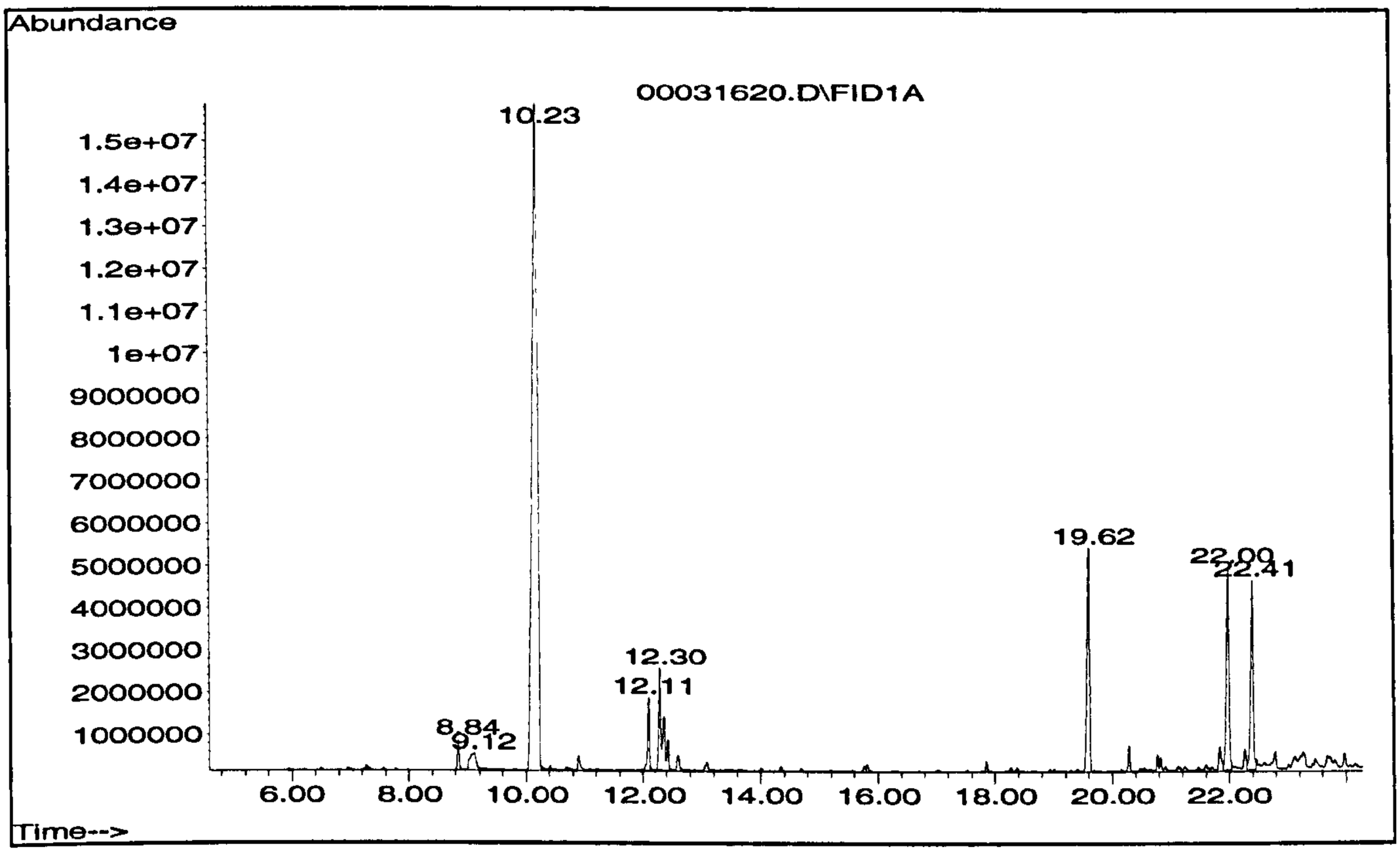


Figure 5.6 Ultra 1 column, extract sample, ramp rate 8 ℃/minute

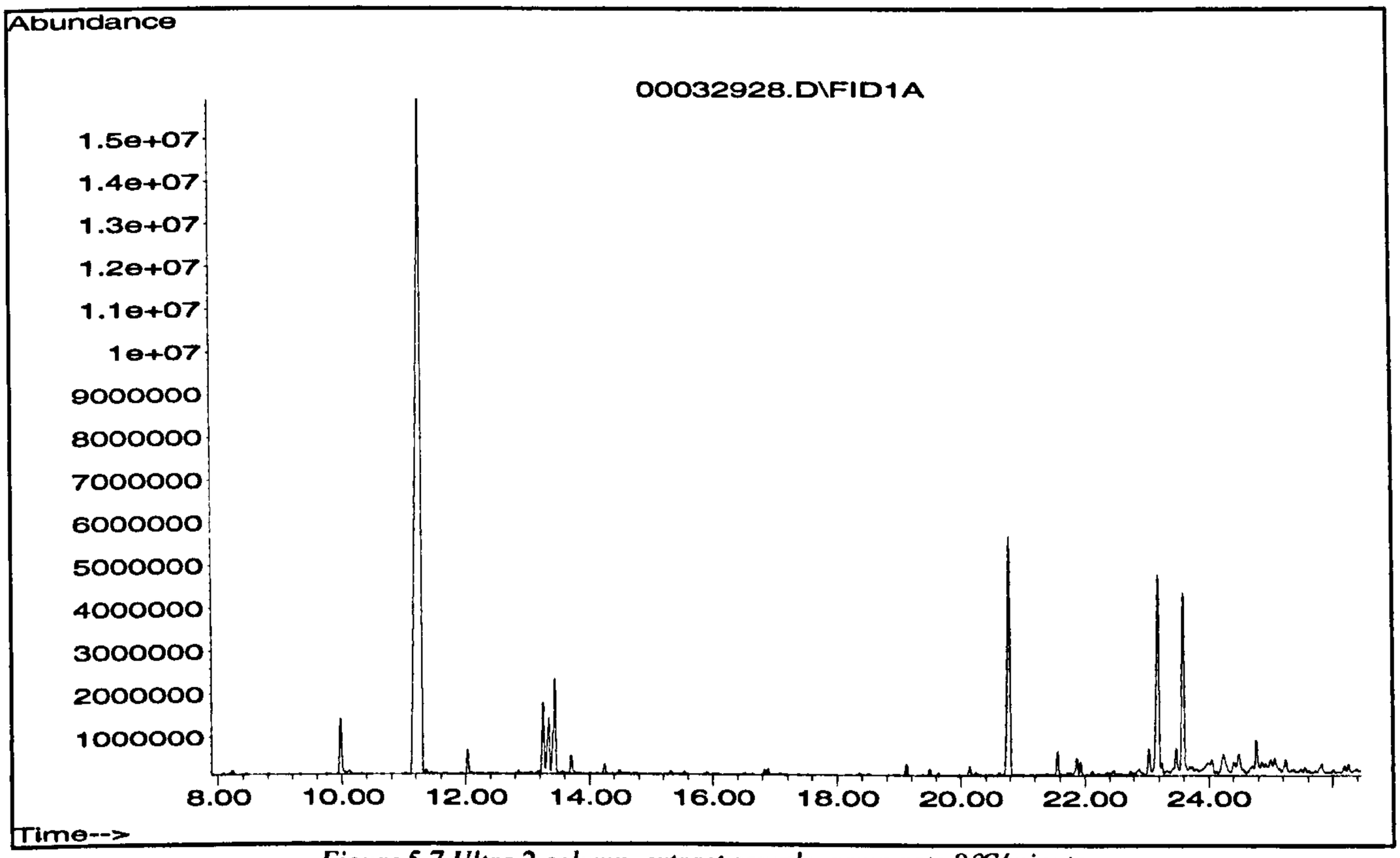


Figure 5.7 Ultra 2 column, extract sample, ramp rate 8 ℃/minute

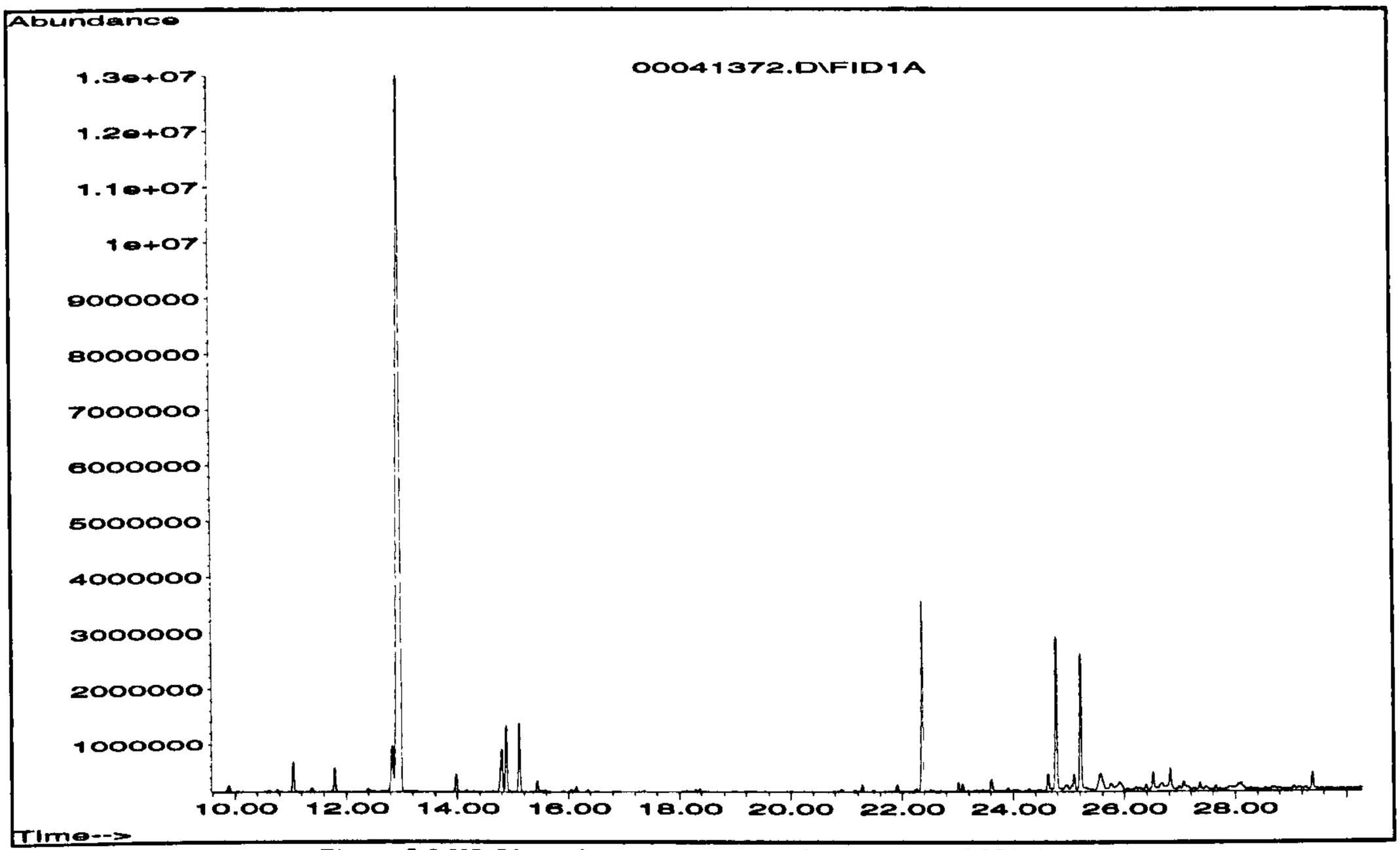


Figure 5.8 HP 50+ column, extract sample, ramp rate 8 ℃/minute

The tables below show the tailing values as evaluated for the five largest peaks in each amphetamine extract chromatogram using each ramp rate for each column. The optimum value for tailing is 0.9-1.2 therefore the column and temperature program which provides an average value for tailing closest to this value should provide the most suitable system for profiling.

Ultra 2	2°C	4°C	6°C	8°C	10°C	12°C
Peak 1	0.12	0.09	0.07	0.08	0.1	N/A
Peak 2	0.53	0.39	0.51	0.53	0.56	
Peak 3		0.13	0.21	0.24	0.23	
Peak 4		0.38	0.28	0.24	0.22	
Peak 5		0.73	0.22	0.26	0.34	
Average		**0.344**	0.258	0.27	0.29	

Table 5.8 Tailing values for 5 largest peaks in extract using Ultra 2 column

HP 50+	2°C	4°C	6°C	8°C	10°C	12°C
Peak 1	0.08	0.16	0.13	0.15	0.11	0.18
Peak 2	0.61	0.56	0.67	0.68	0.5	0.74
Peak 3	0.73	0.25	0.36	0.43	0.22	0.47
Peak 4		0.29	0.47	0.53	0.3	0.52
Peak 5		0.29	0.37	0.58	0.28	0.57
Average		0.31	0.40	0.47	0.28	0.50

Table 5.9 Tailing values for 5 largest peaks in extract using HP-50+ column

The values which are absent from the 2°C per minute data are because of a software problem with the Easy Id section of Chemstation software which would not allow evaluation of tailing values after 60 minutes. The table below shows a comparison of the average tailing of the columns at each ramp rate using this extract sample.

	Ultra 2	HP 50+
2°C	Unknown	Unknown
4°C	0.34	0.31
6°C	0.26	0.40
8°C	0.27	0.47
10°C	0.29	0.28
12°C	Unknown	0.50

Table 5.10 Average tailing values for peaks using each column and ramp rate

From these results it may be concluded that, overall the HP 50+ column gives the best results in terms of peak tailing and fronting. Practically, the only real options are 6, 8, 10 and 12°C per minute. From these results it may be seen that the best option in terms of inertness is the HP 50+ at 8 or 12°C per minute.

The resolution for the HP50+ column was then evaluated for each temperature program using the Chemstation software. The ideal value for resolution is 100 %. This value is defined as the percentage height of the valley between two adjacent peaks.

In terms of resolution, two sets of peaks which were of similar area and which eluted in quick succession were examined to determine whether they could be completely resolved at any of the temperature programs using the HP 50+ column. The results are shown below. The values quoted are the height of the valley between two adjacent peaks as a percentage of the mean height of the peaks.

IIP 50+	Peak set 1	Peak set 2	Average
2°C	101.13	98.58	99.9
4°C	96.16	103.26	99.7
6°C	96.74	107.16	103.0
8°C	96.89	112.52	**104.7**
10°C	95.84	110.9	103.4
12°C	94.55	98.78	96.7

Table 5.11 Resolution values for two peak sets at each ramp rate using HP-50+

From this data it can be seen that the greatest resolution using the HP 50+ column can be achieved using a ramp rate of 8°C per minute.

5.4. Interim Conclusions

The GC method to be used in the next stage of the project was as follows:

Inlet Temperature - 260°C

Injection Method – 4 µL fast injection

Split Ratio - 1:20

Column - HP 50+

Column Configuration - 2.5 m retention gap to Y shaped splitter to two 25m lengths.

Temperature programme - 60°C for 1 minute, ramped at 8°C per minute to reach a maximum of 300°C then held for 10 minutes.

5.5. Small Injection Port Temperature Study

Although the split injection was found to produce optimum results in the previous study, the group as a whole decided to use a splitless injection. A small temperature study was carried out in order to confirm that the temperature of the injection port, which had been chosen, was indeed at an optimum in terms of peak area and reproducibility of results even with a changed injection method. The injection port temperature was varied to determine the most suitable vaporising temperature for our nitrostyrene sample.

5.5.1. Experimental Detail

A single extract was obtained following the optimised extraction procedure (see Chapter 6) and placed in a GC vial. A 2 µL aliquot of the extract was injected in triplicate at five different temperatures in splitless mode. All other variables are as described in Section 5.4.

The temperatures used were 220, 240, 260 and 280°C as well as 250°C which was the proposed value of the group as a whole. The overall impurity profile, as well as the variability of the results for the amphetamine peak, were studied.

As may be seen from the table below, the higher injection port temperature, in general, results in a higher relative peak area for amphetamine. However all RSD values fall within the acceptable analytical range of less than 5 %.

No.	Time	220 mean values	RSD	240°C mean values	RSD	250°C mean values	RSD	260°C mean values	RSD	280°C mean values	RSD
amphetamine		17.59	1.88	17.53	3.35	18.26	3.08	18.94	2.78	18.93	2.52
1	5.36	0.408	5.48	0.537	1.54	0.655	2.06	0.710	2.54	0.718	1.45
2	6.20	0.665	4.71	0.713	4.20	0.770	3.96	0.806	4.69	0.794	3.25
3	7.78	1.285	2.39	1.340	2.92	1.496	3.30	1.568	3.59	1.568	3.05
4	7.92	1.132	1.98	1.141	3.59	1.208	4.07	1.266	3.22	1.261	2.43
5	8.64	0.427	3.23	0.421	5.80	0.431	3.15	0.478	2.53	0.467	4.30
6	8.74	0.599	3.29	0.562	5.55	0.581	2.90	0.591	5.25	0.618	9.63
7	10.40	1.790	1.06	1.741	1.60	1.726	4.96	1.646	1.66	1.666	3.12
8	10.48	0.793	0.55	0.788	1.75	0.778	5.00	0.758	1.83	0.763	2.91
9	12.18	2.718	1.46	2.686	2.07	2.757	2.29	2.809	1.35	2.834	0.81
10	13.94	1.091	1.37	1.065	4.04	1.015	7.39	0.975	0.31	0.910	12.50
11	14.12	0.348	25.36	0.282	3.76	0.273	2.75	0.268	0.90	0.272	4.33
12	16.56	0.364	2.64	0.371	2.19	0.384	2.78	0.386	1.37	0.386	0.45
13	16.86	2.641	1.96	2.680	3.48	2.664	4.05	2.681	2.13	2.627	0.83
14	17.10	1.160	1.69	1.169	2.90	1.182	3.13	1.172	2.64	1.161	0.51
15	17.53	1.956	6.03	2.074	2.91	2.043	1.18	2.231	5.03	2.117	2.20
16	17.76	0.162	8.45	0.180	1.12	0.200	3.65	0.208	1.94	0.219	6.80
17	18.08	7.635	1.64	7.705	3.45	7.868	3.34	7.846	2.33	7.764	0.57
18	18.33	0.950	3.84	0.956	3.05	0.977	3.20	0.975	2.19	0.965	0.65
19	18.88	2.819	2.23	2.827	4.51	2.684	3.20	2.807	3.37	2.686	1.36
20	19.09	1.642	9.02	1.600	3.22	1.643	3.31	1.733	11.32	1.629	0.75
21	19.23	1.892	2.29	1.943	3.30	1.947	3.61	1.953	2.94	1.913	1.18
22	19.40	1.215	2.68	1.281	2.84	1.346	3.46	1.352	3.17	1.330	1.56
23	19.92	1.205	3.03	1.166	5.34	1.009	4.65	1.099	4.65	1.015	2.27
	total	34.897	4.19	35.227	3.27	35.639	3.54	**36.316**	3.08	35.683	**2.91**

Table 5.12 Mean impurity relative peak areas against temperature

From the results shown above, it is clear that the temperature chosen for the injection port does not have a vast impact on the overall profile. The total areas of the selected peaks does, however, follow a trend upwards from the 220°C results to the 260°C values. This upward trend does not continue with the 280°C results. The general reproducibility of the results appears to follow a downward trend from the lower to higher temperatures with 220°C providing the most variable results and 280°C, the least. However, looking at a plot of both 220 and 280 impurity data sets shows that, even given these differences in impurity levels, the overall impurity profiles are almost identical.

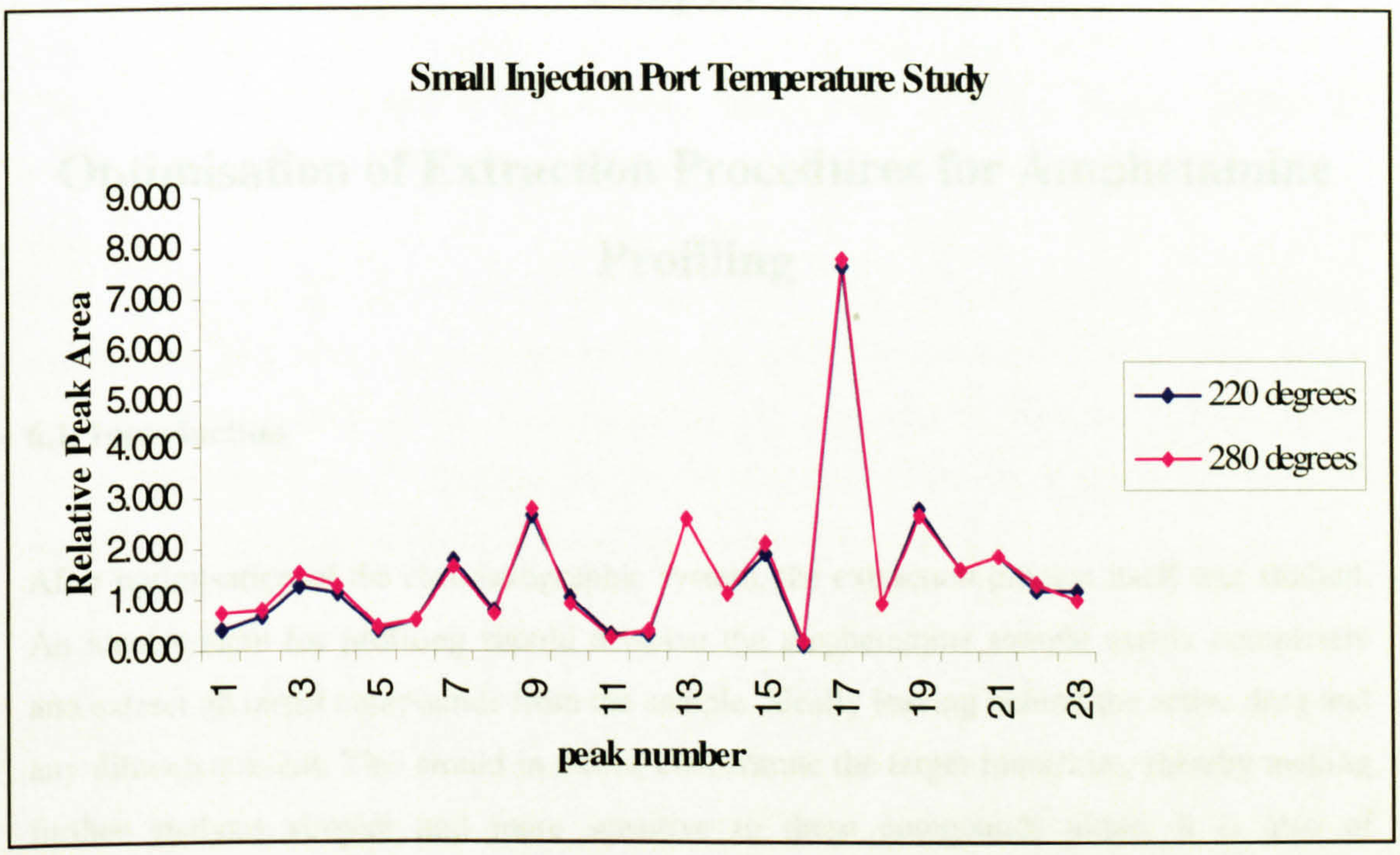


Figure 5.9 220 and 280°C injection port impurity levels

In conclusion, the choice of 250°C as injection port temperature was one based on a compromise between a high enough temperature to volatilise the sample and a level low enough not to cause sample decomposition. However, it has been shown that the impurity peak areas across the whole profile are increased using higher temperatures and the reproducibility of the chromatography is not compromised by increasing the injection port temperature. Therefore, the most suitable temperature, basing the decision solely on the impurity profile from this batch of nitrostyrene, would be either 260°C because it has the highest level of impurities in the profile or 280°C because it has the most reproducible peak area values.

Chapter 6

Optimisation of Extraction Procedures for Amphetamine Profiling

6.1. Introduction

After optimisation of the chromatographic system, the extraction process itself was studied. An ideal system for profiling should dissolve the amphetamine sample matrix completely and extract all target compounds from the sample, ideally leaving behind the active drug and any diluents present. This would in effect, concentrate the target impurities, thereby making further analysis simpler and more sensitive to these compounds alone. It is also of fundamental importance that the impurities and target compounds are extracted out of the bulk sample in a reproducible manner to ensure repeatability of the analysis.

Ideally, the process of extraction would be simple enough to be carried out routinely without many time-consuming or labour-intensive steps. Moreover, the possibility that the extraction could be fully automated should also be considered. In an operational lab, the high number of samples available for profiling on a routine basis may necessitate the automation of the liquid-handling section of the extraction thereby minimising the number of manual steps in the process.

Initially, a method, which had been used in trials in Holland and Sweden [12], was used as a starting point for the study. The process is a liquid–liquid extraction (LLE) using a pH7.4 phosphate buffer with solvent extraction into iso-octane. In this sub-section of the project, a number of alternative methods were evaluated to determine if this procedure was indeed the most sensitive, reproducible and effective extraction procedure available.

Solid phase extractions were evaluated by others involved in the project but with limited success. Therefore, only liquid-liquid extractions were tested to any great extent, to determine which technique would provide the most effective and practical sample preparation method for the profiling of organic impurities in amphetamine samples.

Amphetamine sulphate synthesised using the nitrostyrene route was prepared as the main test sample. The method used to prepare this sample has already been described in Chapter 3. This sample was used 'pure' and additionally bulked with commonly observed diluents to study possible matrix effects, which may develop as a result of the sample composition.

The experiments developed for the optimisation of each component of the system were structured in such a way as to determine the most robust, repeatable and practically feasible method for the extraction of target impurities. The repeatability, reproducibility, accuracy and sensitivity of the methods were assessed after analysis of the resulting extracts using the GC-FID and GC-MSD methods optimised in the previous chapter.

6.2. Liquid-Liquid Extractions

Liquid-liquid extractions can be explained most effectively in a stepwise diagram of the process.

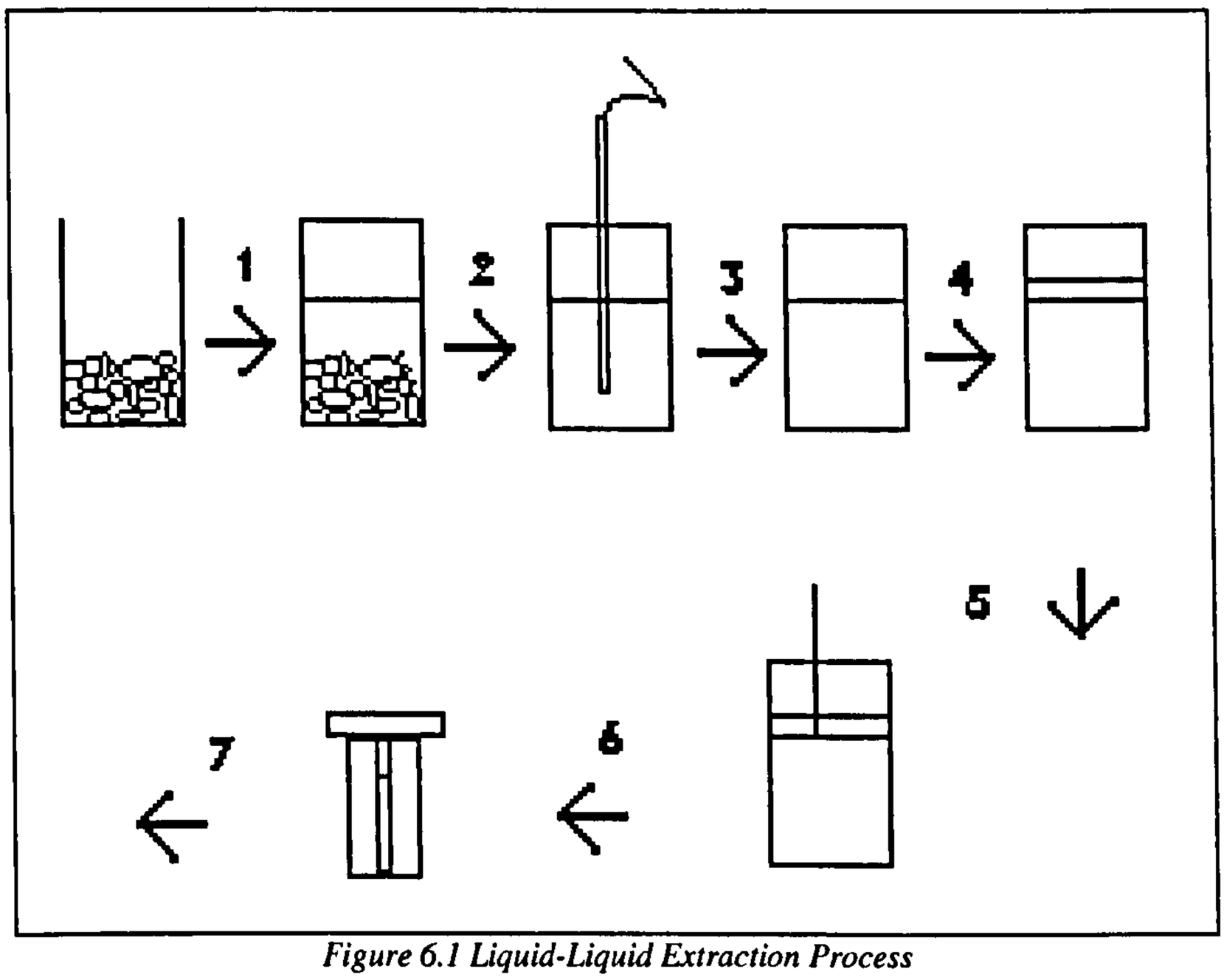


Figure 6.1 Liquid-Liquid Extraction Process

1. Amphetamine powder weighed into a suitable vial
2. Known volume of buffer solution added to vial, 30 minutes mixing
3. pH tested and readjusted to default value
4. 30 minutes mixing
5. Known volume of extraction solvent added to vial, 30 minutes sonication
6. Extraction solvent removed from surface of buffer into GC vial
7. Analysis by GC-FID or GC-MSD

The optimisation of the liquid-liquid extraction is best divided into two or three discrete sections. Firstly, the best buffer in which to dissolve the sample must be determined. The type of buffer, its pH, concentration and the volume required must be assessed for their effect on the extraction of target analytes. Secondly, the extraction solvent, which most successfully and reproducibly pulls out impurities from the buffer, should be assessed. Finally, the pairings of buffer and solvent should be tested to ensure that no adverse effects are found to result from the use of this combination.

6.2.1. Buffers

Aqueous buffers are used to dissolve the amphetamine sample before extraction into the organic phase ready for injection into the GC. The buffer is used to maintain a specific pH even when the sample (which may be basic, neutral or acidic) is added thus ensuring reproducible pH values for the extraction. The buffer is selected for its ability to 'push' the impurities of an amphetamine sample out of the aqueous phase and into the organic phase.

It is likely that different buffer types will be more suitable for particular sample matrices and therefore an assessment of different commercially available buffers is essential. That is to say, one buffer may prove better at dissolving the impurities present in a Leuckart synthesised amphetamine than a nitrostyrene amphetamine since these routes have different target impurities. Other buffers may have difficulty in dissolving bulking agents in samples. The buffer currently in favour in some operational laboratories is a phosphate salt buffer. However, to determine if this is suitable for the majority of amphetamine sample and matrix types, two other buffers, namely, citrate and TRIS® were assessed.

Another parameter to consider is buffer concentration since the dissolution of compounds in a concentrated buffer solution may be more problematic than a dilute buffer. This is simply due to the fact that, for example a 1M solution of phosphate buffer is almost completely saturated with buffer salt. However, a more concentrated solution would provide better

buffering capacity and therefore be more practical for use in a high sample throughput situation. If the buffering capacity is at an optimum, the pH of the buffer should not change when many different samples are dissolved which would minimise the need for pH adjustment of samples.

Seized amphetamine samples may be slightly acidic, basic or neutral depending on the methods used for production, the amount of active drug present and what impurities and diluents have been introduced. The pH of batches of drug made by the same method may also vary depending on the concentrations of certain impurities present. Therefore, when the sample is dissolved in the buffer, it must be checked and readjusted to the default pH value of the buffer to ensure a reproducible extraction. In a busy operational laboratory, this pH adjustment procedure is a particularly time-consuming and labour-intensive stage in the extraction and ideally it should be either simplified or avoided.

In addition, the optimum volume of buffer required to dissolve the amphetamine must be assessed. Here, a compromise must be reached since while a larger buffer volume would be likely to give a more reproducibly dissolved solution it will also prove more difficult to bring the smaller extraction solvent volume into contact with a larger buffer volume. It is also technically more difficult to remove a smaller extraction solvent volume from a larger surface area of the buffer.

Ideally, the buffer should dissolve a relatively large sample mass in a relatively small volume. This should enable the use of a quantity of amphetamine, which is sufficient to allow detection sensitivity for trace level concentrations of impurities. A partially dissolved sample will inevitably have a different profile to that of a completely dissolved sample since selected components in the mixture may preferentially dissolve, leading to a larger relative peak area in a chromatogram when compared to an internal standard.

In addition, since clandestine laboratories producing amphetamine may be using sophisticated methods and purification techniques, particularly clean amphetamine samples containing few impurities at very low concentrations are now commonly found. Therefore, any extraction technique developed must be sensitive and selective for impurities in preference to the active drug and matrix diluents.

6.2.2. Solvents

The next stage of the process is the selection of the optimum solvent parameters to be used in the extraction. Possibilities for the solvent type and the factors influencing the choice of solvent type are many and varied. The solvent should be compatible with the GC-FID and GC-MSD analysis. It must not cause the sample to degrade, and therefore all solvents which were previously tested in the stability study as detailed in Chapter 4, which proved unsuitable should be disregarded. The extraction solvent must not be overly volatile since the small extraction volume could evaporate quickly in the mixing process. The solvent must also be completely immiscible with the buffer solution to enable the extract to be removed from the surface of the buffer without taking in any of the aqueous phase.

Perhaps the most important consideration in choosing a suitable extraction solvent is the ability to extract all of the target impurities in as high a concentration as possible. This should be achieved without also extracting high levels of amphetamine or diluents ie. the solvent must be selective in extraction. Additionally, while the extraction efficiency must be high, the extraction must be as reproducible as possible.

The final choice of buffer and solvent system will be determined following a general study of combinations of buffers and solvents. These combinations will be were narrowed down gradually through a series of experiments. Every set of experiments will be based on the results of the previous set. Each sub-section of this chapter has its own experimental details, results and discussion before outlining the next experimental detail.

6.3. Choice of Buffer and Solvent

Samples prepared to mimic 'street' amphetamine and pure amphetamine sulphate, were tested to determine the dissolution power of five different buffers. The three buffer types – citrate, TRIS® and phosphate were chosen because they cover the range of pH 6 – 8 over their pKa values. Since every salt has its highest buffering capacity at the pKa value of the salt, buffers were tested at or near their optimum buffering capacity. Citrate buffer was tested at pH 6 and 6.5 (pKa is 6.4), TRIS® at pH 8 (pKa is 8.1), phosphate at pH 7 and 7.5 (pKa is 7.2). Buffers were prepared at two different concentrations - 0.1M and 1M which will have given a high enough buffering capacity without decreasing their dissolution power.

Because the buffer never operates alone in an extraction procedure, solvent type was also evaluated in conjunction with the buffer. Solvents tested were iso-octane, toluene, dichloromethane, diethyl ether and ethyl acetate. This range of solvents was used because it encompasses a wide range of organic solvents. Although problems had already been identified in the stability study with the use of dichloromethane (profile changes over time) and diethyl ether (sample concentration changes through evaporation), these solvents were included in case their extraction ability is significantly better than the alternatives and a case might be made for their consideration as extraction solvents in the optimised method.

The extracts obtained from the samples were then analysed by GC-FID and MSD using the injection and analysis technique as optimised in Chapter 5.

In addition, the matrix effects of the sample were evaluated in each of the extraction techniques. This allows for the fact that illicit samples are very rarely found without diluents of one sort or another. Caffeine and lactose were used as the main bulking agents in the samples although, in practice, the sample composition would perhaps be more complex.

The method parameters to be optimised were: pH of buffer, concentration of buffer, buffer salt and type of extraction solvent. In addition, the influence of the sample matrix on the extraction was evaluated by looking at the extracts from the 'pure' amphetamine and amphetamine bulked with caffeine and sugar (lactose).

6.3.1. Buffer Preparation

Preparation of 0.1 M citrate buffer – pH 6.00

Citric acid, 21 g was diluted with distilled water in a 1 L volumetric flask (solution A).
Sodium citrate, 29.4 g was diluted with distilled water in a 1 L volumetric flask (solution B).

Solution A (190 mL) and solution B (810 mL) were mixed thoroughly and NaOH (0.1 M) or HCl (0.1 M) was added to adjust pH to exactly 6.00.

Preparation of 0.1 M citrate buffer – pH 6.50

As for pH 6.00 buffer, solutions A and B were prepared.

Solution A (40 mL) and solution B (960 mL) were mixed thoroughly and NaOH (0.1 M) or HCl (0.1 M) was added to adjust pH to exactly 6.50.

Preparation of 0.1 M phosphate buffer – pH 7.00

Dibasic sodium phosphate, 13.8 g was diluted with distilled water in a 1 L volumetric flask (solution A).
Monobasic sodium phosphate, 26.8 g was diluted with distilled water in a 1 L volumetric flask (solution B).

Solution A (390 mL) and solution B (610 mL) were mixed thoroughly and NaOH (0.1 M) or HCl (0.1 M) was added to adjust pH to exactly 7.00.

Preparation of 0.1 M phosphate buffer – pH 7.50

As for pH 7.00 buffer solution A and B prepared.

Solution A (230 mL) and solution B (770 mL) were mixed thoroughly and NaOH (0.1 M) or HCl (0.1 M) was added to adjust pH to exactly 7.50.

0.1 M TRIS buffer – pH 8.00

TRIZMA® base, 12.11 g was diluted with distilled water in a 1 L volumetric flask.

TRIZMA® base solution (50 mL) and 0.1M hydrochloric acid solution, (29.2 mL) were mixed thoroughly and NaOH (0.1 M) or HCl (0.1 M) was added to adjust pH to exactly 8.00.

6.3.2. Sample Preparation

STANDARD sample – 10 μg/mL concentration of standard impurities (benzaldehyde, ketoxime, benzaldimine, nitrostyrene and aziridines) in each solvent being tested.

TEST 1 sample – 'home-made' amphetamine of at least 95 % purity as measured by HPLC analysis against a standard SIGMA amphetamine sample.

TEST 2 sample – TEST 1 amphetamine (20 % w/w), lactose (40 % w/w) and caffeine (40 % w/w) mixed thoroughly.

6.3.3. Preparation of Solvents

Eicosane, $C_{20}H_{42}$ was added as an internal standard to each extraction solvent at a concentration of 10 μg/mL.

All samples were kept refrigerated when not in use and allowed to come to room temperature for an hour before analysis.

All samples were homogenised thoroughly using an ether slurry method followed by drying using a 'cement-mixer' approach using a rotary evaporator without vacuum or heating.

6.3.4. Experimental Design

The variables studied were:

Buffer type – citrate (pH 6), citrate (pH 6.5), phosphate (pH 7), phosphate (pH 7.5), TRIS (pH 8.1)

Buffer concentration – 0.1 M, 0.5 M, 1 M

Buffer volume – 2 mL, 15 mL

Solvent type – iso-octane, toluene, dichloromethane, ether, ethyl acetate

Solvent volume – 200 μL, 600 μL, 200 μL x 3, 600 μL x 3

Matrix – pure amphetamine, bulked amphetamine

Taking all of these variables into account in every conceivable combination would result in 3600 individual experiments (5x3x2x5x4x2x3) which was practically impossible within the allotted time.

It was concluded that buffer type, concentration, solvent type and matrix effects are dependant on each other and all have an influence on the extraction of each target compound. The solvent and buffer volumes, however, will possibly behave in a more predictable manner and therefore were maintained at a constant level throughout the optimisation of the other parameters. It was assumed that the buffer and solvent volume would probably affect the overall recovery of the entire set of target compounds and would therefore be optimised in a second set of experiments.

Therefore, the first experimental design was as follows –

Factor 1 **Buffer pH** – 5 possibilities
Factor 2 **Buffer Concentration** – 3 possibilities
Factor 3 **Solvent Type** – 5 possibilities
Factor 4 **Matrix Effect** – 2 possibilities

This design requires only 150 extractions and analyses. No replicates of extracts are included in this section of the experimental work since these will be evaluated in the later stages of the optimisation of the extraction procedure.

6.3.5. Extraction Procedure

200 mg of either TEST 1 or TEST 2 (see 6.3.2 for explanation) sample was dissolved in 4 mL of buffer (of specific pH and concentration). Samples were sonicated for 30 minutes, the pH was measured and readjusted to the default value (the original value of the buffer) using 1 M NaOH or HCl as appropriate. Samples were sonicated for a further 30 minutes. 200 µL of solvent was added to the buffer and sample sonicated again for 30 minutes. The solvent was removed from the surface of the buffer and placed in a GC vial ready for analysis.

6.3.6. Results and Discussion

6.3.6.1. Initial Difficulties Encountered with Buffers and Solvents

Buffers

The use of 1 M phosphate buffer was problematic. When prepared, the salts are difficult to dissolve in distilled water. Although they do eventually dissolve, leaving the buffer on a bench over a weekend results in the crystallisation of the salts in the buffer and the solution then required gentle heating to re-dissolve the crystals. Since this solution was almost saturated, the ability of this buffer to dissolve the amphetamine sample was greatly diminished and often, the sample completely solidified after sonication.

The readjustment of the sample to the original pH after adding the amphetamine should also be avoided. This stage is time consuming and, since it involves placing a pH electrode in the sample, adds an extra element of error into the experiment as inevitably, some of the sample adheres to the electrode. This will affect the extraction result since it does not occur in a reproducible manner. In addition this also introduces the issue of cross contamination of samples in a working laboratory. Also, the readjustment of pH involves the addition of more volume to the sample so the 4 mL original buffer volume is altered and therefore affects the true comparability of extractions. The only way to avoid the pH readjustment is to improve the buffering capacity of the buffer by increasing the concentration of the buffer salt although this would also lead to saturation of the buffer and solubility problems when samples are dissolved. Most problems in pH readjustment were found when using the citrate buffers. The pH took longer to stabilise in this buffer and pH readjustment took longer than either phosphate or TRIS®.

Solvents

Since this project was collaborative it was the decision of the group as a whole to include dichloromethane, ethyl acetate and diethyl ether in this section. Even although some practical problems may arise from the use of these solvents it was thought that if they proved to be excellent in terms of extraction, the practical problems could be worked around. The use of dichloromethane in the extraction process was almost impossible. Dichloromethane, which sinks below the buffer was difficult to remove without also removing some of the buffer. In addition, when using the bulked up amphetamine, the dichloromethane extracted a

huge proportion of caffeine and lactose. This caused the syringe to stick, causing the autosampler to stop and the sequence could not be run smoothly leading to failed injections and analysis had to be performed manually. Ether also caused problems because of its volatility. The 200 µL volume of solvent, which was added originally, was greatly diminished by evaporation during the sonicating step (which causes a slight increase in the temperature of the samples) even though the vials used for extraction were sealed. Since the solvent volume remaining was small and spread over a relatively large surface area, it was difficult to remove. Subsequently, the decision was made to avoid using these solvents from further experiments.

6.3.6.2. Combinations of Buffer and Solvents

The chromatograms in Fig 6.2, 6.3, 6.4 and 6.5 show examples of the extracts obtained using different buffer and solvent combinations.

Tables 6.1- 6.3 contain the relative peak areas for each of the target impurities using each of the different buffers for iso-octane, toluene and ethyl acetate solvent extractions. The values for each identified impurity are given for the TEST 1 sample. The total peak area for the TEST 1 sample is also shown (total extract) as well as the total peak area for the TEST 2 sample (excluding amphetamine and caffeine peaks). Dichloromethane and ether chromatograms were not analysed further for reasons discussed previously.

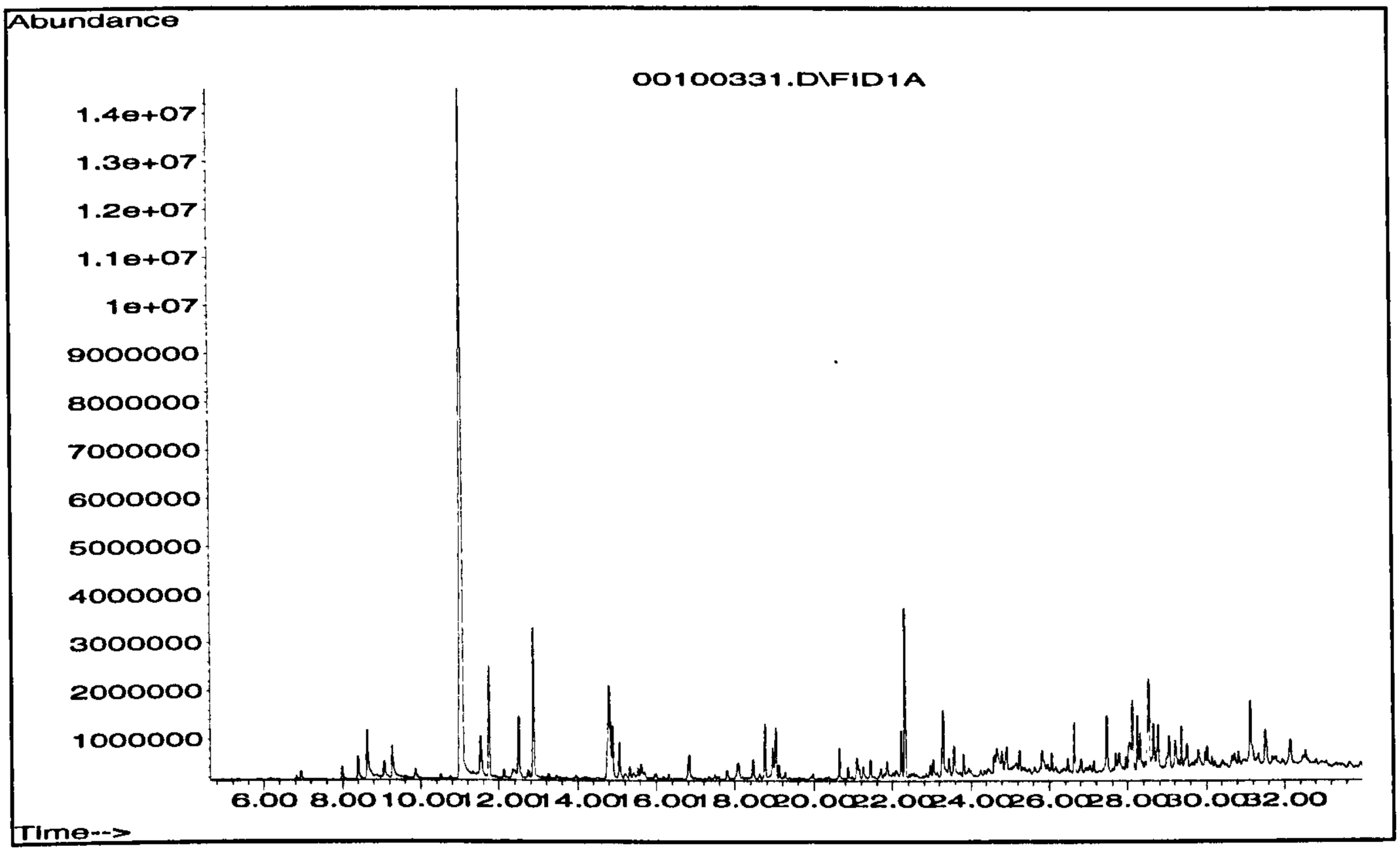


Figure 6.2 Citrate buffer, pH 6.2, 1 M, dichloromethane extract, TEST 2 sample

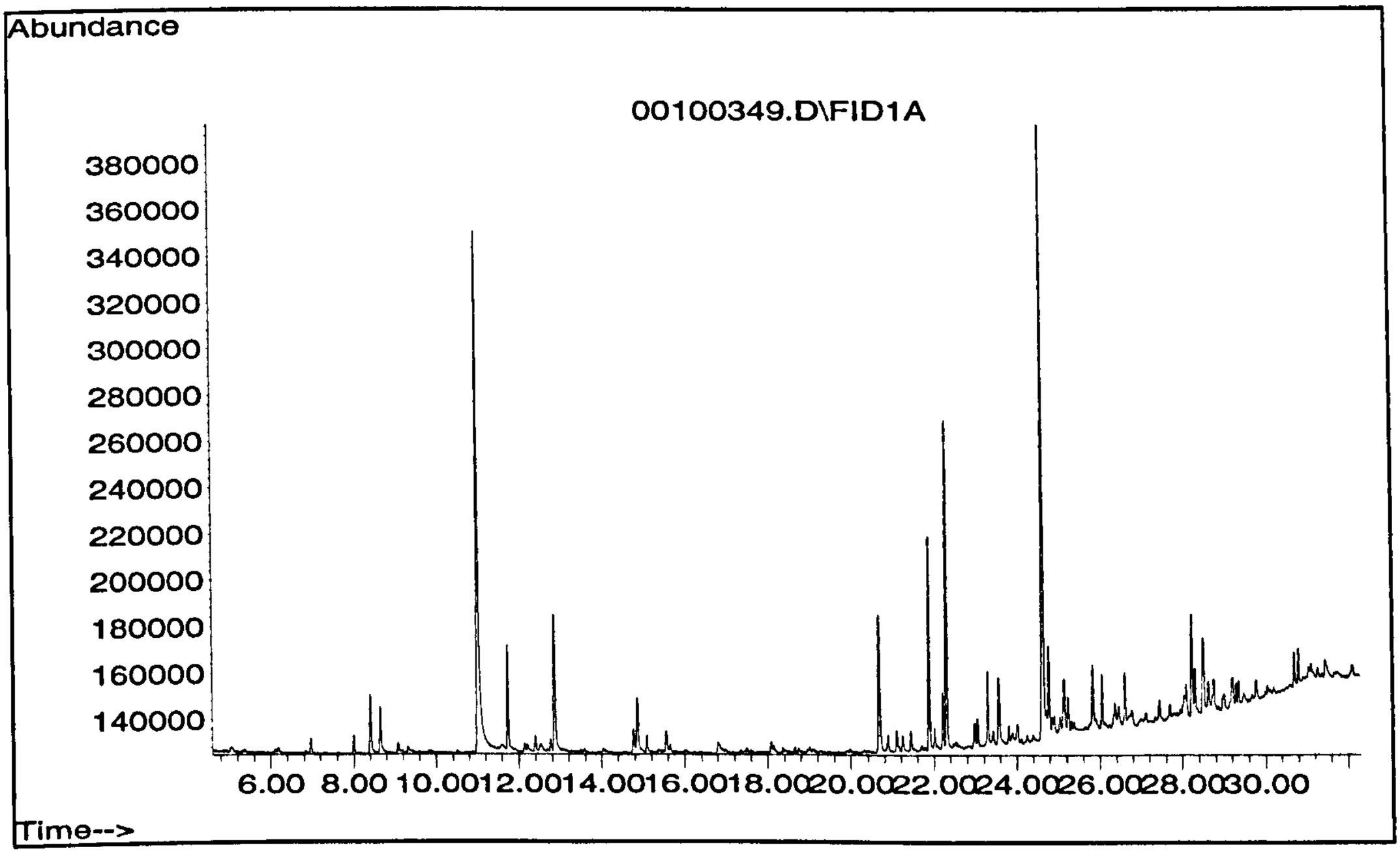


Figure 6.3 Phosphate buffer, pH 7, 0.1 M, iso-octane extract, TEST 2 sample

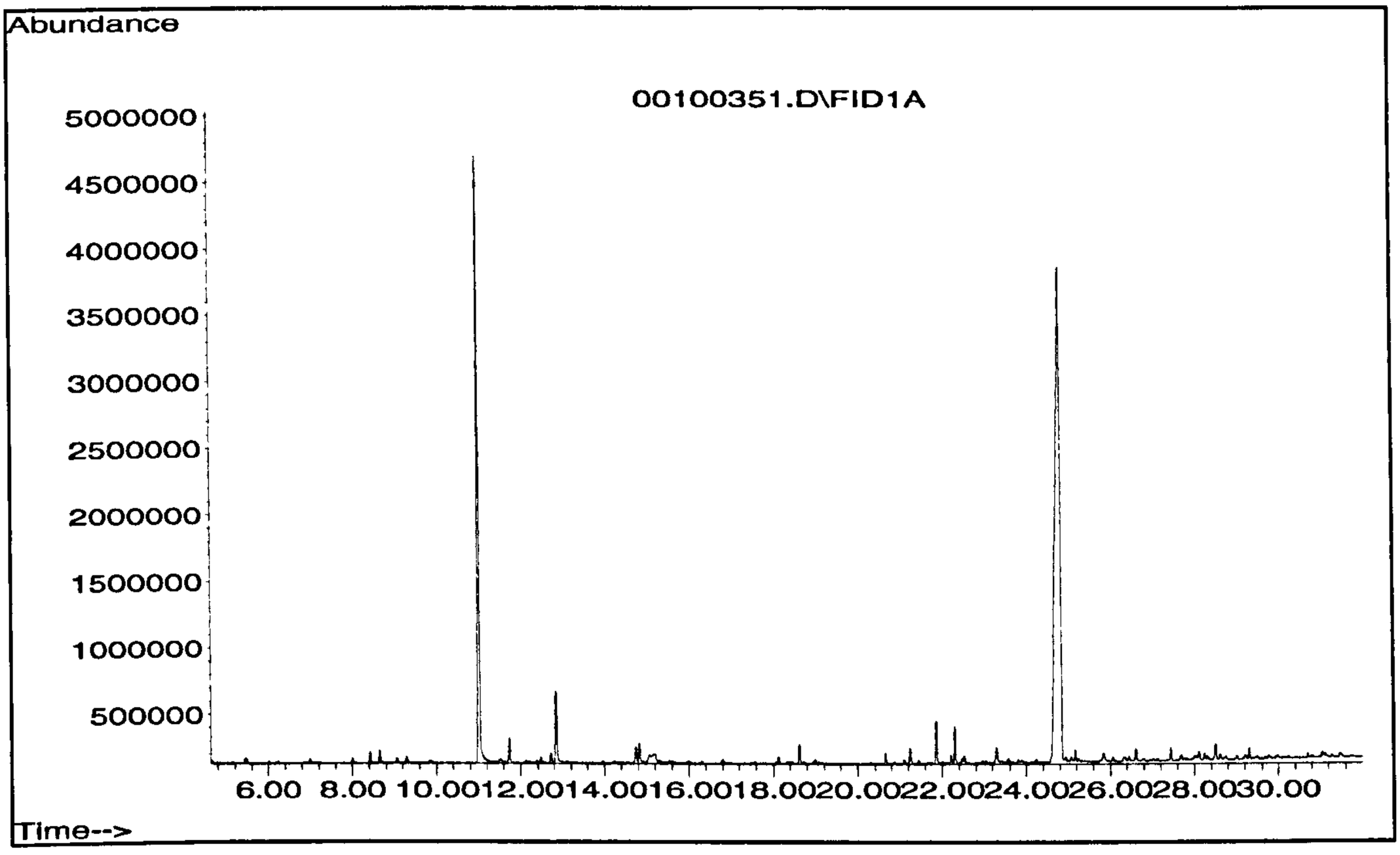


Figure 6.4 TRIS buffer, pH 7.9, 0.1 M toluene extract, TEST 2 sample

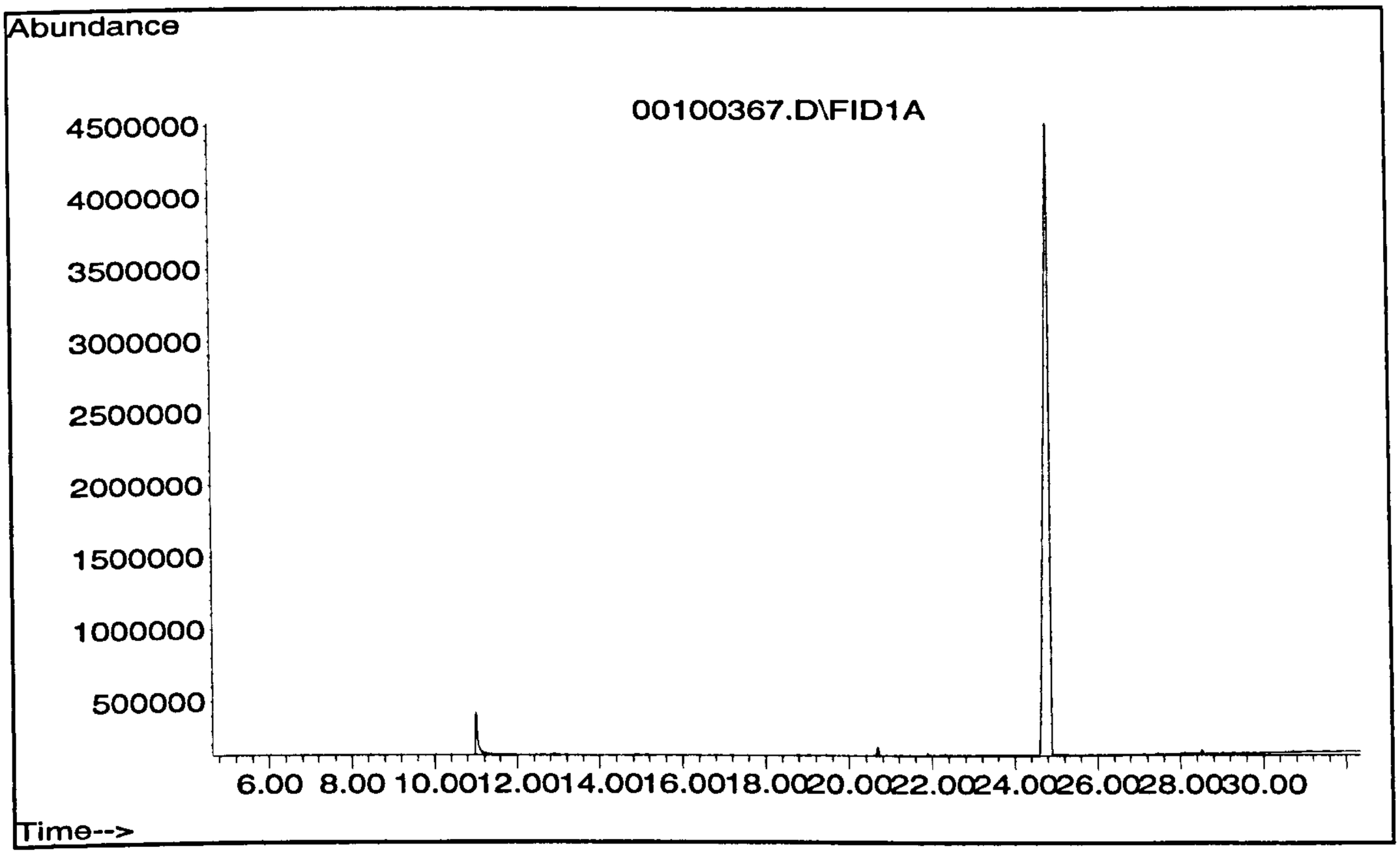


Figure 6.5 Citrate buffer, pH 6.2, 1 M ethyl acetate extract, TEST 2 sample

Iso-octane data – TEST 1 and 2 samples

	citrate pH6.2 0.1M	citrate pH6.2 1M	phosphate pH7 0.1M	phosphate pH7 1M	tris pH7.9 1M	tris pH7.9 0.1M
benzaldehyde	0.680	0.520	2.469	0.529	0.545	0.541
amphetamine	7.062	6.642	34.133	20.235	24.924	47.013
aziridine 1	0.000	0.000	0.000	0.000	0.000	0.000
aziridine 2	2.789	2.648	2.850	5.097	8.886	9.238
ketoxime	3.684	4.914	2.393	5.418	2.565	4.892
nitrostyrene	0.107	0.135	0.000	0.055	0.044	0.090
benzaldiimine	4.570	4.595	17.469	2.617	7.226	2.650
reduced form	4.962	6.522	2.130	9.075	10.904	10.416
total extract	16.792	19.334	27.312	22.791	30.170	27.827
TEST 2 total	2.218	1.716	5.077	1.093	2.981	7.594

Table 6.1 peak areas for impurities in iso-octane relative to internal standard

From the data in Table 6.1, it can be seen that one of our target compounds, an aziridine, is missing from these extracts. The solubility of the compound in iso-octane was then tested and it was found to be only partially soluble. This could explain why other compounds in the sample are preferentially extracted into the solvent layer. The problems in using the saturated solution of phosphate buffer can be seen and the results indicate that when the concentration of the buffer is decreased, the relative peak areas of extracted analytes from the bulked up sample (Test 2) is increased almost five-fold. From this data, the TRIS buffer appears to give the greatest concentration of target analytes at both 1M and 0.1M concentrations when using iso-octane as an extraction solvent.

Toluene data – TEST 1 and 2 samples

	citrate pH6.2 0.1M	citrate pH6.2 1M	phosphate pH7 0.1M	phosphate pH7 1M	Tris pH7.9 1M	tris pH7.9 0.1M
Benzaldehyde	1.436	2.590	0.871	0.861	0.373	0.486
Amphetamine	36.231	53.854	139.096	111.709	235.631	300.809
aziridine 2	13.912	13.086	19.961	21.637	13.928	24.743
ketoxime	16.874	12.499	17.489	18.333	9.677	13.490
aziridine 1	2.509	1.279	2.721	1.981	0.281	1.692
nitrostyrene	0.346	0.232	0.287	0.255	0.000	0.193
benzaldiimine	1.816	12.821	0.903	1.552	2.020	0.966
reduced form	10.687	8.909	11.314	10.973	7.883	10.268
Total extract	47.580	51.416	53.545	55.592	64.161	51.837
TEST 2 total	12.915	6.638	10.854	15.296	21.661	7.319

Table 6.2 Peak areas for impurities in toluene relative to internal standard

The data contained in Table 6.2 shows that the toluene appears to extract target analytes from the buffers much more effectively than iso-octane, especially when used in combination with the TRIS buffer. However, this buffer again appears to allow more

amphetamine into the solvent rather than holding it in the aqueous phase. This large amphetamine peak could have the effect of masking impurities that elute at a similar retention time.

From the data in Table 6.3, it can be seen that using the TRIS buffer again gives the highest concentration of target impurities. However, from the results of the stability study (Chapter 4) it was known that ethyl acetate could degrade samples if left for a few days. Therefore, even although the results seemed to suggest that the ethyl acetate was relatively successful as a solvent in the extraction study, the iso-octane and toluene were preferred.

Ethyl acetate data – TEST 1 and 2 samples

	citrate pH6.2 0.1M	Citrate pH6.2 1M	phosphate pH7 0.1M	phosphate pH7 1M	tris pH7.9 1M	tris pH7.9 0.1M
Benzaldehyde	0.238	0.575	0.147	0.505	1.253	1.475
Amphetamine	27.223	34.259	91.559	126.577	150.515	119.777
aziridine 2	6.000	1.689	1.245	8.931	13.817	14.672
Ketoxime	5.687	2.187	1.356	9.100	11.289	9.027
aziridine 1	0.481	0.614	0.171	1.410	1.592	2.296
Nitrostyrene	0.000	0.000	0.000	0.084	0.000	0.054
benzaldiimine	0.246	0.757	0.339	0.704	0.647	0.750
reduced form	3.092	0.963	0.871	4.900	6.579	6.532
Total extract	15.744	6.783	4.129	25.634	35.176	34.805
TEST 2 total	8.605	0.606	6.652	6.546	13.382	2.259

Table 6.3 peak areas for impurities in ethyl acetate relative to internal standard

From these results along with the results of the other laboratories, the use of the TRIS buffer emerged as the buffer of choice since the recovery of target analytes using the toluene, iso-octane and ethyl acetate was, generally higher using the TRIS buffer. Also, the recovery of impurities using toluene as extraction solvent was higher overall than the other solvents. This could be explained by the fact that toluene is an aromatic compound as are most of our target analytes. However, iso-octane was also an option since, although the recovery of target analytes was lower, the chromatography was 'cleaner' because caffeine and amphetamine extraction was lower.

6.4. Further Optimisation of Extraction Procedure

6.4.1. Introduction

After the results of the previous section were considered, it was decided to further optimise the system by studying the 'best' conditions in more detail. The buffer type was narrowed down to TRIS and phosphate buffers in the pH range 7-8 and using only 1 M (note that we had already experienced solubility problems with 1 M phosphate buffer in the previous section but the group as a whole wanted to include this buffer) and 0.5 M concentrations to increase their buffering capacity. The solvent types now under consideration were toluene and iso-octane along with the possibility of using a mixture of both solvents to minimise the extraction of amphetamine and caffeine but maximise target impurity extraction.

6.4.2. Experimental Design

The buffers studied were TRIS at pH 8.28, 8.10, 7.65 and phosphate at pH 7.01, 7.20 and 7.65. Both solvents were considered individually and as mixtures at 50 % toluene, 14.6 % toluene and 85.4 % toluene in iso-octane. The experimental design consists of star points – buffers at pH 7.01 and 8.28 or solvents at 100% toluene or iso-octane. These conditions are the extremes of possible pH values. The cube points comprise buffers at pH 7.2 and 8.1 with solvents at 14.6 % or 85.4 % toluene. Centre samples were also studied at pH 7.65 and with 50 % toluene. In this experiment, the buffer and solvent volumes are maintained at a constant level since, as in the previous section, they have are expected to have a more simple impact on extraction behaviour.

A diagrammatic explanation of this experimental design in Figure 6.6 shows more simply how analysis of different buffer/solvent combinations should establish the optimum conditions for extraction.

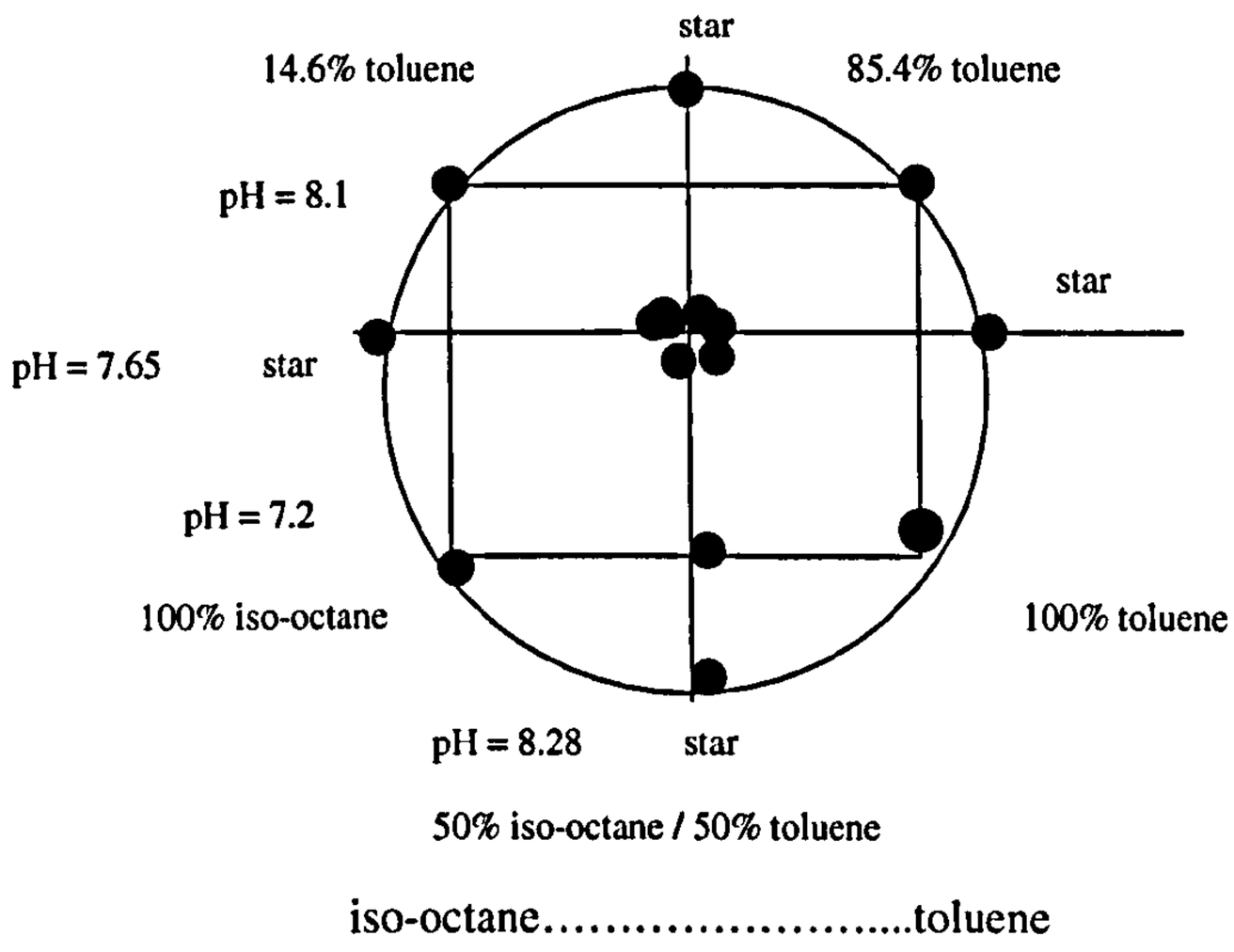


Figure 6.6 Diagrammatic view of experimental design

The pH meter in use was calibrated before preparation of every buffer. Buffers were prepared as in the previous section. Iso-octane and toluene solvents were prepared with internal standard at 10 µg/mL. $C_{20}H_{42}$ was selected as the internal standard for this stage of the experiment.

Only TEST 2 sample (amphetamine, caffeine and lactose matrix- see 6.3.2) was used. 400mg of sample was dissolved in 4 mL of buffer and 400 µL of solvent was used. Each combination of parameters was analysed in duplicate. The extraction combinations are outlined in Table 6.4.

Extracts were analysed using the optimised GC method using FID and MSD detection. Inlet temperature was 250°C (as decided by the group as a whole) and the temperature program at 8°C/min from 60°C to 300°C. The split method was used in the first instance with a 1:20 split ratio. In addition, a splitless method was also used since the level of impurities being extracted was very low. Relative peak areas were then calculated using the peak areas of the target compounds integrated using the Chemstation software and manually as a further check. Peak purity was also checked using the MSD traces to ensure that no co-elution had occurred.

Sample No.	Sample Type	Buffer Type	Buffer pH	% toluene
1	Star point 1	Phosphate	7.01	50
2	Star point 2	TRIS	8.28	50
3	Star point 3	Phosphate	7.65	0
4	Star point 4	Phosphate	7.65	100
5	Star point 5	TRIS	7.65	0
6	Star point 6	TRIS	7.65	100
7	Cube point 7	Phosphate	7.2	14.6
8	Cube point 8	TRIS	8.1	14.6
9	Cube point 9	Phosphate	7.2	85.4
10	Cube point 10	TRIS	8.1	85.4
11	Centre point 11	Phosphate	7.65	50
12	Centre point 12	Phosphate	7.65	50
13	Centre point 13	Phosphate	7.65	50
14	Centre point 14	TRIS	7.65	50
15	Centre point 15	TRIS	7.65	50
16	Centre point 16	TRIS	7.65	50

Table 6.4 Experimental Design

Blank samples were included after every third sample injection. These blank samples consisted of either iso-octane or toluene, treated in the same way as an extract except that the buffer, which they had been in contact with, contained no sample. This was to check that no impurities that may have been present in the buffer were being pulled out by the solvent. In addition, Grob samples were run at the beginning and end of the sample sequence to ensure that the instrument was performing correctly.

6.4.3. Results and Discussion

Figure 6.7 shows graphically the average relative peak area of the benzaldimine impurity for each extraction method with the data label on each point showing the extraction method used. The 'centre phos' and 'tris' labels are the average values for the 'centre' samples using phosphate and tris buffers respectively. The same graphs were plotted for each of the target analytes and the six best sets of conditions (1^{st}, 2^{nd}, 3^{rd} etc) are listed in Table 6.5 below. The best set of conditions for benzaldimine therefore are 'star 6', followed by 'star 4', 'centre phos', 'star 5', 'star 1' and 'cube 9'.

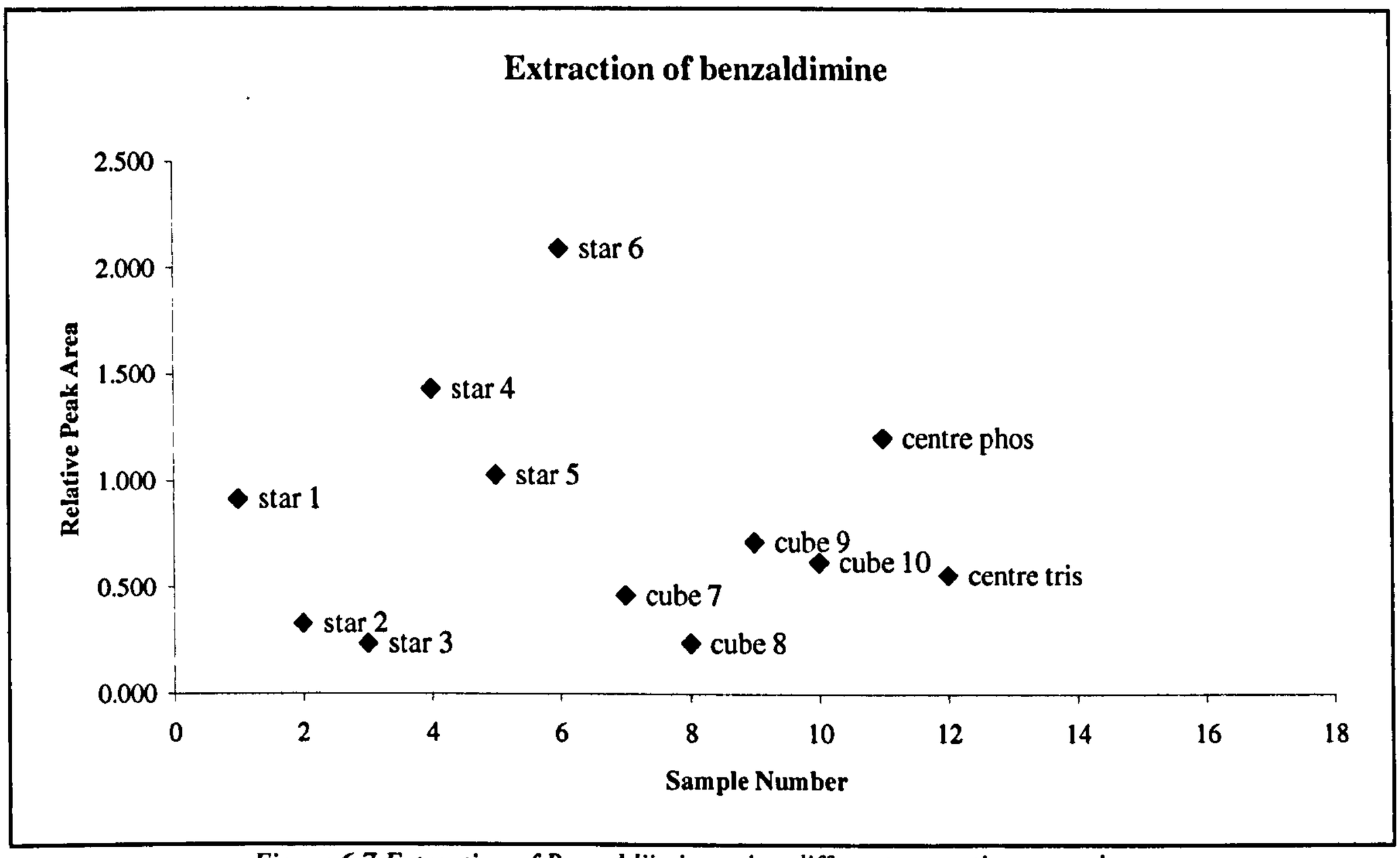


Figure 6.7 Extraction of Benzaldiimine using different extraction procedures

Compound	1st	2nd	3rd	4th	5th	6th
benzaldehyde	Star 4	Star 6	Cube 9	Cube 10	Star 1	Centre phos
aziridine 2	Centre phos	Star 6	Centre tris	Cube 10	Star 1	Cube 9
ketoxime	Star 6	Centre phos	Cube 10	Star 1	Cube 9	Centre tris
aziridine 1	Star 6	Star 1	Cube 10	Star 4		
nitrostyrene	Star 1	Star 6	Cube 10	Centre phos		
benzaldiimine	Star 6	Star 4	Centre phos	Star 5	Star 1	Cube 9
Reduced form	Centre phos	Star 6	Star 5	Star 1	Centre tris	Cube 9

Table 6.5 Six optimum conditions for extraction procedure

It can be seen from these results that the extraction method – 'star 6' appears to have the best overall conditions for the extraction of all target analytes. This method used tris buffer at pH 7.65 and toluene as extraction solvent. It should also be noted that some extraction methods, for example the 'centre phosphate' extraction are good for some analytes but not others. The intention of this experiment, however was to determine the best overall extraction method. When combined with the data from the other laboratories, it was found that a pH slightly higher than 7.65 was necessary for the other routes of synthesis but toluene was the best extraction solvent in terms of pulling out the highest concentration of impurities overall. Using a higher pH buffer would involve using pH 8.1 buffer which would be the equivalent of a Cube 10 sample which while not the optimum for our route was still one of the better

buffer-solvent combinations. As a consequence, of the collaboration with the other laboratories, buffer at pH 8.1 was used in further work [93].

6.5. Optimisation of Buffer and Solvent Volume

6.5.1. Introduction

The aim of this subtask was to determine whether an increase or decrease in the buffer and solvent volumes from those which had been used throughout the previous part of the project would improve the extraction efficiency or repeatability.

After the previous subtask and discussion between labs had been completed, the two 'best' options for solvent and buffer were as follows. 1 M TRIS at pH 8.1 with toluene as solvent gave the best overall recovery of target analytes. 0.5 M phosphate at pH 7.2 with iso-octane as solvent gave a lower impurity recovery but was seen in chromatograms during the previous section to extract less caffeine into the solvent and may, therefore be better for low amphetamine content matrix.

As well as a comparison of large and small buffer and solvent volumes this subtask also investigated the merits, or otherwise, of multiple extractions versus single extractions. A three-step extraction was also included in the study with both buffer-solvent systems which should extract with almost 100% efficiency. The likelihood is that a three-step extraction would not be practically possible in a busy laboratory and would be unlikely to be implemented in a harmonised method for profiling. However, it was thought that the possibility should be investigated since, as in most routine analysis, the process may eventually be automated and therefore the labour-intensive three-step extraction might be used if it was found to give the best overall extraction efficiency and repeatability.

However, it was also thought that the three-step extraction procedure would increase the relative standard deviation between replicate samples. The increase in human error resulting from three additions of solvent and the necessity of removing as much as possible of the previous solvent from the buffer could result in large errors between replicates.

6.5.2. Experimental Design

The TRIS buffer at pH 8.1 was used in this section. The buffer volumes considered were 2 mL and 8 mL. The solvent volumes used were 200 µL and 600 µL in both single and triple extractions.

Using full factorial design, eight possible combinations result as illustrated in Table 6.6.

Sample Number	Buffer Type	Buffer Vol (mL)	Solvent	Vol (µL)
1	TRIS pH 8.1	2	toluene	200
2	TRIS pH 8.1	2	toluene	3x200
3	TRIS pH 8.1	2	toluene	600
4	TRIS pH 8.1	2	toluene	3x600
5	TRIS pH 8.1	8	toluene	200
6	TRIS pH 8.1	8	toluene	3x200
7	TRIS pH 8.1	8	toluene	600
8	TRIS pH 8.1	8	toluene	3x600

Table 6.6 Experimental design

4 mL or 8 mL of 1 M, pH 8.1 TRIS buffer was added and the sample sonicated for 30 mins. 200 µL of toluene (containing $C_{20}H_{42}$) was added and again the sample was sonicated for 30 mins. At this stage, 50 µL of toluene was removed for analysis of the 1 step procedure.

In the 3 step procedure, 50 µL of the solvent was removed and placed in a GC vial. The remainder of the solvent was removed and discarded. A further 200 µL of solvent was added and the mix sonicated for a further 30 mins. Another 50 µL of this solvent was then removed and added to the first aliquot. This process was repeated once more. The total volume in the GC vial for analysis was then 150 µL. Every possible combination of solvent and buffer volume was carried out in triplicate.

The extracts were then analysed by GC-FID and the peak areas evaluated relative to the internal standard.

6.5.3. Results and Discussion

Table 6.7 illustrates the average relative peak area for each target analyte using each of the possible TRIS buffer/toluene volume combinations and extraction procedures for a TEST 2 (6.3.2) sample. 'total extract' here refers to the sum of the relative peak areas of the identified impurities (excluding caffeine) to obtain an overall estimation of how successful the extraction was.

Analyte	2mL buffer 200uL solvent	2mL buffer 3x200uL solvent	2mL buffer 600uL solvent	2mL buffer 3x600uL solvent	8mL buffer 200uL solvent	8mL buffer 3x200uL solvent	8mL buffer 600uL solvent	8mL buffer 3x600uL solvent
Benzaldehyde	0.86	0.92	0.28	0.356	0.52	0.71	0.19	0.24
Amphetamine	64.10	84.45	52.51	61.22	20.812	40.80	25.16	22.55
Aziridine 2	6.73	4.05	2.22	0.712	4.392	3.08	1.90	0.68
Ketoxime	1.21	0.26	0.43	0.18	0.58	0.30	0.24	0
Aziridine 1	0.22	0.016	0	0	0.20	0	0	0
Nitrostyrene	0.10	0	0	0	0.03	0	0	0
benzaldiimine	11.61	3.17	4.28	2.09	5.39	1.52	3.22	0.95
reduced form	3.18	1.74	1.36	0.509	3.49	1.51	1.28	0.36
Caffeine	108.81	129.07	100.34	109.67	53.28	90.62	62.64	77.02
total extract	**23.923**	10.13	8.57	3.83	14.60	7.12	6.83	2.22

Table 6.7 relative peak areas for target analytes using different volumes of TRIS and toluene

As expected, a 200 µL extraction volume provided a more concentrated sample than a 600 µL extraction volume although this is simply a dilution factor based on 1/3 volume of solvent. Tables 6.8 and 6.9 below show a comparison of the total peak areas of impurities obtained using different buffer volumes with the same solvent volumes and different solvent volumes with the same buffer volumes (B= TRIS buffer and S = toluene).

2mL B	**8 mL B**	
200uL S	200uL S	**% difference**
23.92	**14.60**	**164**
600uL S	600uL S	
8.57	**6.83**	**126**
3x200uL S	3x200uL S	
10.13	**7.20**	**142**
3x600ul S	3x600ul S	
3.83	**2.22**	**172**

Table 6.8 buffer comparison

2mL B 200uL S	2mL B 600uL S	rel peak area	% difference
23.92	**8.57**	**25.71**	**7.48**
2mL B 3x200uL S	2mL B 600uL S		
10.13	**8.57**		
8mL B 200uL S	8mL B 600uL S		
14.58	**6.83**	**20.49**	**40.5**
8mL B 3x200uL S	8mL B 600uL S		
7.12	**6.83**		

Table 6.9 solvent comparison

These results show that the smaller buffer volume gives better results with the concentration of target analytes increasing in the solvent. This is to be expected since the small solvent volume comes into contact with a small buffer volume much more easily than a large buffer volume even with good mixing.

The smaller solvent volume obviously gives a more concentrated sample but the larger solvent volume does pull out a higher concentration of target analytes per unit volume. Here, a compromise must be reached. Using a small volume of solvent may not pull out the maximum quantity of target analytes, the higher concentration of the sample makes it easier to analyse, especially if the extract is fairly 'clean'. Also, the percentage gain in using the larger solvent volume is relatively small (7% gain in analyte concentration for a 200% increase in solvent volume).

The use of the triple extraction method does give a more concentrated sample. Using 600 µL in three 200 µL steps, as opposed to one single step, results in increased peak areas. However, the gain in concentration of analytes is offset by increased extraction time (3 sonication and extraction steps) and practical problems associated with the triple extraction.

The relative standard deviation between the triplicate extraction was also evaluated in this experiment to test the theory that the triple extraction method would have a higher RSD between samples, simply because of the higher levels of human error which could result. Table 6.10 shows the RSD values for each of the extraction methods (B= buffer and S= solvent.

	2mL B 200uL S	2mL B 3x200uL S	2mL B 600uL S	2mL B 3x600uL S		8mL B 3x200uL S	8mL B 600uL S	8mL B 3x600uL S
Benzaldehyde	3.5	5.1	10.1	2.9		8.9	21.1	0.54
Amphetamine	0.023	1.2	17.1	2.0		11.9	25.0	2.4
aziridine 2	2.5	3.3	26.7	3.2		4.0	21.1	0.051
Ketoxime	5.4	24.4	20.9	0.21		31.1	47.2	-
aziridine 1	22.1	-	-	-		-	-	-
nitrostyrene	1.2	-	-	-		-	-	-
benzaldiimine	11.8	13.3	26.0	4.0		8.7	18.4	0.098
reduced form	3.3	2.8	2.7	2.9		5.7	4.3	3.0
Caffeine	2.3	6.0	9.8	4.6		9.0	15.1	4.8
Average RSD	3.8	8.0	16.2	2.8		11.3	21.7	1.8

Table 6.10 RSD values for each extraction method, XmL buffer, XμL solvent

As may be seen from the RSD values obtained for these extracts, the largest RSD values appear to be from the single step 600 μL extraction volume. This may be simply because the actual sample concentration is lower, the impurity levels are lower and the peak areas are so small that, in some cases, peak shape is poor and accurate integration is difficult. Nitrostyrene and an aziridine isomer are only seen in the extract which uses a 200μL solvent volume possibly because this sample provides the most concentrated extract and the nitrostyrene and aziridine levels are low enough in the dilute samples so as not to be detected.

In conclusion, the single 200 μL toluene extraction from 2 mL TRIS buffer gives a concentrated sample with low levels of variation in extraction with RSD values less than 15% for the detected impurities (except benzaldiimine which was already shown to be variable) and was therefore selected as the extraction method of choice for this specific sample matrix.

6.6. The influence of sample matrix

6.6.1. Introduction

The aim of this part of the project was to determine whether the matrix containing amphetamine would have any influence on the effectiveness of the extraction procedure or the analysis. Amphetamine was present at 15%, 50% and 100% of the sample, these concentrations being thought to cover both 'street' amphetamine and amphetamine possibly found at the site of a clandestine lab. The proportion of amphetamine present in the sample

was chosen to represent a wide range of possible sample types. For example 100% TEST 1 sample with no caffeine or sugar added contains around 95% pure amphetamine sulphate which is supposed to represent the sample type which may be found at the scene of a clandestine lab where the amphetamine has not yet been cut. 15% amphetamine is a relatively low amphetamine concentration sample such as may be found in an illicit street sample. 50% amphetamine is relatively high concentration for a street sample but may be found in the case where a main 'dealer' is apprehended before having the opportunity to further dilute the active drug content. In all of these cases, the premise is that, the concentration of the drug present in the sample should make no difference to the overall profile of the organic trace impurities. In other words, the impurity profile of the target compounds should be unaffected by the diluents. The remainder of the matrix was caffeine. No sugar was involved as a bulking agent at this stage since sugars were not extracted into the organic solvent and could not be detected in the chromatograms. The buffer used was pH 8.1 TRIS with toluene used as extraction solvent.

The pH of the buffer used was narrowed down to allow a more rapid analysis of the influence of various matrices on the extraction efficiency and repeatability of the procedure. The TRIS buffer had been shown in the previous subtask to give the most reproducible results. 1M TRIS buffer at pH 8.1 was used, the buffer volume varied between 4 and 8mL and 1 and 3 stage 200 µL toluene extractions used.

The extraction procedure was identical to that in the previous subtask. However, the GC program was changed slightly to give an initial oven temperature of 100°C and the injection volume was increased to compensate for a loss of sensitivity due to the age of the column.

6.6.2. Experimental Detail

The appropriate amount of amphetamine was weighed into a test tube (either 30 mg, 100 mg or 200 mg depending on the matrix under investigation) and the appropriate amount of caffeine added to give a total weight of 200 mg per sample. The sample was then capped and mixed by manual inversion.

The extraction procedure which followed was identical to that described in the previous section. 2 x pH, 2 x buffer volume, 2 x solvent, 2 x processes, 3 x matrix types, 3 x replicates

(144 samples total). The 1 step solvent extraction was 200 μL solvent and the 3 step was 3x200 μL solvent extractions with the combination of the extracts.

Sample	**Sample Matrix**	**Buffer Type**	**Solvent**	**Buffer (mL)**	**Extraction Steps**
5	15%amphetamine	TRIS pH 8.1	Iso-octane	4	1 and 3 step
6	15%amphetamine	TRIS pH 8.1	Iso-octane	8	1 and 3 step
7	15%amphetamine	TRIS pH 8.1	Toluene	4	1 and 3 step
8	15%amphetamine	TRIS pH 8.1	Toluene	8	1 and 3 step
13	50%amphetamine	TRIS pH 8.1	Iso-octane	4	1 and 3 step
14	50%amphetamine	TRIS pH 8.1	Iso-octane	8	1 and 3 step
15	50%amphetamine	TRIS pH 8.1	Toluene	4	1 and 3 step
16	50%amphetamine	TRIS pH 8.1	Toluene	8	1 and 3 step
21	100%amphetamine	TRIS pH 8.1	Iso-octane	4	1 and 3 step
22	100%amphetamine	TRIS pH 8.1	Iso-octane	8	1 and 3 step
23	100%amphetamine	TRIS pH 8.1	Toluene	4	1 and 3 step
24	100%amphetamine	TRIS pH 8.1	Toluene	8	1 and 3 step

Table 6.11 Experimental design

6.6.3. Results and Discussion

Before any numerical analysis of the peak areas obtained for the target impurities was undertaken, the chromatograms of each sample were assessed. The extraction procedure which was thought to be the best overall, ie. a TRIS-toluene extraction, was assessed. Exemplar chromatograms showing the 100%, 50% and 15% amphetamine extracted using this extraction process are shown in Figures 6.8, 6.9 and 6.10. The chromatographic profile is enlarged in Figures 6.11, 6.12 and 6.13.

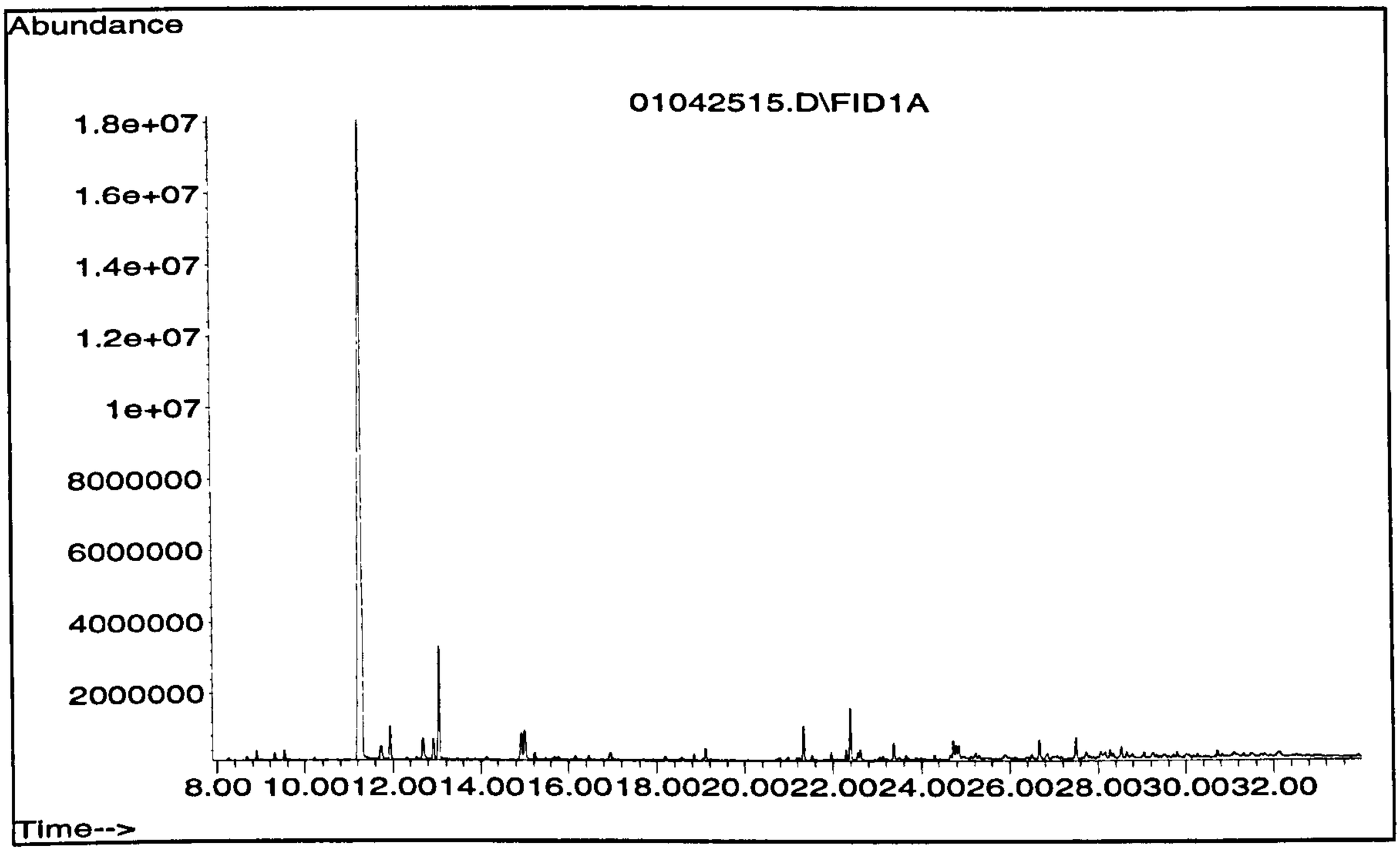


Figure 6.8 100% amphetamine matrix – 4ml tris/ 200µL toluene extraction

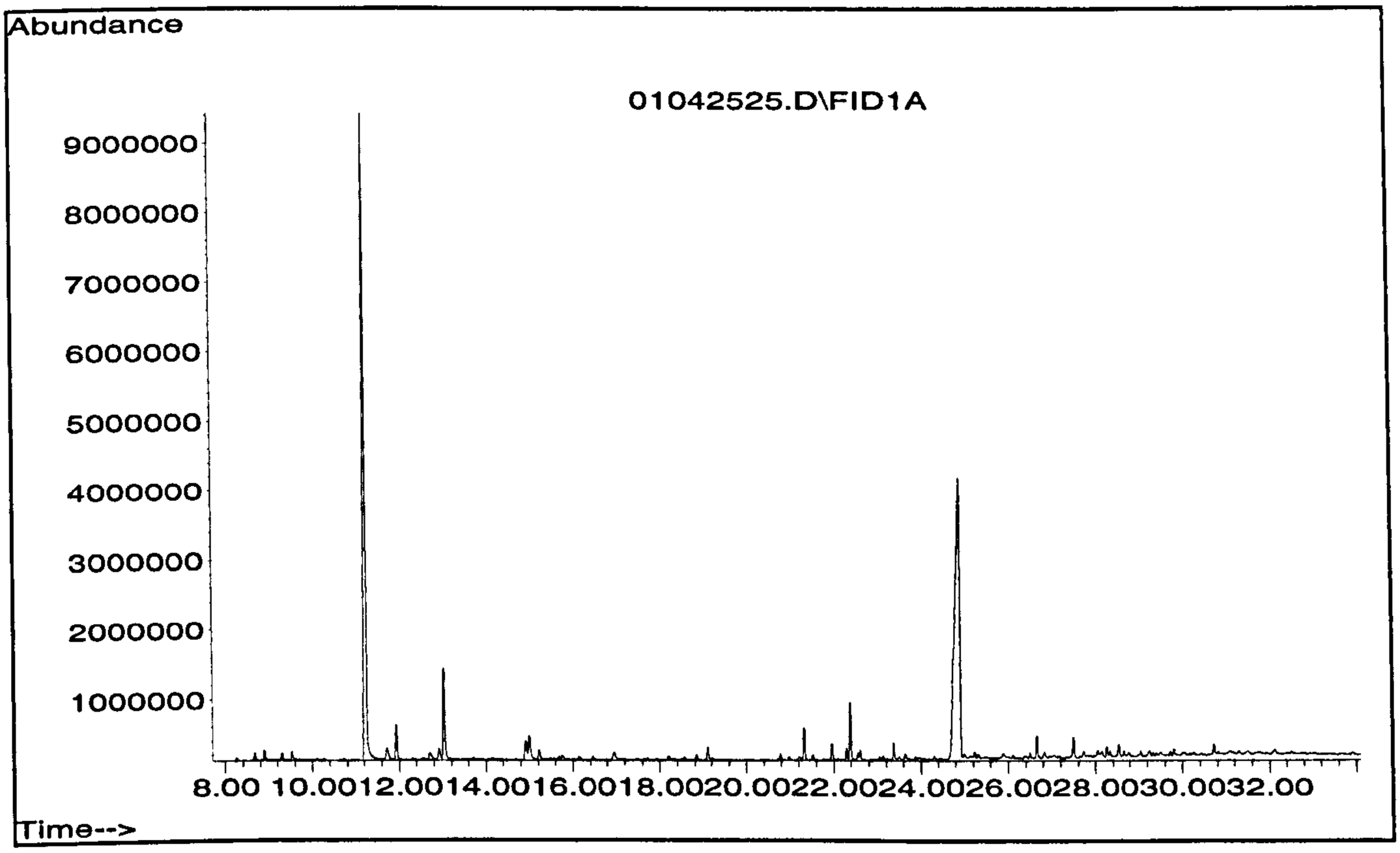


Figure 6.9 50% amphetamine matrix – 4mL tris/200µL toluene extraction

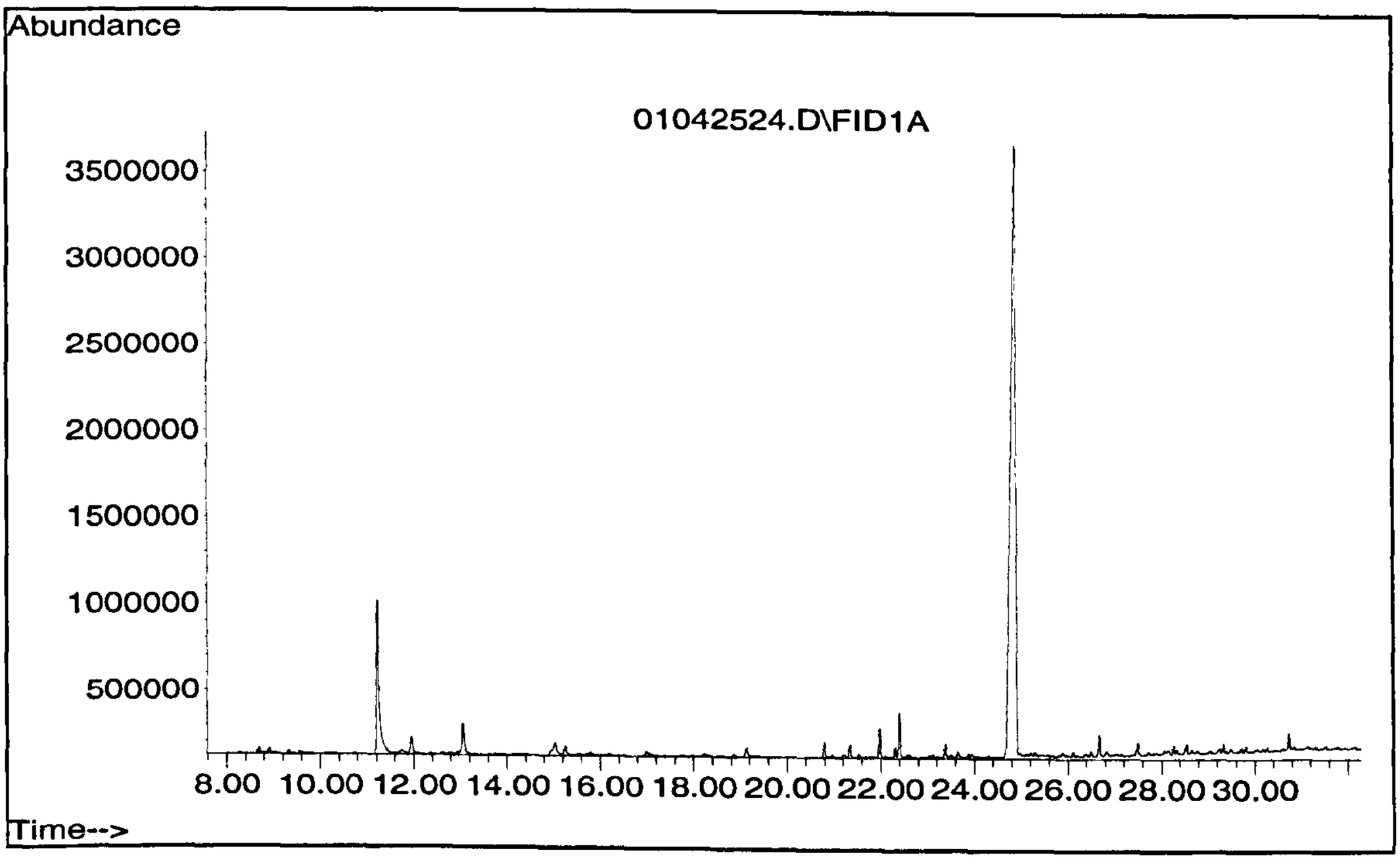


Figure 6.10 15% amphetamine matrix – 4mL tris/200uL toluene extraction

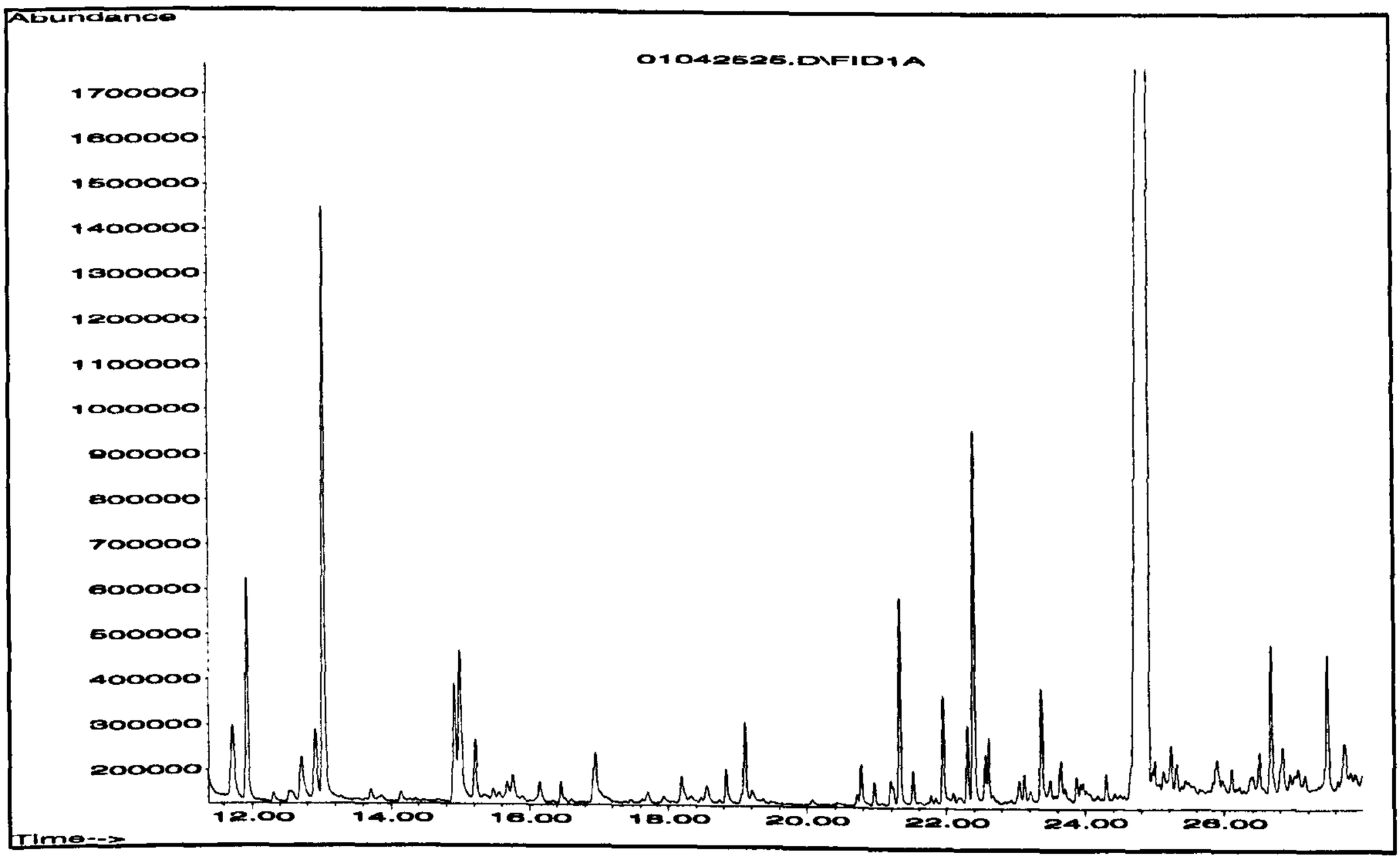


Figure 6.11 Enlarged profile, 100% amphetamine – no diluents

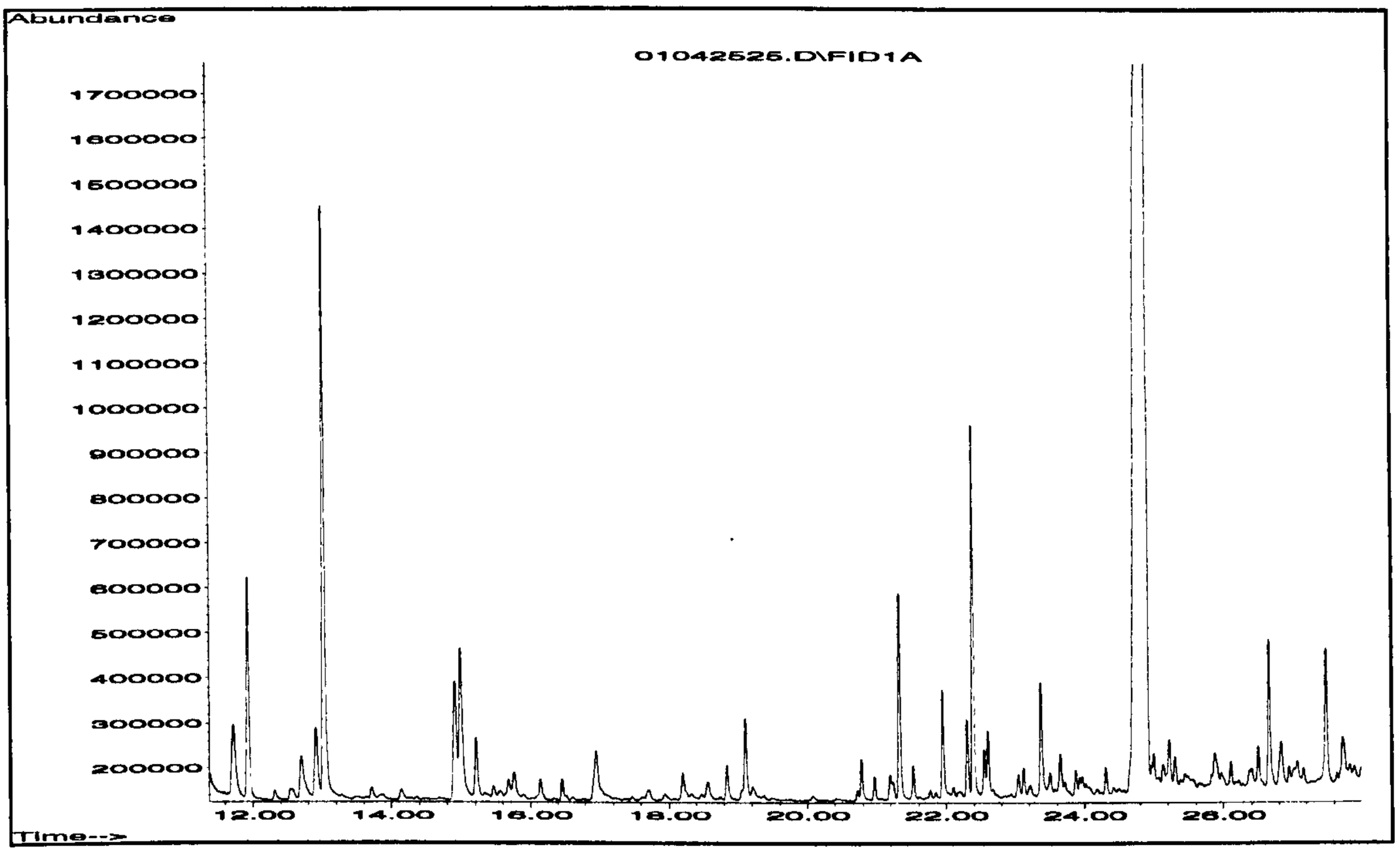


Figure 6.12 Enlarged profile, 50% amphetamine, 50% caffeine

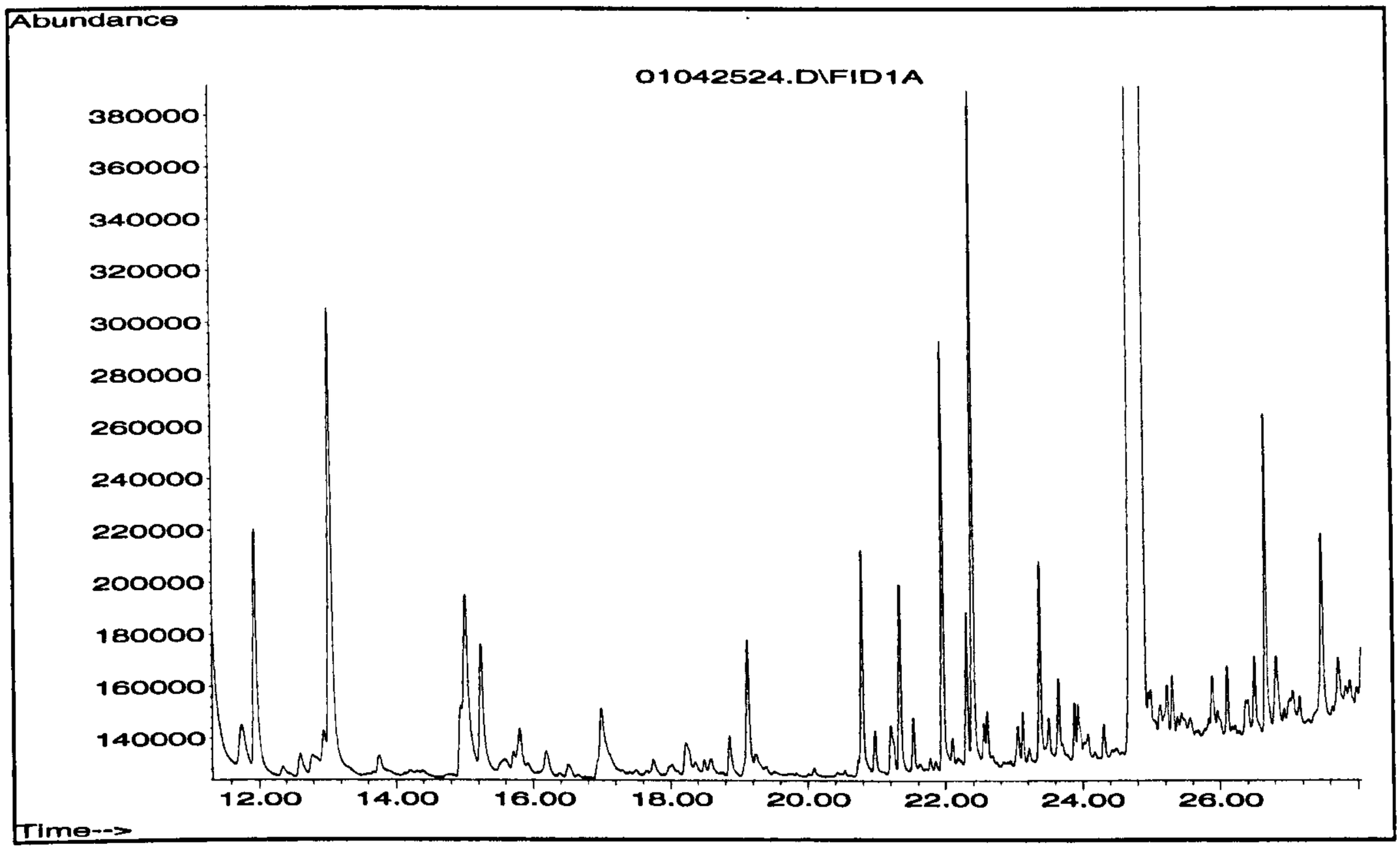


Figure 6.13 Enlarged profile, 15% amphetamine, 85% caffeine

The chromatograms in Figures 6.14, 6.15, 6.16, 6.17 highlight the difference in impurity profile caused by a difference in buffer volume – either a 4 mL or 8 mL TRIS buffer.

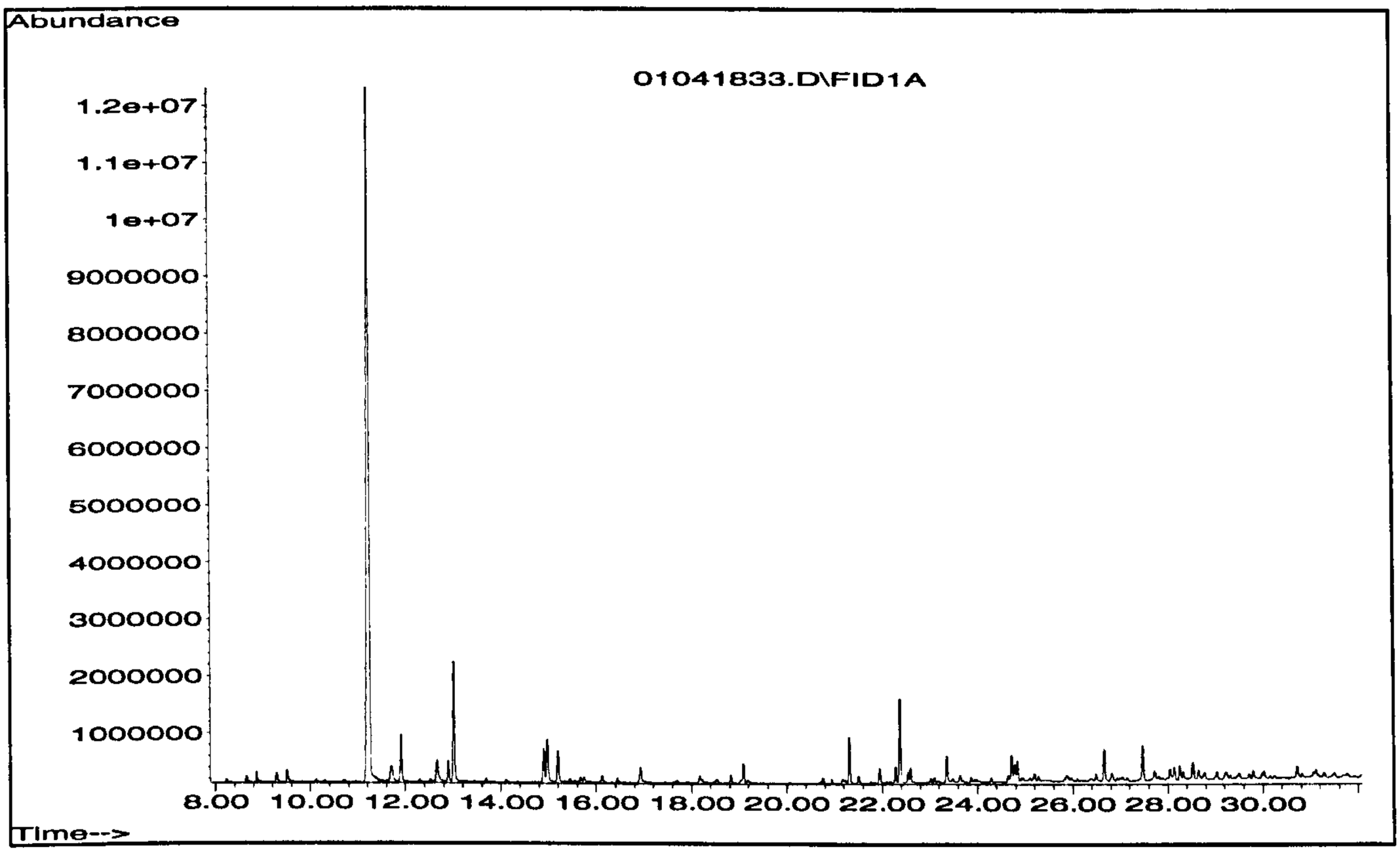


Figure 6.14 100% amphetamine matrix 8mL tris/200uL toluene

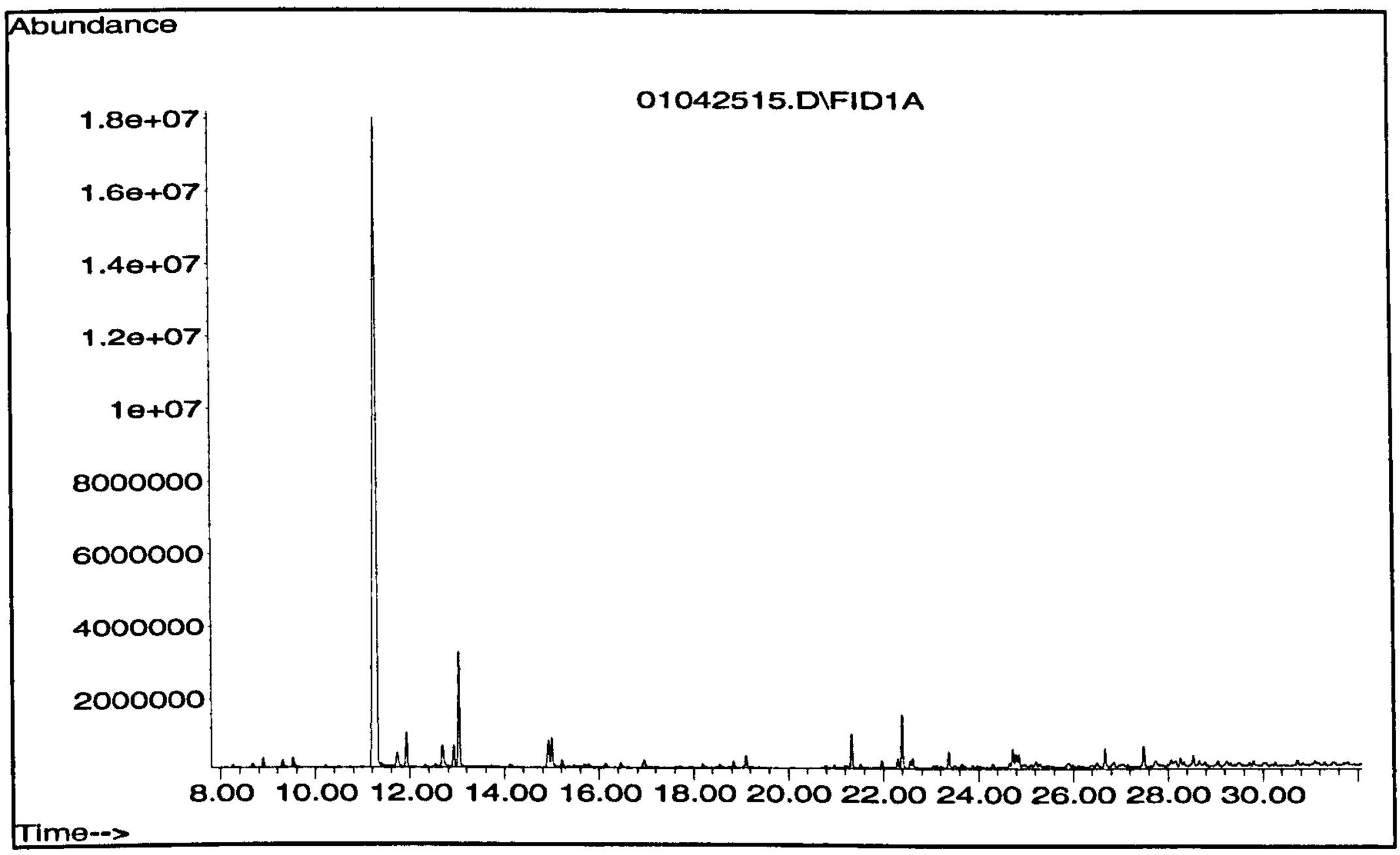


Figure 6.15 100% amphetamine matrix 4mL tris/200uL toluene

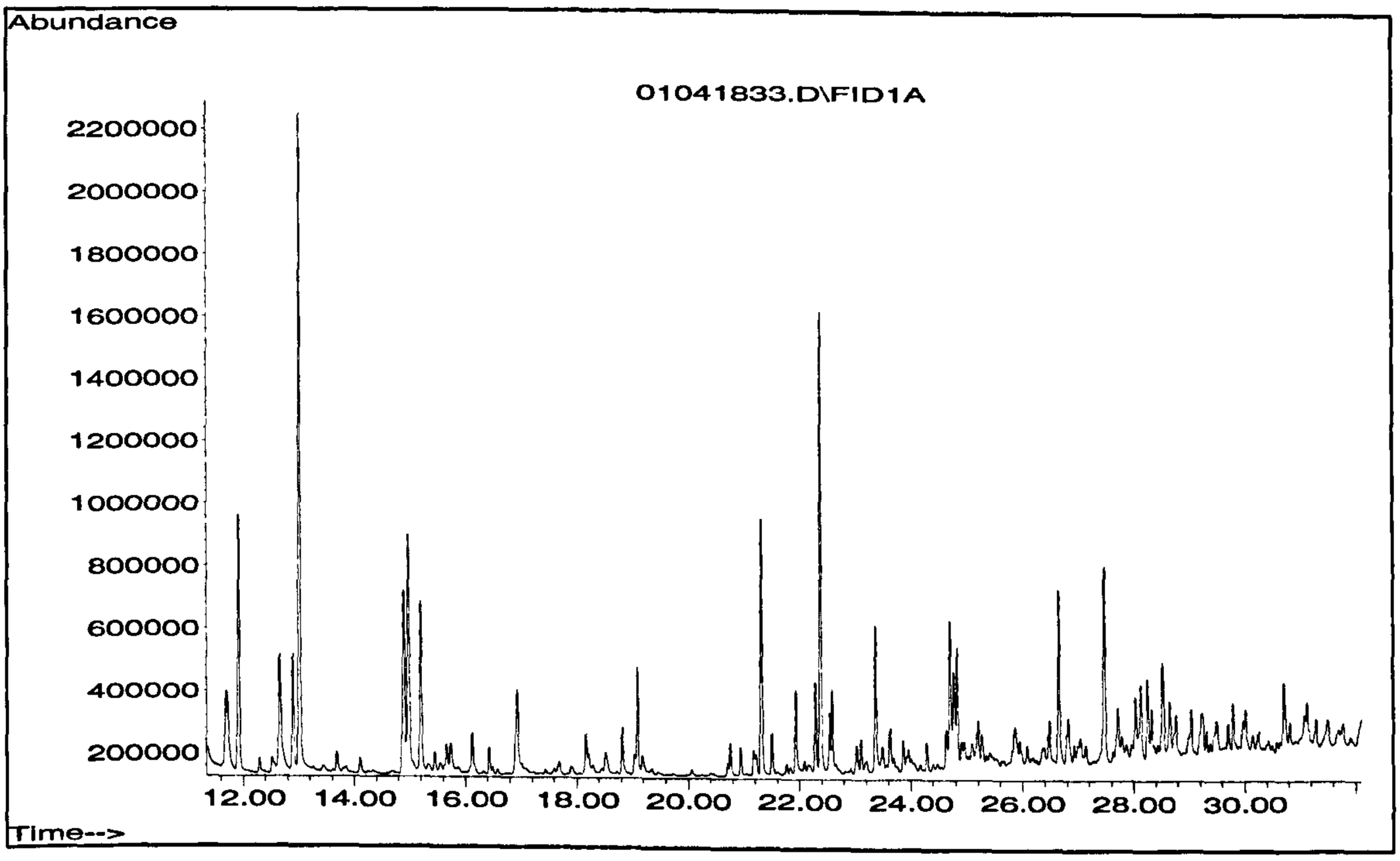


Figure 6.16 Enlarged 100% amphetamine matrix, 8mL tris/200uL toluene

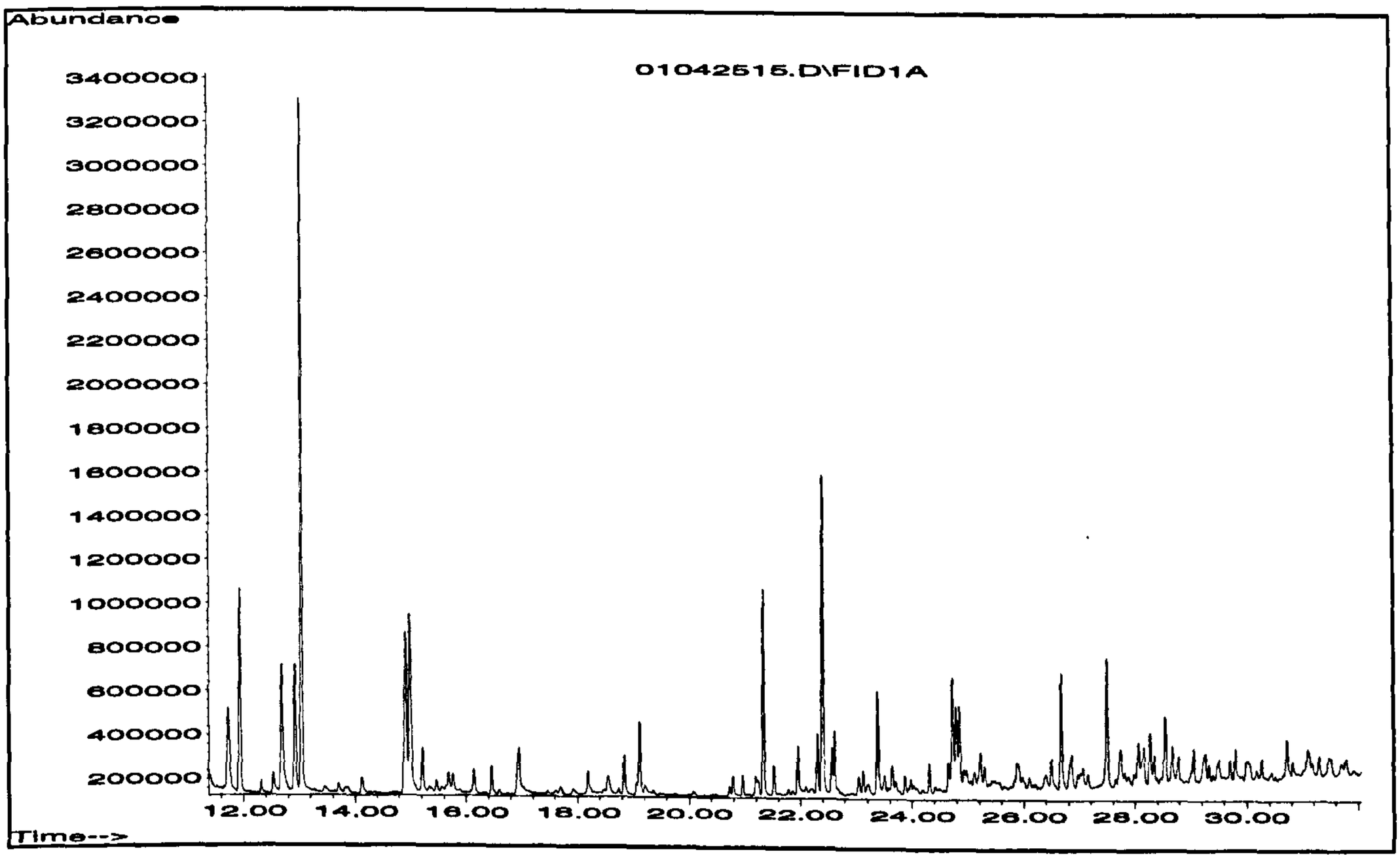


Figure 6.17 Enlarged 100% amphetamine matrix, 4mL tris/200uL toluene

The chromatograms in Figures 6.18 and 6.19 show the difference in impurity profile obtained when using the same buffer but different solvents.

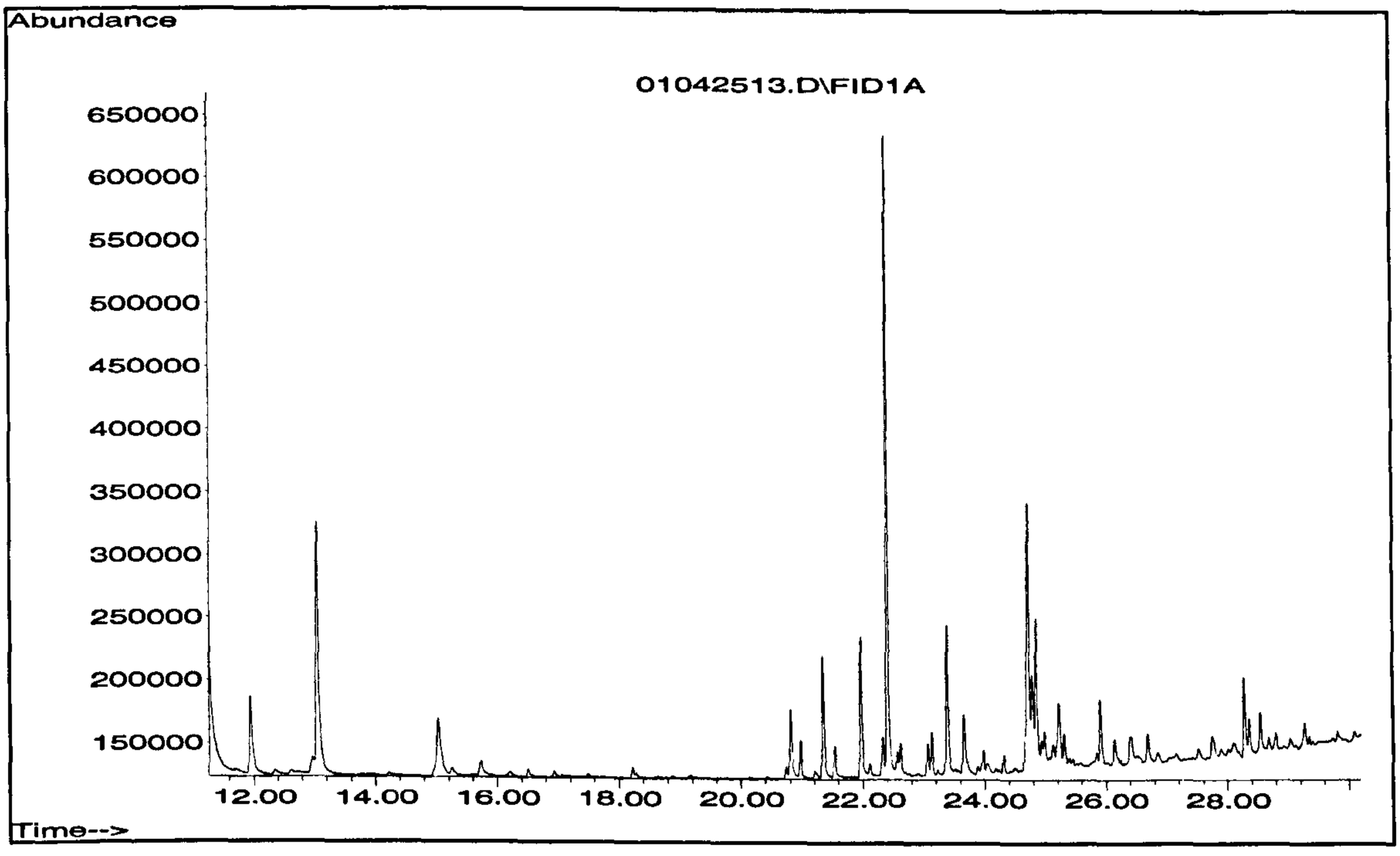


Figure 6.18 50% amphetamine matrix 4mL tris/200uL iso-octane

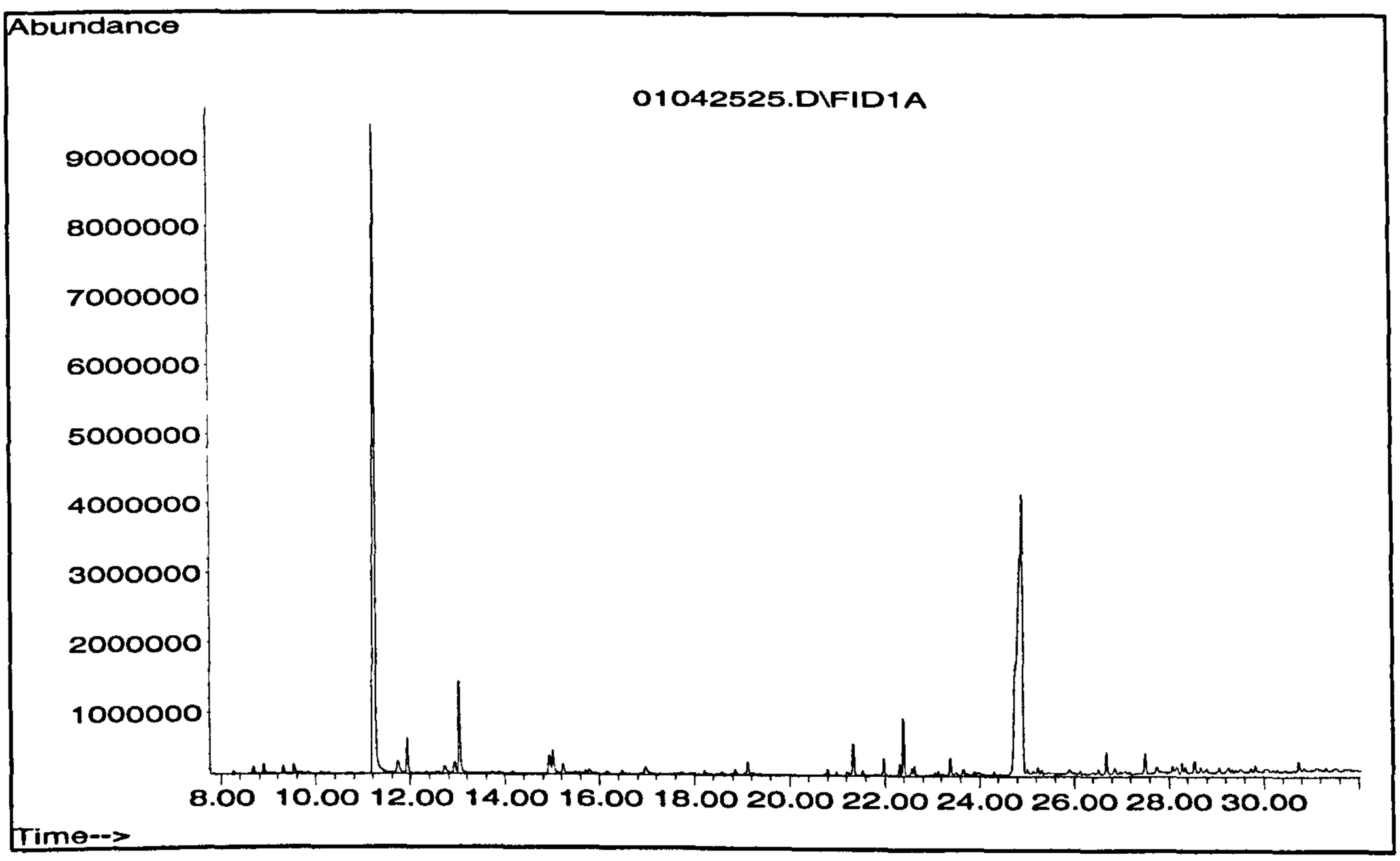


Figure 6.19 50% amphetamine matrix 4mL tris/200uL toluen

6.7. Extended Matrix Study

6.7.1. Aims

The premise which this particular set of experiments were based on, was the theory that the extraction and analysis method should work equally well with concentrated and extremely dilute amphetamine samples. In order that street seized samples may be traced to samples seized in their raw form (straight after production), the impurity profile extracted from both these types of seizures should be easily linked.

This particular study was set up in order to determine if the active drug content or the concentration of diluents added to a sample has an influence on the amphetamine impurity profile. This study also encompasses a review of the buffer volume used as well as the solvent in conjunction with different sample matrices. A multiple extraction was set up in the same way as the previous matrix study. However, in this case, the three extracts were retained and analysed separately to evaluate how successful the first extraction is in pulling out the majority of the impurities and whether subsequent extractions yield enough impurities to merit a multiple-step extraction procedure.

6.7.2. Experimental Detail

The buffer chosen for inclusion in this study was 1M, pH 8.1, TRIS buffer. Each buffer was evaluated using both 4 mL and 8 mL volumes. 200 µL toluene was used in conjunction with both buffer volumes. Each combination of buffer and solvent were extracted and analysed in three individual stages.

In stage one, the 200 µL solvent was combined with the buffer containing the sample, (200 mg of each sample matrix). The mixture was then sonicated for 30 minutes, and centrifuged for 5 minutes. As much as possible of the toluene layer was removed and placed in a GC-vial with insert. A further 200 µL solvent was added to the buffer and the procedure repeated. A third extraction step was carried out and this portion retained separately.

Each of the combinations of matrix, buffer type, volume and solvent type were extracted in multiple steps and replicated three times.

The solvent extracts were analysed using the optimised GC-MS method.

Matrix	Buffer	Volume	Solvent	Extractions
100% amphetamine 0% caffeine	1M, pH 8.1 tris buffer	4mL buffer	Toluene	1 x 200µL solvent
50% amphetamine 50% caffeine		8mL buffer	Iso-octane	2 x 200µL solvent
15% amphetamine 85% caffeine				3 x 200µL solvent

Table 6.12 Experimental Design Extended Matrix Study

6.7.3. Results and Discussion

There are several aspects to consider when assessing the results of these experiments. Firstly, the solvent-buffer combination which extracts the highest concentration of impurities must be considered to be the most suitable to cover a variety of different samples with high levels of amphetamine as well as very dilute samples with few impurities present.

Ideally, the buffer and solvent extract must cope with a high level of diluents or a high level of amphetamine and be able to dissolve both types of matrix to create a homogeneous solution.

The profile of the impurities should be identical, no matter what level of diluents are present, with only the actual concentration of impurities being relative to the amphetamine concentration.

6.7.3.1. Amphetamine Content - Toluene

It was thought that if the amphetamine extraction was reproducible that this would be a good indication that the extraction was working reproducibly in general. In addition, the possibility of using the amphetamine peak as a standard which the impurity peak areas could be compared to (and therefore no internal standard added), would depend on the

reproducibility of the amphetamine peak area and would be directly related to the actual amphetamine content in the matrix.

In order to assess if the amphetamine content of the extractions was variable across replicate samples using a single 200 µL extraction, the amphetamine peaks were integrated and the RSD determined. The relative amphetamine content of each of the extracts from the 100% (no diluent), 50% and 15% matrix is outlined in Table 6.13 (RSD values below 5% are noted in red).

100%		Toluene 1	RSD	Toluene 2	RSD	Toluene 3	RSD
	Tris 4mL	455	21.3	597	6.6	581	10.3
	Tris 8mL	289	5.0	290	4.4	331	4.7
50%		Toluene 1		Toluene 2		Toluene 3	
	Tris 4mL	174	3.4	259	3.6	318	9.0
	Tris 8mL	126	2.4	145	1.5	171	3.3
15%		Toluene 1		Toluene 2		Toluene 3	
	Tris 4mL	81	6.4	68	9.9	84	9.5
	Tris 8mL	45	8.9	45	0.6	46	7.3

Table 6.13 Mean amphetamine content of toluene extracts and relative variation values

These results suggest that (in terms of reproducibility of amphetamine peak area) the 8mL TRIS buffer would be the most suitable. Graphically the relationship for amphetamine extraction from 15, 50 and 100% amphetamine matrix using TRIS buffer and toluene solvent is shown in Figure 6.20. 1, 2 and 3 refer to replicate samples.

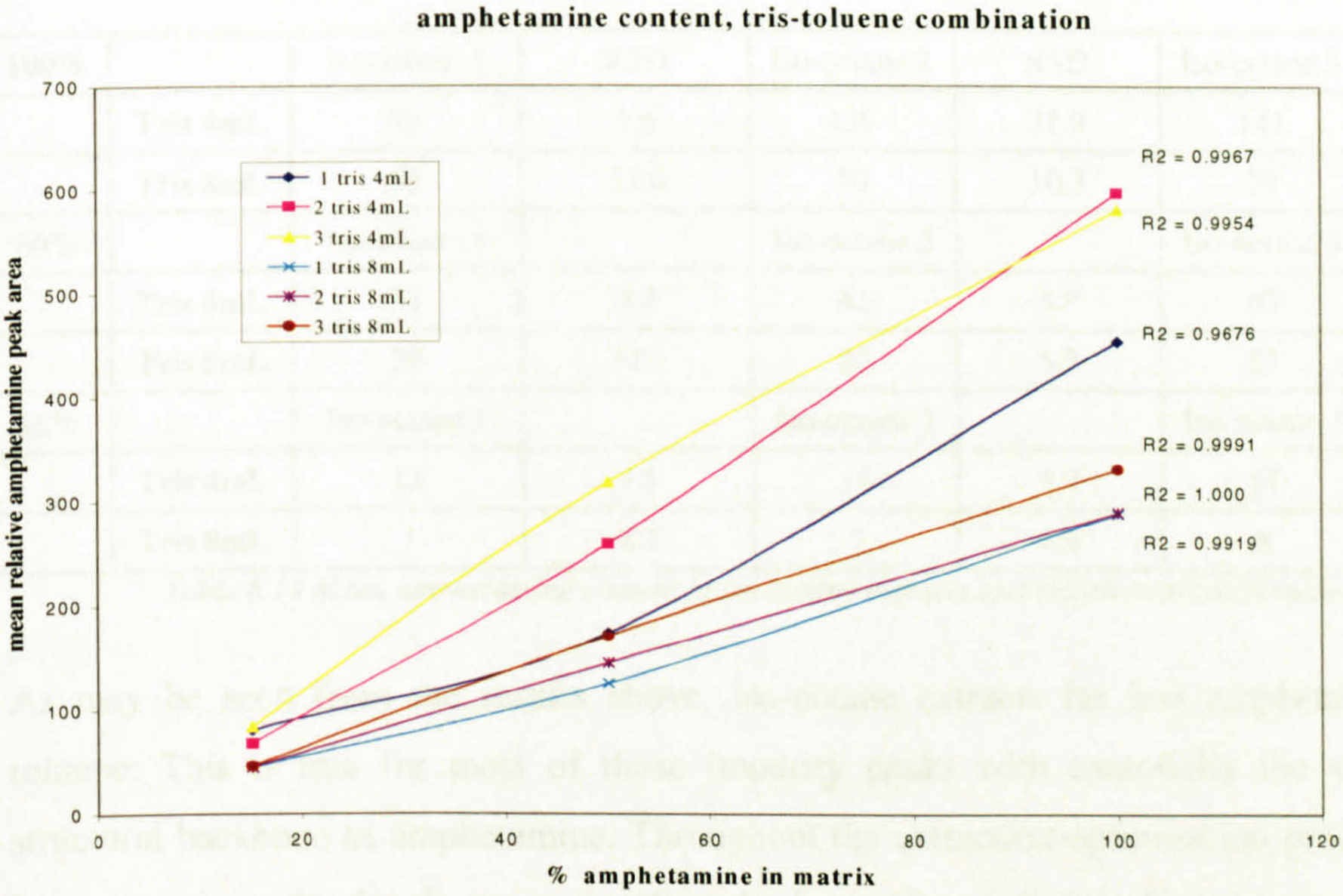


Figure 6.20 First amphetamine extract levels from 15, 50, 100% matrix

The analysed content of amphetamine in the 100%, 50% and 15% samples are not directly proportional to the actual content of the matrix. However, the closest relationship for the amphetamine extraction from the three different matrices is given by the 8mL TRIS buffer with toluene. Simply based on the ability to reproducibly extract the amphetamine, this buffer- solvent combination would be the method of choice.

In general, the toluene in combination with TRIS buffer progressively extracts more amphetamine with each subsequent extraction in the multi-step procedure. The actual level extracted in each subsequent step is also consistent. Using the 8mL rather than 4mL buffer volume allows less amphetamine to pass into the solvent phase but the higher volume buffer tends to give a more reproducible amphetamine peak area. In reality, the significantly lower level of amphetamine extracted by the 8 mL buffer could be an advantage. In terms of chromatographic clarity, the smaller amphetamine peak without a long tail is less likely to obscure impurities with a similar retention time if only an FID is available for use.

6.7.3.2. Amphetamine Content – Iso-Octane

Table 6.14 outlines the amphetamine content of the iso-octane extractions (numbers highlighted in red indicate an RSD less than 5%).

100%		Iso-octane 1	RSD	Iso-octane 2	RSD	Iso-octane 3	RSD
	Tris 4mL	93	1.6	139	31.9	141	34.8
	Tris 8mL	55	13.6	50	10.3	78	15.2
50%		Iso-octane 1		Iso-octane 2		Iso-octane 3	
	Tris 4mL	32	4.3	42	4.9	60	12.3
	Tris 8mL	29	14.3	27	5.7	27	4.2
15%		Iso-octane 1		Iso-octane 2		Iso-octane 3	
	Tris 4mL	13	3.5	18	1.7	17	18.1
	Tris 8mL	7	8.7	7	6.6	8	2.1

Table 6.14 Mean amphetamine content of iso-octane extracts and relative variation values

As may be seen from the results above, iso-octane extracts far less amphetamine than toluene. This is true for most of those impurity peaks with essentially the same basic structural backbone as amphetamine. Throughout the extraction-optimisation procedure, the iso-octane impurity levels were significantly lower than toluene. In iso-octane's favour however, is the fact that the profile is virtually unaffected by the presence of caffeine. The caffeine peak essentially eradicates the region of the chromatogram where it elutes which may mask peaks which could otherwise be significant.

Using 4 mL TRIS buffer in combination with iso-octane provides, for the first extraction, reproducible peak areas for amphetamine. However, subsequent extractions are not generally as predictable. In addition, in contrast to the results for toluene, the use of 8 mL buffer, generally causes more variation in the amphetamine concentration.

In terms of reproducible amphetamine extraction and analysis only, excluding, at this stage, any consideration of impurities, the most successful procedure for all three matrices is the combination of 8 mL TRIS buffer with 200 µL of toluene. All three extractions of the same sample and of the three different matrix have RSD less than 5% for 100% and 50%, and less than 10% for the 15% matrix.

6.7.3.3. Impurity Extraction – Toluene

In drug profiling, the most important consideration, are the impurities themselves, without reference to amphetamine or caffeine content. There are a few ways to consider these results. One way is to consider only a set number of peaks chosen from the entire profile. This method is simple enough when, as in this case, we have many extracts of essentially the same sample with different diluent levels and only slight variation due to inhomogeneity. In reality, selecting important and inconsequential peaks would not be possible as the sample would be 'unseen' and therefore, choosing which peaks to study in the profile and which to disregard, would prove problematic. Choosing to study only identified peaks, in the case of the nitrostyrene route, only provides five route-specific peaks with which to build a profile, which doesn't allow for much variation between samples.

Instead, at this stage, it was decided to look only at specific peaks selected from the entire profile which had a peak area equal to or greater than that of the internal standard in the three replicate 100% matrix extracts. Although these peaks were not necessarily route specific, they were present at a sufficiently high concentration to believe that they would also be easily detected in a sample of only 15% this concentration.

In total, 36 peaks were chosen from the whole range of the chromatogram. They varied in retention time from 5 to 25 minutes and with peak areas from 1 to 30 times the peak area of the internal standard and were therefore, thought to be representative of many possible impurities in a the impurity profile. The total impurity content of the first extract was studied for each possible combination of buffer and toluene. The retention times and mean relative impurity concentration for each buffer/volume combination is shown in Table 6.15. In addition, the RSD for the replicate extractions is shown (values below 10% are highlighted in red). This value is important since the ability to yield a high level of impurities must be set against the variability of the results. The final row shows the mean total extract from the 100% matrix. The value for the amphetamine peak is not shown since this has already been discussed.

No	RT	100%, 4mL tris buffer	RSD	100%, 8mL tris buffer	RSD
1	5.21	1.22	**40.0**	1.12	**5.6**
2	5.73	2.17	**69.2**	1.59	**12.9**
3	7.68	3.69	**96.2**	1.65	**3.4**
4	7.75	4.96	**32.7**	5.78	**8.8**
5	7.89	2.68	**13.8**	1.83	**4.9**
6	8.37	4.23	**33.1**	3.88	**6.1**
7	10.41	25.81	**39.1**	24.53	**6.7**
8	10.49	4.96	**44.7**	4.73	**3.8**
9	10.68	2.48	**29.3**	2.36	**4.8**
10	10.88	2.83	**30.2**	2.84	**15.9**
11	11.19	3.81	**51.6**	1.09	**23.2**
12	12.19	0.71	**40.7**	1.01	**12.3**
13	14.39	1.76	**22.3**	1.39	**4.4**
14	14.66	0.90	**45.1**	0.61	**2.8**
15	16.90	1.55	**29.4**	1.06	**12.8**
16	17.57	5.63	**32.5**	6.42	**6.5**
17	18.10	2.58	**41.7**	4.15	**20.5**
18	18.21	8.15	**20.3**	13.35	**6.4**
19	18.91	4.02	**18.2**	0.11	**13.5**
20	19.13	1.17	**29.7**	1.34	**39.8**
21	20.28	2.91	**34.5**	3.03	**7.2**
22	20.34	3.01	**28.4**	4.03	**6.9**
23	20.42	1.22	**28.4**	1.46	**7.2**
24	20.77	4.70	**23.7**	6.93	**6.5**
25	20.87	1.83	**40.1**	2.25	**22.9**
26	21.45	2.33	**35.9**	2.97	**0.3**
27	21.80	1.45	**46.0**	1.46	**6.4**
28	22.06	1.42	**60.7**	1.24	**9.7**
29	22.26	2.83	**41.7**	3.15	**5.6**
30	22.52	1.77	**28.5**	1.85	**14.3**
31	23.16	3.46	**22.6**	3.90	**8.2**
32	23.33	1.43	**23.7**	1.12	**19.7**
33	23.60	1.72	**34.2**	1.81	**6.3**
34	23.72	2.48	**51.5**	2.12	**9.6**
35	23.82	1.52	**43.8**	1.46	**7.3**
36	23.98	1.43	**51.2**	1.28	**3.9**
	Total extract 1	**120.80**	**1<15%**	**120.89**	**30<15%**
	Total extract 1+2	**268.91**		**244.28**	
	Total extract 1,2,3	**355.12**		**323.70**	

Table 6.15 100% Matrix Mean relative peak areas of selected impurities relative to $C_{20}H_{42}$ and RSD values from first extraction

From these results it may be seen that, considering only those impurities chosen for profiling, the highest total level of impurities is obtained by using the 8mL phosphate buffer with toluene as solvent. By the second extract, however, the 4 mL TRIS buffer appears to extract more impurities (Total Extract 1 + 2). In the third extract, the 4 mL TRIS again gives the highest level of impurities (Total Extract 1,2,3).

This information when combined with the previous experimental data for amphetamine content leads to the conclusion that, for 100% matrix samples, the use of the 8 mL TRIS buffer combined with toluene would be the most effective extraction method for a single extraction.

The mean values for each selected impurity across the three replicate extractions for the 100% matrix using 8 mL TRIS and toluene are plotted in Figure 6.21. Viewing the first, second and third extract graphically, it is clear that some impurities are extracted preferentially to others and their concentration in subsequent extractions decreases as one would expect, However, some impurities have a higher concentration in the second extraction than in the first. In general, the profiles for each extraction follow a similar although not identical pattern.

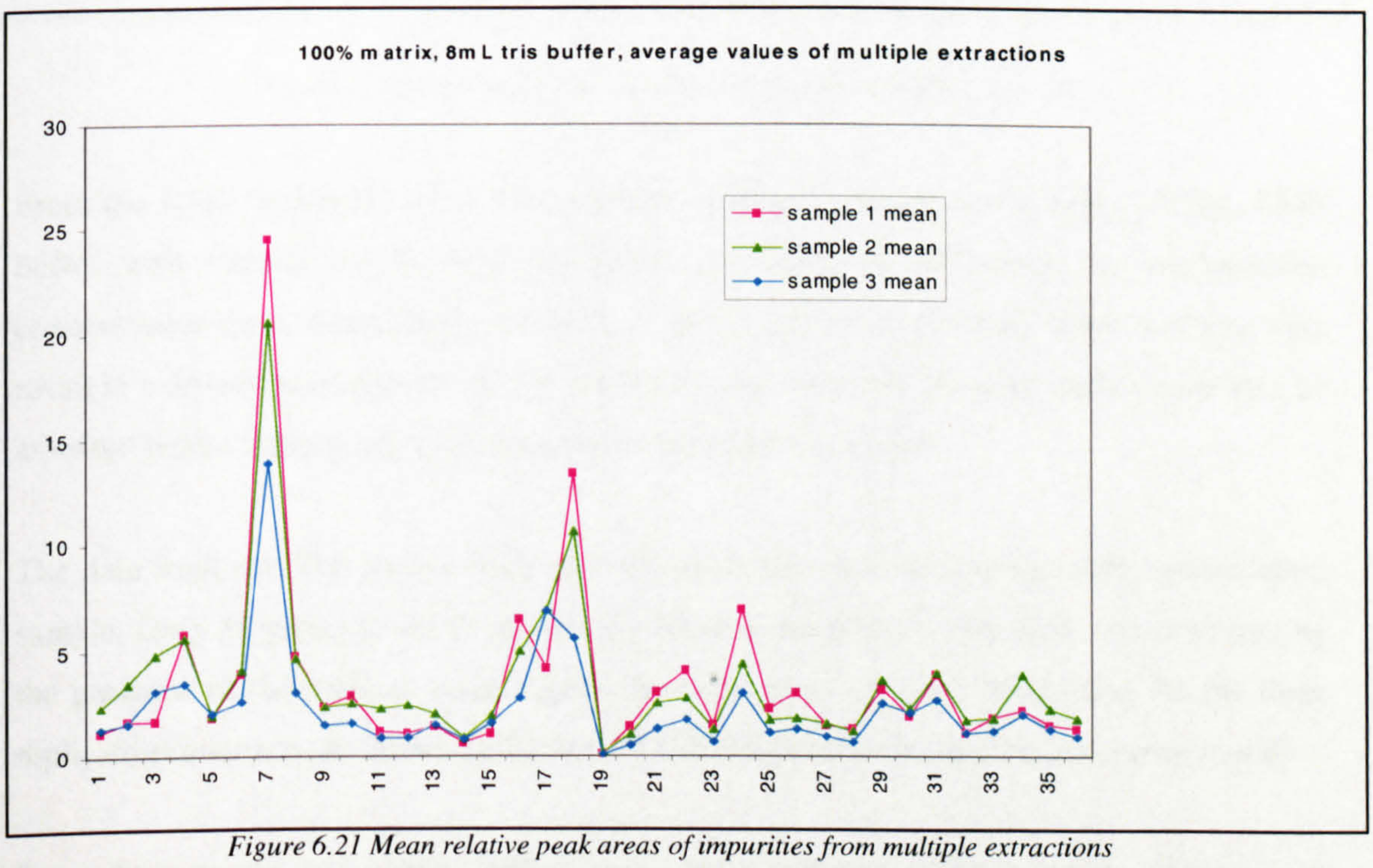


Figure 6.21 Mean relative peak areas of impurities from multiple extractions

The reasoning behind a triple extraction process is not to view each subsequent extraction individually but as a combined sample of the three extracts. Figure 6.22 illustrates this in a plot of the total impurities from three multiple extractions using 8 mL TRIS with toluene. The impurity profiles are similar but the relative peak areas are not identical. The similarity of the profiles after multiple extractions shows that even although the samples when analysed individually after each stage in the process, yield a different profile, the combined total analyte is almost identical.

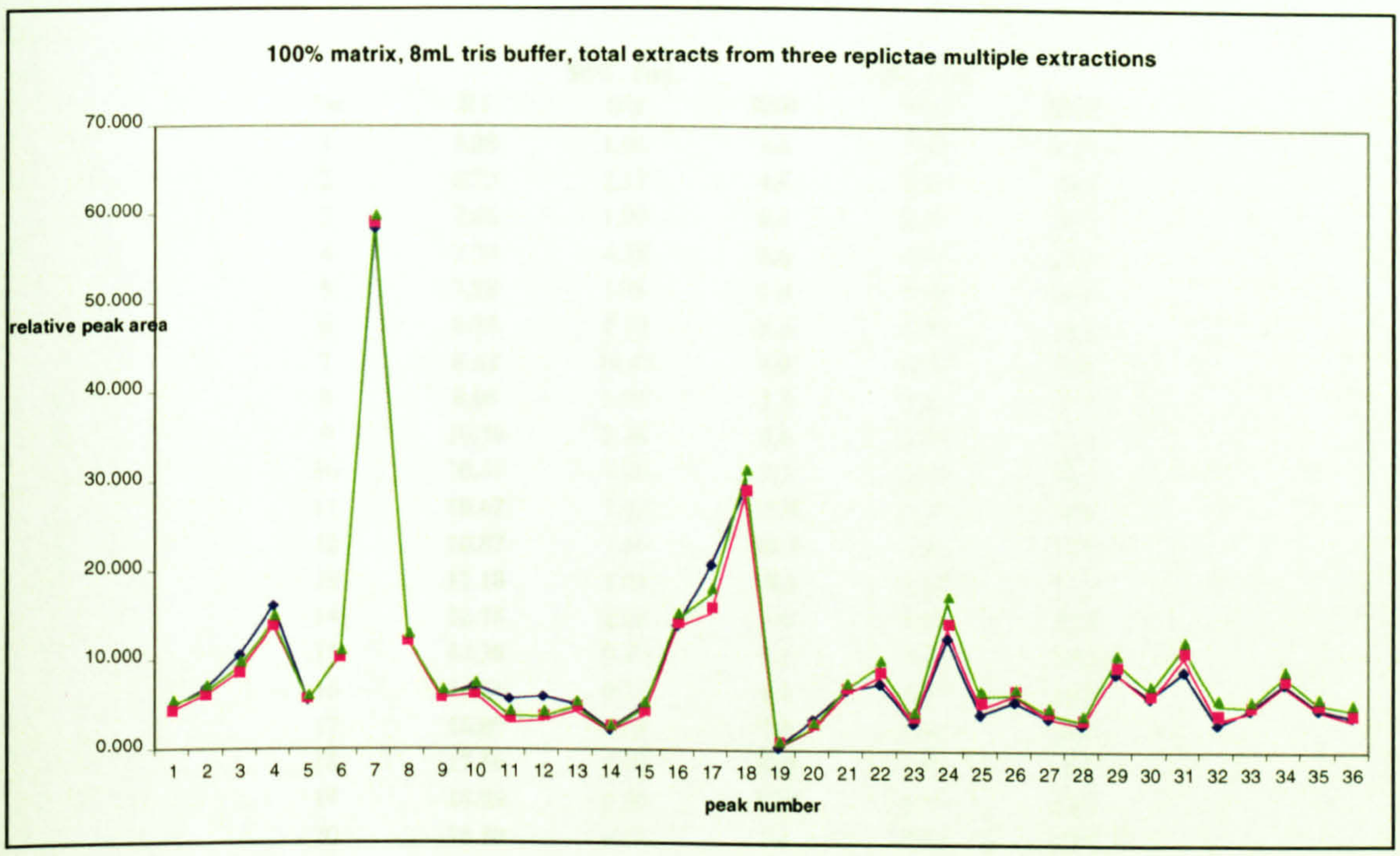


Figure 6.22 Mean total peak area for impurities from multiple extracts

From the 100% matrix data, the recommended method for extraction would be 8 mL TRIS buffer with toluene for the first extraction. However, the differences in amphetamine concentration (and subsequently different impurity levels) in the two other matrices may result in a different conclusion and the analytical information from these studies must also be assessed before making any final decision on how best to proceed.

The data from the 50% matrix study was treated in the same way as the more concentrated sample. Only 35 peaks could be studied for these extracts since one peak was obscured by the presence of the caffeine peak. Again, the mean peak area and RSD value for the three replicate extractions are shown in Table 6.16 (RSD values less than 10% are shown in red).

From these results, the method which shows least variation within replicate samples uses 4 mL TRIS buffer combined with toluene. Problems were encountered with the 8 mL TRIS buffer samples when extracted and analysed, some buffer had been removed with the sample and caused difficulties with the injection. Therefore, the variation seen in the values in Table 6.16 may be more a result of the analysis rather than the extraction process. The 4 mL phosphate buffer variation is relatively good but the 8 mL phosphate buffer extracts fewer impurities and the results are far too variable to be considered as a possible extraction method.

No	RT	50%, 4mL tris	RSD	50%, 8mL tris	RSD
1	**5.20**	1.61	**3.4**	1.92	**47.3**
2	**5.73**	2.17	**4.6**	2.52	**50.3**
3	**7.66**	1.90	**6.6**	2.20	**35.2**
4	**7.74**	4.35	**5.6**	4.15	**11.4**
5	**7.88**	1.31	**1.8**	0.98	**4.0**
6	**8.35**	2.12	**8.6**	1.70	**11.1**
7	**8.62**	14.45	**6.0**	12.17	**9.4**
8	**8.68**	3.91	**3.2**	3.45	**6.4**
9	**10.39**	3.48	**5.8**	2.89	**15.4**
10	**10.47**	1.70	**5.7**	1.56	**20.6**
11	**10.67**	1.47	**19.9**	2.38	**36.6**
12	**10.87**	1.16	**15.7**	2.96	**75.0**
13	**11.18**	1.01	**10.1**	1.20	**47.9**
14	**12.18**	1.05	**3.6**	1.27	**27.1**
15	**14.38**	0.40	**2.1**	0.40	**14.5**
16	**14.66**	0.73	**2.1**	1.05	**39.8**
17	**16.89**	4.53	**7.0**	4.36	**10.3**
18	**17.56**	4.02	**10.9**	3.89	**2.5**
19	**18.09**	6.36	**12.0**	6.89	**11.7**
20	**18.20**	0.82	**5.1**	0.73	**12.4**
21	**18.83**	0.18	**19.2**	0.48	**12.3**
22	**19.12**	1.61	**12.9**	1.73	**15.8**
23	**20.08**	0.36	**8.7**	0.66	**29.0**
24	**20.98**	0.12	**16.0**	1.35	**37.2**
25	**21.46**	0.52	**11.6**	0.38	**43.9**
26	**21.80**	0.79	**9.0**	0.93	**37.4**
27	**22.06**	0.26	**6.4**	0.33	**29.6**
28	**22.26**	0.99	**13.2**	1.47	**45.1**
29	**22.50**	0.50	**77.5**	0.94	**39.0**
30	**23.16**	2.28	**7.0**	2.38	**33.3**
31	**23.34**	0.31	**12.7**	0.27	**36.8**
32	**23.60**	0.78	**14.9**	0.89	**28.7**
33	**23.72**	1.96	**8.4**	2.20	**45.5**
34	**23.84**	0.94	**8.1**	1.08	**38.1**
35	**23.97**	0.60	**7.7**	0.81	**58.7**
	total extract 1	**70.77**	**30<15%**	**74.58**	**11<15%**

Table 6.16 50% Matrix Mean relative peak areas of selected impurities and RSD values for first extraction

The mean values for the chosen impurities for the first second and third multiple extraction for the 4 mL TRIS buffer are shown below. The total extracts combining the three subsequent extractions provide the total extracted profile and the profile for 4 mL TRIS with toluene is shown in Figure 6.23. This displays the same findings as the 100% matrix, that the combined multiple extracts give very similar profiles when using the selected peaks.

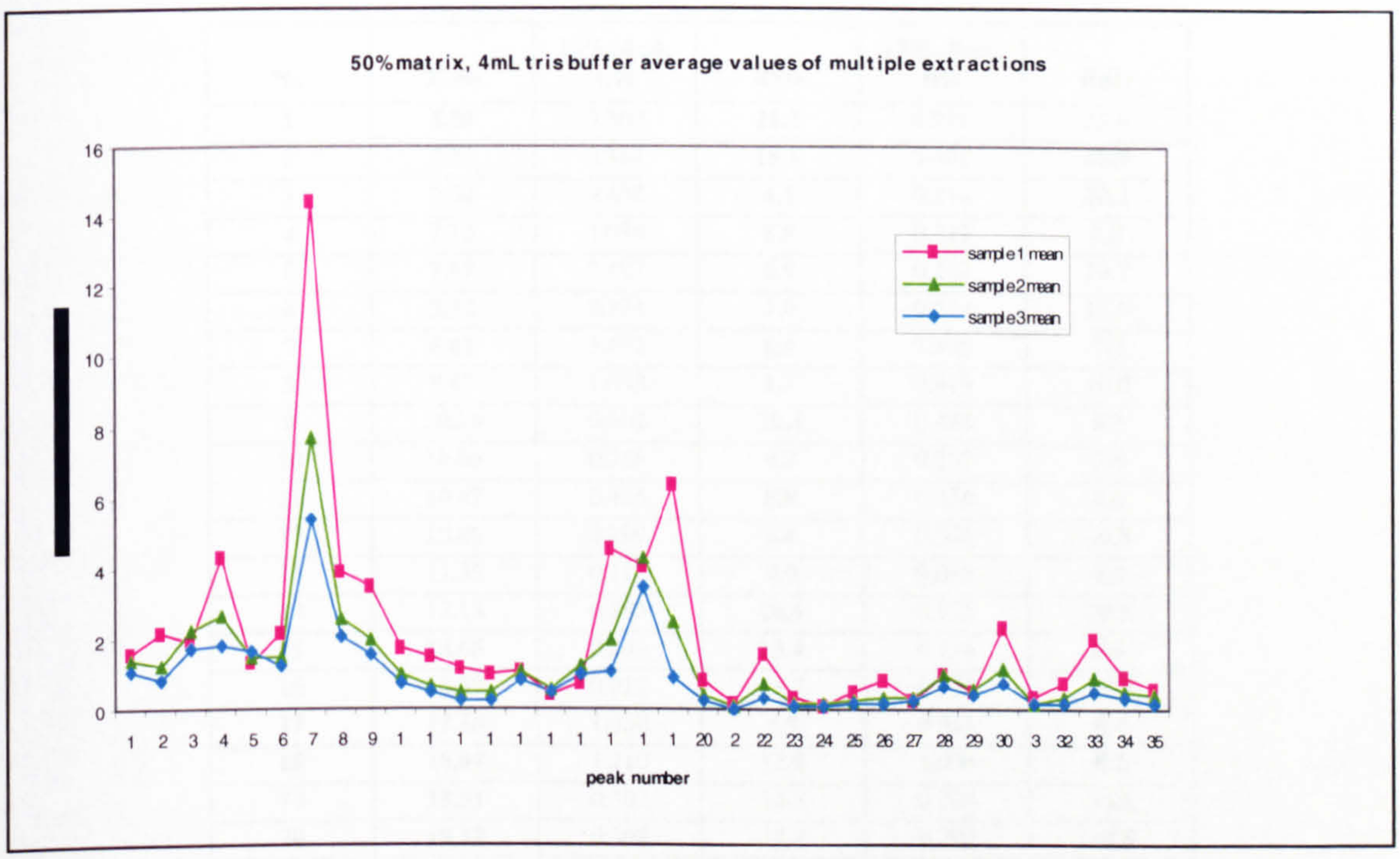


Figure 6.23 Mean relative peak areas for impurities from multiple extractions

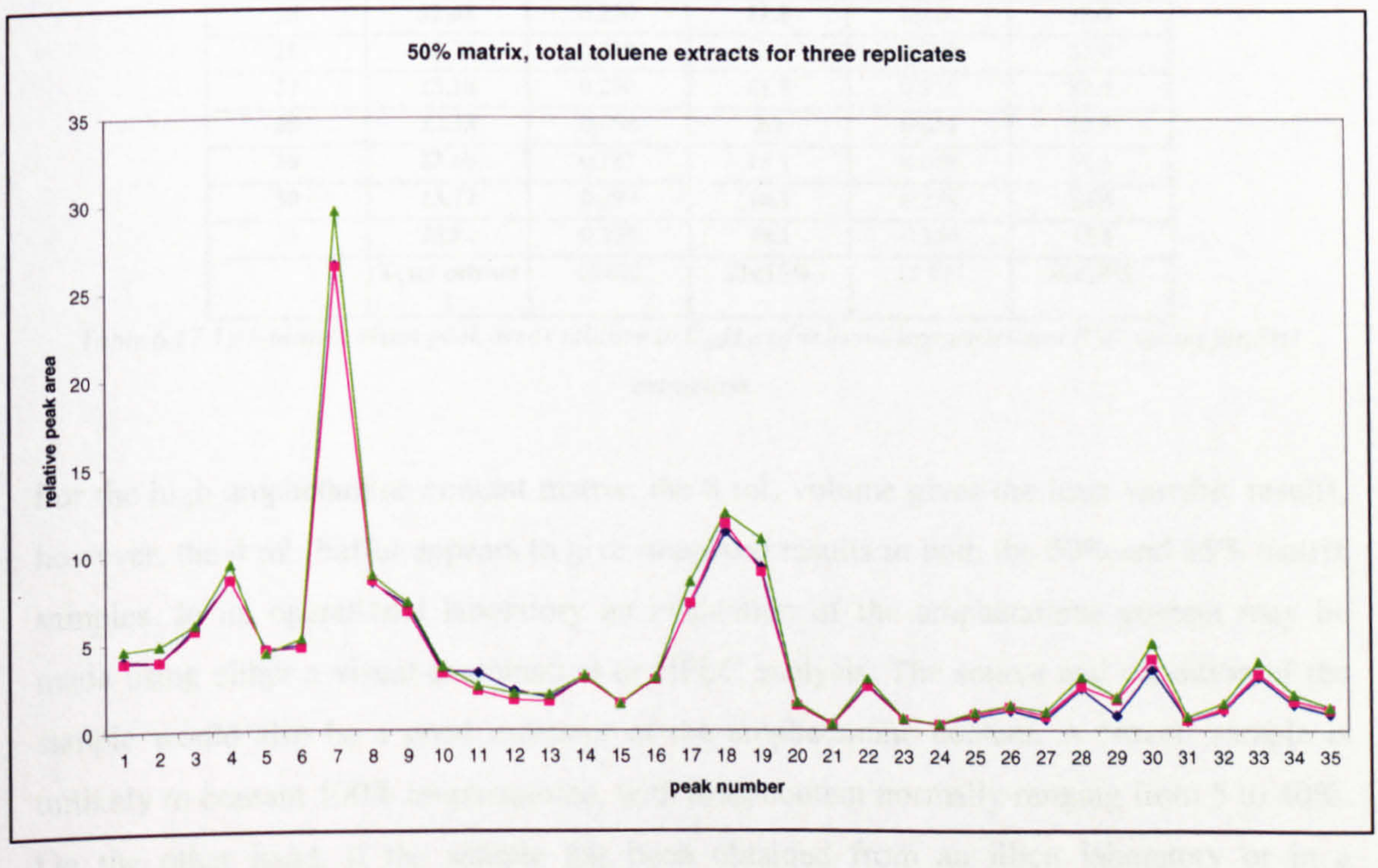


Figure 6.24Mean total peak area for impurities from multiple extractions

In the same way, the 15% matrix was studied and the mean results from the first extractions are shown in Table 6.17.

No	Time	15%, 4mL tris	RSD	15%, 8mL tris	RSD
1	5.20	0.300	21.2	0.971	25.6
2	5.75	0.344	18.6	0.401	48.9
3	7.64	0.452	4.1	0.214	20.2
4	7.72	1.038	5.8	0.711	8.8
5	7.87	0.492	6.9	0.282	10.7
6	8.32	0.374	1.0	0.234	13.9
7	8.61	3.492	6.4	2.405	7.2
8	8.67	1.093	1.7	0.819	10.0
9	10.36	0.662	10.4	0.488	6.2
10	10.46	0.336	9.7	0.257	5.4
11	10.67	0.485	8.8	0.456	4.6
12	10.86	0.299	6.6	0.548	10.8
13	11.35	0.117	8.9	0.085	7.4
14	12.18	0.283	20.6	0.175	20.5
15	14.68	0.292	13.4	0.134	6.6
16	16.89	0.917	11.7	0.789	10.2
17	17.56	1.058	4.9	0.511	8.4
18	18.09	1.210	12.0	1.006	8.2
19	18.91	0.303	14.5	0.200	16.1
20	19.12	0.368	12.4	0.305	10.5
21	20.09	0.082	17.8	0.072	15.6
22	20.90	0.241	13.2	0.289	19.8
23	21.47	0.103	26.0	0.109	26.6
24	21.81	0.130	16.5	0.130	25.0
25	22.07	0.230	11.2	0.214	18.0
26	22.27	0.418	12.4	0.296	23.9
27	23.18	0.270	41.7	0.315	87.3
28	23.33	0.068	3.1	0.053	12.2
29	23.60	0.187	15.1	0.019	31.5
30	23.72	0.293	16.1	0.278	34.8
31	23.84	0.158	19.1	0.150	42.1
	Total extract 1	16.092	21<15%	12.917	16<15%

Table 6.17 15% Matrix Mean peak areas relative to $C_{20}H_{42}$ of selected impurities and RSD values for first extraction

For the high amphetamine content matrix, the 8 mL volume gives the least variable results, however, the 4 mL buffer appears to give improved results in both the 50% and 15% matrix samples. In an operational laboratory an evaluation of the amphetamine content may be made using either a visual examination or HPLC analysis. The source and condition of the sample would also be a good indicator of the amphetamine content. A 'street' sample is unlikely to contain 100% amphetamine, with drug content normally ranging from 5 to 40%. On the other hand, if the sample has been obtained from an illicit laboratory or in a crystalline form, the likelihood is that the amphetamine content will be high. Therefore, a two-tiered system might be most suitable with 4 mL used for small 'street' seizures known to be diluted and 8 mL used for samples which have been seized immediately after production at source.

6.7.3.4. Impurity Extraction – Iso-Octane

The peak areas of impurities observed when iso-octane is used as an extraction solvent have been consistently lower than those of toluene throughout the optimisation of the extraction process. However, the fact that less amphetamine and caffeine is extracted through iso-octane could lead to a cleaner impurity extract and if the results show that iso-octane extracts are less variable than toluene, then a case could be made for its use as an extraction solvent.

The results from iso-octane extracts of 100% matrix samples are shown in Table 6.18 (values highlighted in red are RSD less than 10%).

Looking at the variability of the results for the impurity peak areas, the RSD values for the 4mL volume are high. The 8 mL TRIS buffer gives the most reproducible results with 23 peaks having an RSD less than 15% as compared to 7 peaks using the 4 mL TRIS buffer.

No	Time	100% 4mL tris	RSD	100% 8mL tris	RSD
1	5.15	0.20	55.0	0.27	23.1
2	7.70	2.96	15.3	2.33	1.9
3	7.86	0.96	7.5	0.67	2.7
4	8.30	0.61	18.2	0.39	8.3
5	8.59	8.25	15.3	13.05	7.0
6	10.35	0.79	10.5	0.54	0.8
7	10.44	0.51	12.9	0.34	1.4
8	10.66	2.02	19.6	1.34	2.1
9	12.18	0.35	5.8	0.22	8.8
10	16.89	3.38	13.4	2.64	2.1
11	17.56	1.43	64.6	1.18	4.8
12	18.09	7.72	33.1	7.21	3.6
13	18.20	1.10	7.9	0.70	7.5
14	18.91	0.81	39.1	0.80	12.6
15	19.12	2.13	34.1	2.08	2.7
16	20.28	2.25	25.4	1.79	7.6
17	20.34	0.73	24.2	0.54	5.7
18	20.41	2.97	26.0	2.45	5.0
19	20.77	1.29	34.9	0.95	14.2
20	20.87	1.90	22.9	1.39	3.7
21	21.53	0.64	45.4	0.55	8.7
22	21.80	0.53	62.6	0.44	12.5
23	22.06	0.95	84.8	0.43	17.6
24	22.26	0.75	28.9	0.63	16.3
25	22.54	0.36	7.6	0.34	55.9
26	23.17	0.59	26.7	0.40	38.9
27	23.33	0.26	44.5	0.19	34.3
28	23.72	1.07	66.7	0.87	12.1
29	23.84	0.53	58.8	0.43	19.2
30	23.97	0.60	62.4	0.54	8.0
	total	48.61	7<15%	45.70	23<15%

Table 6.18 100% Matrix Mean peak areas relative to $C_{20}H_{42}$ of selected impurities and RSD values for first extraction

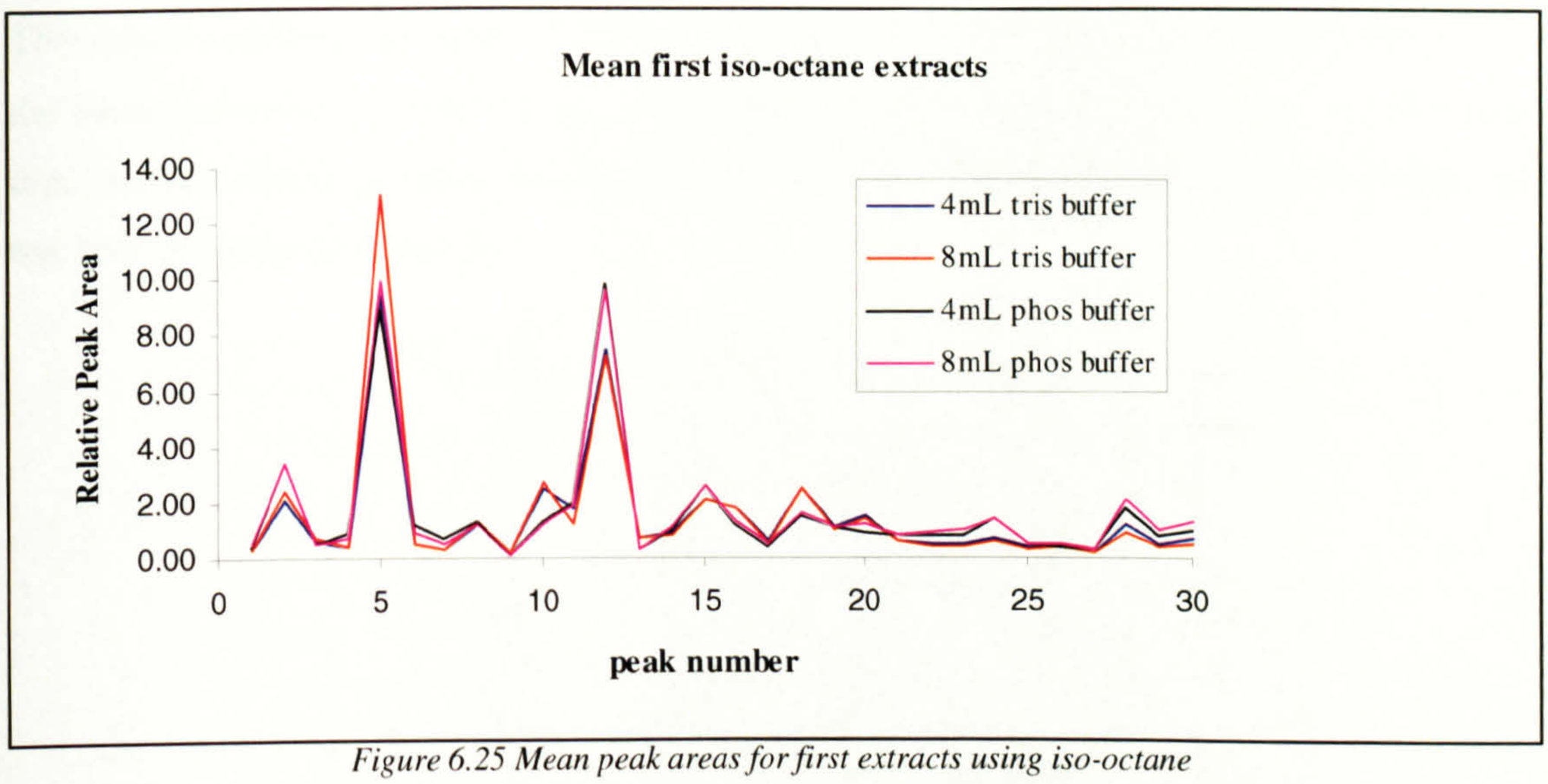


Figure 6.25 Mean peak areas for first extracts using iso-octane

No	Time	50% 4mL tris	RSD	50% 8mL tris	RSD
1	**5.13**	0.205	**37.0**	0.316	**9.0**
2	**7.69**	1.145	**5.8**	0.972	**12.4**
3	**7.85**	0.379	**6.9**	0.349	**15.6**
4	**8.33**	0.081	**7.8**	0.172	**18.5**
5	**8.58**	3.593	**3.2**	4.048	**8.0**
6	**10.34**	0.257	**12.9**	0.267	**17.7**
7	**10.43**	0.154	**15.4**	0.159	**14.2**
8	**10.65**	0.567	**11.8**	0.669	**4.0**
9	**12.17**	0.114	**5.0**	0.113	**11.0**
10	**16.88**	1.422	**3.9**	1.219	**2.1**
11	**17.56**	1.073	**66.5**	0.947	**14.3**
12	**18.08**	4.772	**13.8**	4.892	**7.4**
13	**18.19**	0.223	**31.4**	0.173	**49.8**
14	**18.90**	0.349	**14.7**	0.374	**7.1**
15	**19.12**	1.134	**13.5**	1.146	**9.7**
16	**20.28**	1.577	**8.7**	5.376	**72.9**
17	**20.41**	1.260	**11.7**	1.186	**3.3**
18	**20.76**	0.295	**49.4**	0.391	**12.1**
19	**20.86**	0.327	**45.7**	0.373	**40.6**
20	**21.52**	0.178	**20.4**	0.189	**0.4**
21	**21.79**	0.245	**30.9**	0.307	**5.1**
22	**22.06**	0.038	**17.2**	0.045	**-**
23	**22.25**	0.112	**70.1**	0.240	**10.2**
24	**22.65**	0.036	**77.0**	0.041	**29.6**
25	**23.17**	0.097	**31.4**	0.134	**16.5**
26	**23.33**	0.041	**41.1**	0.047	**2.5**
27	**23.71**	0.685	**54.9**	1.011	**1.1**
28	**23.84**	0.241	**49.5**	0.355	**4.1**
29	**23.97**	0.256	**32.8**	0.348	**17.5**
	Total extract 1	20.854	**13<15%**	25.860	**19<15%**

Table 6.19. 50% Matrix Mean peak areas relative to $C_{20}H_{42}$ of selected impurities and RSD values for first extraction

The values obtained for the 50% matrix, in Table 6.19, were treated in the same way as for the more concentrated sample. Here, the highest total peak area for the extracts and the most reproducible results are given by the use of 8 mL TRIS. Table 6.20 illustrates the results for the 15% amphetamine matrix.

	Time	15% 4mL tris	Rsd	15% 8mL tris	Rsd
1	5.14	0.131	5.6	0.091	64.8
2	7.70	0.353	2.6	0.215	12.4
3	7.86	0.139	5.0	0.076	5.1
4	8.30	0.030	7.3	0.021	34.7
5	8.59	1.704	2.3	1.022	4.5
6	10.36	0.057	5.0	0.042	6.8
7	10.45	0.037	8.1		-
8	10.66	0.253	0.7	0.210	2.9
9	12.18	0.042	2.3	0.026	6.4
10	16.88	0.391	1.8	0.299	14.1
11	17.56	0.166	6.0	0.161	16.5
12	18.09	0.754	14.8	1.012	6.5
13	18.20	0 033	15.8	0.027	20.7
14	18.90	0.063	5.1	0.077	2.9
15	19.12	0.174	10.8	0.234	10.4
16	20.27	1.553	26.5	1.695	37.9
17	20.41	0.237	23.3	0.199	8.9
18	20.76	0.083	2.5	0.110	6.6
19	20.89	0.074	6.5	0.079	2.9
20	21.52	0.032	10.4	0.044	0.8
21	22.25	0.049	16.0	0.064	13.0
22	23.17	0.029	10.8	0.036	4.0
23	23.72	0.134	6.5	0.204	9.0
24	23.84	0.037	15.0	0.075	4.7
25	23.97	0.056	12.7	0.079	16.9
	total	6.608	20<15%	6.099	18<15%

Table 6.20 15% Matrix Mean peak areas relative to $C_{20}H_{42}$ of selected impurities and RSD values for first extraction

The values in Tables 6.18 to 6.20 show that the 4 mL TRIS buffer gives a slightly higher concentration of impurities and slightly better reproducibility than the 8 mL TRIS buffer. This reinforces the fact that a two tiered system might be more suitable since, as with toluene, better results are achieved with 8 mL TRIS for the 100% and 50% matrix and 4mL TRIS for the 15% matrix.

Figure 6.26 shows the average relative peak areas of impurities from the first extraction of the three different matrices using 4 mL TRIS buffer and iso-octane. A similar but not identical pattern can be seen with certain peaks following the pattern more closely than others.

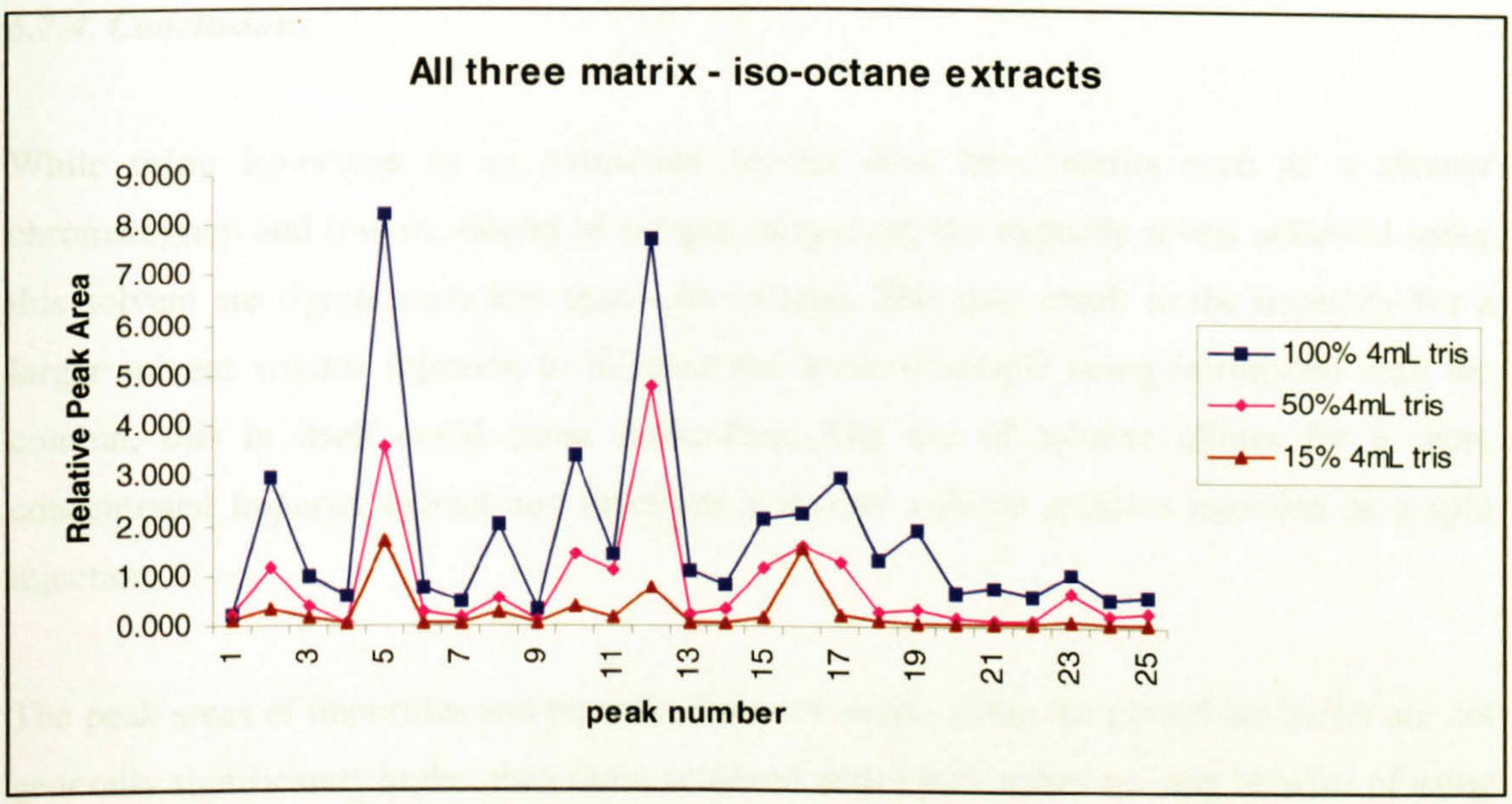


Figure 6.26 15, 50 and 100% amphetamine matrices with 4mL TRIS, iso-octane extracts

Figure 6.27 displays the impurity pattern which emerges when the peak areas of impurities present in extracts from both toluene and iso-octane are compared (only those peaks detected in both iso-octane and toluene extracts are presented). What may be seen is that while toluene consistently extracts a higher concentration of impurities, the overall pattern is very similar.

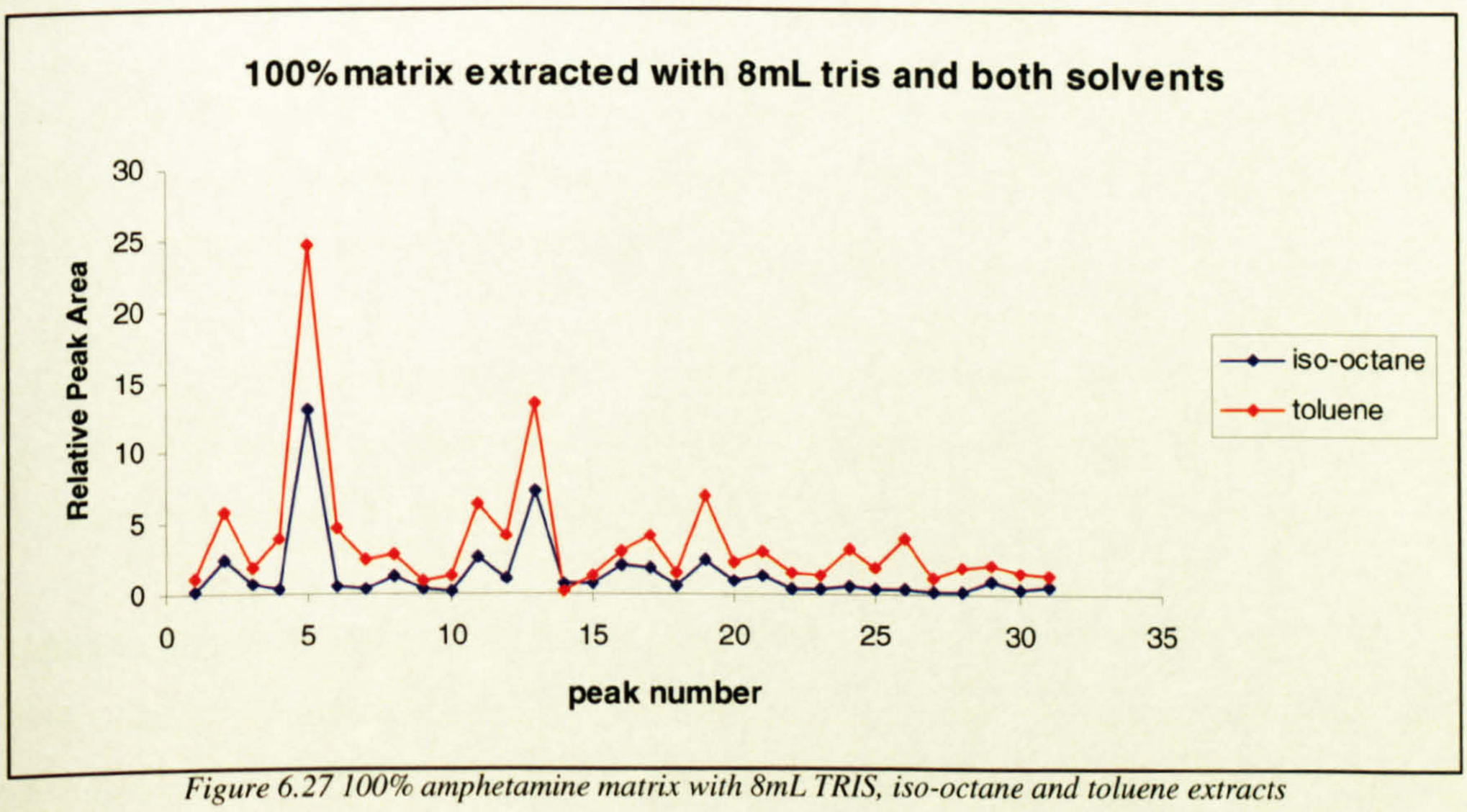


Figure 6.27 100% amphetamine matrix with 8mL TRIS, iso-octane and toluene extracts

6.7.4. Conclusions

While using iso-octane as an extraction solvent does have merits such as a cleaner chromatogram and less likelihood of sample carry-over, the impurity levels achieved using this solvent are significantly less than with toluene. This may result in the necessity for a larger solvent volume injection to increase the level of sample being introduced onto the column, this in itself could cause difficulties. The use of toluene allows for a more concentrated impurity extract and therefore a smaller volume splitless injection or a split injection.

The peak areas of impurities and reproducibility of results using the phosphate buffer are not generally significantly higher than those achieved with TRIS buffer and any benefits of using phosphate are outweighed by the solubility problems associated with it.

As a compromise between the volumes of 4 mL and 8 mL TRIS buffers it is suggested that 8 mL TRIS buffer be used in combination with toluene when a high amphetamine content has been confirmed through an initial amphetamine quantification analysis (>50% amphetamine w/w) and a 4 mL TRIS buffer with toluene be used when the amphetamine content is thought to be lower (<50% w/w).

Chapter 7

Evaluation of the Optimised Extraction Process and Analytical Method

7.1. Reproducibility Study – Nitrostyrene Sample

Having agreed upon the optimum extraction and chromatographic methods as discussed in Chapters 5 and 6, the reproducibility of results obtained using these methods was assessed. Ideally, the RSD values for the relative peak areas of impurities and amphetamine should be within 5%. This value would include; variation resulting from inhomogeneity of the sample, extraction variation, chromatographic variation and possible subjective integration of the chromatograms. If the variation within aliquots of a single sample is significant then the use of profiling becomes more problematic. The reproducibility of impurity profiles obtained using the optimised methods required evaluation in light of these factors.

7.1.1. Experimental Detail

Using the GC and LL extraction methods optimised in the previous sections, aliquots taken from the original sample of combined nitrostyrene amphetamine batches without diluents (TEST 1 sample) were extracted and analysed.

From the previous homogeneity study (Chapter 4), it was established that 200 mg of this concentrated sample tended to result in solubility problems in the buffer. Therefore, 100 mg aliquots were randomly removed from the bulk matrix for inclusion in the experiment.

Fifteen aliquots of 100mg were selected and dissolved in 1M, pH 8.1 TRIS buffer, 4 mL. The solution was placed in a sonic bath for 30 minutes. Toluene, 200 µL, containing internal standard (C_{20} at 10 µg/mL) was added to the mix and the sample placed in a sonic bath for a further 30 minutes. A known proportion of the upper organic phase was removed and placed in a GC vial for analysis by GC-FID and GC-MSD using the method developed previously. The GC-MSD trace was used to identify known impurities. The GC-FID chromatographic signals were integrated using the ChemStation software and the peak areas studied to

determine the variability within extraction and analysis of samples known to originate form a single source.

7.1.2. Results and Discussion

Visually, the chromatograms obtained using FID and MS were similar in profile. Overall, 96 common peaks were present in all 15 extracts. Relative to the internal standard, 29 peaks had an RSD value less than 10% for peak areas. The impurities with a relative peak area over 1 unit are shown in Table 7.1. Those highlighted in red have an RSD less than 10%. Unidentified peaks are numbered in order of elution.

None of the impurities identified by comparison with the prepared standards had an RSD value less than 5% but all had an RSD less than 10%. The relative peak areas were also calculated with respect to the amphetamine content of the sample since it was thought that the amount of amphetamine impurities present should be proportional to the amphetamine content. However, this made no recognisable difference in the overall reproducibility of the results.

The amphetamine content, relative to the internal standard was found to have an RSD of 5.6% over the sample population, which confirms the findings from the original study (Chapter 4) and illustrated homogeneity of the sample. Figure 7.1 shows the impurity peak area relative to the internal standard for the peaks present in all 15 replicates. The impurity profiles look similar enough to be almost completely superimposed. While there is quite significant variation in some peaks there are no obvious outlying sample profiles in the population that could be rejected as being, for example, unsuccessful extractions or low volume injections.

		Relative to Standard		Relative to Amphet.	
Peak	**Time**	**Mean**	**RSD**	**Mean**	**RSD**
1	5.72	1.03	**14.5**	0.0035	**10.6**
2	8.64	1.48	**17.6**	0.0050	**17.4**
3	8.86	1.37	**18.7**	0.0046	**19.0**
4	9.51	1.70	**26.0**	0.0057	**25.2**
Amphetamine	11.24	296	**5.59**	1.0000	**0.00**
6	11.69	2.13	**15.4**	0.0072	**12.5**
7	11.90	7.57	**8.42**	0.0255	**5.18**
8	12.71	3.38	**7.57**	0.0114	**6.89**
9	12.91	3.82	**8.13**	0.0129	**7.42**
Aziridine	13.00	21.7	**7.89**	0.0731	**5.65**
Ketoxime 1	14.99	10.3	**9.33**	0.0348	**7.94**
Ketoxime 2	15.21	2.92	**11.1**	0.0099	**11.3**
13	15.50	1.07	**171**	0.0035	**170**
14	16.15	1.30	**7.33**	0.0044	**3.79**
Benzaldimine	21.31	7.67	**4.99**	0.0259	**5.89**
16	21.93	3.41	**20.3**	0.0115	**18.1**
Red. Form	22.37	10.1	**6.08**	0.0342	**5.28**
18	22.58	1.55	**20.9**	0.0052	**20.3**
19	23.35	3.49	**8.73**	0.0118	**5.70**
20	24.28	1.15	**6.65**	0.0039	**9.89**
21	24.62	1.36	**12.9**	0.0046	**14.8**
22	24.69	5.01	**6.77**	0.0169	**8.01**
23	24.75	3.93	**7.29**	0.0133	**7.48**
24	24.81	2.28	**6.41**	0.0077	**6.33**
25	25.08	1.15	**15.9**	0.0039	**15.1**
26	25.19	2.86	**9.16**	0.0097	**8.45**
27	25.86	1.77	**14.0**	0.0059	**10.3**
28	26.64	2.44	**8.21**	0.0083	**10.3**
29	26.80	1.26	**20.0**	0.0043	**23.2**
30	27.46	4.12	**7.40**	0.0139	**7.70**
31	27.70	1.93	**6.96**	0.0065	**5.50**
32	28.50	2.77	**24.3**	0.0093	**23.9**
33	29.02	1.05	**21.8**	0.0035	**19.6**
34	29.22	1.17	**16.5**	0.0040	**18.9**

Table 7.1 Relative Mean Peak Areas and RSD Values, Reproducibility Study, Nitrostyrene Amphetamine

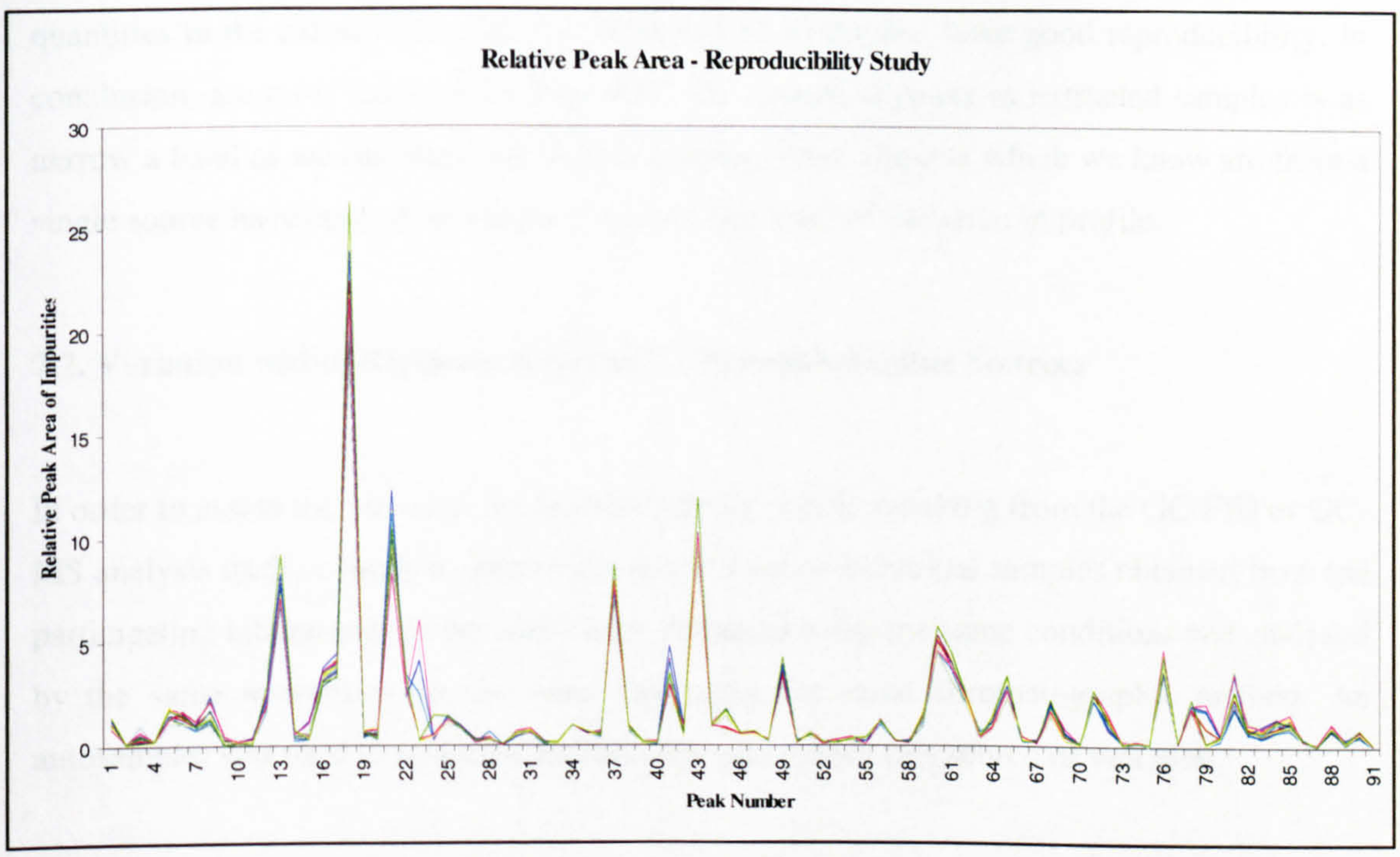


Figure 7.1 Relative Impurity Peak Areas: Reproducibility Study

In terms of the reproducibility of results obtained when a single sample is extracted and analysed, the following conclusions may be drawn

1. That the amphetamine content of the sample has a relative variation of less than 6% after extraction and analysis. This, in a sample combined from individually prepared batches with a variety of amphetamine contents, serves to confirm that the original sample was successfully homogenised.

2. The variability of the results of the identified impurities themselves, being between 5 and 10% is not ideal, as we would rely on these peak area profiles being almost identical in order to make a judgement on samples being from a single source. If the variation in a single sample is 10% then it may be expected that sample aliquots from a single source, which have been divided and distributed differently will have even greater variation.

The use of peaks, which are not identified as route-specific, is justified because as is the case of the nitrostyrene-synthesised amphetamine levels of nitrostyrene and aziridine are so low that they are not always present in extracts. Without these peaks available for profiling, there are only five identifiable route-specific peaks. Of the peaks available in relatively high

quantities in the extracts there are an additional 13 peaks that have good reproducibility. In conclusion, a cut-off value of 15-20% RSD for identified peaks in extracted samples is as narrow a band as we can allow for linked samples, since aliquots which we know are from a single source have been shown to have at least this level of variation in profile.

7.2. Variation within Replicate Injections – 12 Amphetamine Sources

In order to assess the variation in chromatographic profile resulting from the GC-FID or GC-MS analysis itself, a single extract from each of twelve individual samples obtained from the participating laboratories in the study were extracted using the same conditions and analysed by the same instrument on the same day using the same chromatographic method. An autosampler was used to minimise human error and reduce this source of variation.

This experiment set out to determine which of the component parts of each extract gave reproducible relative peak areas when the same extract was analysed four times and thus which components would be suitable for inter and intra-day variation studies. In theory, only impurities shown to have minimal variation between analyses of a single sample could be used to determine variation between separate extractions and between extractions performed on different days. Each participating laboratory produced 3 samples of separately synthesised amphetamine sulphate and bulked these samples using various diluents and bulking agents. NBI produced batches using reductive amination, Strathclyde produced batches using the nitrostyrene route and both ISPC and SKL produced batches using Leuckart methods.

7.2.1. Experimental Detail

200 mg aliquots of each of 12 sample matrices from different sources, covering the three main synthetic routes, were weighed into separate vials. 1M TRIS buffer, pH 8.1, 4 mL, was added and the sample placed in a sonic bath for 15 minutes or until the sample was completely dissolved. In the case of IPSC sample 1, the aliquot was never fully dissolved. This was thought to be due to the sample matrix having unusual diluents present. Although the diluted matrix does have a significant effect on the solubility of the sample in buffer, it was not established whether this affected the extraction efficiency.

Toluene, 200 µL, containing internal standard ($C_{20}H_{42}$) at 10 µg/mL, was added and the sample sonicated for a further 15 minutes. The organic phase was removed and split into four GC vials fitted with inserts for analysis by GC-FID.

The resultant chromatograms were integrated using the ChemStation software and the peak areas of the impurities present were calculated relative to the peak area of the internal standard.

7.2.2. Results and Discussion

The variation in impurity peak areas was calculated and the results are shown in Tables 7.2 to 7.5. The values shown are representative of the total number of peaks that were present in all four replicate injections of the single extract. The number of peaks present in all four replicate analyses with an RSD value of less than 5% and 2% are shown. These results are graphically displayed in Figure 7.2 as a plot of the relative peak area of impurities against peak number for each replicate analysis of a single extract.

7.2.2.1. Three Samples of Nitrostyrene Synthesised Amphetamine

Number of Peaks			
	Sample 1	Sample 2	Sample 3
Peaks in all replicates	**186**	**239**	**223**
Peaks with RSD < 5%	**105**	**121**	**149**
Peaks with RSD < 2%	**42**	**35**	**64**

Table 7.2 Replicate Injections of Extracts from Three Samples of Nitrostyrene Amphetamine

Sample 1 contained 105 peaks having RSD less than 5% within replicate injections. While this level is analytically acceptable, in the case of profiling, an ideal value for variation in replicate injections is nearer the 2% level. In this instance, each sample has at least 30 peaks that would be suitable for use in profiling.

The relative peak areas of all components present in the four replicate analyses of the three samples of nitrostyrene-synthesised amphetamine, is shown graphically in Figures 7.2 to 7.4 below. The y-axis is the relative peak area of a detected peak (relative to the internal standard) and the x-axis is the peak number (peaks have been numbered 1-X in order of elution and detection). The relative peak areas have been shifted upwards since superimposing these patterns makes visual comparison difficult. On comparison of these

profiles, it is clear that visually, the chromatograms obtained from a single extract injected four times are virtually indistinguishable.

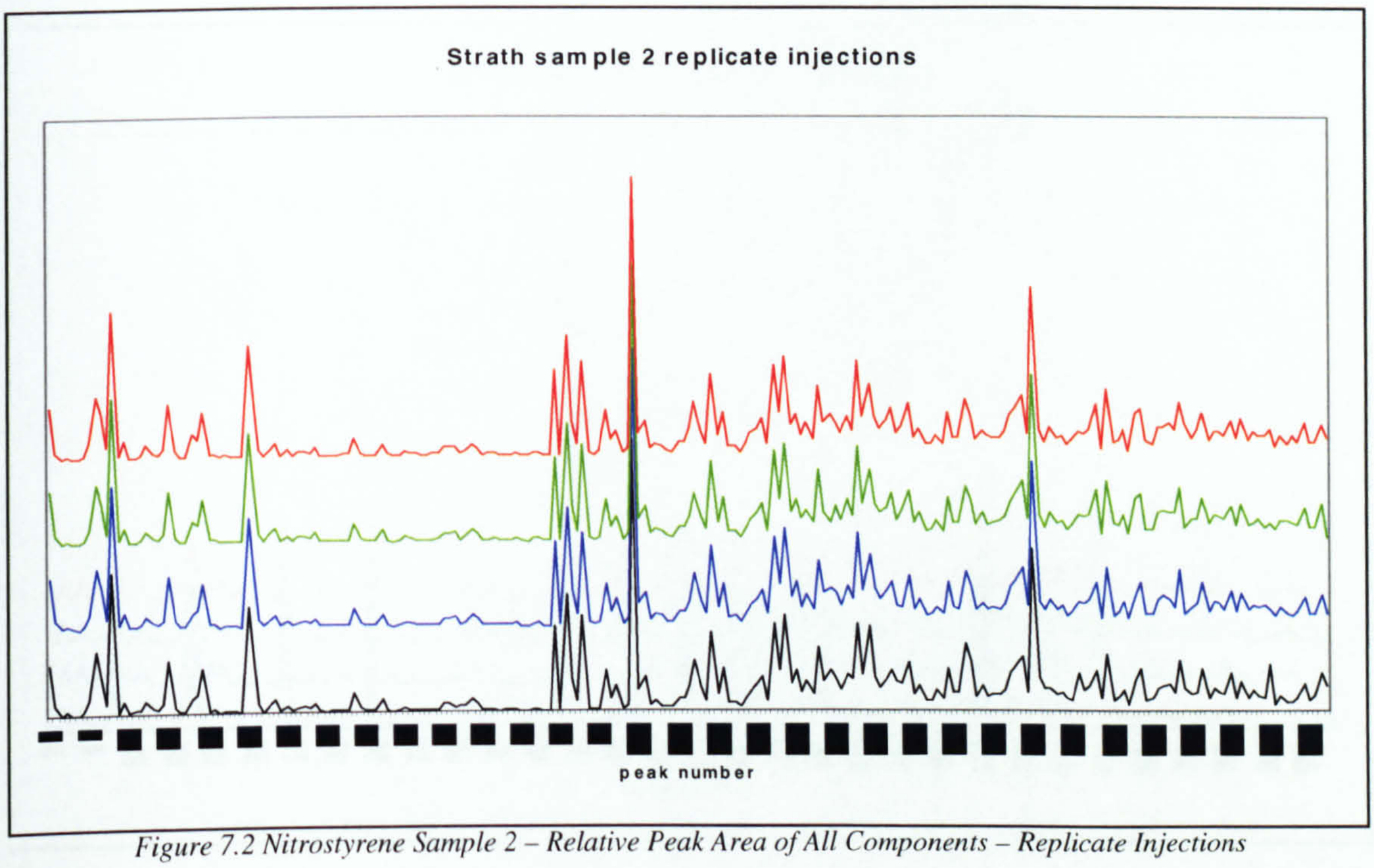


Figure 7.2 Nitrostyrene Sample 2 – Relative Peak Area of All Components – Replicate Injections

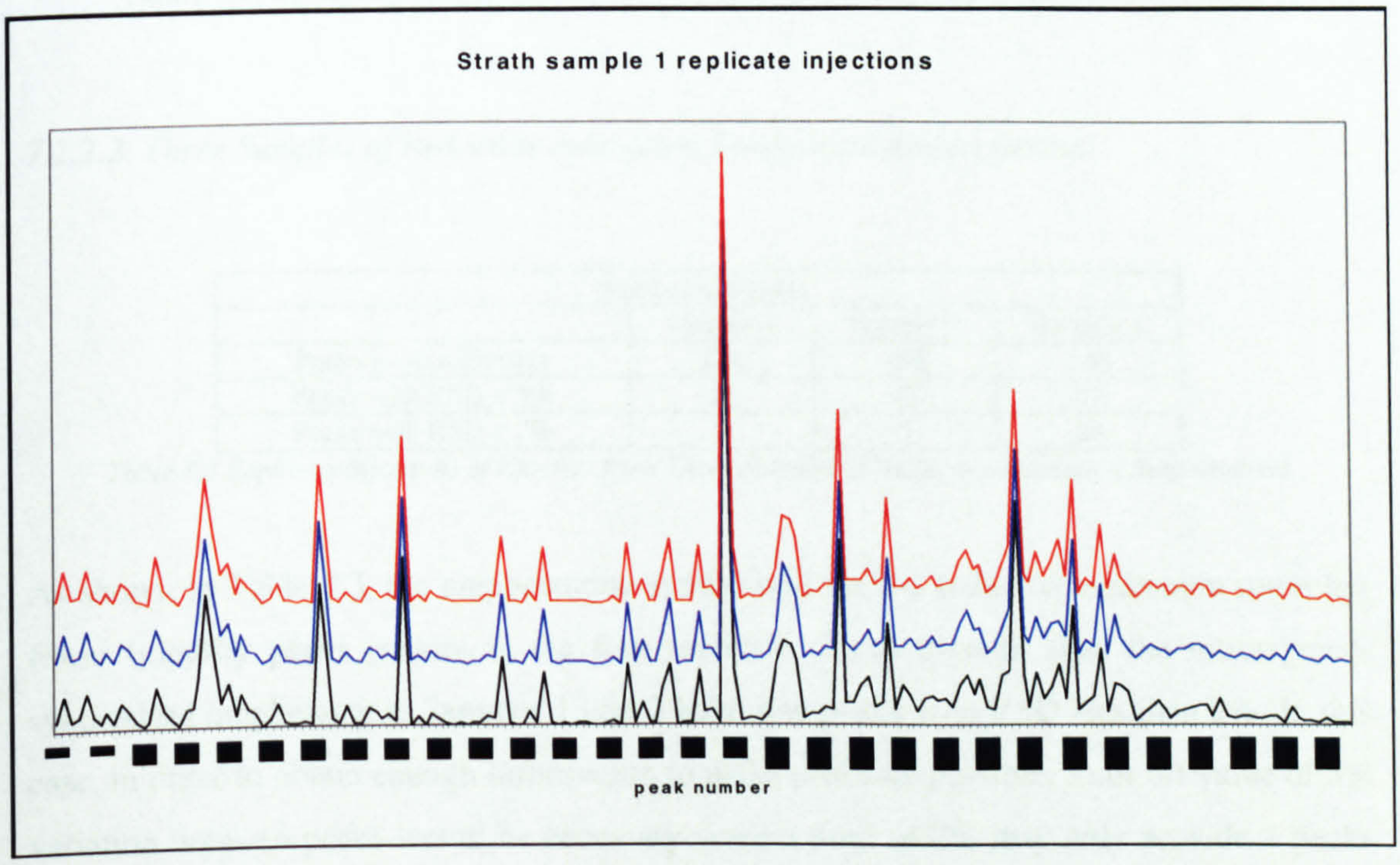


Figure 7.3 Nitrostyrene Sample 1– Relative Peak Area of All Components – Replicate Injections

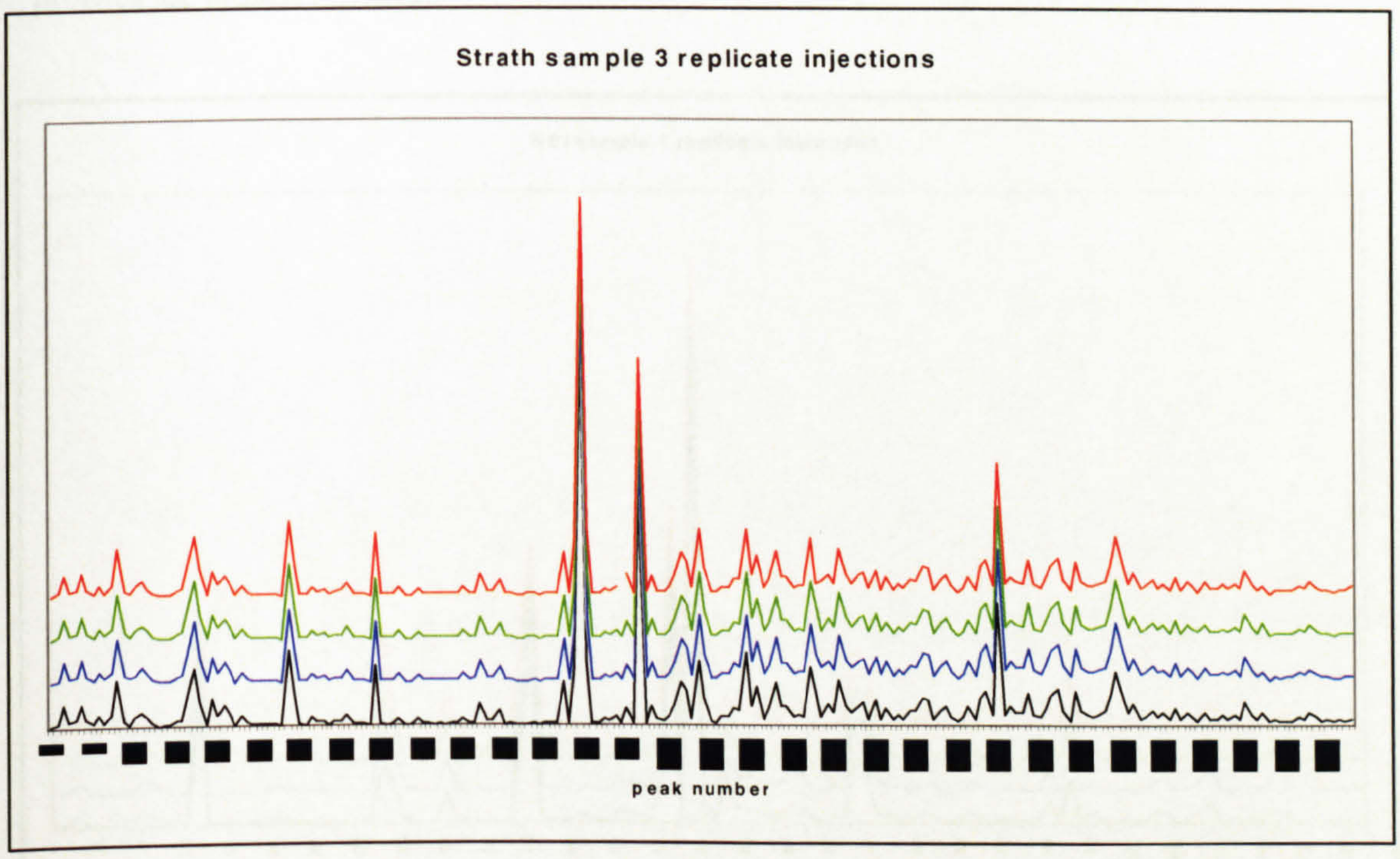


Figure 7.4 Nitrostyrene Sample 3 – Relative Peak Area of All Components – Replicate Injections

7.2.2.2. Three Samples of Reductive Amination Synthesised Amphetamine

Number of Peaks			
	Sample 1	Sample 2	Sample 3
Peaks in all replicates	**126**	**175**	**140**
Peaks with RSD < 5%	**28**	**47**	**87**
Peaks with RSD < 2%	**7**	**9**	**30**

Table 7.3 Replicate Injections of Extracts from Three Samples of Reductive Amination Amphetamine

As shown in Table 7.3, the amphetamine synthesised via the reductive amination route has fewer impurity peaks present in the four replicate chromatograms than the nitrostyrene-synthesised amphetamine. Samples 1 and 2 have few peaks with RSD less than 2%. In this case, in order to obtain enough information to make profiling possible, a cut-off value of 5% variation between peaks would be necessary since a limit of 2% may only provide 7 peaks for comparison. At 5% level variation level, at least 30 peaks are available for profiling in all three samples.

Shown below in Figures 7.5 to 7.7 are the graphical representations of the variation in profile across the replicate analyses. It may be seen that again, although the statistical levels of variation are slightly higher than for the nitrostyrene synthesised amphetamine, visually the profiles are almost identical.

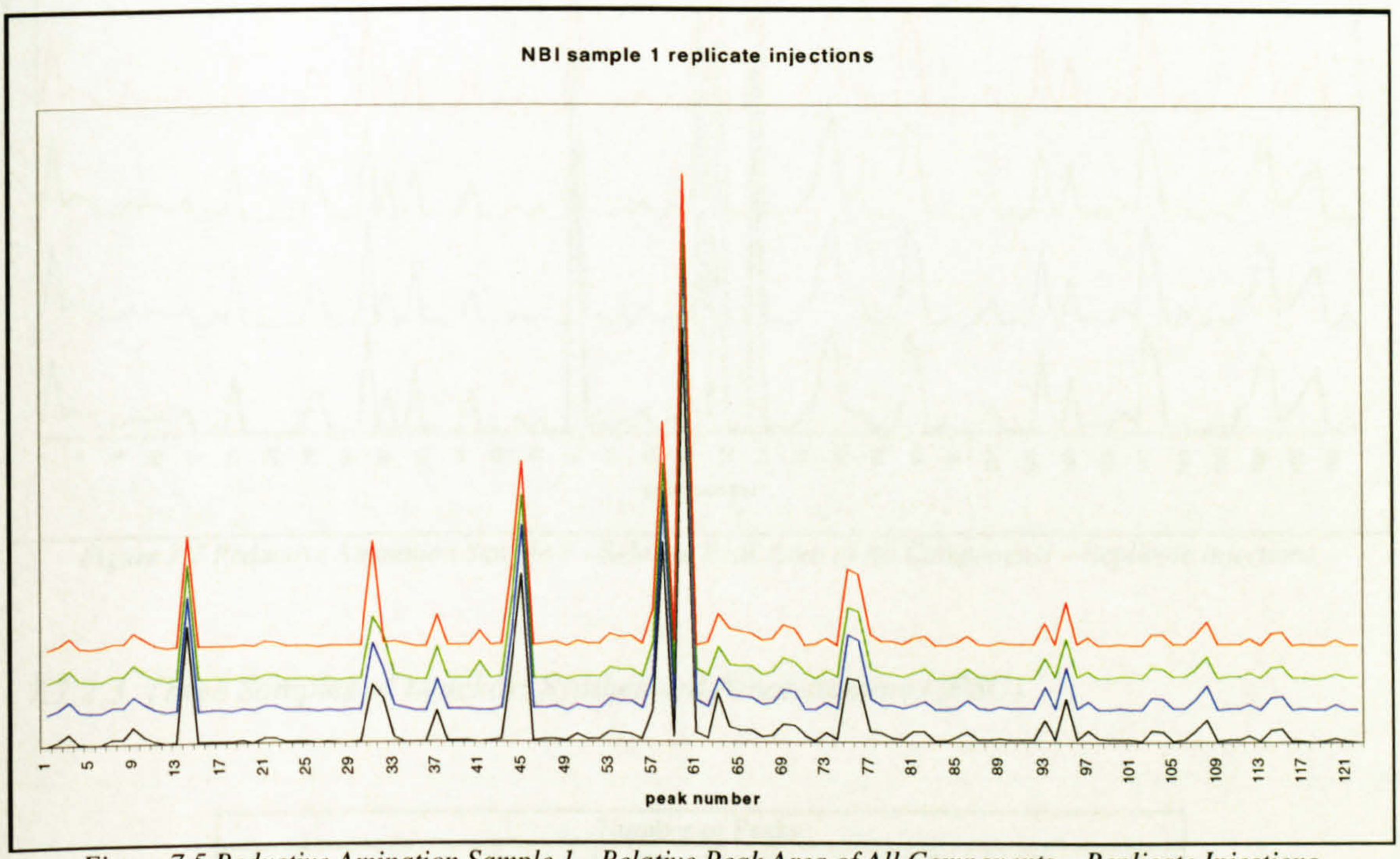


Figure 7.5 Reductive Amination Sample 1 - Relative Peak Area of All Components – Replicate Injections

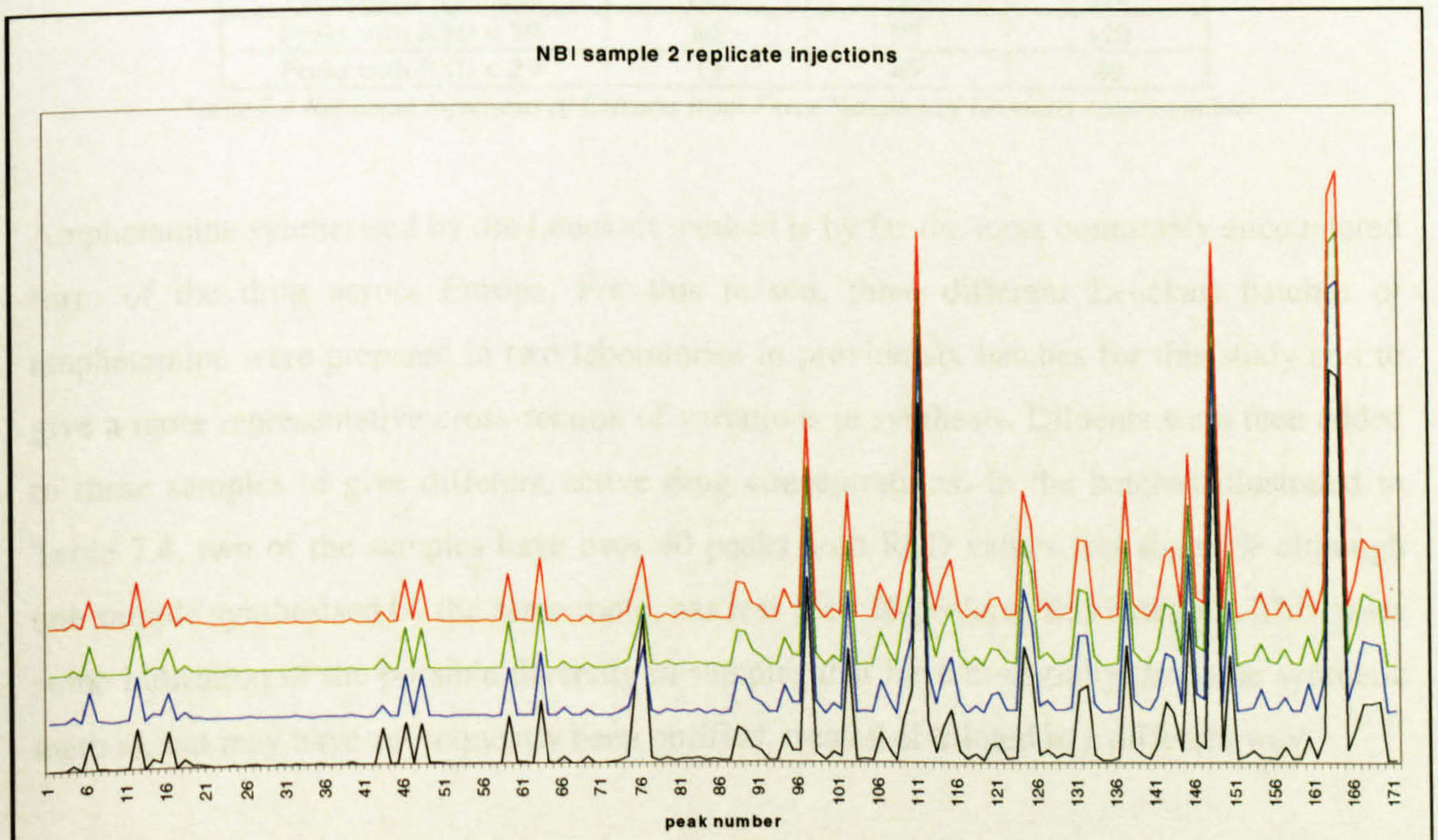


Figure 7.6 Reductive Amination Sample 2 - Relative Peak Area of All Components – Replicate Injections

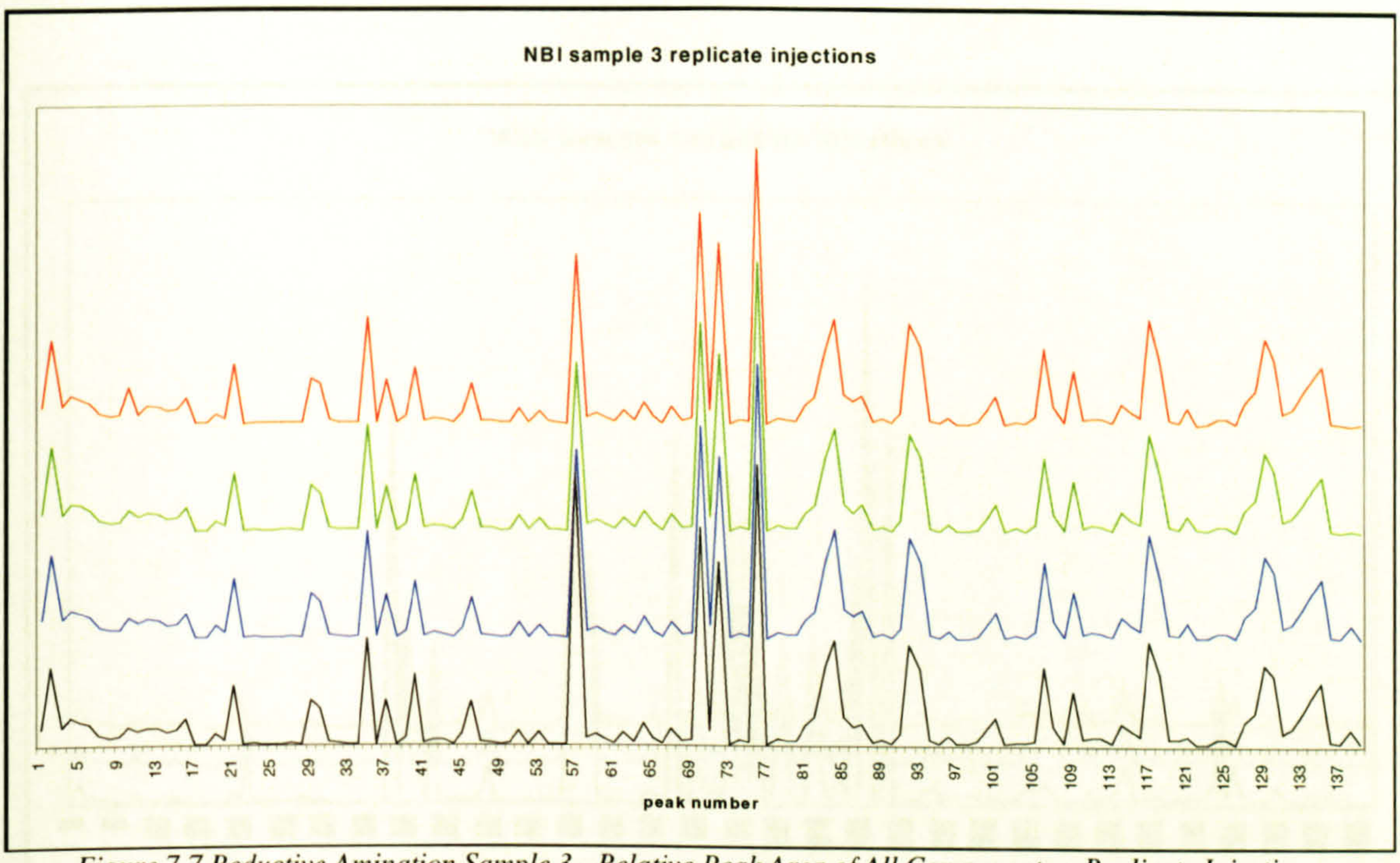


Figure 7.7 Reductive Amination Sample 3 - Relative Peak Area of All Components – Replicate Injections

7.2.2.3. Three Samples of Leuckart Synthesised Amphetamine (IPSC)

Number of Peaks			
	Sample 1	Sample 2	Sample 3
Peaks in all replicates	**192**	**168**	**214**
Peaks with RSD < 5%	**86**	**95**	**120**
Peaks with RSD < 2%	**19**	**49**	**40**

Table 7.4 Replicate Injections of Extracts from Three Samples of Leuckart Amphetamine

Amphetamine synthesised by the Leuckart method is by far the most commonly encountered form of the drug across Europe. For this reason, three different Leuckart batches of amphetamine were prepared in two laboratories to provide six batches for this study and to give a more representative cross-section of variations in synthesis. Diluents were then added to these samples to give different active drug concentrations. In the batches illustrated in Table 7.4, two of the samples have over 40 peaks with RSD values less than 2% although one sample synthesised by the same route, has less than 20 peaks in this category. This gives some indication of the possible diversity in samples that have essentially the same synthetic method, but may have subsequently been purified, treated or diluted in a different way.

Figures 7.8 to 7.10 are plots of the relative peak areas against peak numbers for each of the replicate injections of the same extracts of Leuckart amphetamine. Again, the profiles are almost indistinguishable.

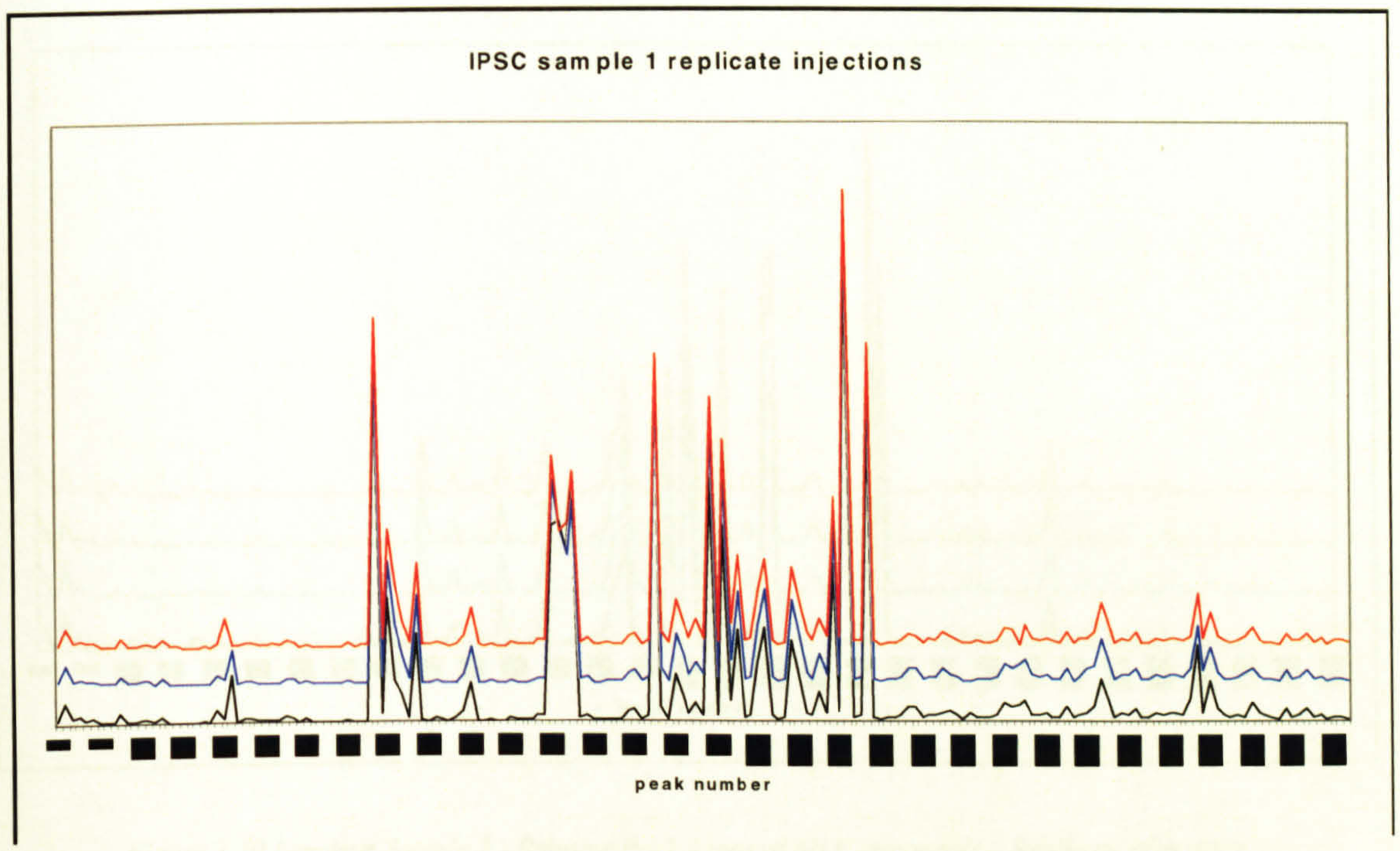


Figure 7.8 Leuckart Sample 1 -Relative Peak Areas of All Components – Replicate Injections

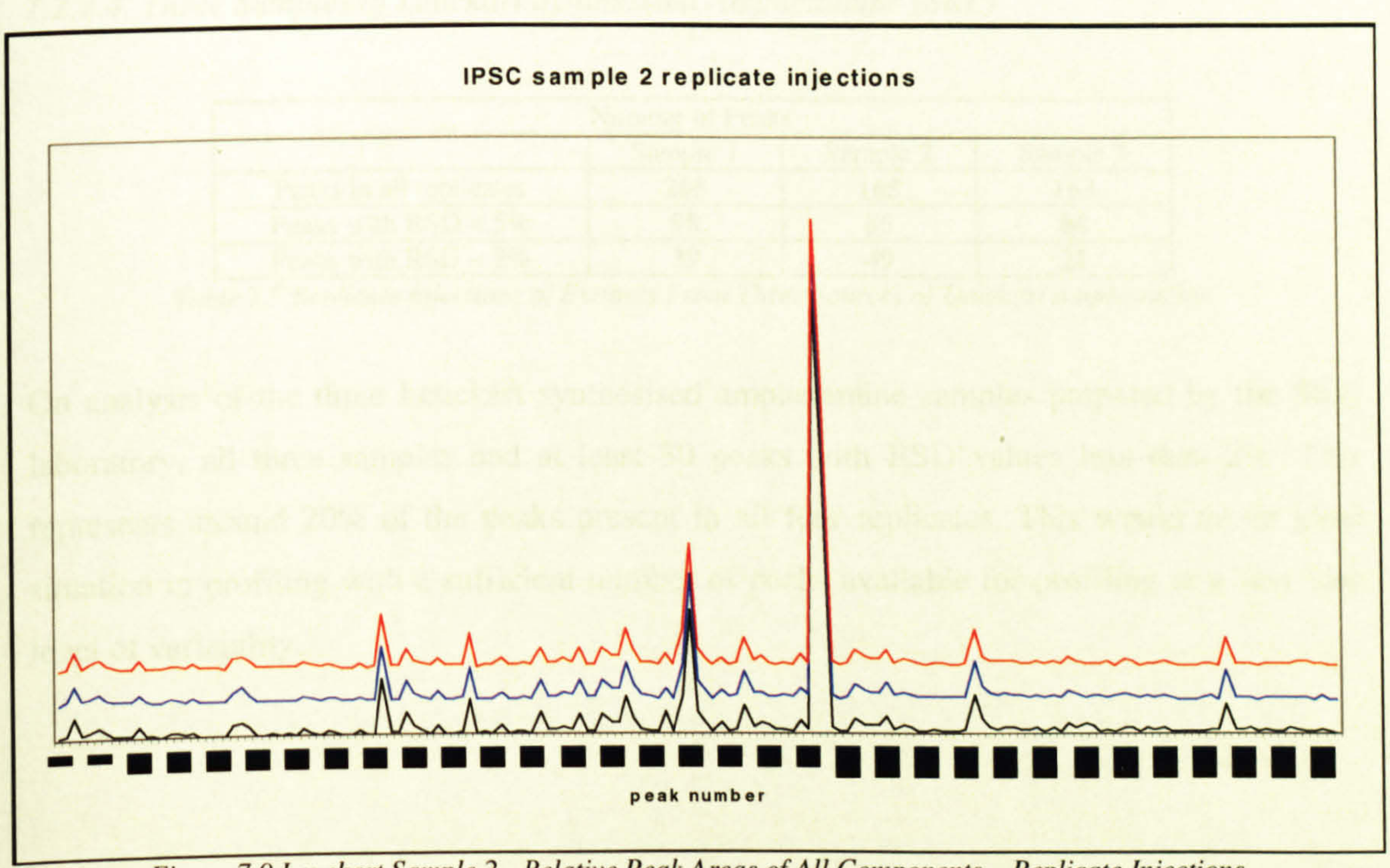


Figure 7.9 Leuckart Sample 2 - Relative Peak Areas of All Components – Replicate Injections

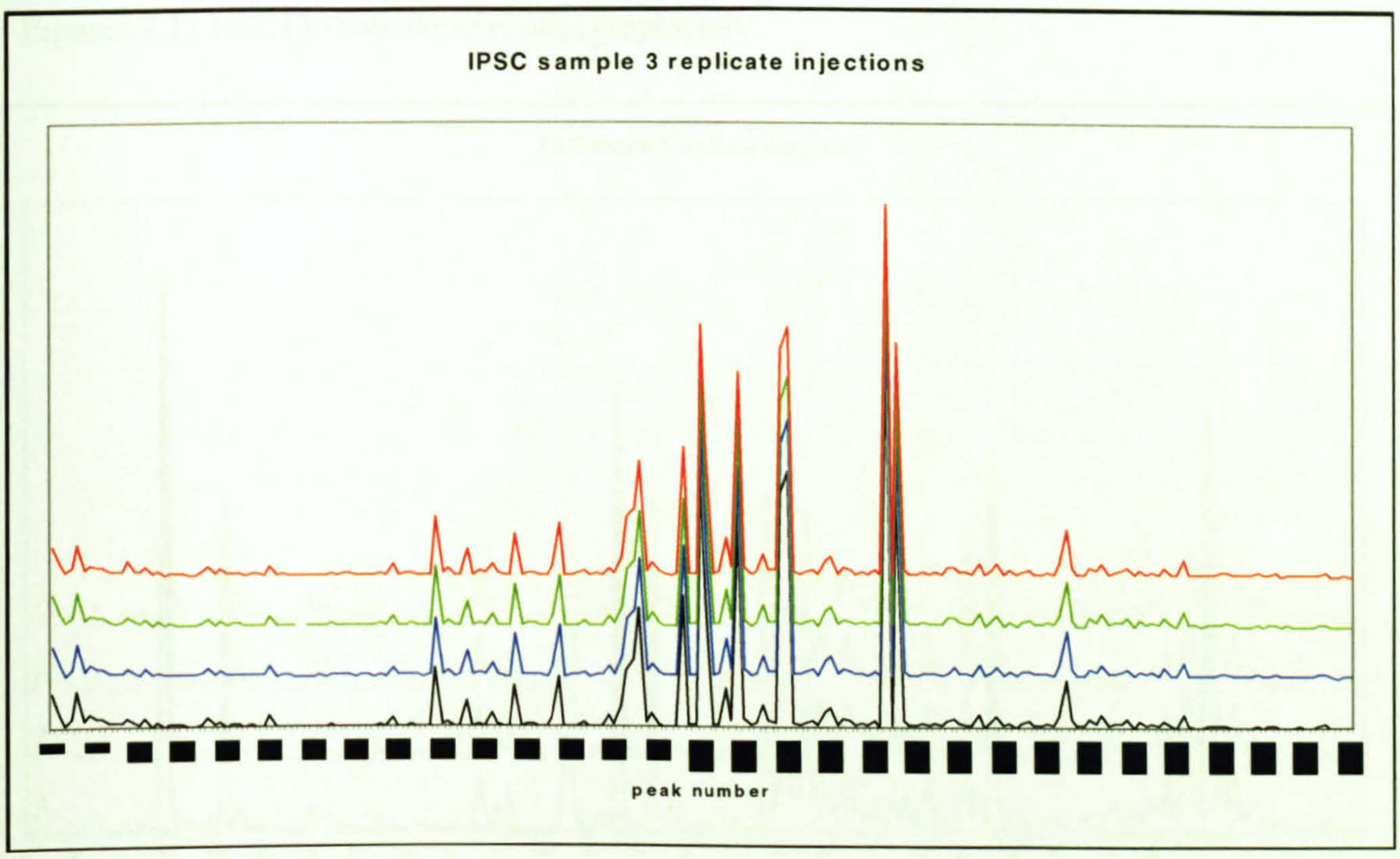


Figure 7.10 Leuckart Sample 3 - Relative Peak Areas of All Components – Replicate Injections

7.2.2.4. Three Samples of Leuckart Synthesised Amphetamine (SKL)

Number of Peaks			
	Sample 1	Sample 2	Sample 3
Peaks in all replicates	**205**	**165**	**163**
Peaks with RSD < 5%	**98**	**85**	**64**
Peaks with RSD < 2%	**39**	**49**	**31**

Table 7.5 Replicate Injections of Extracts From Three Sources of Leuckart Amphetamine

On analysis of the three Leuckart synthesised amphetamine samples prepared by the SKL laboratory, all three samples had at least 30 peaks with RSD values less than 2%. This represents around 20% of the peaks present in all four replicates. This would be an ideal situation in profiling with a sufficient number of peaks available for profiling at a very low level of variability.

Figures 7.11 to 7.13 show these results graphically.

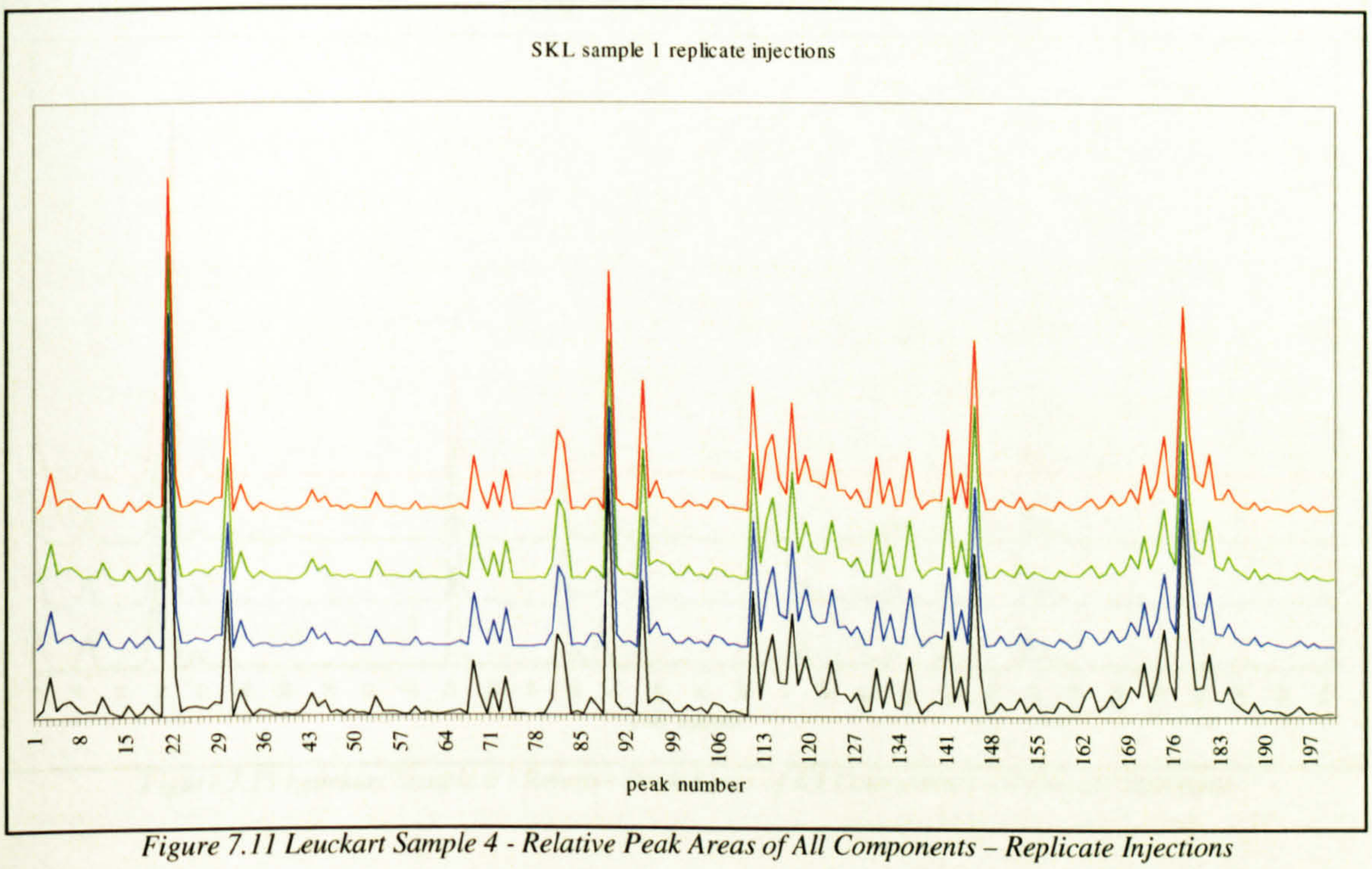


Figure 7.11 Leuckart Sample 4 - Relative Peak Areas of All Components – Replicate Injections

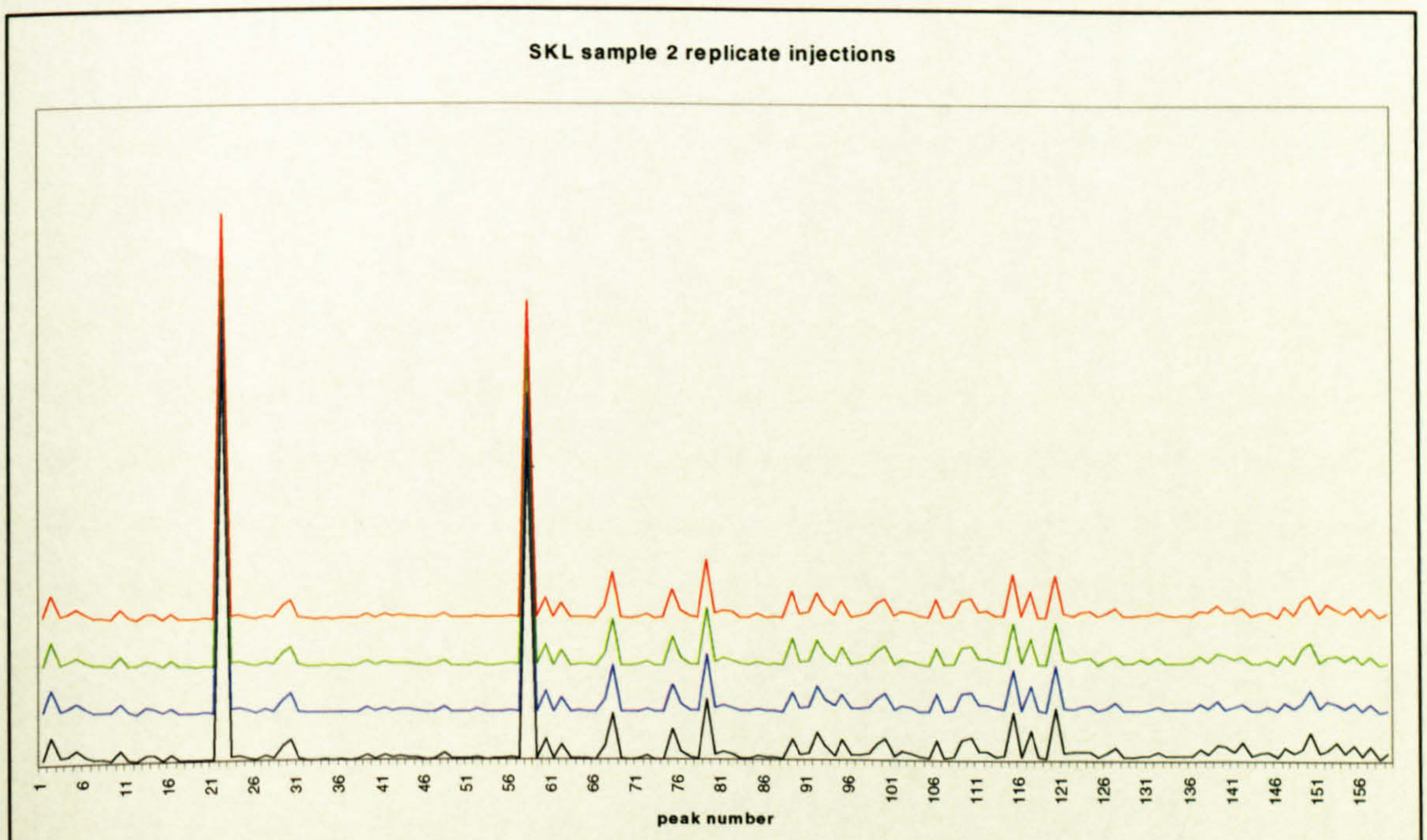


Figure 7.12 Leuckart Sample 5 - Relative Peak Areas of All Components – Replicate Injections

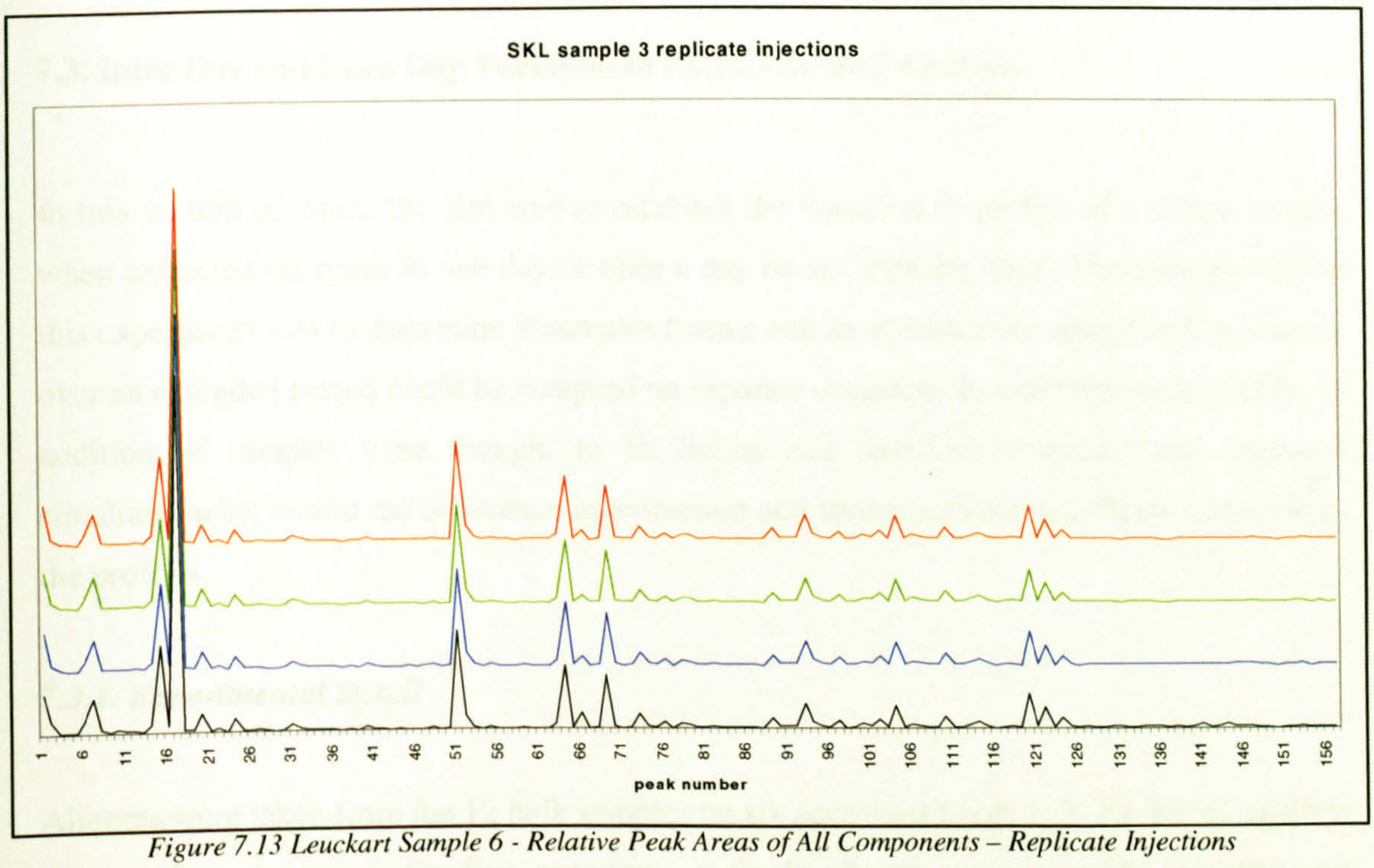


Figure 7.13 Leuckart Sample 6 - Relative Peak Areas of All Components – Replicate Injections

7.3. Inter Day and Intra Day Variation in Extraction and Analysis

In this section of work, the aim was to establish the variation in profile of a single sample when extracted six times in one day or once a day on six separate days. The concept behind this experiment was to determine if samples from a common source but seized independently over an extended period could be analysed on separate occasions to yield the same profile. In addition, if samples were thought to be linked and therefore extracted and analysed simultaneously, would the difference in extraction and analysis cause significant variation in the profiles.

7.3.1. Experimental Detail

Aliquots were taken from the 12 bulk samples on six occasions (days 1, 7, 14, 28, 42 and 56) over an 8-week period. On five occasions, a single aliquot was removed, extracted and analysed. On one occasion, six aliquots were removed and analysed. In total, each of the 12 matrices were analysed 11 times.

The extraction and analysis procedure was identical to that of the injection reproducibility study detailed in section 7.2.

200 mg aliquots were removed from the 12 bulk samples and weighed into a vial. 1M TRIS buffer, pH 8.1, 4 mL, was added and the sample placed in a sonic bath for 15 minutes until the sample was completely dissolved. Toluene (200 µL) containing internal standard at 10 µg/mL, was added and the sample sonicated for a further 15 minutes. The organic phase was removed and placed in a GC vial with insert for analysis by the optimised method.

7.3.2. Results and Discussion

7.3.2.1. IPSC Sample 1 – Toluene Extractions

Amphetamine for this sample was prepared via the Leuckart route in the IPSC lab in Lausanne, Switzerland and bulked using readily available substances commonly used as diluents in amphetamine samples.

In Table 7.6, the total number of peaks common to all replicate extractions in each separate study is shown. Also shown is the number of peaks present with RSD values less than 15%. It is clear, from these results, that although both studies show a large number of peaks are common in the extraction and resulting chromatograms, the relative peak areas of the impurities are quite variable.

	Intra Day Variation	Inter Day Variation
Peaks in all samples	157	165
Peaks with %RSD < 15	56	17

Table 7.6 Number of Peaks Present in Replicate Extractions - Intra and Inter Day Variation

10 peaks were identified as having RSD values less than 15 % in both the intra and inter day variation studies. These peaks are identified in Table 7.7.

IPSC Sample 1 Toluene Extraction Intra Day Variation			IPSC Sample 1 Toluene Extraction Inter Day Variation		
Time	**Mean**	**%RSD**	Time	**Mean**	**%RSD**
7.30	42.2	11.9	**7.30**	37.8	3.18
13.46	8.16	3.84	**13.46**	7.62	7.34
14.08	3.05	5.10	**14.07**	2.94	3.03
15.35	2.37	5.74	**15.36**	2.24	7.89
16.86	0.06	10.9	**16.86**	0.05	7.31
17.69	0.04	10.9	**17.69**	0.04	1.26
18.13	0.17	7.19	**18.12**	0.15	7.99
18.18	0.16	9.66	**18.17**	0.16	10.4
20.30	7.54	10.4	**20.30**	7.43	3.94
20.68	41.9	9.54	**20.72**	42.5	7.67

Table 7.7 IPSC Sample 1 - Toluene Extractions.
Peaks Common to Both Intra and Inter Day Variation Studies with RSD Less Than 15%

A graphical representation of the chromatographic profile of the samples in the intra day variation is shown in Figure 7.14. As may be seen from the superimposition of the chromatographic profiles, a visual comparison highlights the similarities and divergence across the replicates. Figure 7.15 shows the variation of the extraction across 7 days.

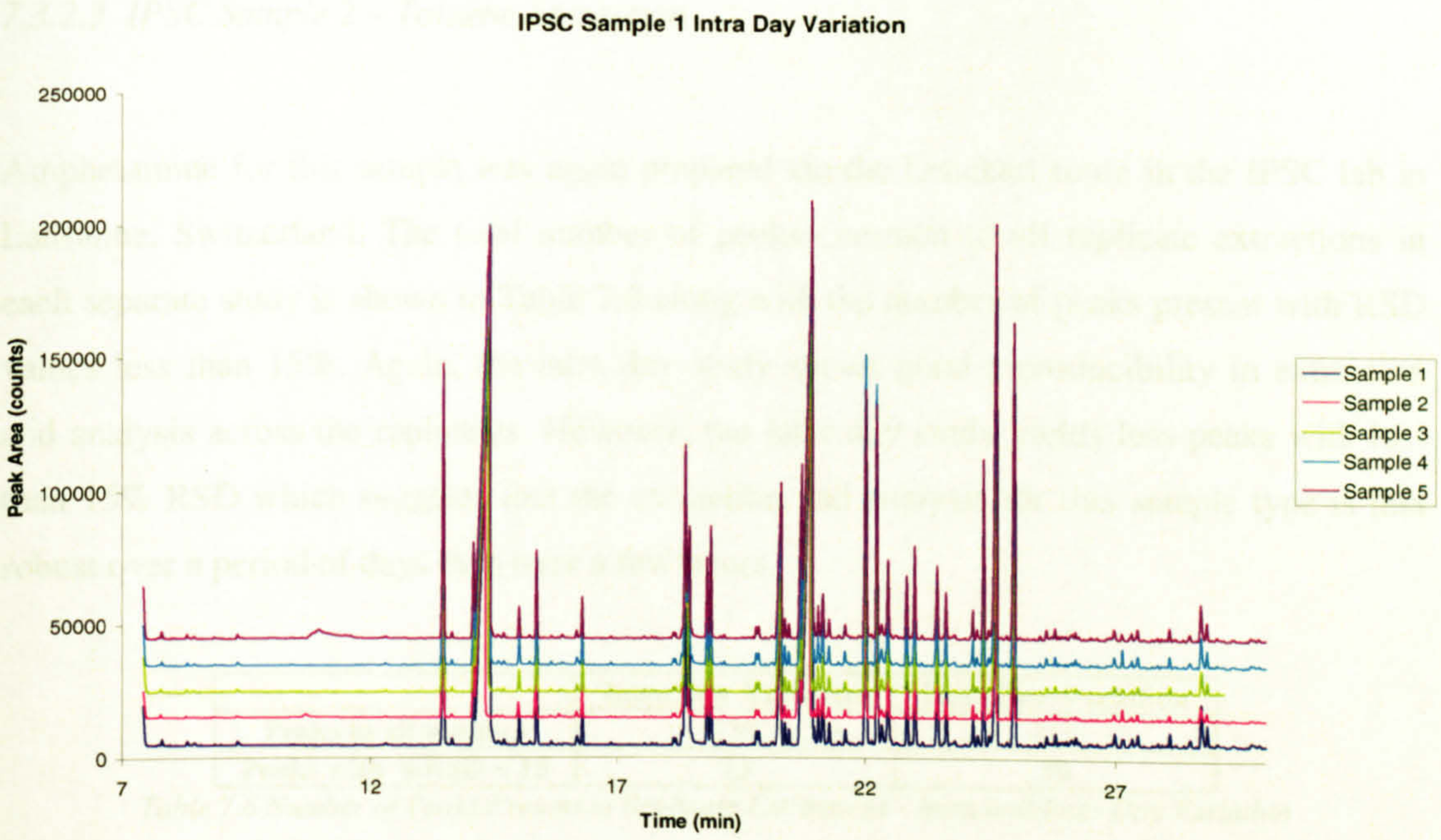


Figure 7.14: IPSC Sample 1 - Toluene Extractions -Intra Day Variation in Replicate Samples

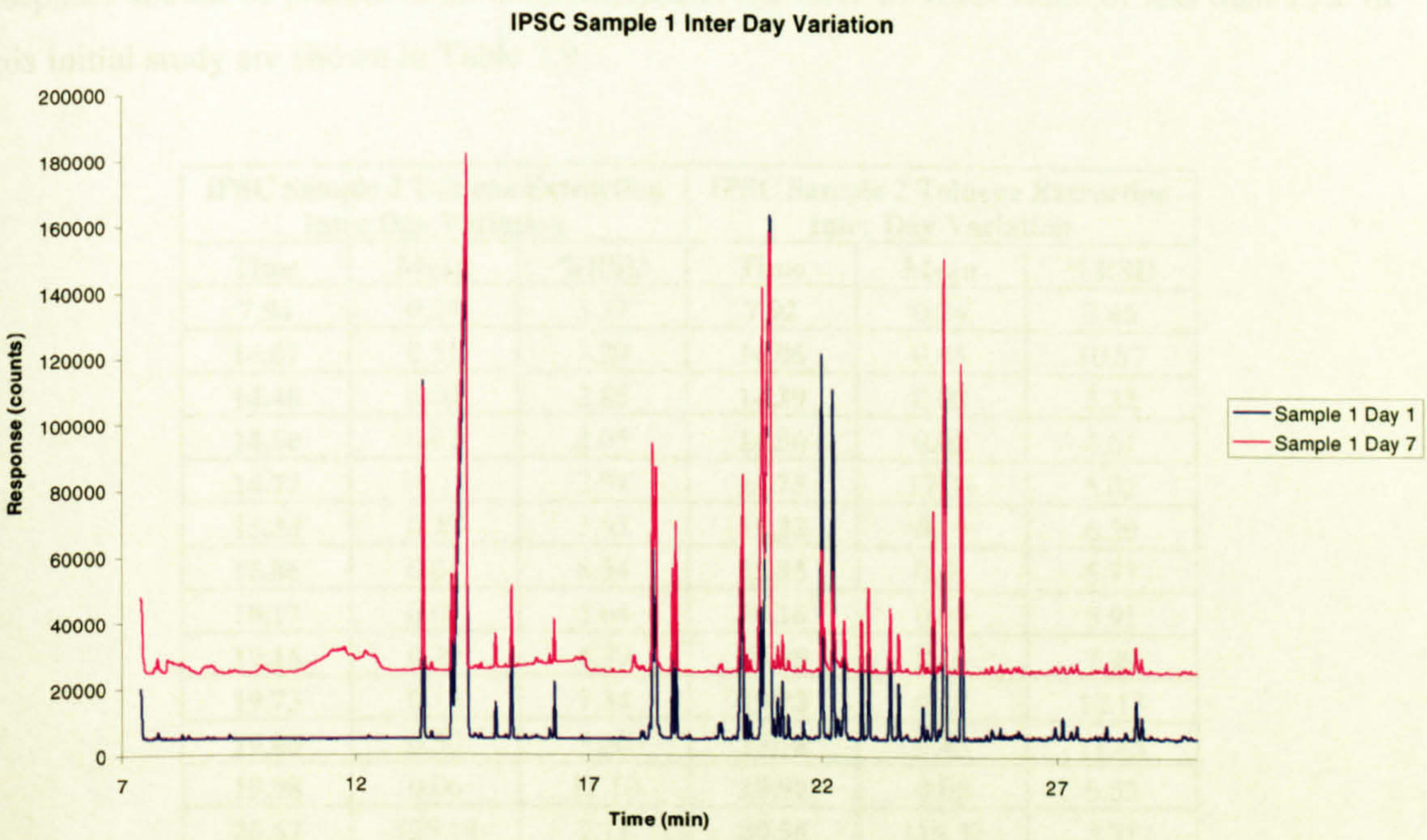


Figure 7.15: IPSC Sample 1 - Toluene Extractions - Intra Day Variation Over 7 Days

7.3.2.3. IPSC Sample 2 – Toluene Extraction

Amphetamine for this sample was again prepared via the Leuckart route in the IPSC lab in Lausanne, Switzerland. The total number of peaks common to all replicate extractions in each separate study is shown in Table 7.8 along with the number of peaks present with RSD values less than 15%. Again, the intra day study shows good reproducibility in extraction and analysis across the replicates. However, the inter day study yields less peaks with less than 15% RSD which suggests that the extraction and analysis for this sample type is less robust over a period of days than over a few hours.

	Intra Day Variation	Inter Day Variation
Peaks in all samples	159	119
Peaks with %RSD < 15	**73**	**19**

Table 7.8 Number of Peaks Present in Replicate Extractions - Intra and Inter Day Variation

14 peaks were identified as common in the intra and inter day variation studies having RSD values of less than 15 %. The peaks which could be considered as being useful for profiling purposes should be present in all chromatograms and have an RSD value of less than 15% in this initial study are shown in Table 7.9

IPSC Sample 2 Toluene Extraction Intra Day Variation			IPSC Sample 2 Toluene Extraction Inter Day Variation		
Time	**Mean**	**%RSD**	**Time**	**Mean**	**%RSD**
7.94	0.24	3.37	**7.92**	0.24	7.46
14.07	0.51	3.29	**14.06**	0.45	10.87
14.40	0.09	2.85	**14.39**	0.08	3.32
14.50	0.02	2.05	**14.50**	0.02	4.61
14.77	18.17	2.94	**14.75**	17.39	5.02
15.33	0.20	3.63	**15.32**	0.19	6.59
15.86	0.01	8.54	**15.85**	0.01	5.77
18.17	0.05	5.04	**18.16**	0.05	5.91
19.16	0.20	4.39	**19.15**	0.18	7.99
19.73	0.12	7.34	**19.72**	0.12	12.13
19.80	0.50	3.40	**19.78**	0.45	11.45
19.98	0.06	11.10	**19.97**	0.05	6.62
20.67	125.14	2.11	**20.55**	116.37	3.21
22.24	0.93	5.80	**22.22**	0.81	14.89

Table 7.9 IPSC Sample 2 - Toluene Extraction
Peaks Common to Both Intra and Inter Day Variation Studies with RSD Less Than 15%

Shown in Figures 7.16 and 7.17 are graphical representations of the intra and inter day variation between sample chromatograms.

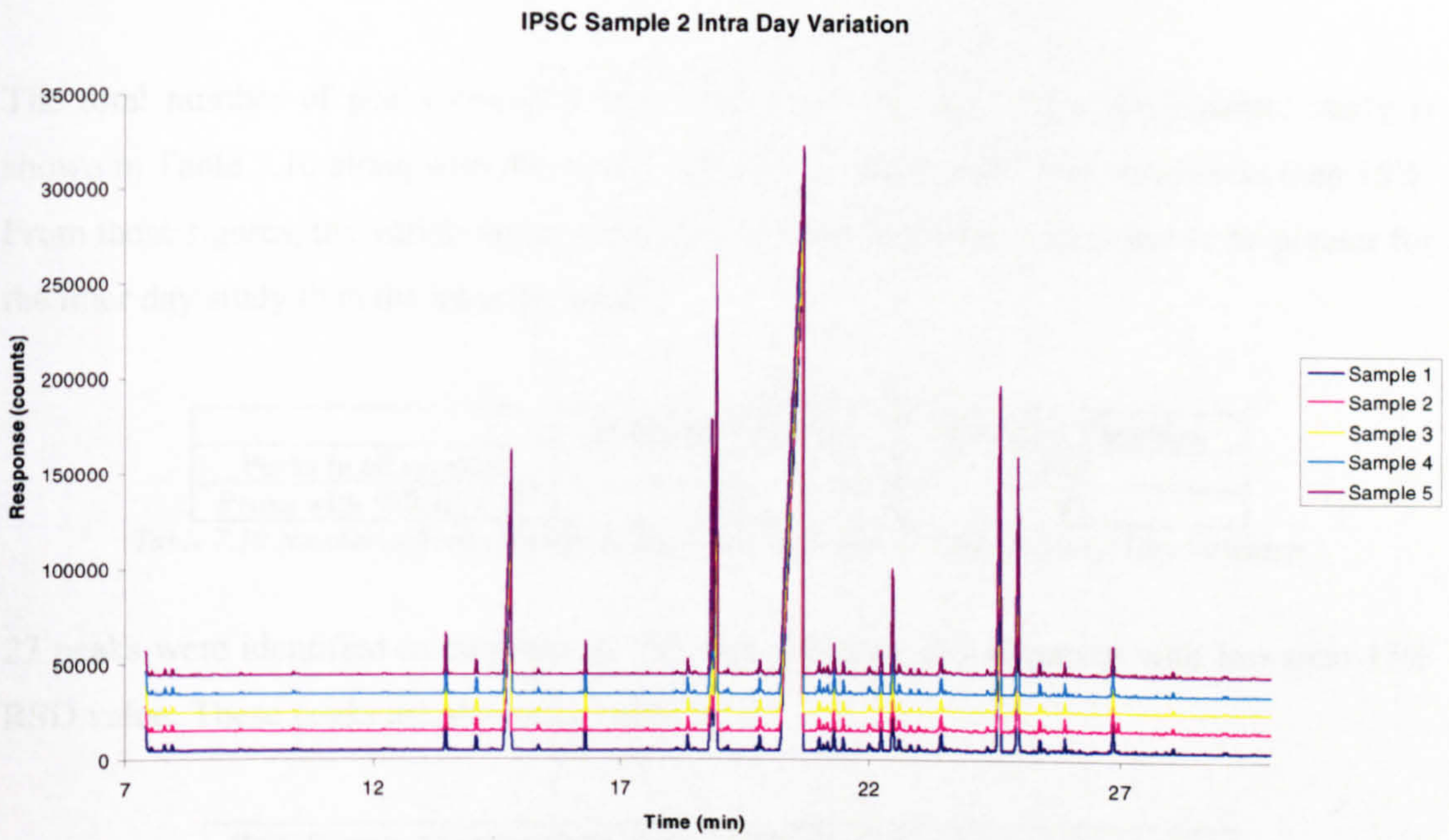


Figure 7.16 IPSC Sample 2 – Toluene Extraction - Intra Day Variation in Replicate Samples

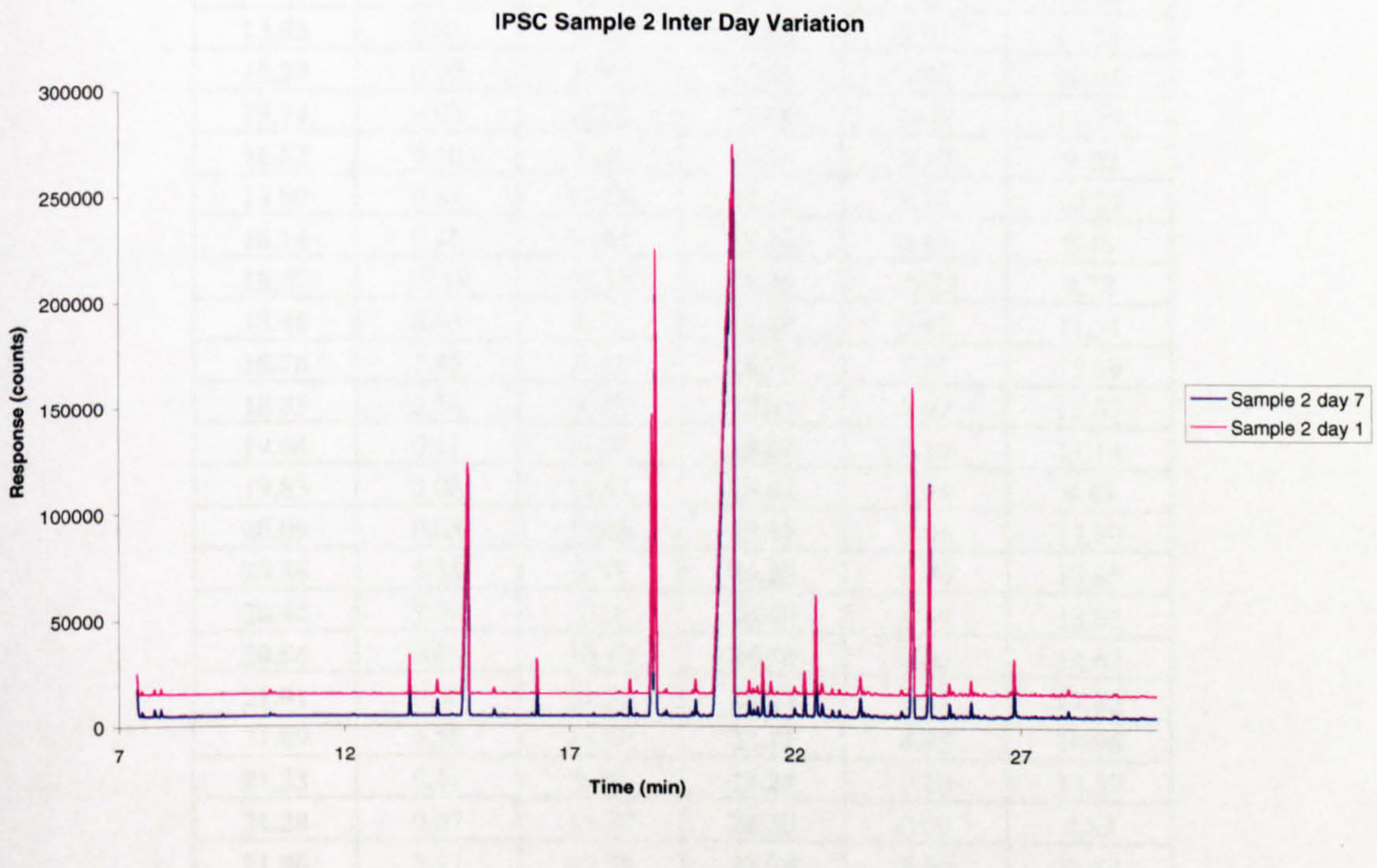


Figure 7.17 IPSC Sample 2 – Toluene Extraction - Inter Day Variation Over 7 Days

7.3.2.4. ISPC Sample 3 – Toluene Extraction

The total number of peaks common to all replicate extractions in each separate study is shown in Table 7.10 along with the number of peaks present with RSD values less than 15%. From these figures, the variability of extraction and analysis does not appear to be greater for the inter day study than the intra day study.

	Intra Day Variation	Inter Day Variation
Peaks in all samples	96	109
Peaks with %RSD < 15	**40**	**43**

Table 7.10 Number of Peaks Present in Replicate Extractions - Intra and Inter Day Variation

27 peaks were identified as common in the intra and inter day variation with less than 15% RSD value. These peaks are shown in Table 7.11

IPSC Sample 3 Toluene Extraction Intra Day Variation			ISPC Sample 3 Toluene Extraction Inter Day Variation		
Time	**Mean**	**%RSD**	**Time**	**Mean**	**%RSD**
7.32	64.63	6.71	**7.34**	66.22	14.74
7.89	0.11	6.45	**7.92**	0.11	14.95
13.42	1.36	8.32	**13.44**	1.23	13.53
13.53	0.03	9.56	**13.55**	0.03	11.21
15.29	0.90	9.94	**15.31**	0.84	10.04
15.74	0.03	12.22	**15.76**	0.03	13.75
16.83	0.10	7.64	**16.85**	0.10	9.70
18.09	0.81	10.19	**18.11**	0.77	10.27
18.14	0.45	10.45	**18.16**	0.43	8.48
18.37	17.18	10.39	**18.39**	16.22	9.72
18.46	0.93	9.32	**18.48**	0.82	11.31
18.78	1.45	9.27	**18.79**	1.37	12.59
18.85	2.54	8.90	**18.87**	2.42	12.37
19.06	0.11	11.77	**19.08**	0.10	11.14
19.83	2.08	11.41	**19.84**	1.99	9.47
20.09	0.05	13.66	**20.11**	0.05	12.80
20.36	5.55	7.35	**20.38**	5.20	13.85
20.44	3.76	7.21	**20.46**	3.49	13.82
20.56	0.06	10.10	**20.58**	0.05	14.63
21.01	0.38	11.22	**21.03**	0.37	11.23
21.09	4.58	13.76	**21.11**	4.45	10.98
21.23	0.11	9.95	**21.25**	0.10	13.39
21.28	0.07	13.70	**21.30**	0.07	8.83
21.96	5.77	12.75	**21.98**	5.60	11.84
22.20	6.08	13.84	**22.21**	5.91	12.82
22.29	0.34	8.08	**22.31**	0.32	9.34
23.11	0.33	13.01	**23.13**	0.32	14.28

Table 7.11: IPSC Sample 3 – Toluene Extraction
Peaks Common to Both Intra and Inter Day Variation Studies with RSD Less Than 15%

Figures 7.18 show a graphical representation of the chromatographic profiles from Day 1 and Day 7 in the inter day variation studies. Once again, the profiles are almost completely superimposed which suggests, at least visually, the impurity profiles could be linked.

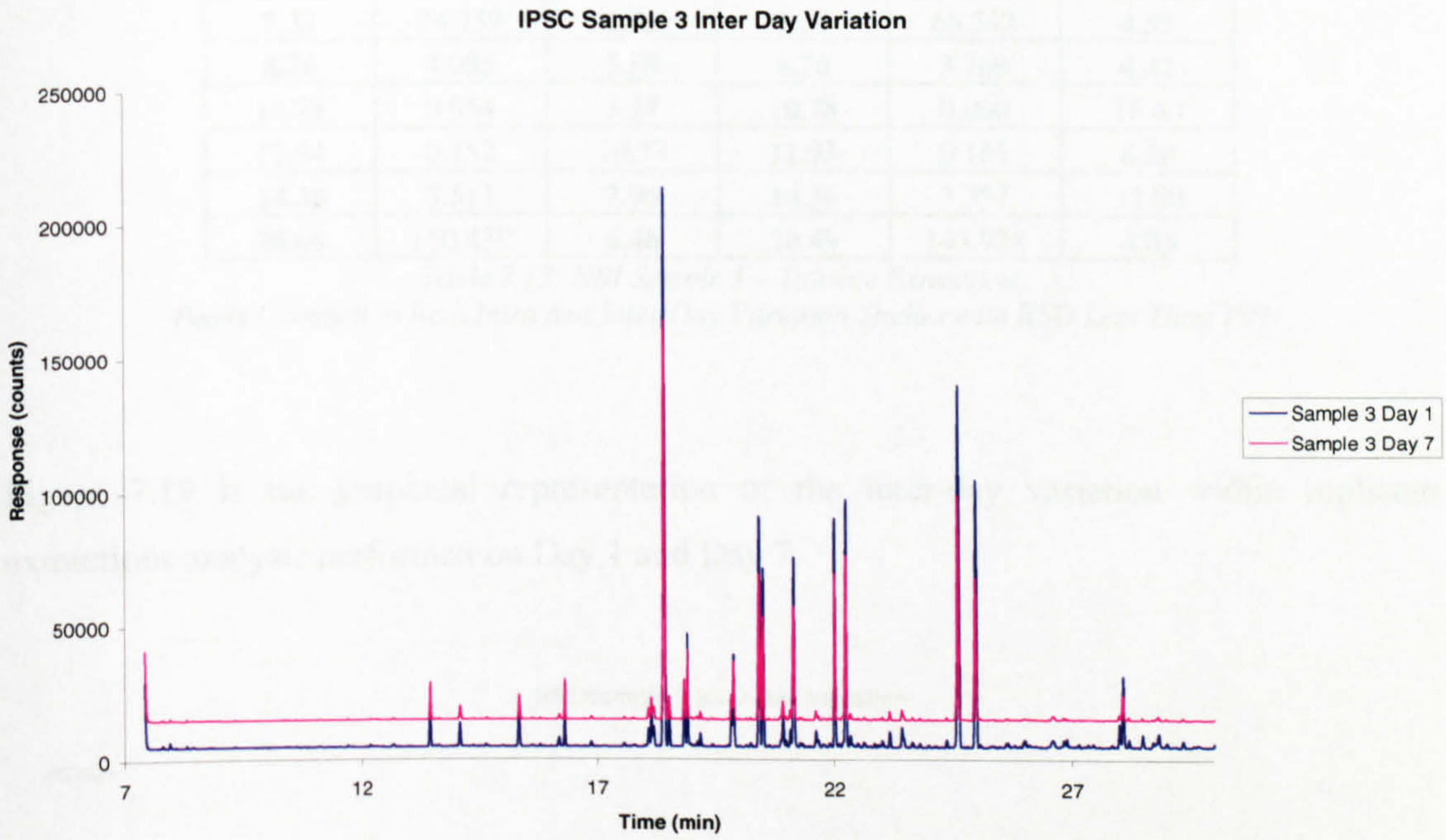


Figure 7.18 ISPC Sample 3 - Toluene Extraction - Inter Day Variation Over 7 Days

7.3.2.5. NBI Sample 1 – Toluene Extracts

Amphetamine for this sample was prepared via the reductive amination route in the NBI lab in Vantaa, Finland. The total number of peaks common to all six replicate extractions in each separate study is shown in Table 7.12 along with the number of peaks present with RSD values less than the requisite 15%. From this data, it appears that the reductive amination samples have fewer impurities common to all the chromatograms than the Leuckart amphetamine samples. In addition, there are less impurity peaks with consistently reproducible results.

	Intra Day Variation	**Inter Day Variation**
Peaks in all samples	84	82
Peaks with %RSD < 15	**25**	**14**

Table 7.12 Number of Peaks Present in Replicate Extractions - Intra and Inter Day Variation

Only 6 peaks were common to both the intra and inter-day studies with an RSD value of less than 15%. These peaks are shown in Table 7.13.

NBI Sample 1 Toluene Extraction Intra Day Variation			NBI Sample 1 Toluene Extraction Inter Day Variation		
Time	**Mean**	**% RSD**	**Time**	**Mean**	**% RSD**
7.33	74.939	**4.95**	**7.34**	66.542	**4.55**
8.70	4.085	**5.58**	**8.70**	3.769	**4.42**
10.79	0.054	**5.27**	**10.78**	0.060	16.40
11.94	0.152	10.33	**11.93**	0.161	**6.28**
14.38	3.511	**7.96**	**14.36**	3.357	12.80
20.68	150.439	**6.46**	**20.49**	143.928	**4.03**

Table 7.13: NBI Sample 1 – Toluene Extraction
Peaks Common to Both Intra and Inter Day Variation Studies with RSD Less Than 15%

Figure 7.19 is the graphical representation of the inter-day variation within replicate extractions analysis performed on Day 1 and Day 7.

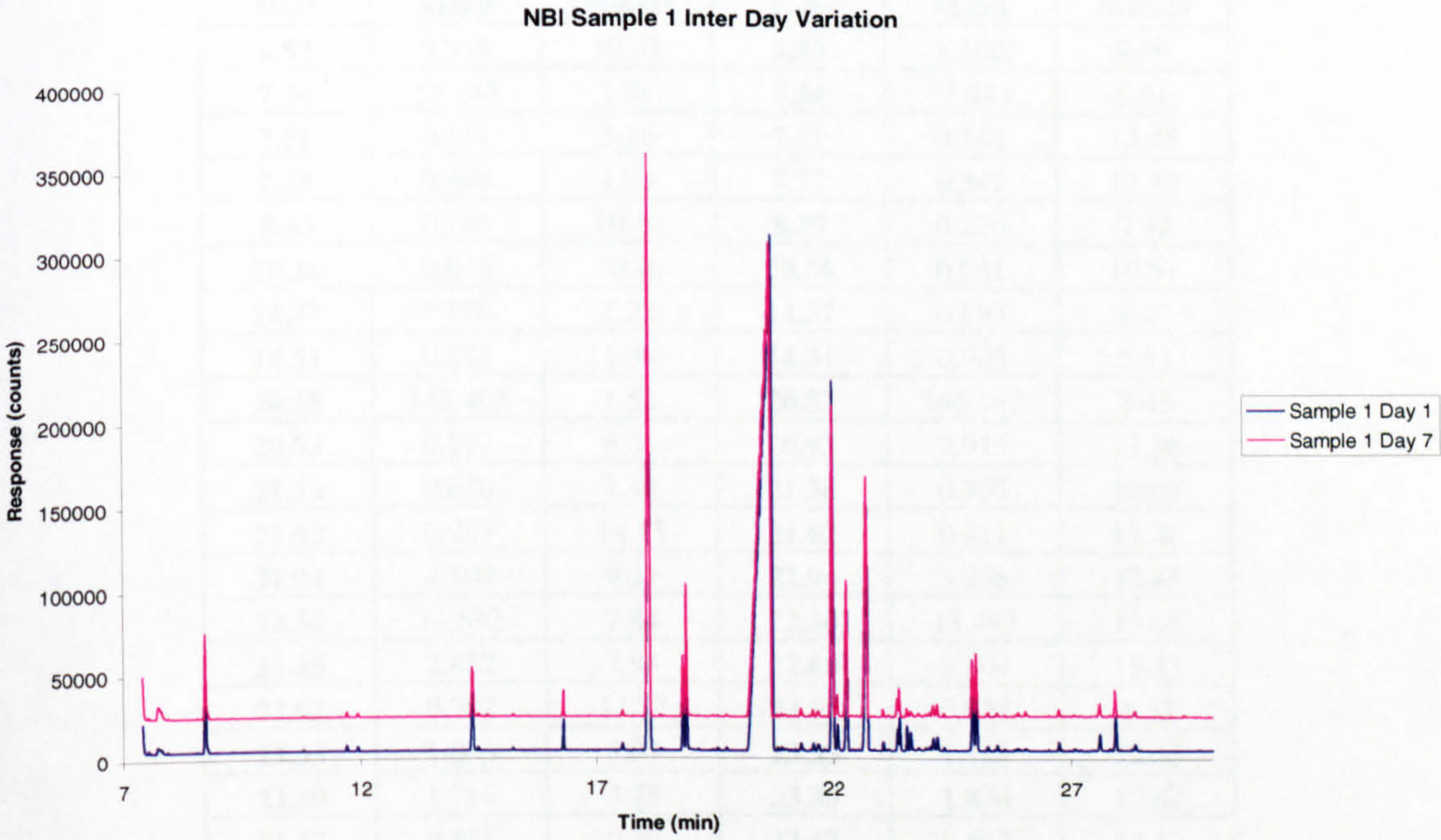


Figure 7.19 NBI Sample 1 - Toluene Extractions - Inter Day Variation Over 7 Days

7.3.2.6. NBI Sample 2 – Toluene Extraction

This sample was also prepared by the reductive amination route. The total number of peaks common to all six replicate extractions in each separate study is shown in Table 7.14 along with the number of peaks present with RSD values less than the requisite 15%.

	Intra Day Variation	Inter Day Variation
Peaks in all samples	146	124
Peaks with %RSD < 15	**58**	**38**

Table 7.14: Number of Peaks Present in Replicate Extractions - Intra and Inter Day Variation

Table 7.15 shows the peaks common to both the intra and inter day studies with an RSD value of less than 15%.

NBI Sample 2 Toluene Extraction Intra-Day Variation			**NBI Sample 2 Toluene Extraction Inter-Day Variation**		
Time	Mean	%RSD	Time	Mean	%RSD
6.83	0.936	10.33	**6.83**	1.108	8.69
7.34	63.243	1.89	**7.34**	61.444	6.01
7.51	0.151	5.16	**7.51**	0.142	12.45
7.78	0.498	1.99	**7.77**	0.542	13.99
8.39	0.186	10.90	**8.39**	0.226	9.32
10.36	0.038	10.40	**10.36**	0.041	10.51
14.37	0.876	1.22	**14.37**	0.893	8.47
14.51	0.883	1.39	**14.51**	0.905	6.53
20.58	142.405	1.52	**20.57**	146.192	8.46
20.93	0.797	6.61	**20.93**	0.915	13.36
21.56	0.670	7.42	**21.56**	0.703	10.60
21.62	0.263	14.13	**21.62**	0.311	13.36
21.94	4.509	9.31	**21.94**	5.298	12.85
22.30	11.692	7.84	**22.30**	13.443	13.46
22.65	2.822	7.98	**22.65**	3.304	13.43
23.02	0.782	13.77	**23.02**	0.937	5.33
23.33	1.035	5.01	**23.33**	1.118	12.33
23.39	1.716	3.75	**23.39**	1.838	12.87
23.57	9.585	9.39	**23.57**	9.517	13.67
24.08	1.239	6.25	**24.08**	1.366	13.01
24.91	2.840	6.46	**24.91**	2.445	12.42
28.20	8.236	10.39	**28.21**	8.967	9.16

Table 7.15 NBI Sample 2 - Toluene Extractions
Peaks Common to Both Intra and Inter Day Variation Studies with RSD Less Than 15%

Figure 7.20 is the graphical representation of the inter-day variation within replicate extractions analysis performed on Days 1 and 7.

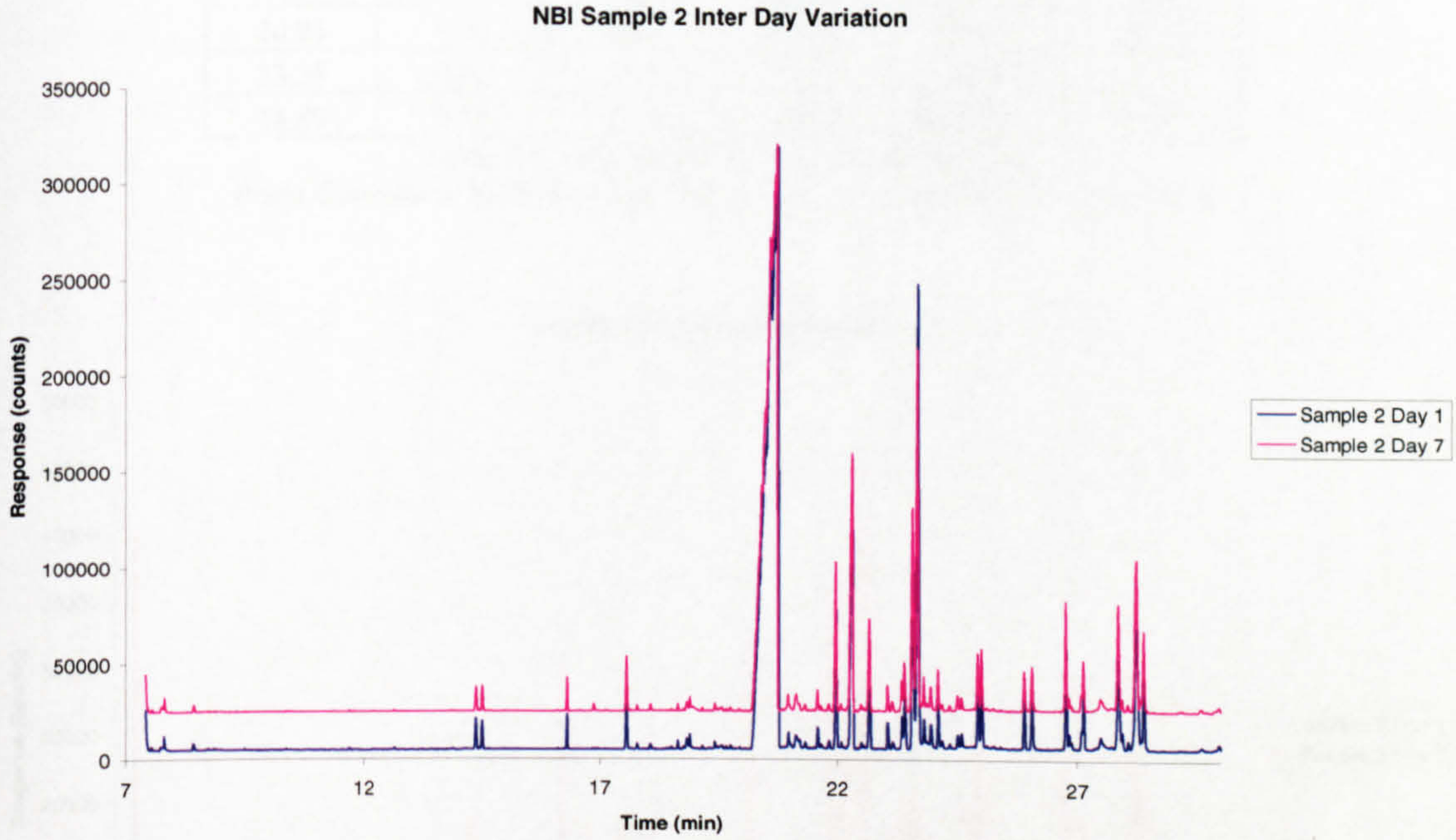


Figure 7.20 NBI Sample 2 - Toluene Extraction - Inter Day Variation Over Days 1 and 7

7.3.2.7. NBI Sample 3 – Toluene Extractions

Amphetamine for this sample was prepared via the reductive amination route in the NBI lab in Vantaa, Finland. The total number of peaks common to all six replicate extractions in each separate study is shown in Table 7.16 along with the number of peaks present with RSD values less than the requisite 15%.

	Intra Day Variation	**Inter Day Variation**
Peaks in all samples	144	74
Peaks with %RSD < 15	**106**	**6**

Table 7.16 Number of Peaks Present in Replicate Extractions - Intra and Inter Day Variation

Table 7.17 shows the peaks present in both the intra and inter day studies with an RSD less than 15%. Only 5 peaks fulfil this criteria. A graphic representation of the chromatographic profiles of replicate samples taken on Day 1 and Day 7 is shown in Figure 7.21.

NBI Sample 3 Toluene Extraction Intra Day Variation			NBI Sample 3 Toluene Extraction Inter Day Variation		
Time	**Mean**	**%RSD**	**Time**	**Mean**	**%RSD**
7.34	66.81	**2.53**	**7.34**	71.67	**5.89**
14.50	0.33	**2.43**	**14.49**	0.33	**1.58**
20.93	0.05	**5.59**	**20.92**	0.04	10.97
23.35	0.22	**1.69**	**23.35**	0.22	14.45
24.89	0.78	**0.63**	**24.89**	0.82	11.73

Table 7.17 NBI Sample 3 - Toluene Extractions
Peaks Common to Both Intra and Inter Day Studies with RSD Less Than 15%

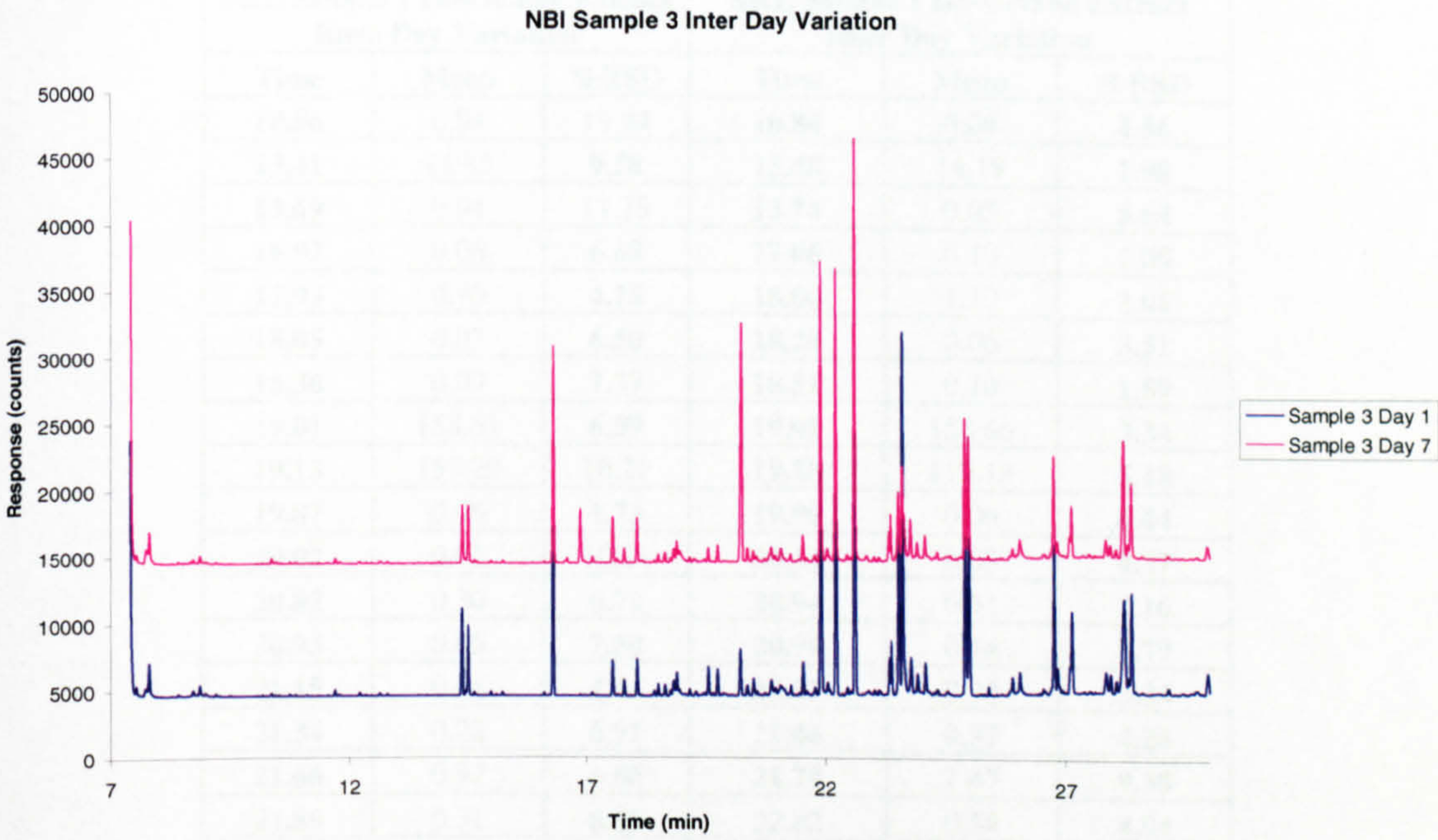


Figure 7.21 NBI Sample 3 - Toluene Extraction - Inter Day Variation Over Days 1 and 7

7.3.2.8. SKL Sample 1 – Iso-octane Extractions

Amphetamine for this sample was prepared via the Leuckart route in the SKL lab in Linköping, Sweden. The total number of peaks common to all six replicate extractions in each separate study is shown in Table 7.18 with the number of peaks present with RSD values less than 15%.

	Intra Day Variation	Inter Day Variation
Peaks in all samples	79	124
Peaks with %RSD <15	**48**	**82**

Table 7.18 Number of Peaks Present in Replicate Extractions - Intra and Inter Day Variation

The results in this table are unexpected. Essentially, the conclusion that may be drawn from these results, is that the samples would be best extracted on separate days. The number of peaks which are reproducibly extracted and analysed over 56 days, (at the 15% RSD level) is almost double the amount when analysed on a single day. Table 7.19 shows the peaks present in both intra and inter day studies with an RSD value less than 15%.

SKL Sample 1 Iso-Octane Extract Intra Day Variation			SKL Sample 1 Iso-Octane Extract Inter Day Variation		
Time	**Mean**	**%RSD**	**Time**	**Mean**	**%RSD**
10.86	0.04	19.94	**10.84**	0.04	**4.56**
13.41	14.85	**9.58**	**13.48**	14.18	**1.98**
13.69	0.04	11.78	**13.74**	0.05	**5.64**
16.92	0.06	**6.68**	**17.06**	0.10	**2.08**
17.93	0.90	**4.78**	**18.06**	1.10	**2.06**
18.05	0.03	**6.50**	**18.20**	0.06	**3.51**
18.36	0.09	**7.37**	**18.51**	0.10	**1.59**
19.01	158.61	**6.59**	**19.01**	158.66	**3.34**
19.15	155.29	10.21	**19.10**	112.13	**5.18**
19.87	0.06	**1.73**	**19.99**	0.06	**6.44**
20.02	0.02	10.21	**20.14**	0.03	**4.37**
20.82	0.30	**6.72**	**20.94**	0.31	**2.16**
20.93	0.06	**7.50**	**20.99**	0.14	**8.79**
21.15	0.64	**4.44**	**21.27**	0.95	**3.34**
21.34	0.22	**6.97**	**21.46**	0.37	**4.28**
21.66	0.92	**6.86**	**21.78**	1.47	**9.38**
21.89	0.31	**8.86**	**22.02**	0.54	**4.04**
21.99	0.10	13.03	**22.12**	0.22	10.94
22.06	0.52	13.70	**22.18**	0.71	**9.94**
22.85	0.09	13.29	**22.98**	0.16	**7.66**
23.32	0.31	**7.80**	**23.45**	0.54	**8.03**
23.76	0.02	12.54	**23.89**	0.05	**3.73**
23.82	0.05	**2.40**	**23.95**	0.05	**6.06**
24.23	0.05	**7.55**	**24.36**	0.11	10.93
24.34	0.04	**5.23**	**24.47**	0.07	**5.30**
24.44	0.50	**9.18**	**24.57**	0.88	**5.24**
25.10	0.20	**5.63**	**25.22**	0.24	**4.76**
25.94	0.07	11.33	**26.07**	0.12	10.02
27.82	0.08	**8.66**	**27.97**	0.14	**7.52**

Table 7.19: SKL Sample 1 - Iso-Octane Extraction
Peaks Common to Both Intra and Inter Day Variation Studies with RSD Less Than 15%

7.3.2.9. SKL Sample 1 – Toluene Extraction

SKL Sample 1 was also extracted into toluene to determine the most suitable solvent in terms of reproducibility. The total number of peaks common to all six replicate extractions in each separate study is shown in Table 7.20 along with the number of peaks present with RSD values less than 15%. The results obtained for the toluene profiles are closer to what would be expected in inter and intra day studies ie. that the extractions and analyses are more reproducible if carried out on a single day. Figure 7.22 shows the inter-day variation of the chromatograms on Day 1 and Day 7.

	Intra Day Variation	**Inter Day Variation**
Peaks in all samples	152	134
Peaks with % RSD < 15	**119**	**41**

Table 7.20 Number of Peaks Present in Replicate Extractions - Intra and Inter Day Variation

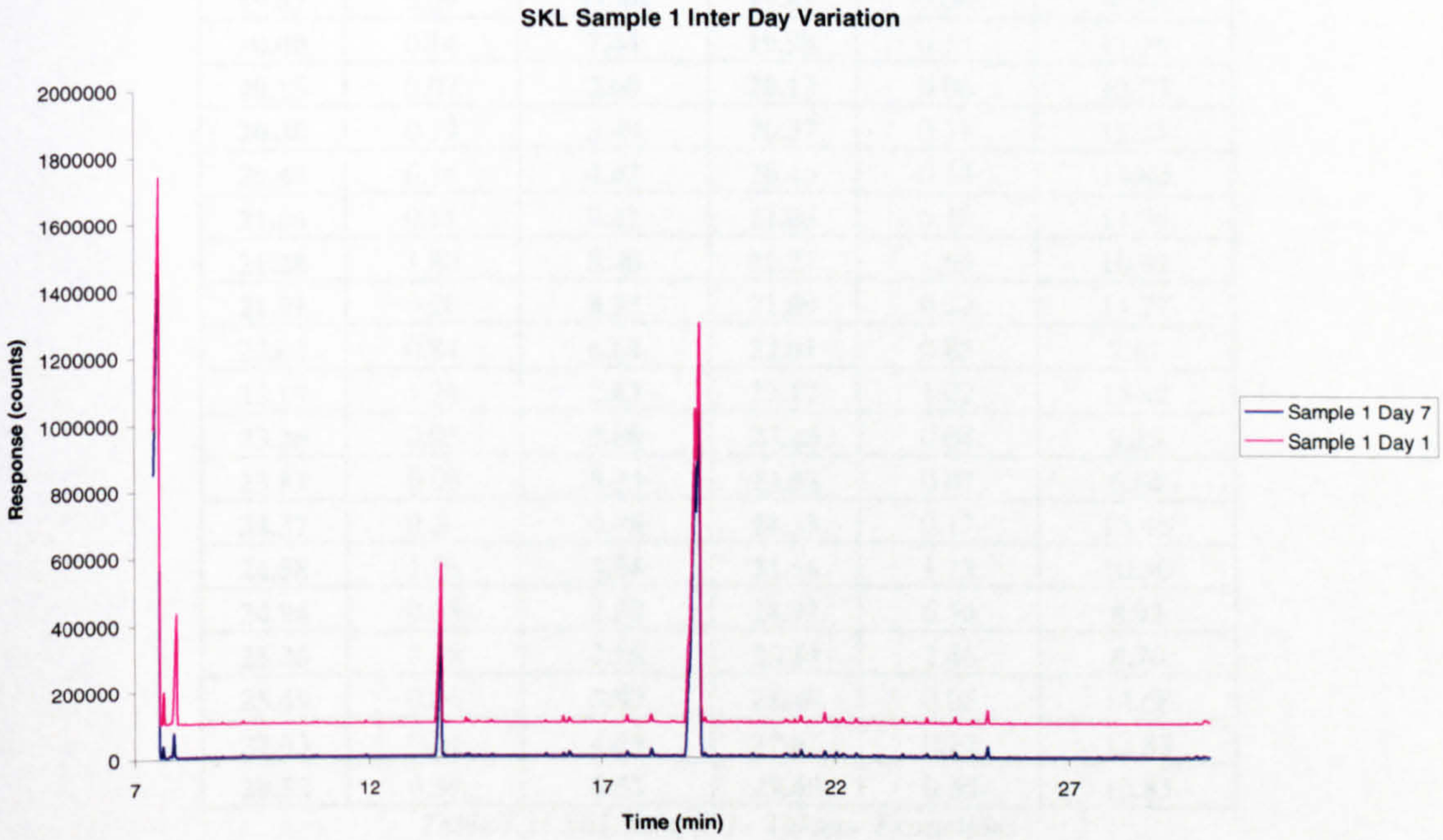


Figure 7.22 SKL Sample 1 - Toluene Extraction - Inter Day Variation Over Days 1 and 7

Table 7.21 shows the peaks common to both the intra and inter day study with RSD values less than 15%.

SKL Sample 1 Toluene Extraction Intra Day Variation			SKL Sample 1 Toluene Extraction Inter Day Variation		
Time	**Mean**	**%RSD**	**Time**	**Mean**	**%RSD**
10.79	0.12	**5.92**	**10.79**	0.12	**7.03**
10.86	0.22	**3.11**	**10.86**	0.21	**3.82**
11.77	0.05	**3.56**	**11.76**	0.04	13.62
11.93	0.35	**4.06**	**11.92**	0.31	14.63
13.02	0.04	**8.17**	**13.02**	0.04	10.49
13.57	65.65	**4.17**	**13.56**	61.94	**6.91**
13.68	0.03	**9.32**	**13.68**	0.03	10.92
13.83	0.09	**7.13**	**13.83**	0.10	**7.57**
14.36	0.22	**4.90**	**14.36**	0.20	**7.43**
14.55	0.03	10.25	**14.55**	0.03	**6.29**
14.64	0.34	**8.54**	**14.64**	0.34	**4.15**
17.64	0.16	**2.15**	**17.63**	0.15	12.82
18.07	1.76	**3.58**	**18.06**	1.63	**9.65**
18.51	0.38	**2.43**	**18.50**	0.34	13.04
19.05	231.76	**3.70**	**19.01**	208.07	13.83
19.14	139.78	**3.25**	**19.10**	136.40	**7.11**
19.23	1.06	13.80	**19.20**	0.92	19.06
19.82	0.09	11.44	**19.81**	0.08	**9.27**
20.00	0.14	**7.44**	**19.98**	0.13	11.79
20.15	0.07	**2.60**	**20.13**	0.06	13.27
20.38	0.19	**3.49**	**20.37**	0.17	12.26
20.47	0.16	**4.03**	**20.46**	0.14	14.80
21.06	0.18	**7.41**	**21.05**	0.16	11.76
21.28	1.80	**8.40**	**21.27**	1.65	10.97
21.91	0.28	**8.33**	**21.89**	0.27	11.27
22.02	0.84	**6.64**	**22.01**	0.85	**7.67**
22.19	1.24	**2.63**	**22.17**	1.02	15.42
23.26	0.05	**5.68**	**23.25**	0.04	**8.15**
23.81	0.09	**5.23**	**23.82**	0.07	**6.64**
24.37	0.20	**4.79**	**24.35**	0.17	13.46
24.58	1.28	**3.24**	**24.56**	1.13	10.50
24.94	0.63	**2.33**	**24.92**	0.56	**8.92**
25.26	2.75	**2.16**	**25.24**	2.46	**8.76**
25.49	0.06	**3.09**	**25.48**	0.05	14.68
27.63	0.24	**4.59**	**27.61**	0.22	12.83
29.52	0.39	**8.52**	**29.49**	0.30	12.83

Table 7.21 SKL Sample 1 - Toluene Extractions
Peaks Common to Both Intra and Inter Day Variation Studies with RSD Less Than 20%

7.3.2.10. SKL Sample 2 – Iso-Octane Extracts

Again, this Leuckart amphetamine was analysed after extraction with both toluene and iso-octane for comparison. The total number of peaks common to all six replicate extractions in each separate study is shown in Table 7.22 with the number of peaks present with RSD values less than 15%. It may be seen that, although both sets of chromatograms have almost identical numbers of peaks common in both intra and inter day studies, the intra day study gives the most reproducible profiles by far.

	Intra Day Variation	Inter Day Variation
Peaks in all samples	110	113
Peaks with % RSD < 15	**86**	**30**

Table 7.22 Number of Peaks Present in Replicate Extractions - Intra and Inter Day Variation

Table 7.23 highlights the peaks present in both inter and intra day studies with RSD values less than 15 %.

SKL Sample 2 Iso-Octane Extracts Intra Day Variation			SKL Sample 2 Iso-Octane Extracts Inter Day Variation		
Time	**Mean**	**%RSD**	**Time**	**Mean**	**%RSD**
7.17	6.40	**4.77**	**7.17**	6.31	**8.47**
7.41	1.41	10.32	**7.41**	1.37	10.78
7.70	8.72	**7.59**	**7.71**	9.44	**9.13**
10.85	0.01	12.78	**10.84**	0.01	10.57
11.32	0.02	**6.72**	**11.31**	0.02	**9.98**
12.67	0.03	11.18	**12.67**	0.03	10.53
13.47	11.26	**4.18**	**13.48**	11.45	**6.57**
13.83	0.03	**9.35**	**13.82**	0.03	13.99
14.07	0.75	**4.61**	**14.06**	0.77	**4.76**
18.06	0.70	**1.93**	**18.05**	0.74	12.80
18.50	0.04	**2.00**	**18.49**	0.04	**6.19**
19.01	69.67	**4.48**	**19.04**	72.22	10.92
19.13	0.07	**4.53**	**19.14**	0.07	14.64
19.29	0.02	**6.67**	**19.29**	0.02	11.06
19.35	0.01	**9.71**	**19.35**	0.01	**9.33**
20.14	0.01	14.59	**20.13**	0.01	14.11
20.24	0.88	**4.10**	**20.23**	0.84	**9.47**
21.27	0.28	**2.57**	**21.26**	0.29	**7.85**
21.46	0.20	**2.38**	**21.45**	0.20	11.36
21.63	0.04	**2.91**	**21.62**	0.05	12.60
21.77	0.27	**3.42**	**21.75**	0.29	14.12
24.46	0.03	**1.47**	**24.45**	0.03	13.45

Table 7.23 SKL Sample 2 - Iso-octane Extractions
Peaks Common to Both Intra and Inter Day Variation Studies with RSD Less Than 15%

7.3.2.11. SKL Sample 2 – Toluene Extractions

Sample 2 was also extracted into toluene. The total number of peaks common to all six replicate extractions in each separate study and with RSD values less than 15% may be seen in Table 7.24. This data again suggests that the extraction and analysis of linked samples on a single day produces more reproducible results than those studies on separate occasions. Table 7.25 shows the peaks present in the inter and intra day studies with an RSD value less than 15 %.

	Intra Day Variation	Inter Day Variation
Peaks in all samples	101	71
Peaks with % RSD < 15	**69**	**24**

Table 7.24Number of Peaks Present in Replicate Extractions - Intra and Inter Day Variation

SKL Sample 2 Toluene Extractions Intra Day Variation			SKL Sample 2 Toluene Extractions Inter Day Variation		
Time	Mean	%RSD	Time	Mean	%RSD
7.57	8.71	**3.26**	**7.57**	8.60	**6.78**
7.88	45.56	**2.99**	**7.88**	42.79	13.92
9.30	0.25	**6.76**	**9.30**	0.31	14.89
11.33	0.06	**4.85**	**11.33**	0.06	10.63
11.47	0.04	**1.51**	**11.47**	0.04	10.14
11.75	0.04	**1.94**	**11.75**	0.04	**7.88**
11.93	0.11	**3.42**	**11.93**	0.12	12.94
13.54	51.83	**2.75**	**13.55**	52.36	**8.68**
14.10	6.39	**2.65**	**14.10**	6.45	**8.76**
14.37	0.38	**2.14**	**14.37**	0.39	12.08
17.06	0.08	**2.58**	**17.06**	0.08	11.56
17.64	0.17	**2.39**	**17.64**	0.17	13.13
18.06	1.22	**3.23**	**18.06**	1.21	12.13
18.95	201.17	**2.99**	**18.96**	198.95	13.39
19.74	0.08	**4.92**	**19.74**	0.07	**8.56**
19.99	0.05	**8.36**	**19.99**	0.05	12.87
20.70	146.33	**2.21**	**20.71**	131.58	10.45
21.79	0.41	**4.44**	**21.79**	0.38	14.85
22.14	0.13	**3.93**	**22.14**	0.13	11.76
22.99	0.13	10.71	**22.99**	0.12	14.97
23.45	0.41	**6.89**	**23.45**	0.40	13.89

Table 7.25 SKL Sample 2 - Toluene Extractions
Peaks Common to Both Intra and Inter Day Variation Studies with RSD Less Than 15%

The profiles of replicate extracts in the intra and inter day studies may be seen graphically in Figures 7.23 and 7.24. There are certain peaks that, while present in all extracts, are particularly variable but for most peaks, the profiles are very similar.

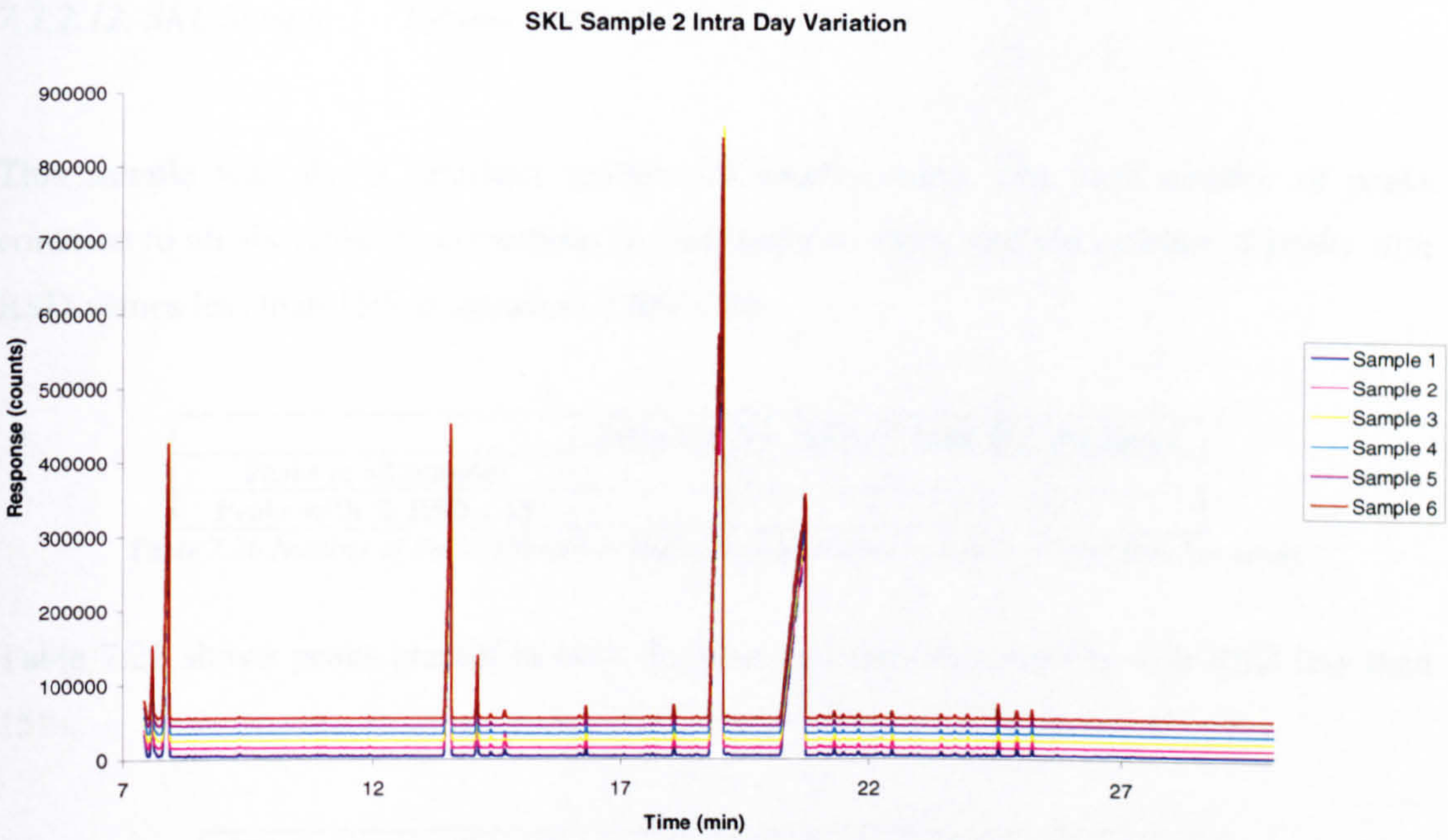


Figure 7.23 SKL Sample 2 – Toluene Extraction – Intra Day Variation in Replicate Samples

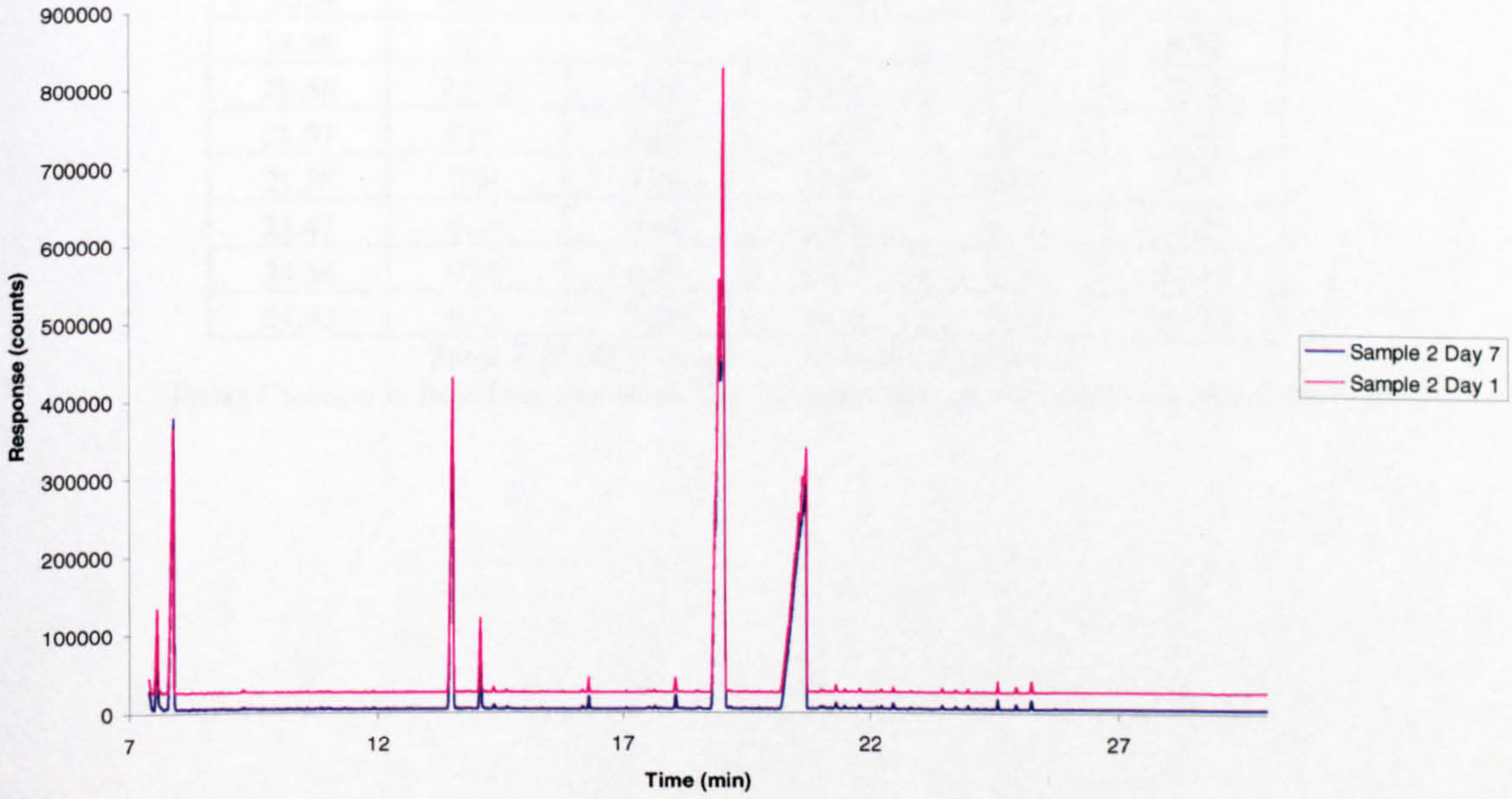


Figure 7.24 SKL Sample 2 – Toluene Extraction - Inter Day Variation Over days 1 and 7

7.3.2.12. SKL Sample 3 – Toluene Extractions

This sample was also a Leuckart synthesised amphetamine. The total number of peaks common to all six replicate extractions in each separate study and the number of peaks with RSD values less than 15% is shown in Table 7.26.

	Intra Day Variation	Inter Day Variation
Peaks in all samples	59	47
Peaks with % RSD < 15	**47**	**19**

Table 7.26 Number of Peaks Present in Replicate Extractions - Intra and Inter Day Variation

Table 7.27 shows peaks present in both the inter and intra day studies with RSD less than 15%.

SKL Sample 3 Toluene Extractions intra Day Variation			**SKL Sample 3 Toluene Extractions Inter Day Variation**		
Time	**Mean**	**%RSD**	**Time**	**Mean**	**%RSD**
7.40	114.43	**5.20**	**7.40**	152.20	11.49
10.86	0.05	**2.84**	**10.86**	0.05	**5.91**
13.48	12.38	**2.96**	**13.48**	11.30	**6.61**
14.07	1.69	**2.89**	**14.07**	1.57	**5.10**
14.13	0.25	**3.21**	**14.13**	0.24	12.87
18.86	27.30	**3.82**	**18.85**	24.41	11.41
18.96	40.59	**2.63**	**18.95**	36.30	11.99
19.98	0.03	10.37	**19.97**	0.03	**9.92**
20.56	74.34	**2.55**	**20.55**	87.91	12.78
21.07	0.05	**4.13**	**21.06**	0.04	12.93
21.28	0.44	**4.76**	**21.28**	0.44	10.31
21.47	0.14	**8.41**	**21.46**	0.13	13.59
24.56	0.66	**6.81**	**24.55**	0.65	11.40
24.93	0.38	**7.24**	**24.92**	0.39	14.42

Table 7.27: SKL Sample 3 - Toluene Extractions
Peaks Common to Both Intra and Inter Day Variation Studies with RSD Less Than 15%

Figures 7.25 shows the profiles of inter day variation for SKL sample 3.

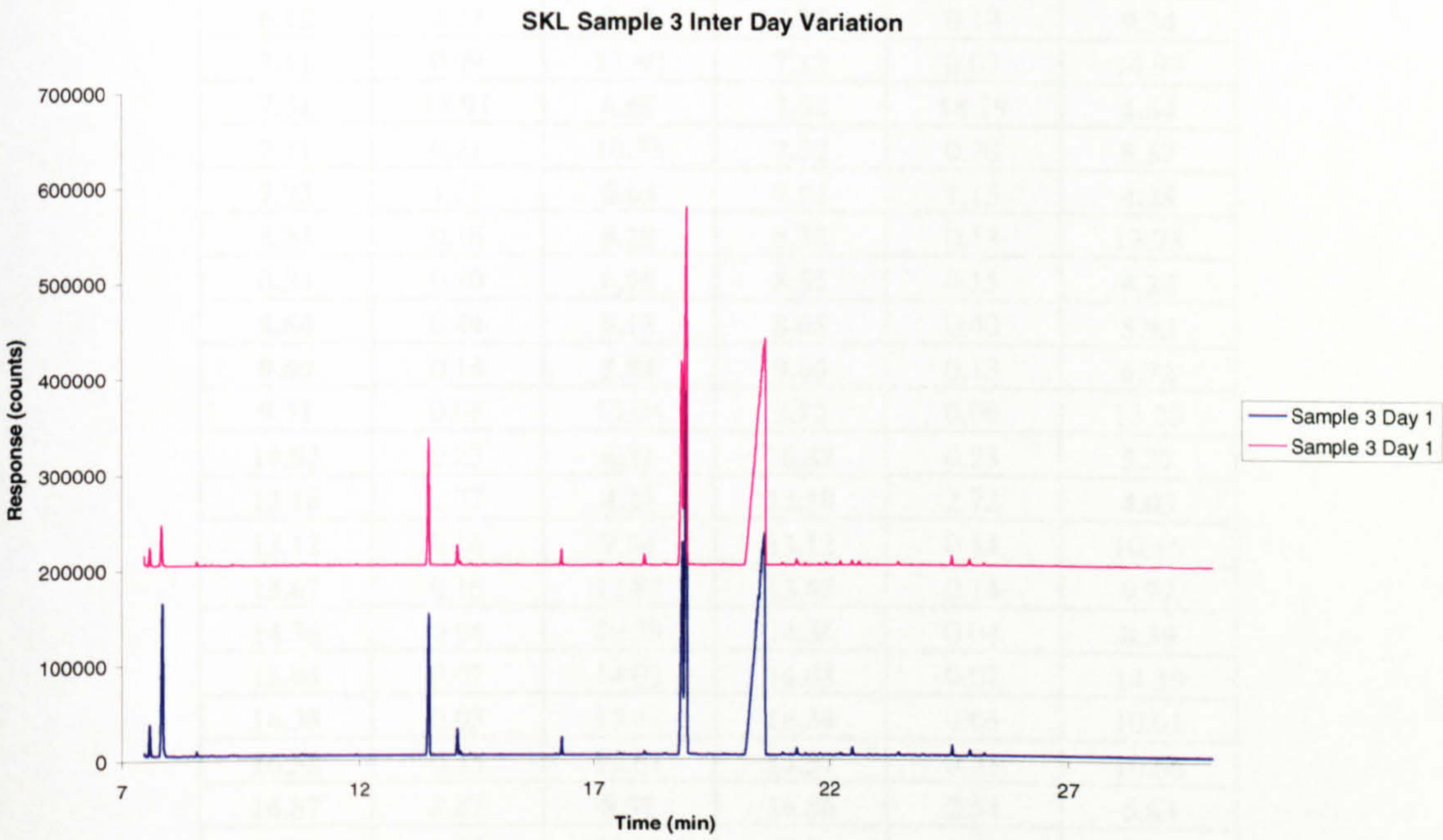


Figure 7.25 SKL Sample 3 – Toluene Extraction – Inter Day Variation Over 56 Days

7.3.2.13. Strathclyde Sample 1 – Toluene Extractions

Amphetamine for this sample was prepared via the nitrostyrene route in the lab in Strathclyde. The total number of peaks common to all six replicate extractions in each separate study is shown in Table 7.28 with the number of peaks present having RSD values less than 15%. It is clear that although there are more impurity peaks in this sample than any of the previous samples, very few of them appear at a consistent level. However, the inter day and intra day variation results are very similar.

	Intra Day Variation	**Inter Day Variation**
Peaks in all samples	207	192
Peaks with % RSD < 15	**48**	**66**

Table 7.28 Number of Peaks Present in Replicate Extractions - Intra and Inter Day Variation

Table 7.29 show those peaks present in both the inter and intra day study with an RSD value of less than 15 %.

Strathclyde Sample 1 Toluene Extraction Intra Day Variation			Strathclyde Sample 1 Toluene Extraction Inter Day Variation		
Time	Mean	%RSD	Time	Mean	%RSD
6.21	0.84	10.02	**6.23**	0.78	**8.74**
6.72	0.22	**8.45**	**6.73**	0.19	**9.34**
7.11	0.09	13.80	**7.12**	0.07	14.97
7.31	18.91	**6.55**	**7.32**	18.79	**4.64**
7.51	0.21	10.33	**7.52**	0.20	**8.57**
7.93	1.21	**6.63**	**7.94**	1.15	**4.28**
8.34	0.16	**8.28**	**8.35**	0.13	13.75
8.54	0.16	**6.98**	**8.55**	0.15	**4.24**
8.64	0.44	**8.13**	**8.65**	0.40	**5.93**
9.60	0.14	**5.84**	**9.60**	0.13	**6.72**
9.71	0.08	13.04	**9.72**	0.06	13.20
10.82	0.23	**6.31**	**10.82**	0.23	**5.37**
12.18	2.77	**4.24**	**12.18**	2.72	**4.03**
13.12	0.16	**7.84**	**13.12**	0.14	10.15
13.67	0.16	12.84	**13.67**	0.14	**9.77**
14.36	0.04	19.59	**14.36**	0.04	**8.39**
16.04	0.07	14.02	**16.03**	0.07	14.39
16.35	0.03	15.44	**16.34**	0.03	10.01
16.58	0.35	12.61	**16.57**	0.33	10.66
16.87	2.87	**8.51**	**16.86**	2.54	**6.63**
17.12	1.05	13.09	**17.11**	1.05	11.91
18.09	7.69	12.57	**18.08**	7.28	10.34
18.14	0.12	**7.83**	**18.13**	0.10	**6.72**
18.18	0.38	**6.81**	**18.17**	0.30	12.84
18.34	0.91	14.09	**18.33**	0.88	13.34
18.89	3.08	**6.89**	**18.88**	2.64	10.80
19.10	1.55	13.06	**19.09**	1.44	10.80
19.25	1.91	14.24	**19.23**	1.79	11.02
19.84	0.27	11.84	**19.83**	0.24	10.80
19.93	1.43	11.92	**19.92**	1.04	12.61
20.60	81.78	**2.98**	**20.58**	78.61	**4.90**
20.90	1.91	**9.35**	**20.89**	1.55	10.02
21.27	0.39	11.43	**21.26**	0.32	**7.57**
21.59	0.28	11.96	**21.57**	0.24	**4.42**
21.94	0.23	14.84	**21.93**	0.18	11.42
22.35	0.21	14.99	**22.33**	0.19	12.69
23.30	0.27	14.87	**23.29**	0.23	11.45

Table 7.29 Strathclyde Sample 1 - Toluene Extractions
Peaks Common to Both Intra and Inter Day Variation Studies with RSD Less Than15%

The results for this sample are shown in a graphical representation of the chromatograms of replicate samples on one occasion in Figure 7.26.

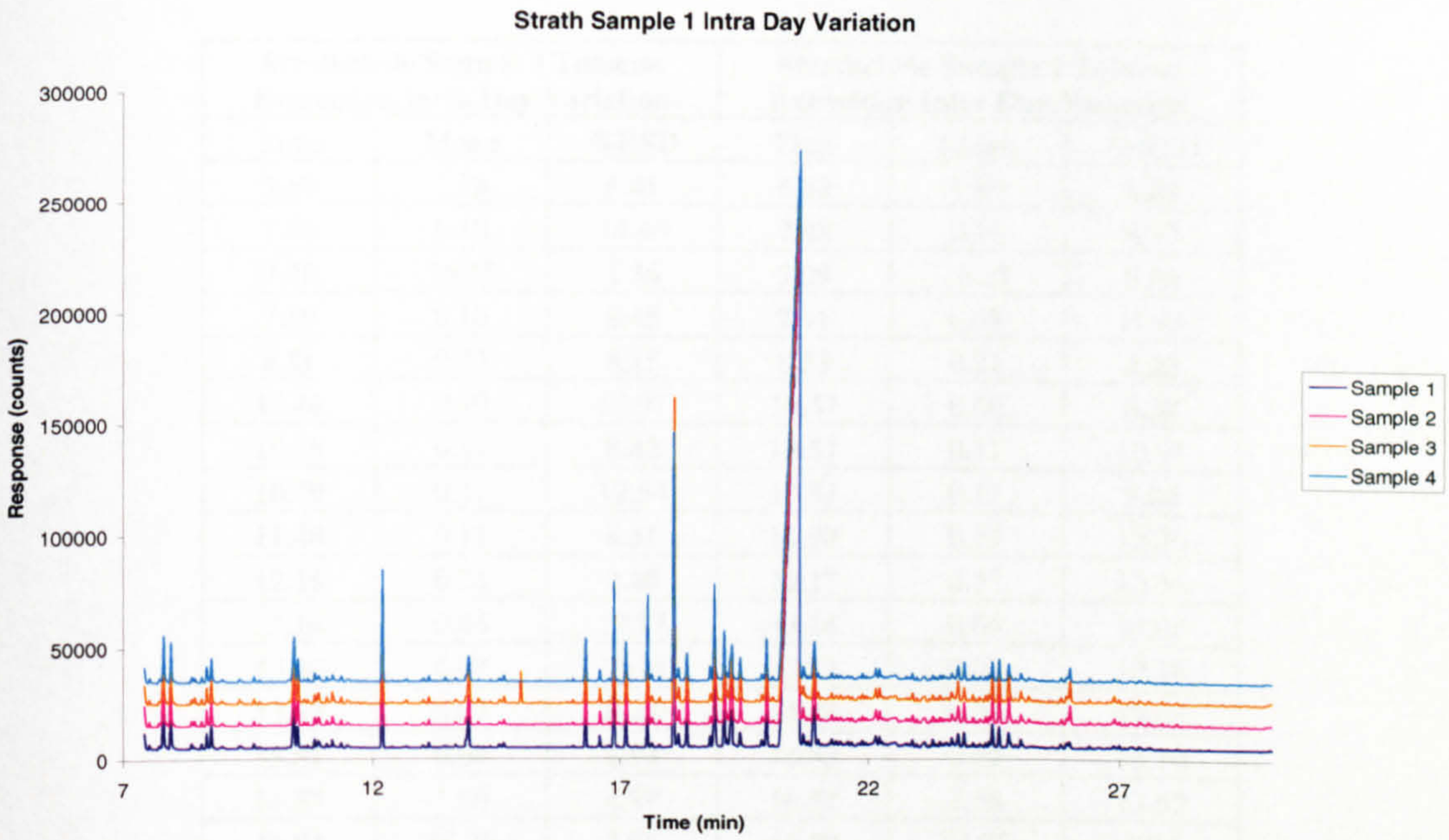


Figure 7.26 Strathclyde Sample 1 – Toluene Extractions – Intra Day Variation in Replicate Extractions

7.3.2.14. Strathclyde Sample 2 – Toluene Extractions

Again this sample was synthesised by the nitrostyrene route. The total number of peaks common to all six replicate extractions in each separate study may be found in Table 7.30 with RSD values less than 15%. The results for the intra-day study are encouraging for profiling purposes, with 142 peaks having an RSD less than 5%. However, the assay does not appear to be so robust for this particular sample if analyses are carried out on separate days.

	Intra Day Variation	**Inter Day Variation**
Peaks in all samples	222	182
Peaks with % RSD < 15	**142**	**86**

Table 7.30 Number of Peaks Present in Replicate Extractions - Intra and Inter Day Variation

Table 7.31 shows the peaks present in both the inter and intra day studies with an RSD value less than 15 %. Figure 7.27 shows the variation in profiles obtained on Day 1 and Day 7.

Strathclyde Sample 2 Toluene Extraction Intra Day Variation			Strathclyde Sample 2 Toluene Extraction Inter Day Variation		
Time	Mean	%RSD	Time	Mean	%RSD
5.80	1.78	6.41	5.82	1.80	6.82
7.06	0.18	14.40	7.08	0.16	8.97
7.28	16.37	1.36	7.29	16.37	9.94
7.89	0.10	8.45	7.91	0.08	11.61
8.51	0.23	8.15	8.53	0.21	8.22
10.46	0.70	13.97	10.47	0.66	6.38
10.55	0.11	8.42	10.57	0.11	10.91
10.79	0.12	12.54	10.81	0.11	5.04
11.48	0.11	6.31	11.50	0.11	12.76
12.15	0.24	2.40	12.17	0.23	10.36
13.14	0.04	12.77	13.16	0.04	14.06
13.30	0.02	13.14	13.32	0.02	10.16
13.42	0.03	8.24	13.44	0.03	12.68
15.81	0.04	8.54	15.83	0.04	13.10
16.55	1.60	4.79	16.57	1.56	13.62
16.89	14.76	2.06	16.90	14.91	6.94
17.10	1.32	2.16	17.11	1.27	11.01
17.23	0.05	5.78	17.25	0.05	14.89
17.75	0.40	1.79	17.77	0.35	14.39
17.89	0.04	8.09	17.91	0.03	13.27
17.96	0.12	6.45	17.97	0.11	13.75
18.05	3.79	2.34	18.06	3.65	10.35
18.10	0.29	13.21	18.12	0.29	10.46
18.22	0.09	3.71	18.24	0.09	7.99
18.27	0.16	4.71	18.29	0.16	6.93
18.38	0.13	2.73	18.40	0.13	8.54
18.73	0.19	5.61	18.75	0.18	9.41
18.77	0.32	2.00	18.79	0.32	12.34
19.06	0.33	5.07	19.08	0.32	12.04
19.13	0.17	6.14	19.14	0.16	13.31
19.22	1.24	2.16	19.23	1.15	12.11
19.65	0.06	6.25	19.66	0.06	6.73
19.70	0.17	3.85	19.72	0.16	11.53
19.81	0.26	6.41	19.83	0.27	6.86
20.48	68.03	6.76	20.56	66.56	5.88
20.76	0.54	1.84	20.76	0.54	9.46
20.96	0.41	1.95	20.96	0.38	10.91
21.02	0.59	8.93	21.03	0.54	10.58
21.33	0.23	9.48	21.34	0.23	10.98
21.42	0.92	3.45	21.43	0.88	9.78
21.75	0.29	5.22	21.76	0.28	11.53
22.01	1.39	10.42	22.02	1.32	14.31
22.22	0.99	11.70	22.33	0.48	12.46
22.33	0.50	2.51	22.41	0.36	14.02
22.40	0.45	2.90	22.47	0.46	13.56
22.56	0.61	8.05	22.57	0.57	10.87
22.88	0.74	12.24	22.88	0.69	9.98
22.96	0.19	4.00	22.96	0.21	13.88
23.09	0.37	5.47	23.10	0.34	6.26

23.17	0.13	**8.13**	**23.17**	0.12	**9.25**
23.23	0.14	**5.84**	**23.24**	0.14	**8.64**
23.28	0.26	19.71	**23.29**	0.25	12.29
23.34	0.18	14.99	**23.35**	0.15	14.59
23.39	0.57	**4.44**	**23.41**	0.54	**7.92**
23.53	0.22	**2.17**	**23.54**	0.22	**8.59**
23.65	0.62	**5.07**	**23.66**	0.63	12.14
23.85	0.20	**7.82**	**23.86**	0.20	10.60
23.90	0.33	**6.00**	**23.91**	0.33	13.67
24.06	0.20	11.49	**24.07**	0.20	10.43
24.13	0.21	**3.73**	**24.14**	0.22	11.19
24.27	0.30	**3.42**	**24.28**	0.29	**9.09**
24.33	0.55	**5.77**	**24.34**	0.53	13.13
24.46	0.62	12.48	**24.47**	0.58	10.57
24.58	0.80	**6.86**	**24.59**	0.79	13.28
25.02	0.40	**2.79**	**25.02**	0.37	12.09
25.19	0.25	11.14	**25.19**	0.23	11.75
25.24	0.15	**6.63**	**25.25**	0.13	12.70
25.57	0.27	11.68	**25.69**	0.28	**9.99**
25.67	0.29	13.39	**25.80**	0.52	11.89
25.98	0.71	**5.80**	**25.99**	0.72	10.53
26.20	0.21	**1.29**	**26.20**	0.19	**8.31**
26.87	0.29	**5.13**	**26.80**	0.32	**8.31**
26.94	0.61	**2.19**	**26.87**	0.32	14.43
27.10	0.29	14.82	**27.11**	0.28	12.82
27.51	0.25	**9.03**	**27.52**	0.24	12.78
27.89	0.29	**5.81**	**27.91**	0.28	**8.90**

Table 7.31 Strathclyde Sample 2 - Toluene Extraction
Peaks Common to Both Intra and Inter Day Variation Studies with RSD Less Than 15%

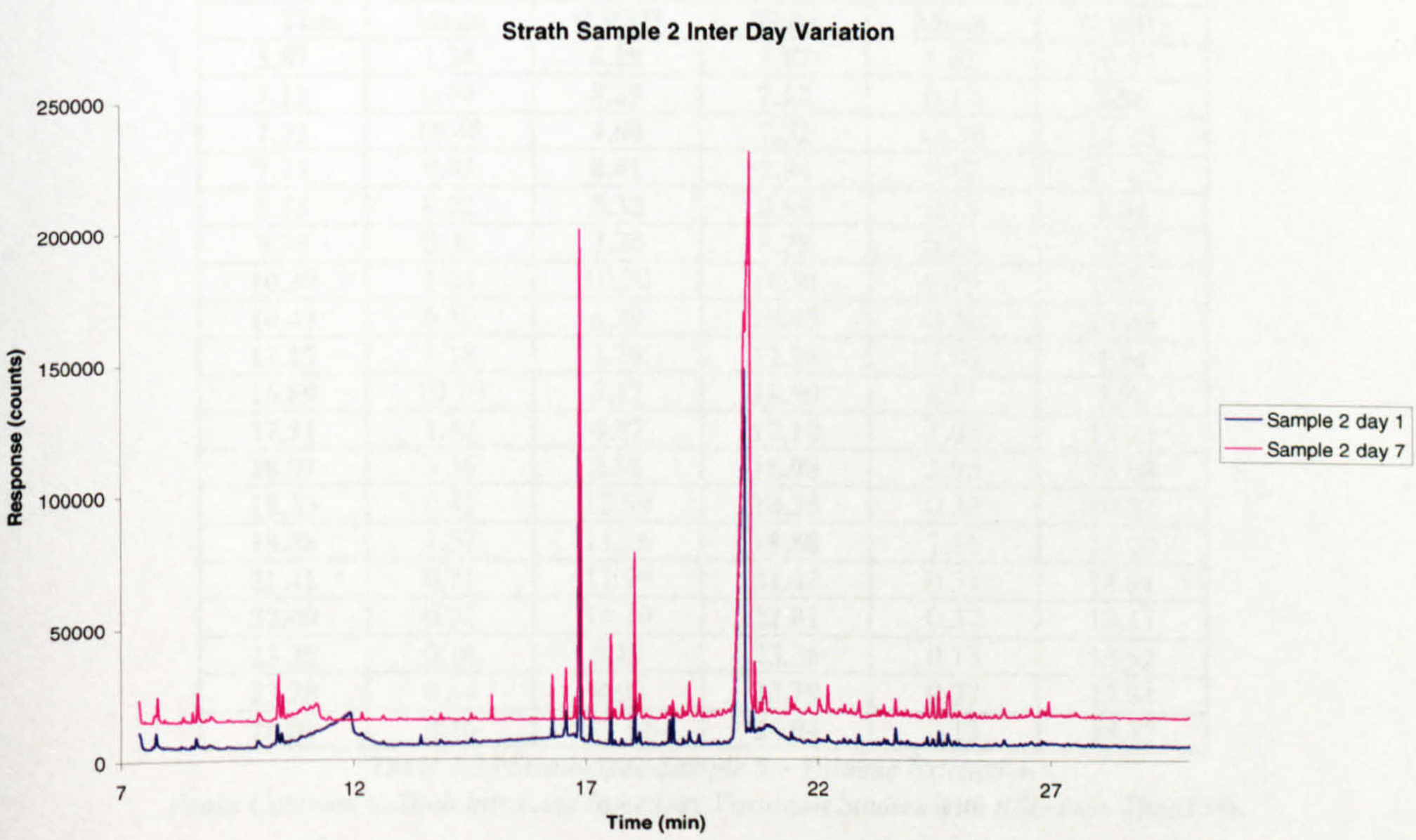


Figure 7.27 Strathclyde Sample 2 – Toluene Extractions – Inter Day Variation Over Days 1 and 7

7.3.2.15. Strathclyde Sample 3 – Toluene Extractions

Again, this sample was synthesised via the nitrostyrene route. The total number of peaks common to all six replicate extractions in each separate study is shown in Table 7.32 with the number of peaks present with RSD values less than 15%. It may be seen that although the extracts have approximately the same number of peaks present in all replicates as Sample 2, the impurity levels are far more variable.

	Intra Day Variation	Inter Day Variation
Peaks in all samples	209	185
Peaks with % RSD <15	**100**	**23**

Table 7.32 Number of Peaks Present in Replicate Extractions - Intra and Inter Day Variation

Table 7.33 shows those peaks present in both the inter and intra day studies with RSD values less than 15 %.

Strathclyde Sample 3 Toluene extractions Intra Day Variation			Strathclyde Sample 3 Toluene extractions Inter Day Variation		
Time	Mean	%RSD	Time	Mean	%RSD
5.87	1.24	**6.28**	**5.87**	1.02	10.54
7.12	0.14	**8.25**	**7.12**	0.12	**9.96**
7.32	15.48	**4.60**	**7.32**	16.14	11.28
7.94	0.43	**8.41**	**7.94**	0.42	13.17
8.55	0.22	**5.32**	**8.55**	0.18	**6.91**
8.75	0.30	**3.28**	**8.75**	0.24	10.22
10.39	1.04	10.20	**10.38**	0.79	14.77
10.47	0.50	**6.39**	**10.47**	0.38	12.48
12.17	1.18	**3.70**	**12.18**	1.00	**1.94**
16.89	10.70	**5.12**	**16.90**	8.51	**7.90**
17.11	1.42	**4.77**	**17.12**	1.05	13.23
18.07	5.36	**2.91**	**18.08**	3.96	13.68
18.33	0.42	12.59	**18.35**	0.32	10.32
18.88	1.57	11.26	**18.88**	1.16	14.20
21.41	0.71	11.99	**21.42**	0.53	14.91
22.00	0.63	14.19	**22.01**	0.37	13.17
23.35	0.16	**8.19**	**23.36**	0.13	13.52
23.78	0.54	**8.93**	**23.79**	0.37	14.84
28.01	0.16	11.12	**28.04**	0.13	14.37

Table 7.33 Strathclyde Sample 3 – Toluene Extraction
Peaks Common to Both Intra and Inter Day Variation Studies with RSD Less Than15%

Figures 7.28 show the results of the inter-day study on Day 1 and Day 7 graphically, highlighting the similarities and variations between samples.

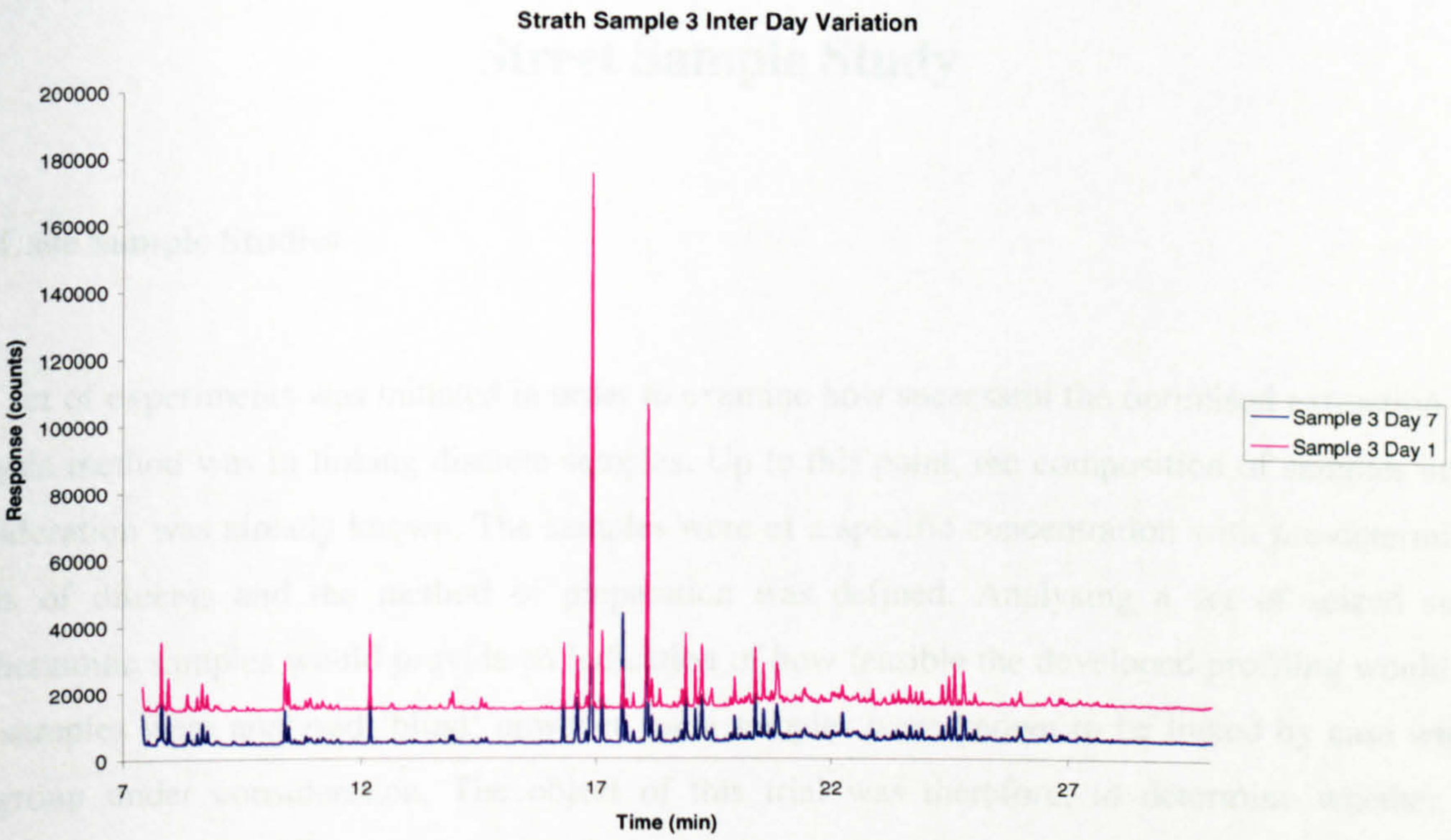


Figure 7.28 Strathclyde Sample 3 – Toluene Extractions – Inter Day Variation On Days 1 and 7

Chapter 8

Street Sample Study

8.1. Case Sample Studies

This set of experiments was initiated in order to examine how successful the optimised extraction and analysis method was in linking discrete samples. Up to this point, the composition of samples under consideration was already known. The samples were of a specific concentration with pre-determined levels of diluents and the method of preparation was defined. Analysing a set of seized street amphetamine samples would provide an indication of how feasible the developed profiling would be. The samples were analysed 'blind' however some samples were known to be linked by case within the group under consideration. The object of this trial was therefore, to determine whether the proposed impurity profiling system would highlight relationships between samples that could be confirmed or rejected, using previous knowledge of the cases.

Street samples were thought to have a more complex matrix with a range of diluents and possible additional drug content which may affect either the solubility of the samples in the buffer or be themselves be extracted preferentially into the solvent. The actual amphetamine content was also more variable in these samples since, until this stage, only three different concentrations had been used in the simulated study which were all relatively high, from 100% to 15% active drug. A total amphetamine content of as little as 2% is not unusual in some 'street' samples and it would be interesting to see whether this method could be used when the content is so low and the impurity content possibly even lower. The route used to synthesise these batches was also unknown, however since the Leuckart route is the most commonly encountered in the UK it was thought that this was the most likely synthetic method for many of the samples.

Case samples were obtained from the Procurator Fiscal in Glasgow. Of the samples available, those analysed were selected because of their variation in appearance from off-white crystalline powders to yellow and some almost brown crystalline samples. The amphetamine content of the samples had previously been quantified by HPLC but this data was not available to me prior to analysis [94].

In addition to analysing individual samples, some seizures had sufficient quantities to enable multiple extractions and analyses. This allows for comparison between separate extractions of a single sample

batch. One case sample had also been seized pre-weighed in small cling-film wraps so, although these batches were linked by similar wrapping and appearance, the content of these wraps was not known. If the samples had been thoroughly homogenised from a single batch of amphetamine then the active drug content and the impurity profile should theoretically be similar.

8.2. Initial Small Case Sample Study

8.2.1 Aims

This small experiment was set up to determine what possible levels of amphetamine we could be expected to see in a set of samples thought to be representative of those seized in Glasgow at that time.

8.2.2. Experimental Procedure

In this initial small-scale experiment, eight different samples were chosen as representative of different matrix types with varied crystalline and powder appearances. 200mg aliquots of the samples were extracted using the optimised method and analysed using GC-FID.

8.2.3. Results

Table 8.1 below shows the relative peak areas of amphetamine and caffeine peaks present in the samples (as compared to the internal standard peak area). The variation between the samples is significant and gives some impression of the wide range of amphetamine content and diluents present in street samples seized in Glasgow at that time.

Sample Name	Amphetamine Relative Peak Area	Caffeine Relative Peak Area
NND 51	12.9	205
NND 134	23.8	186
NND 15	35.9	18.6
NND 33	95.9	557
NND 118	37.6	28.6
NND 107	25.8	178
NND 117	31.6	63.9
NND 128	4.09	35.5

Table 8.1 Relative Peak Areas of Compounds in Selected Case Samples

8.3. Case Sample NND 117

8.3.1 Aims

Sample NND 117 was obtained as a selection of smaller samples pre-wrapped in cling film ready for supply. This experiment set out to determine if samples linked by the similar packaging and allegedly from the same supplier, were also linked by their organic impurity profile.

8.3.2. Experimental Detail

10 of these cling-film wraps were chosen at random from 15 available samples, the bags were cut and the contents removed to sample vials. The sample appeared to have been split into smaller quantities and the actual sample weight in each wrap varied from 187 mg to 330 mg. Since it may have changed the sample matrix to interfere with the bulk by homogenising each small sub-sample, the entire content of each wrap was dissolved in buffer and weight recorded.

The NND 117 samples were extracted and analysed individually using the methods optimised in the previous experiments. However, at this stage only the FID was employed since the MS source was not available. The amphetamine and caffeine relative peak areas from the chromatograms obtained from each separate sample was determined.

8.3.3. Results and Discussion

Figures 8.1 and 8.2 are representative chromatograms obtained from 2 of the extracts from NND 117 samples and show the differences in relative amphetamine (*ca* 6 min) and caffeine (*ca* 24 min) content in samples linked by case but do not have the same impurity profile.

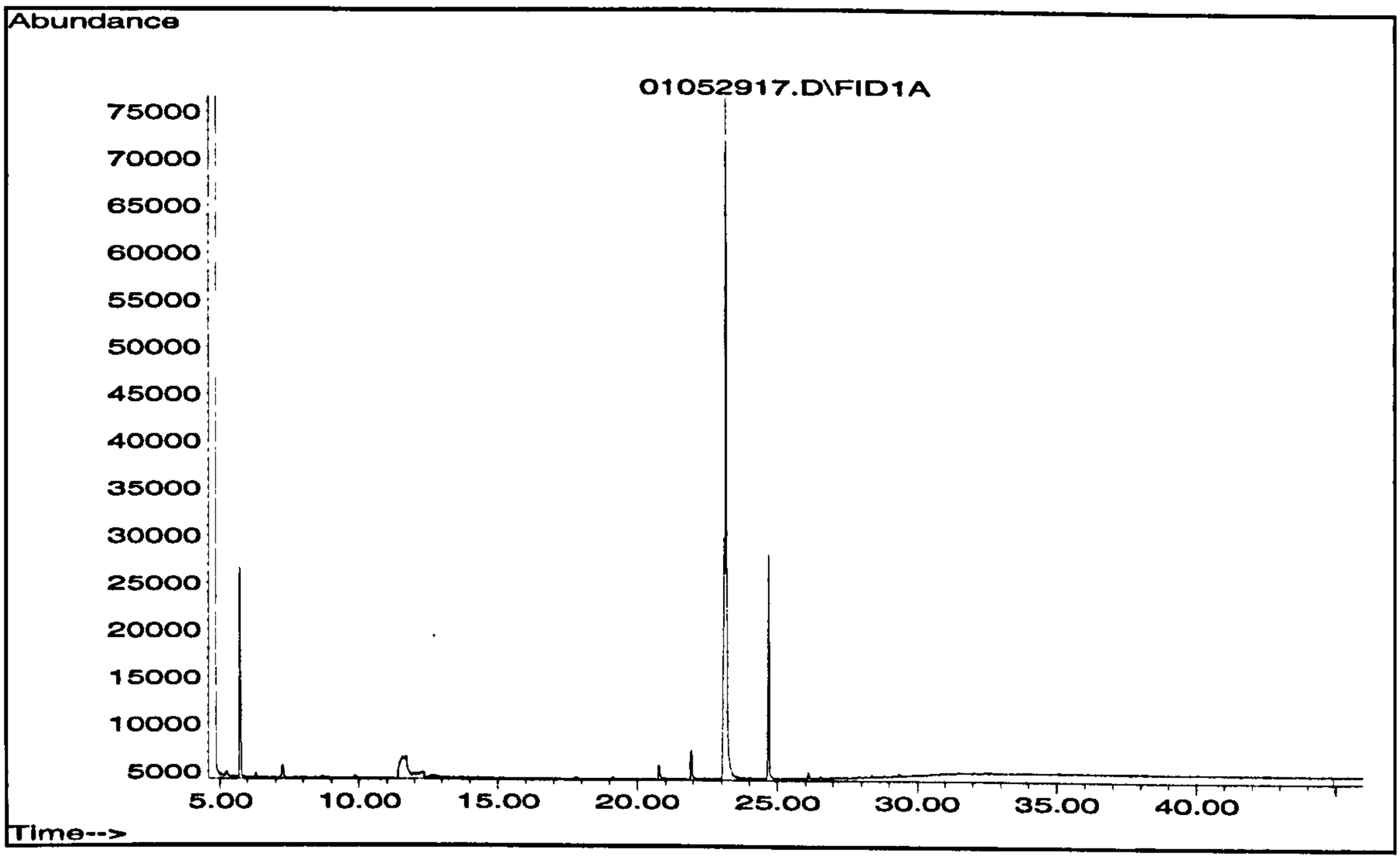


Figure 8.1 Sample NND117 Aliquot Number 5 (187.4mg)

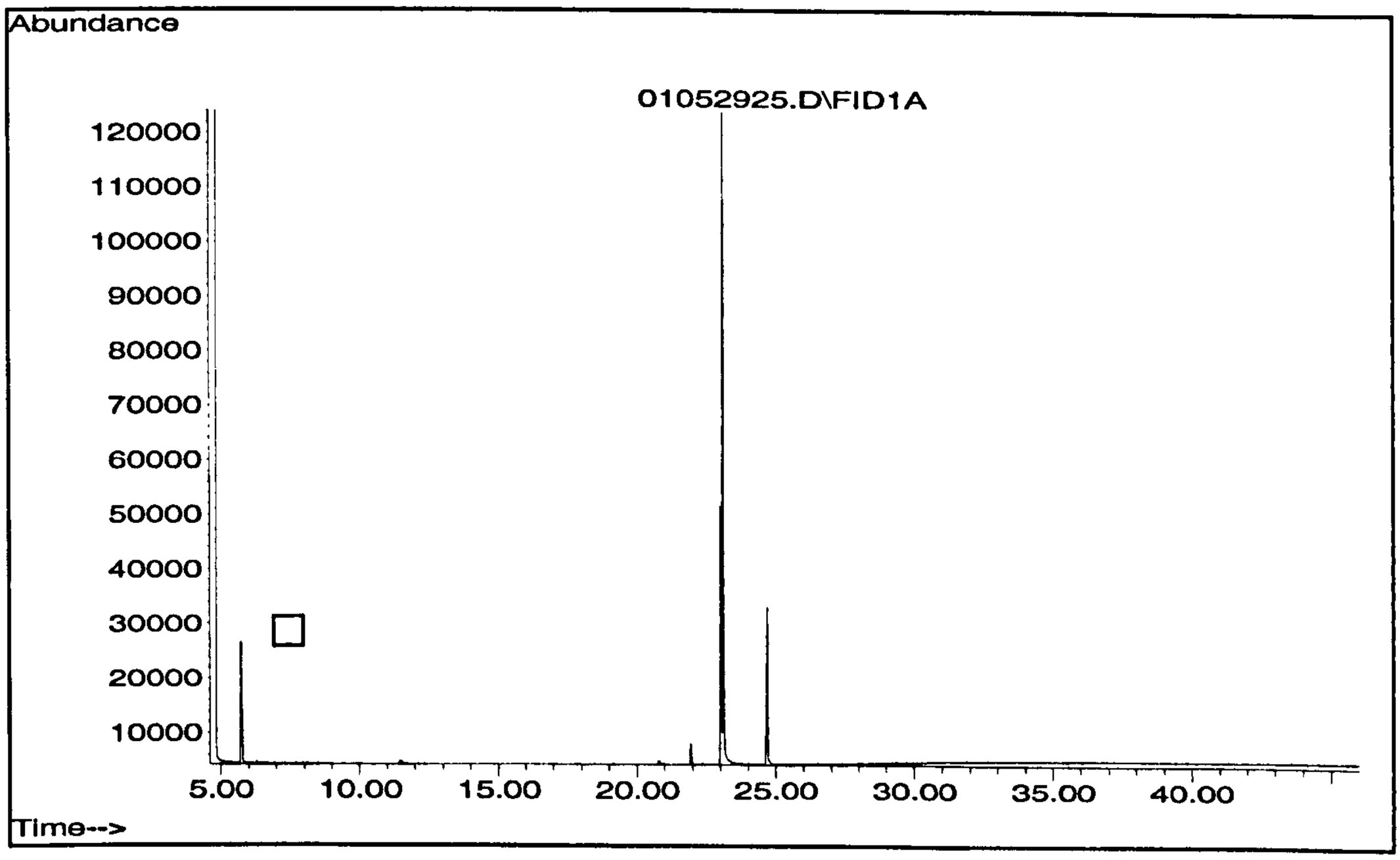


Figure 8.2 Sample NND117 Aliquot Number 10 (300.2mg)

From the data in Table 8.2, in 5 of the 10 samples, no amphetamine was detected in the profile. Caffeine was present in all but one of the samples. When amphetamine was identified, the relative response varied considerably. The bulk sample, which had been split to create the smaller samples, may not have been homogenised thoroughly since the drug content appeared to vary so considerably between the small wraps. Alternatively, the amphetamine content and bulking agent may have been added separately and using only rough approximations of weight, resulting in extremely variable sample matrices. When the active drug concentration is so different, the levels of impurities are so variable that profiling samples by their impurities is problematic. In addition to amphetamine and caffeine, the only other peak present in any significant quantity was one at 24.7 minutes that was not easily identifiable as only the FID was employed at this stage, and no mass spectral data could be obtained. Essentially, this experiment highlighted the fact that samples assumed to contain amphetamine and impurities may contain very small quantities of amphetamine and therefore almost undetectable levels of impurities.

NND 117	Amphetamine	Caffeine
Sample 1	33613082	67879165
Sample 2	22726082	23902782
Sample 3	29930103	143975220
Sample 4	18090487	58960651
Sample 5	7772143	106331148
Sample 6	None	62989927
Sample 7	None	11598134
Sample 8	None	70608641
Sample 9	None	63550204
Sample 10	None	None

Table 8.2 Peak Areas of Selected Peaks in Samples NND117 (samples 1-10)

8.4. Case Sample NND 128

8.4.1. Aims

Sample NND 128 was sufficiently large enough to allow several extractions and analysis of individual aliquots of this powder and could therefore be used to assess if the extraction and analysis could link samples from a single source.

8.4.2. Experimental Detail

8 x 200 mg aliquots of homogenised NND128 were extracted using the same method as previously optimised. The solvent extracts were injected in duplicate and the variation between injection and analyses was determined in addition to the variation between aliquots.

8.4.3. Results and Discussion

Table 8.3 shows the relative peak areas of selected impurities as the mean of the two replicate injections alongside the overall mean value of the relative peak area and the overall RSD of the impurity. Those impurities with an overall RSD of less than 15 % are highlighted in red. The overall reproducibility of these extractions and analyses is reasonable with half of the selected peaks having an RSD of less than 15 %.

Time	Mean 1	Mean 2	Mean 3	Mean 4	Mean 5	Mean 6	Mean 7	Mean 8	Overall mean	Overall RSD
6.27	0.158	0.161	0.161	0.150	0.152	0.164	0.154	0.152	0.157	**3.3**
8.66	2.557	2.299	2.294	2.075	2.025	2.118	2.174	2.062	2.20	**8.0**
Amp.	30.605	30.200	29.568	34.525	36.442	26.957	34.587	31.310	31.8	**9.9**
11.92	0.634	0.426	0.494	0.495	0.471	0.372	0.514	0.261	0.458	23.9
19.15	0.183	0.041	0.072	0.067	0.100	0.061	0.096	0.068	0.0860	50.6
20.77	0.993	1.018	1.011	1.000	0.991	0.992	0.986	0.974	0.996	**1.4**
21.93	0.817	0.373	0.610	0.677	0.451	0.509	0.517	0.554	0.564	24.5
22.36	0.117	0.094	0.112	0.104	0.102	0.091	0.113	0.115	0.106	**9.3**
22.71	0.061	0.145	0.175	0.159	0.159	0.188	0.157	0.155	0.150	25.5
23.02	4.822	5.330	4.928	5.423	4.882	4.080	4.745	4.564	4.85	**8.7**
23.10	13.017	10.419	12.337	12.674	12.849	10.595	13.288	16.473	12.7	**14.7**
24.68	16.962	14.045	14.355	13.736	14.786	12.838	13.458	13.864	14.3	**8.7**
26.52	0.063	0.041	0.043	0.051	0.066	0.052	0.048	0.059	0.0529	17.2
28.14	0.052	0.051	0.068	0.066	0.066	0.053	0.052	0.054	0.0578	**12.9**
28.56	0.452	0.368	0.416	0.379	0.518	0.356	0.314	0.386	0.399	15.8
28.82	0.088	0.102	0.134	0.091	0.060	0.104	0.102	0.113	0.0993	21.4
29.34	0.041	0.033	0.022	0.055	0.054	0.078	0.056	0.064	0.0504	35.3
29.79	0.320	0.490	0.515	0.499	0.667	0.461	0.497	0.565	0.502	19.4

Table 8.3 Relative Peak Areas of Selected Impurities of Individual Extracts of Sample NND128

The variation in the amphetamine content is around 10% between replicates and aliquots, which suggests that the sample is relatively homogeneous in amphetamine content. The impurity peak areas present, however, are not so reproducible, even although there were visual similarities in the profiles produced as seen in Figures 8.3 and 8.4.

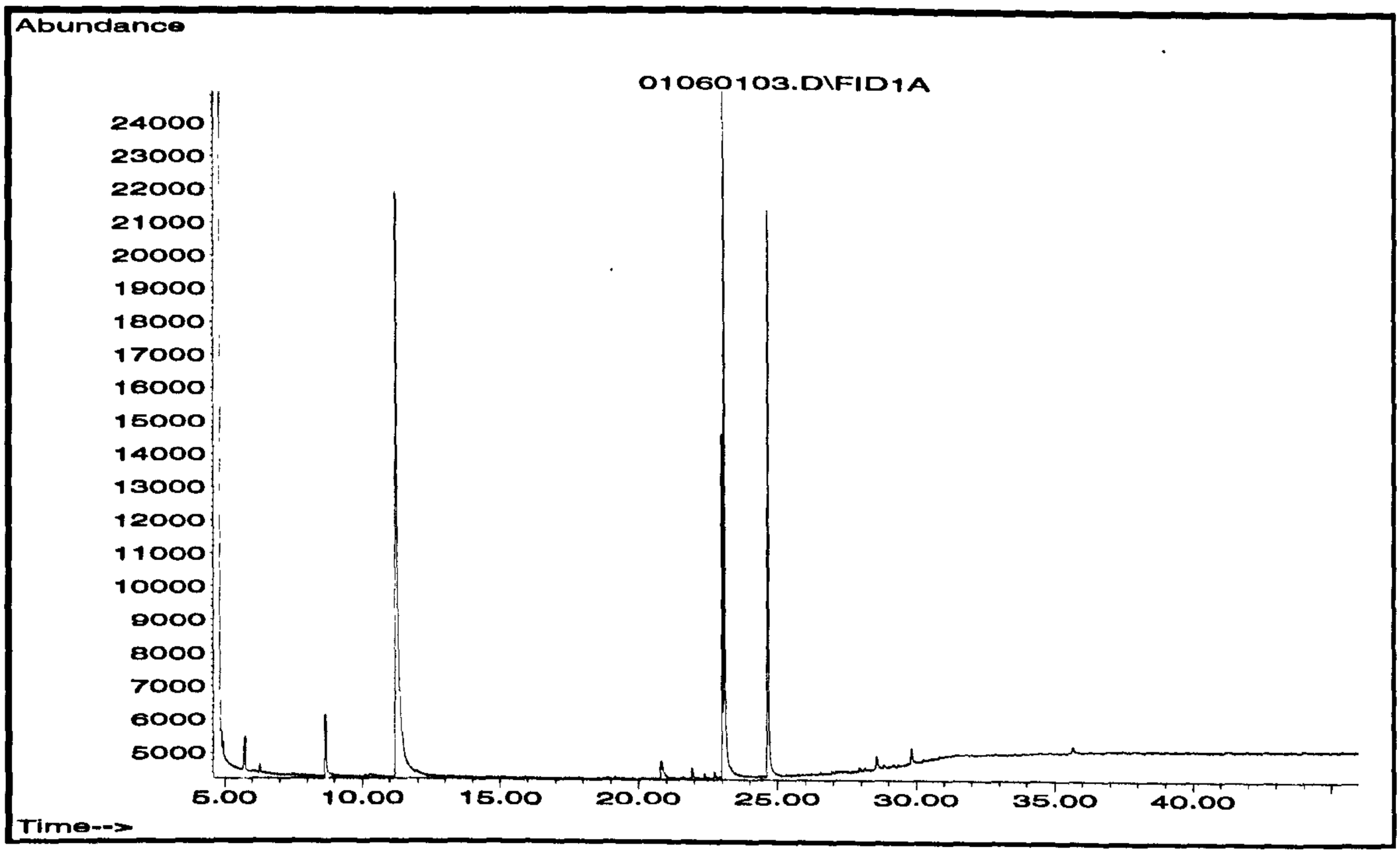


Figure 8.3 Sample NND 128 Aliquot Number 3

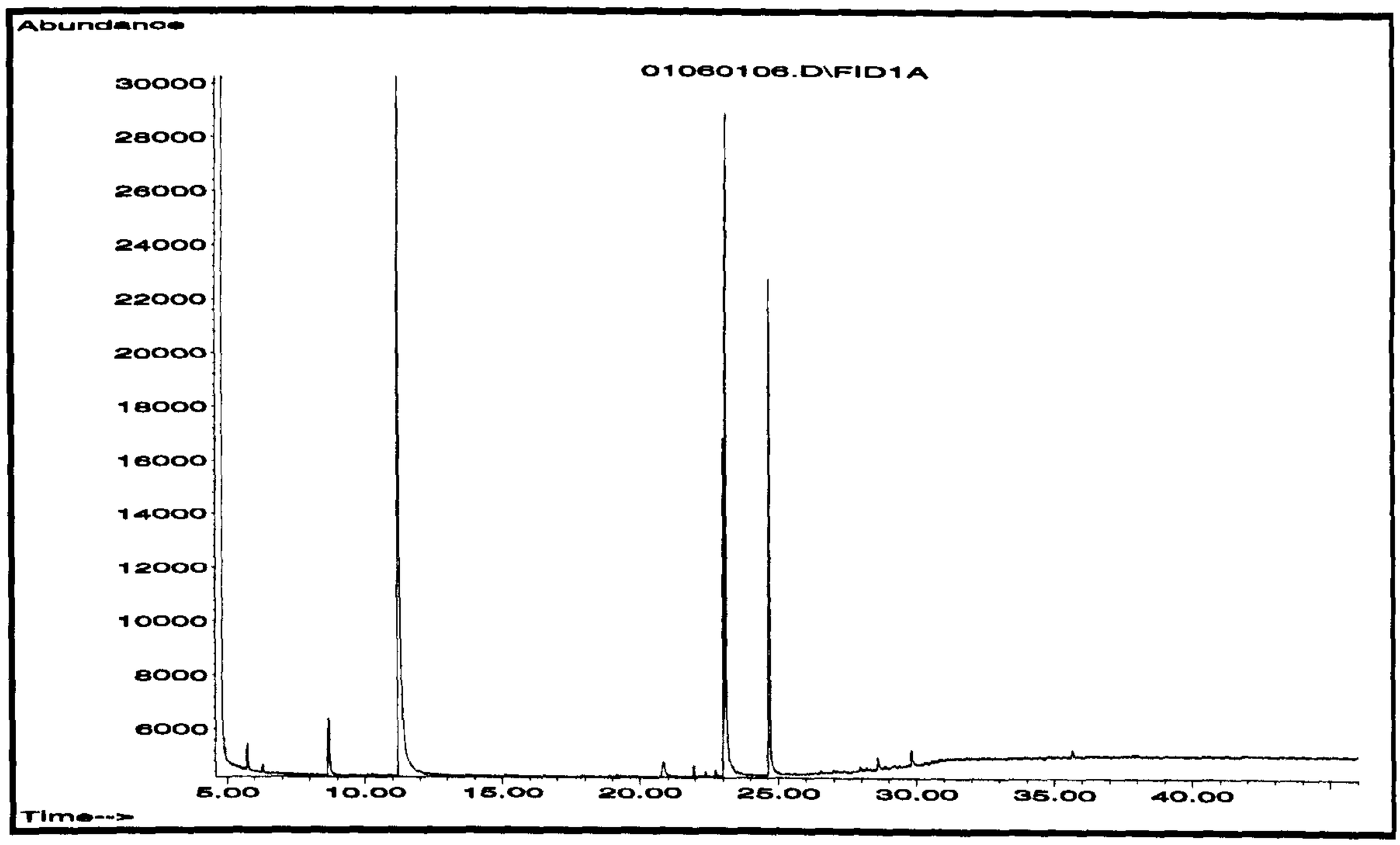


Figure 8.4 Sample NND 128 Aliquot Number 6

8.5. Larger Scale Study

8.5.1. Introduction

In this final experiment, a larger representative sample of 92 seizures taken in Glasgow during the period 1996-1997 were obtained, details on their appearance were noted and the samples extracted and analysed following the optimised procedures. Links between samples were made using both visual and statistical comparisons of the profiles.

The samples varied considerably in size, colour and general appearance. A small number of samples appeared as though they had been seized straight from a reaction vessel while others were packaged in small quantities ready for distribution. Those which had not been purified were yellow to brown sticky lumps. Those further down the distribution chain were bulked with white powders resembling crystalline sugars or matt powders similar in appearance to powdered milk.

The majority of samples were already bulked and prepared for supply and in most cases there were no unusual characteristics or distinguishing features by which visual links could be made.

8.5.2. Experimental Procedure

After noting the original appearance of the sample, every sample was ground in a pestle and mortar to thoroughly homogenise the bulk material such that sub-samples removed for analysis would be representative of the whole.

Where possible, 200 mg aliquots were removed from the bulk. In a very small number of cases, 200 mg was not available. In these cases, the maximum amount possible was weighed and this noted.

The extraction and analysis procedure used 4 mL, pH 8.1 TRIS buffer into which the sample was dissolved, with 200 µL toluene containing internal standard (C_{20} at 10 µg/mL added to extract. A 3 µL volume was injected splitless into the GC system, and FID and MSD detection was used throughout.

8.5.3. Samples with Few Impurities

Samples with too few impurity peaks to allow comparison proved especially difficult. However, this is an important aspect of the profiling process. Of the samples tested, some had very little amphetamine present and few identifiable impurity peaks present. In these cases, profiling using organic impurities is virtually impossible, since the samples have virtually no distinguishing characteristics that allow us to differentiate between seizures.

8.5.3. Visual Linking via Chromatographic Similarities

Where organic impurities were present in significant quantities in the samples, a visual comparison of chromatograms was possible by overlaying and superimposing chromatograms. Table 8.4 lists those seizures thought to be linked using this approach.

Seizures
NND13 and NND14
NND55, NND63, NND70
NND150 and NND152A
NND152B and NND153
NND103 – NND108
NND119 – NND125
NND130 – NND139
NND45 and NND46
NND15 and NND15B
NND52 and NND53
NND54 and NND57
NND65 and NND73
NND98 and NND99
NND109 and NND110 (108)
NND51 and NND71
NND93, NND94, NND95

Table 8.4 Seizures thought to be linked using visual comparison of chromatograms

When these links had been identified using a visual comparison of chromatograms, details of those seizures known to be linked were released and the success of the experiment could be established.

8.6. Confirmed Links

The list of linked samples was not made available before the extraction and analysis of all samples. It should be noted, however, that although some samples were linked by case, they may not necessarily have the same level of diluents or may not even be from one production batch and their profiles would be different.

8.6.1. Samples Linked By Case

Number of Samples	Sample Numbers
2	NND13 and NND14
2	NND15 and NND16
2	NND33 and NND34
4	NND43 – NND46
26	NND51 – NND76
5	NND93 – NND97
2	NND98 and NND99
8	NND103 – NND110
2	NND112 and NND113
7	NND119 – NND125
2	NND127 and NND128
10	NND130 – NND139
4	NND150 – NND153
2	NND155 and NND156

Table 8.5 Samples Linked by Case

8.6.2. Samples NND13 and NND14

Although on first viewing the chromatographic profile of NND13 and NND14 there appears to be few impurities as shown in the FID chromatogram in Figure 8.7, when you zoom in on the baseline, the profile is a complicated mix of many impurities at low levels. The samples were thought to be synthesised via the Leuckart route since some route-specific impurities were detected. Figure 8.7 shows the FID impurity profile of NND 13 and Figure 8.8 shows the TIC impurity profile of NND 14 with the baseline enlarged to show the complicated mixture of impurities present in this sample. Figure 8.9 shows the impurity profiles of both NND 13 and NND 14 superimposed to highlight the similarities in the profiles.

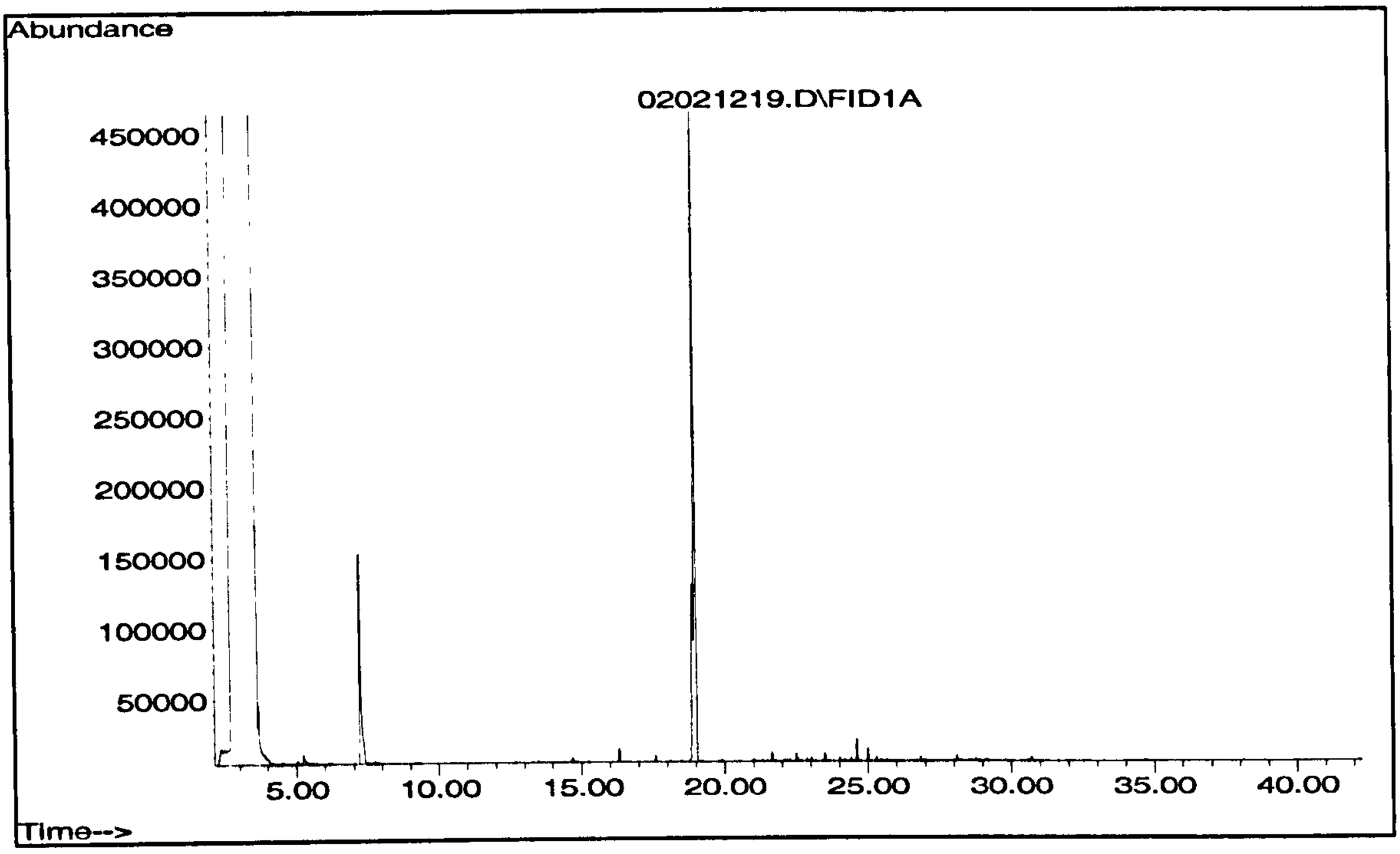


Figure 8.7 NND13 Impurity Profile

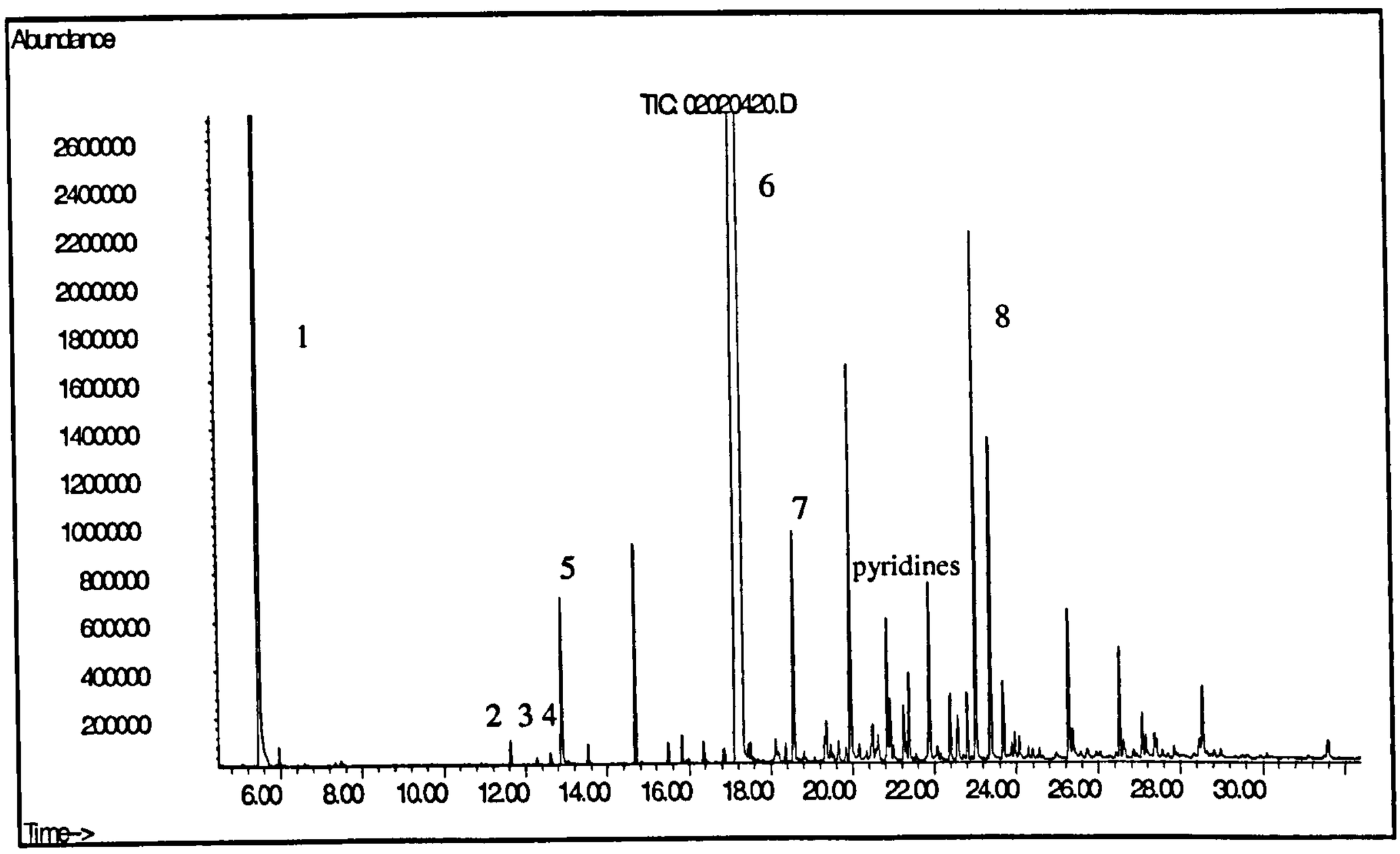


Figure 8.8 NND14 Impurity Profile zoomed on baseline

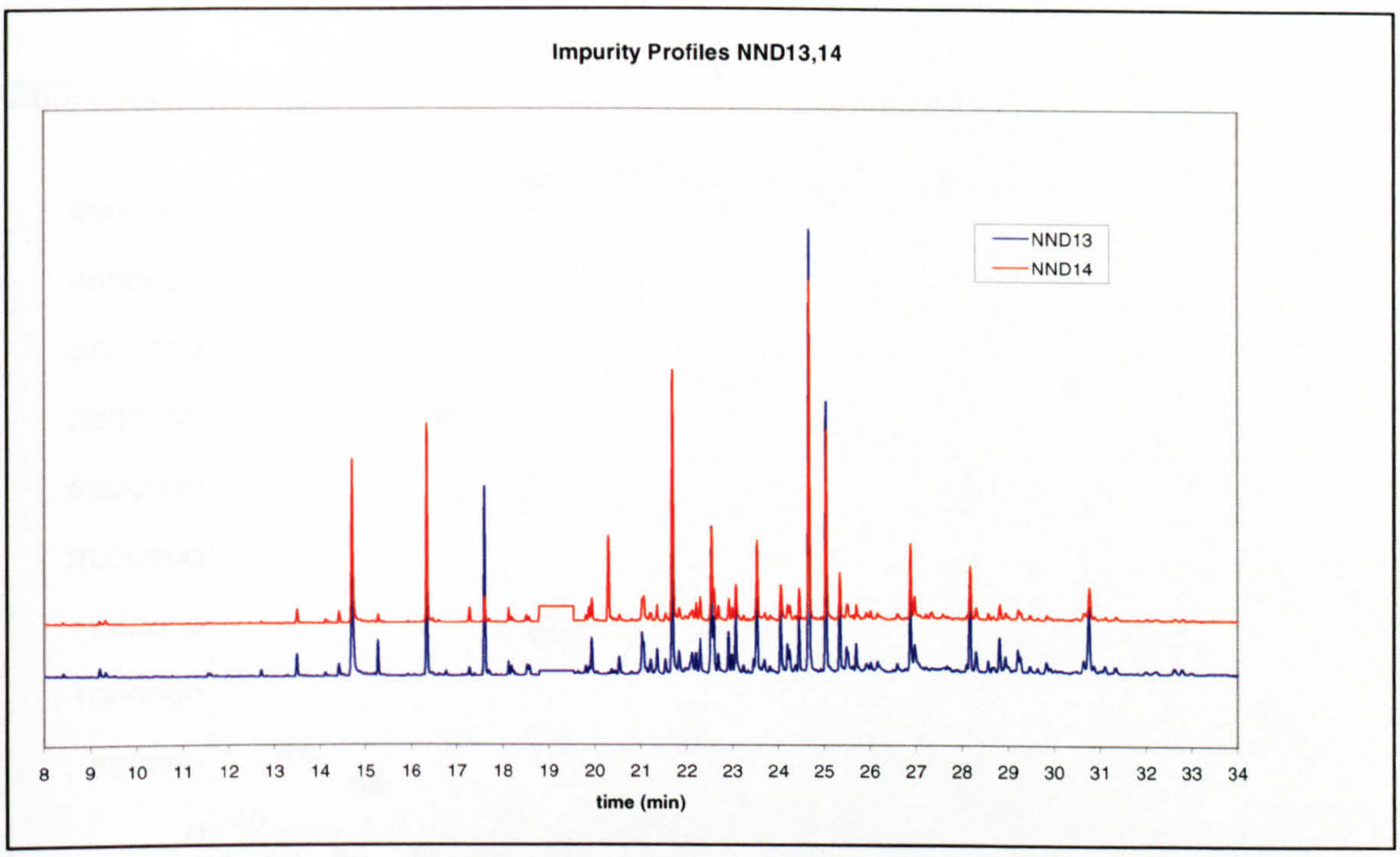


Figure 8.9 Impurity Profiles of NND13 and NND14

Figures 8.10 to 8.20 show the mass spectra of impurities identified in NND 13 and NND 14. Amphetamine (Fig 8.8, 1) was detected at 5.5 min, pyrimidines (Fig 8.8, 2 and 3) at 11.6 min and 12.3 min, acetylamphetamine (Fig 8.8, 4) at 12.6 min, N-formylamphetamine (Fig 8.8, 5) at 12.9 min, DPIA (Fig 8.8, 6) at 17.4 min, caffeine(Fig 8.8, 7) at 18.6 min, pyridines at 21.2, 21.4, 21.9 and 22.0, 22.8 min and DPIF at 23 min. Note that these retention times are those detected by the MSD and not the FID. Also, Figure 8.15 shows the enlarged region where the pyridines elute around 21-23min.

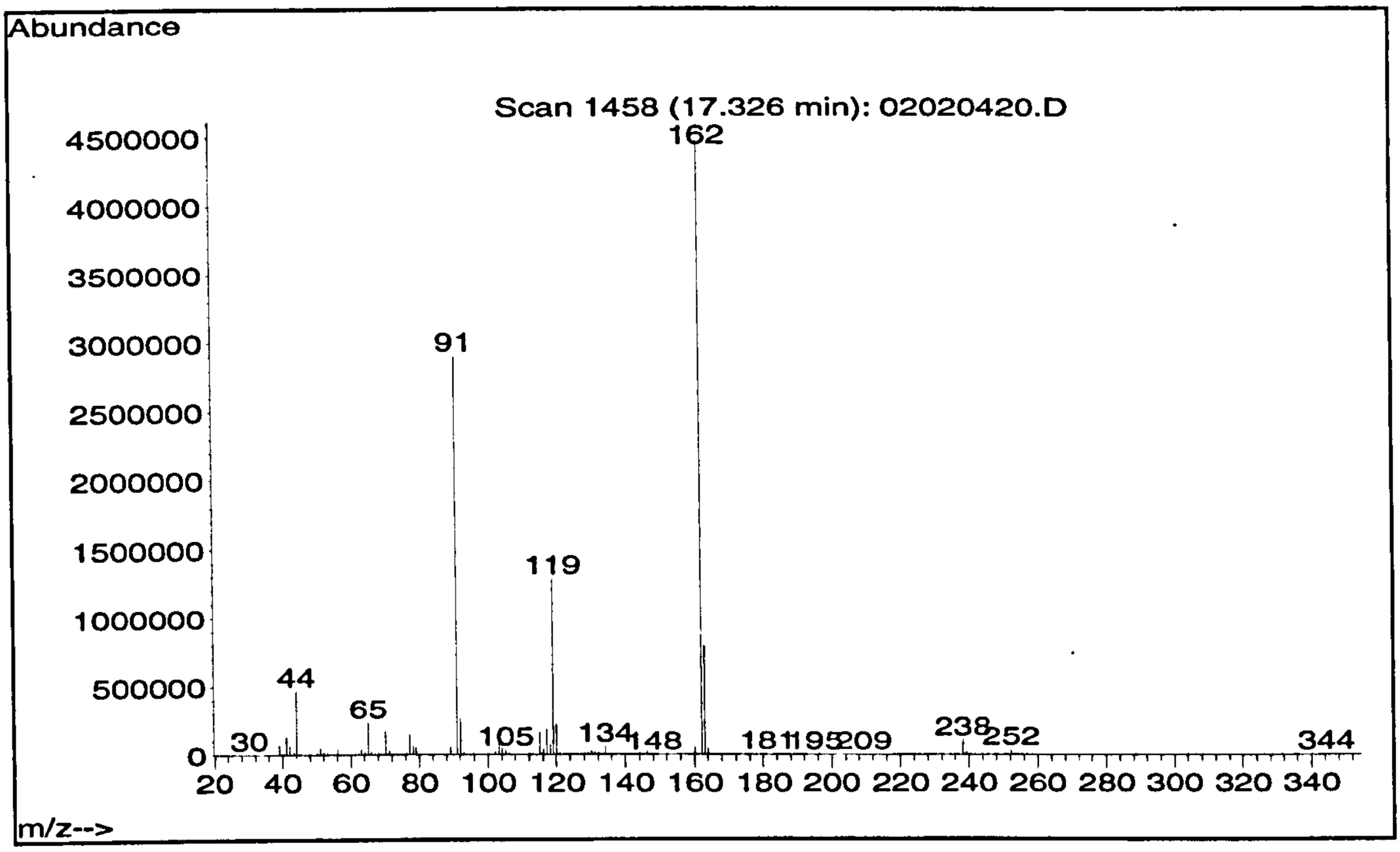


Figure 8.10 Major Impurity in NND13 and NND14 N,N-Di(B-phenylisopropyl)amine, (DPIA) at 17.4 min

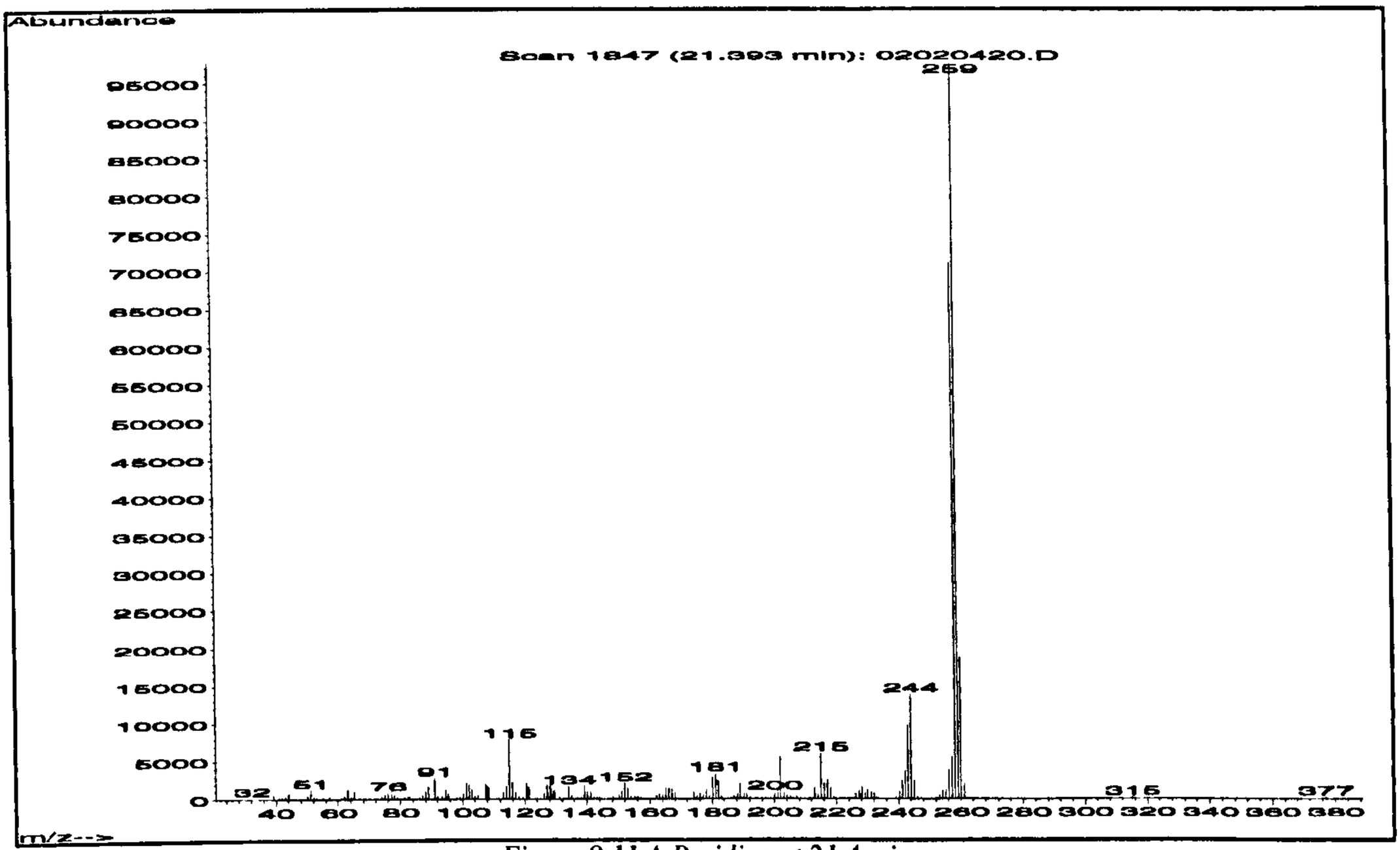


Figure 8.11 A Pyridine at 21.4 min

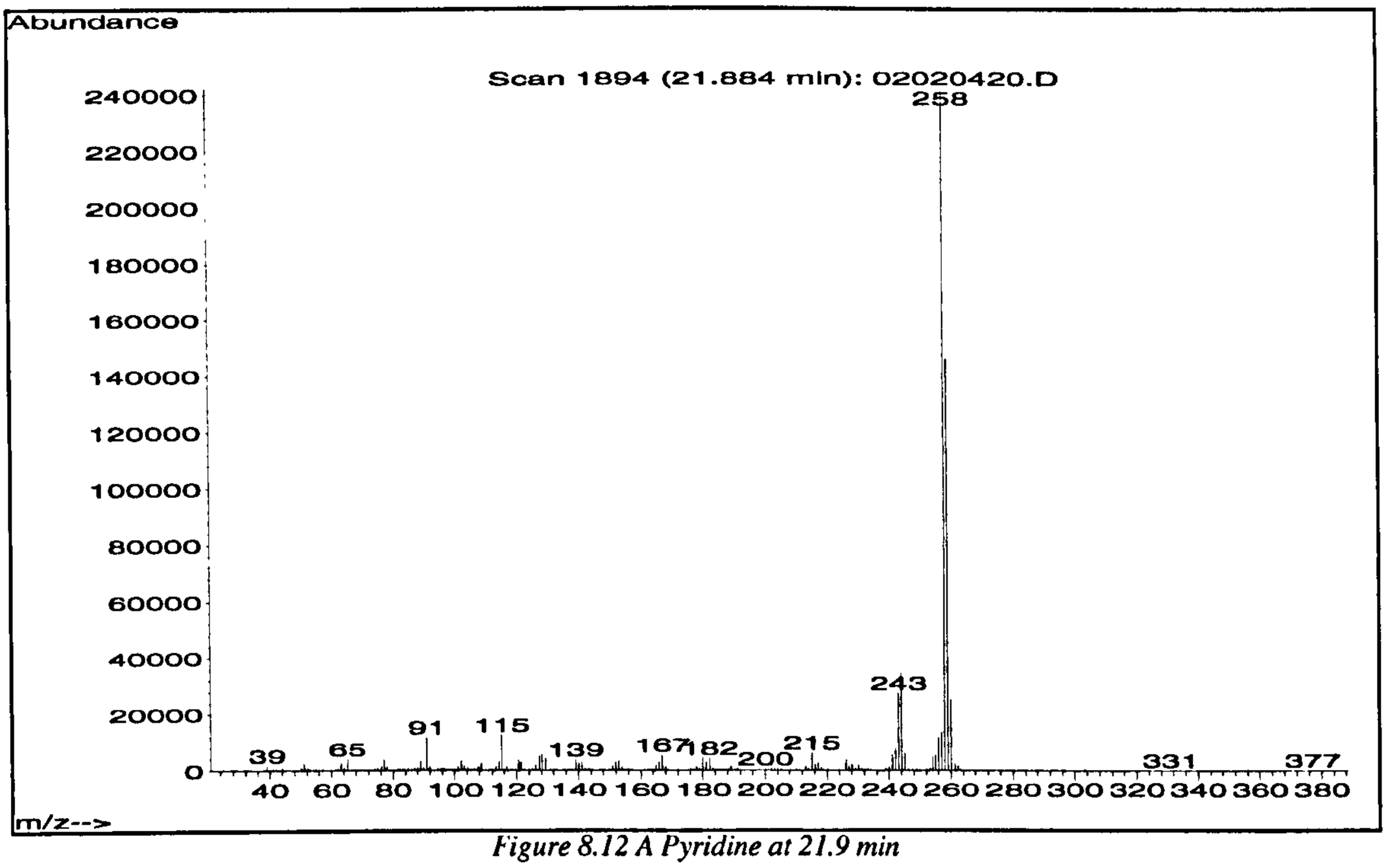


Figure 8.12 A Pyridine at 21.9 min

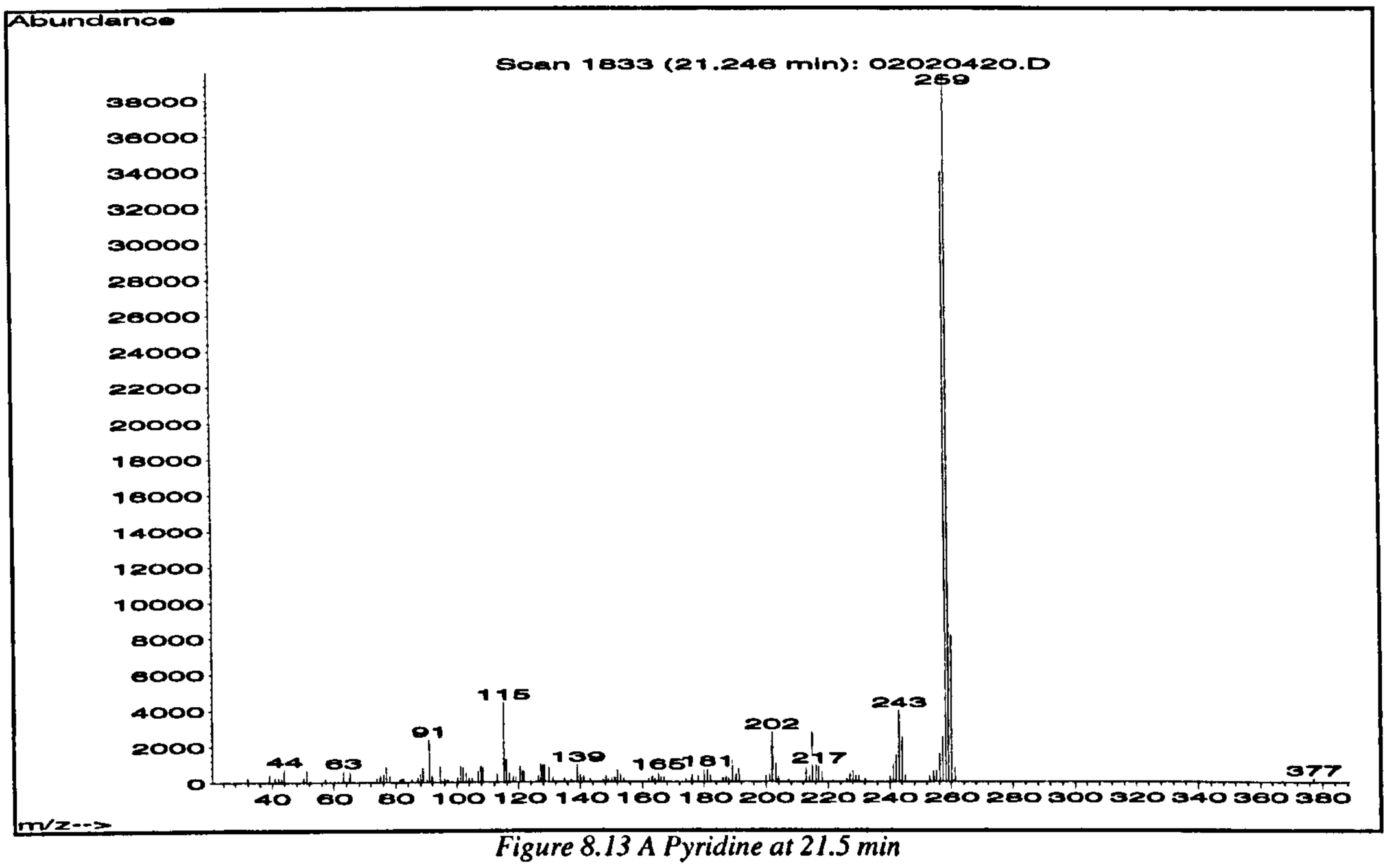


Figure 8.13 A Pyridine at 21.5 min

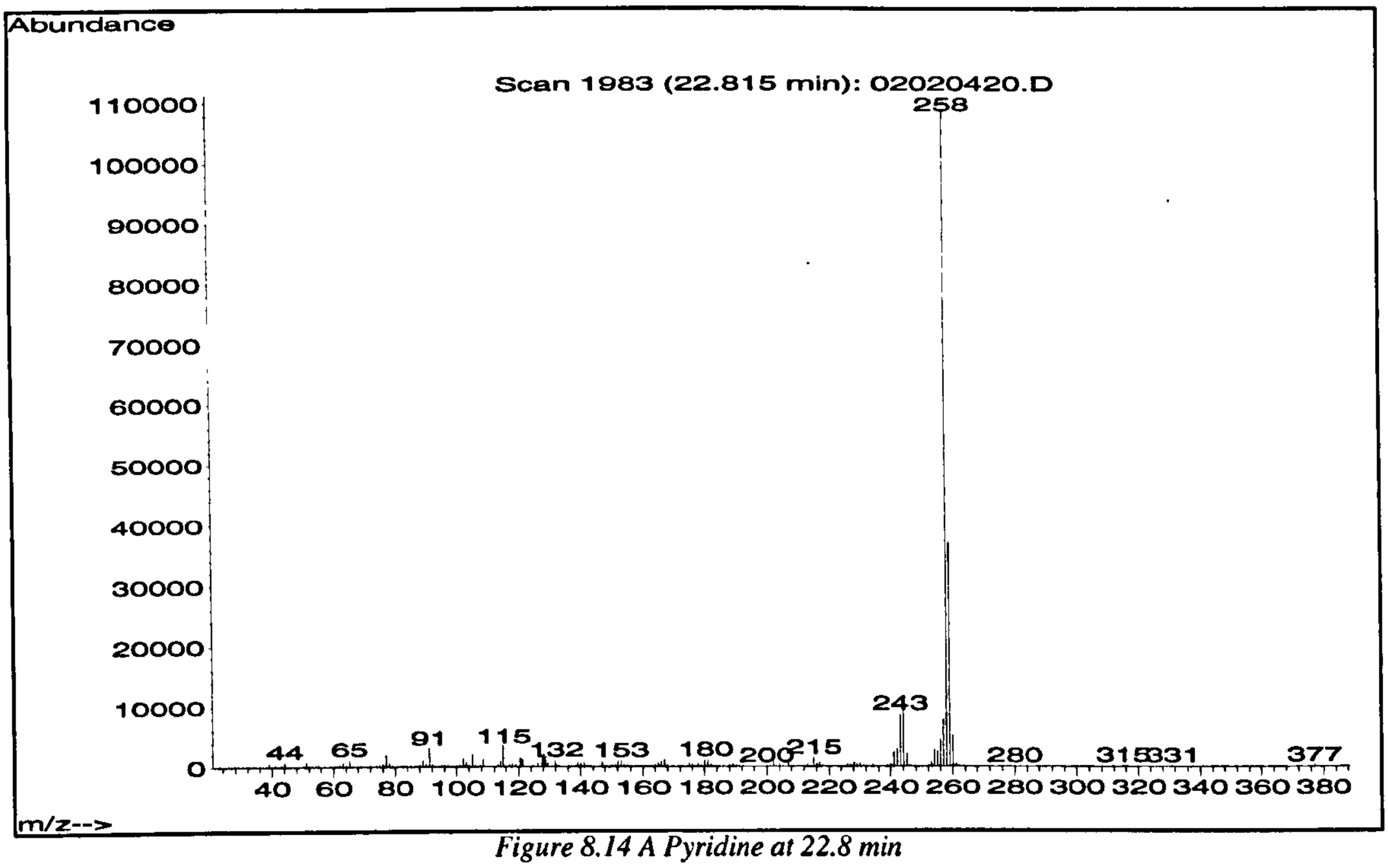


Figure 8.14 A Pyridine at 22.8 min

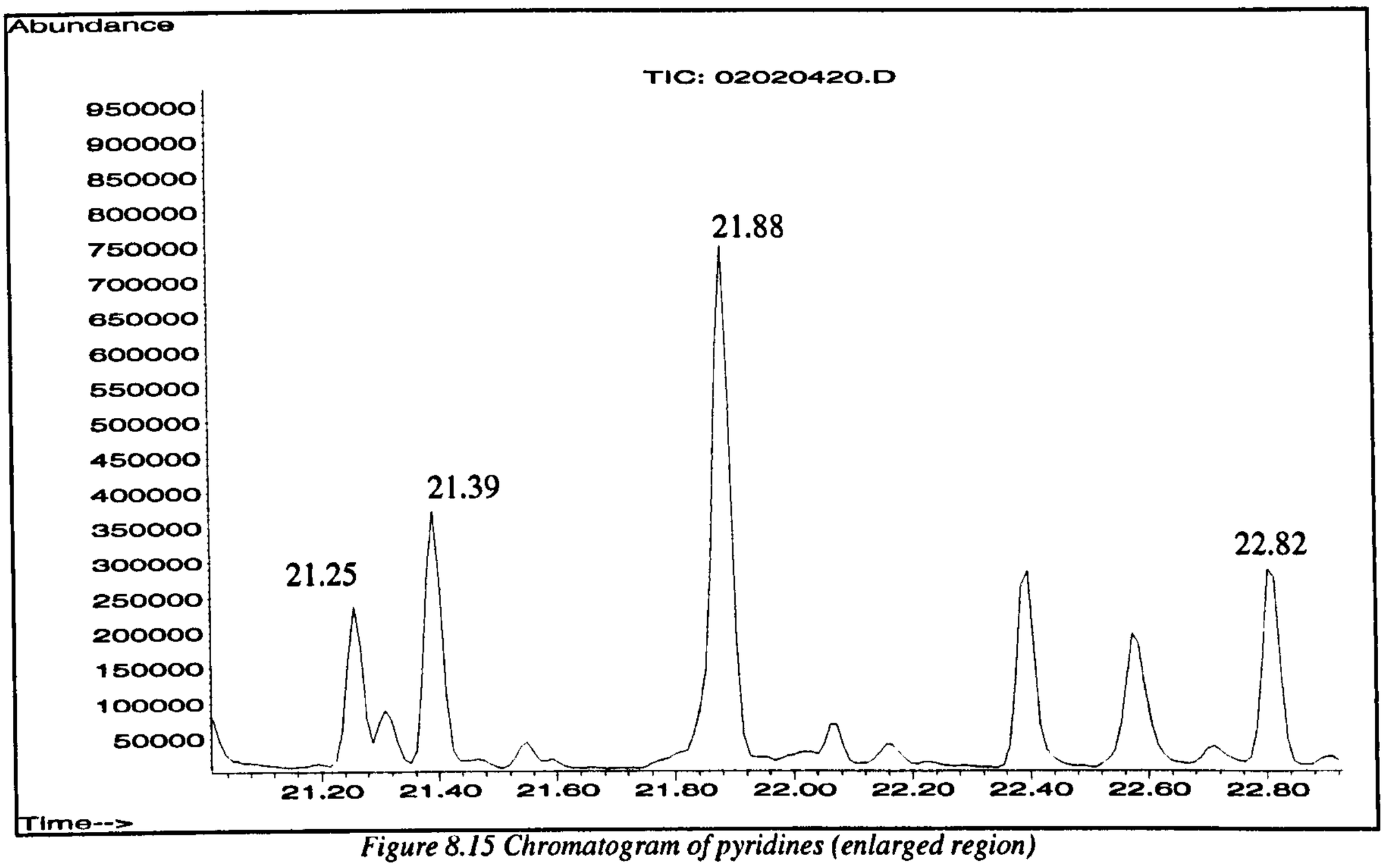


Figure 8.15 Chromatogram of pyridines (enlarged region)

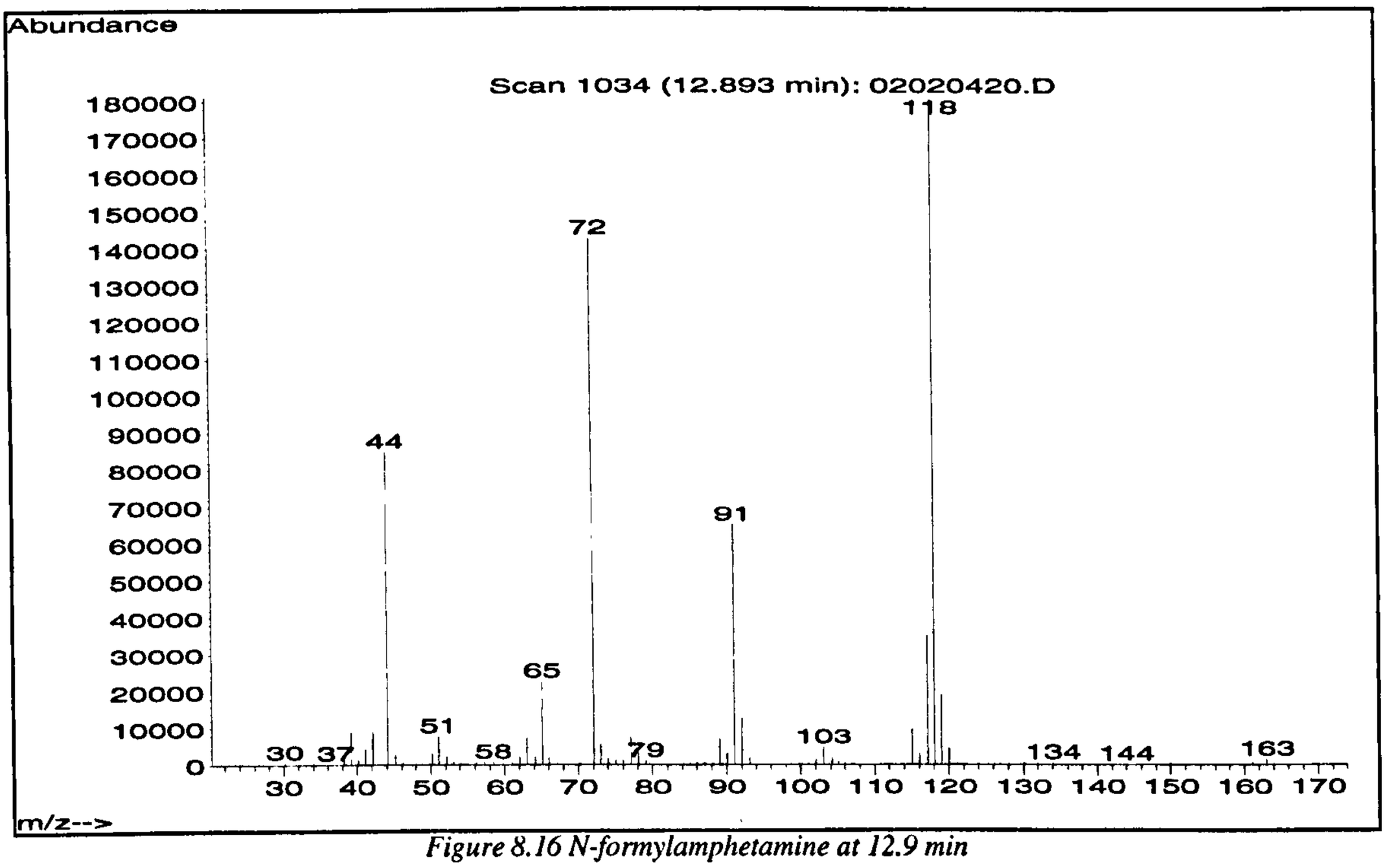


Figure 8.16 N-formylamphetamine at 12.9 min

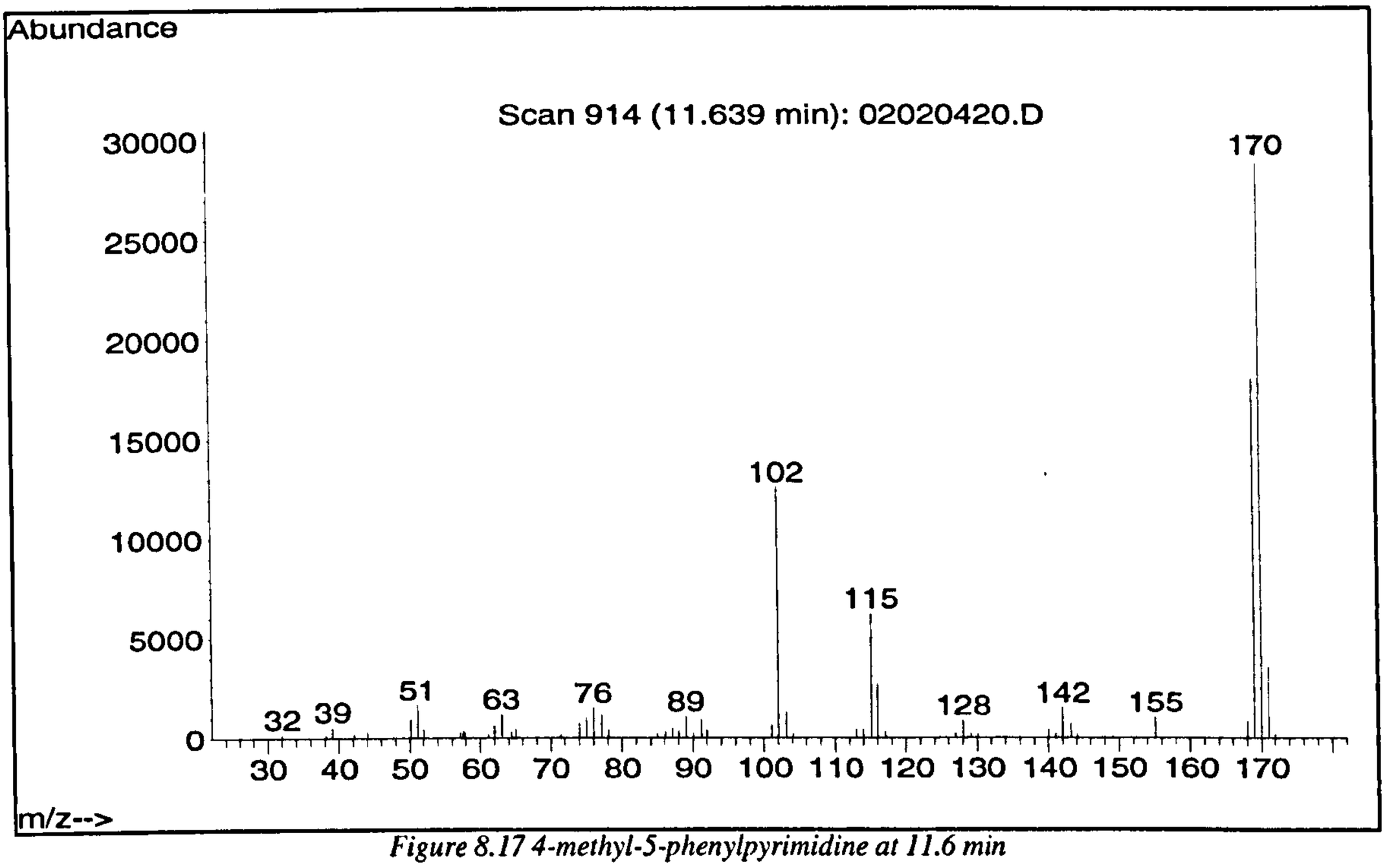


Figure 8.17 4-methyl-5-phenylpyrimidine at 11.6 min

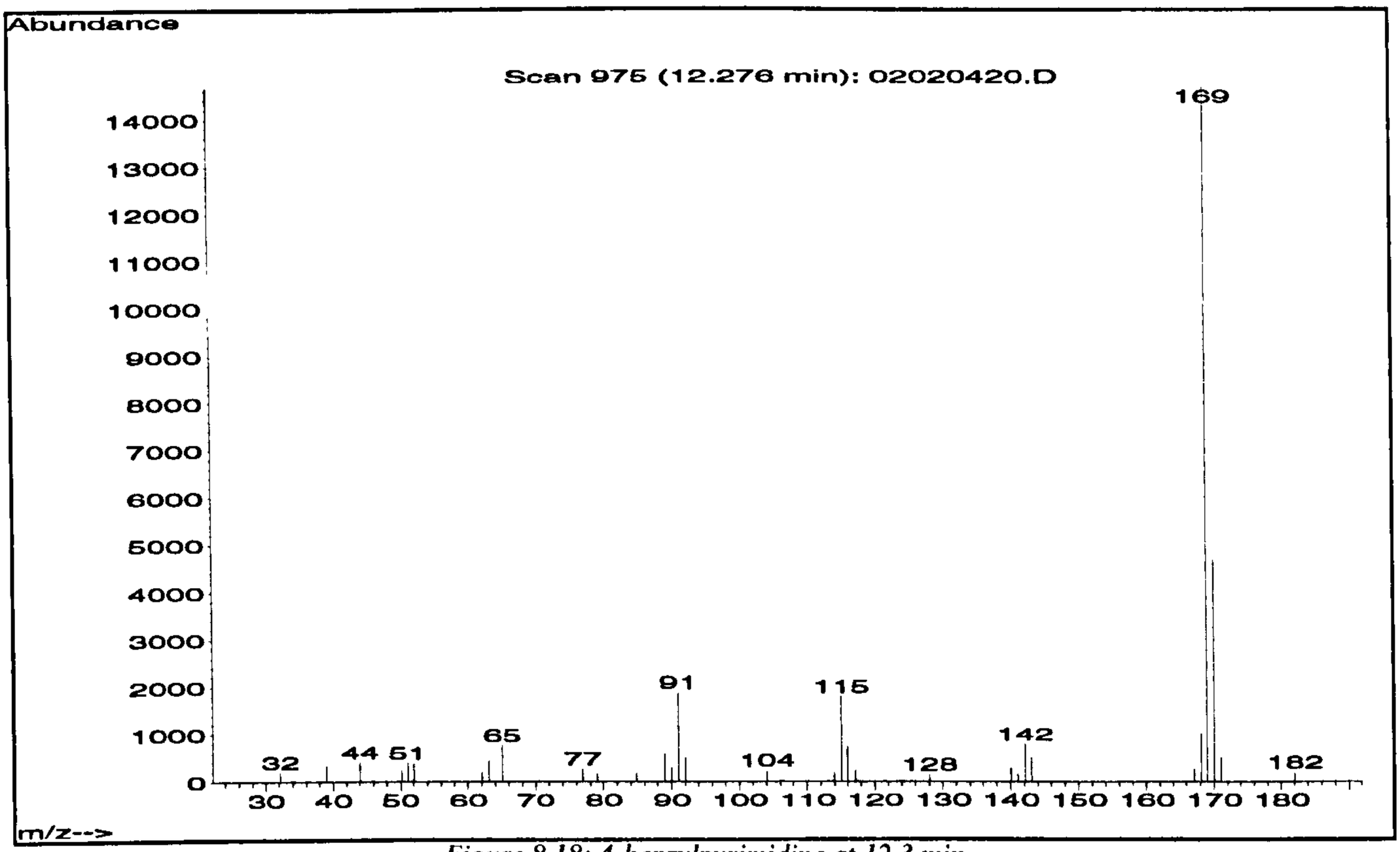


Figure 8.18: 4-benzylpyrimidine at 12.3 min

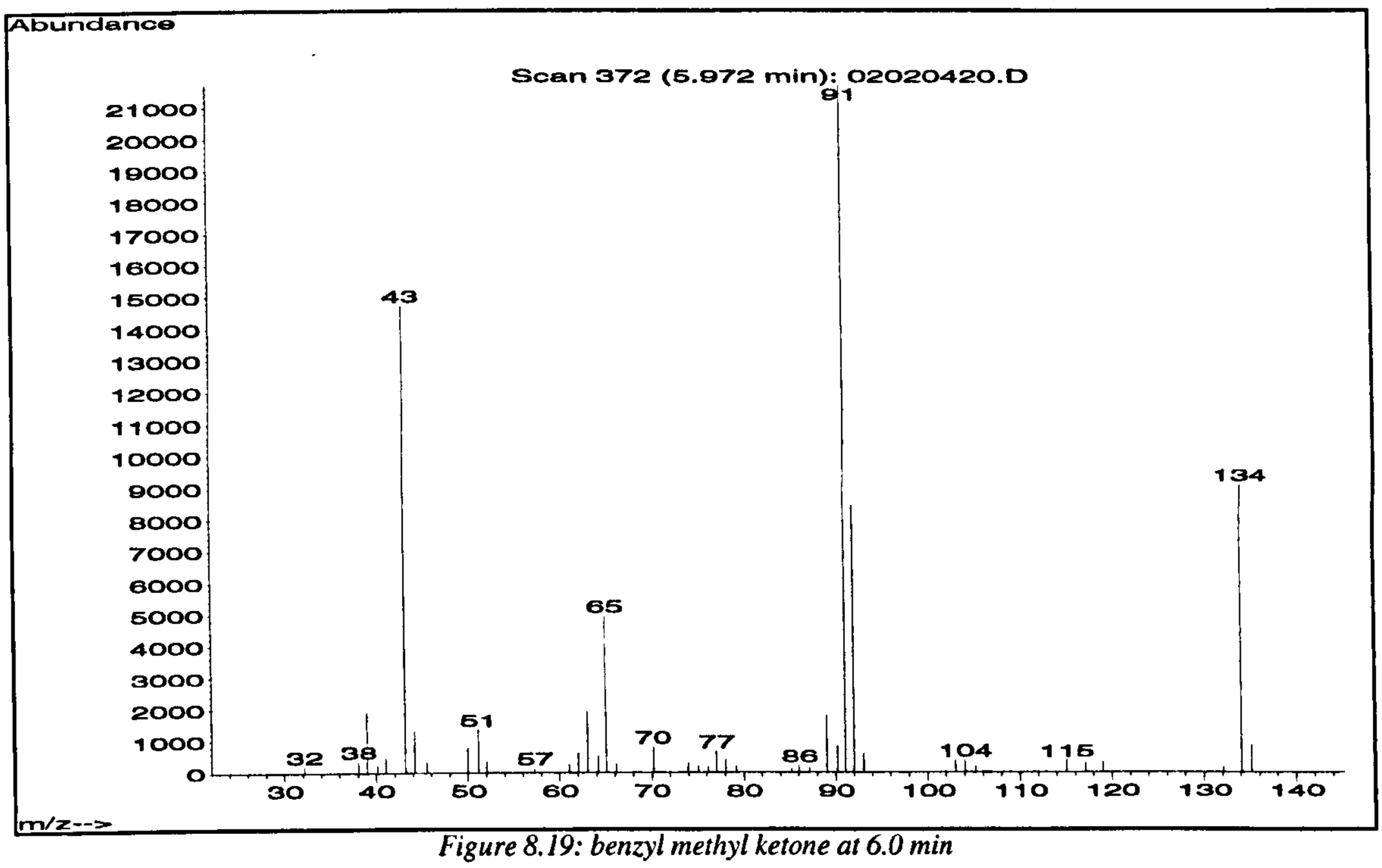


Figure 8.19: benzyl methyl ketone at 6.0 min

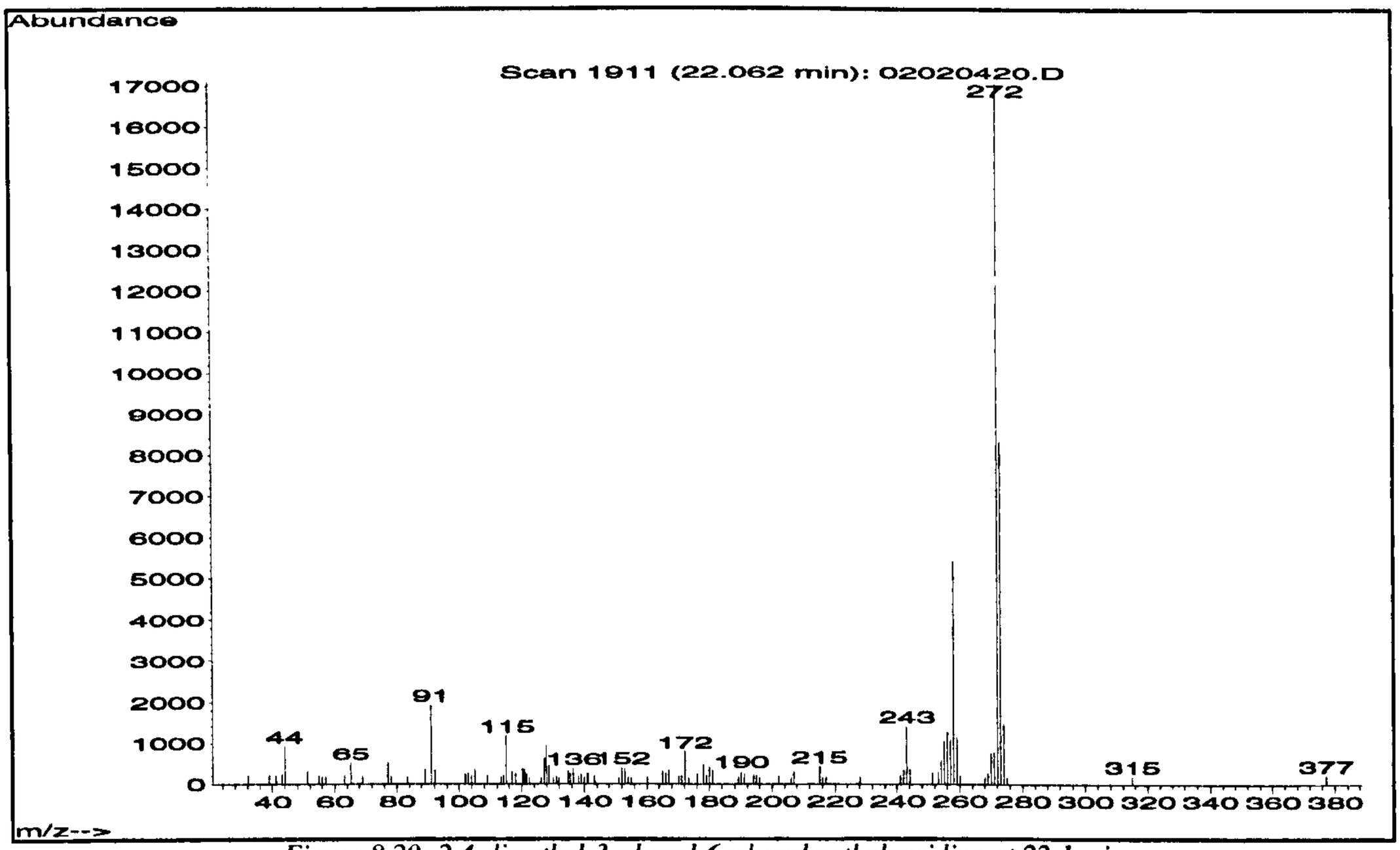


Figure 8.20: 2,4-dimethyl-3-phenyl-6-phenylmethylpyridine at 22.1 min

8.6.3. NND 15 and NND 15B

On extraction and analysis of NND 15 and NND 15B it was established that the impurity profiles of the individual samples showed similar profiles (Figure 8.22) which would link them to a common source of amphetamine although there are slight differences in the profiles around 23 and 25 mins. The presence of both pyrimidines (Fig 8.21, 2 and 3) was established, therefore both samples were thought to be synthesised by the Leuckart route. N-formylamphetamine (4), DPIA (5) and caffeine (6) were also detected but no pyridines were observed in the profile as shown in Figure 8.21.

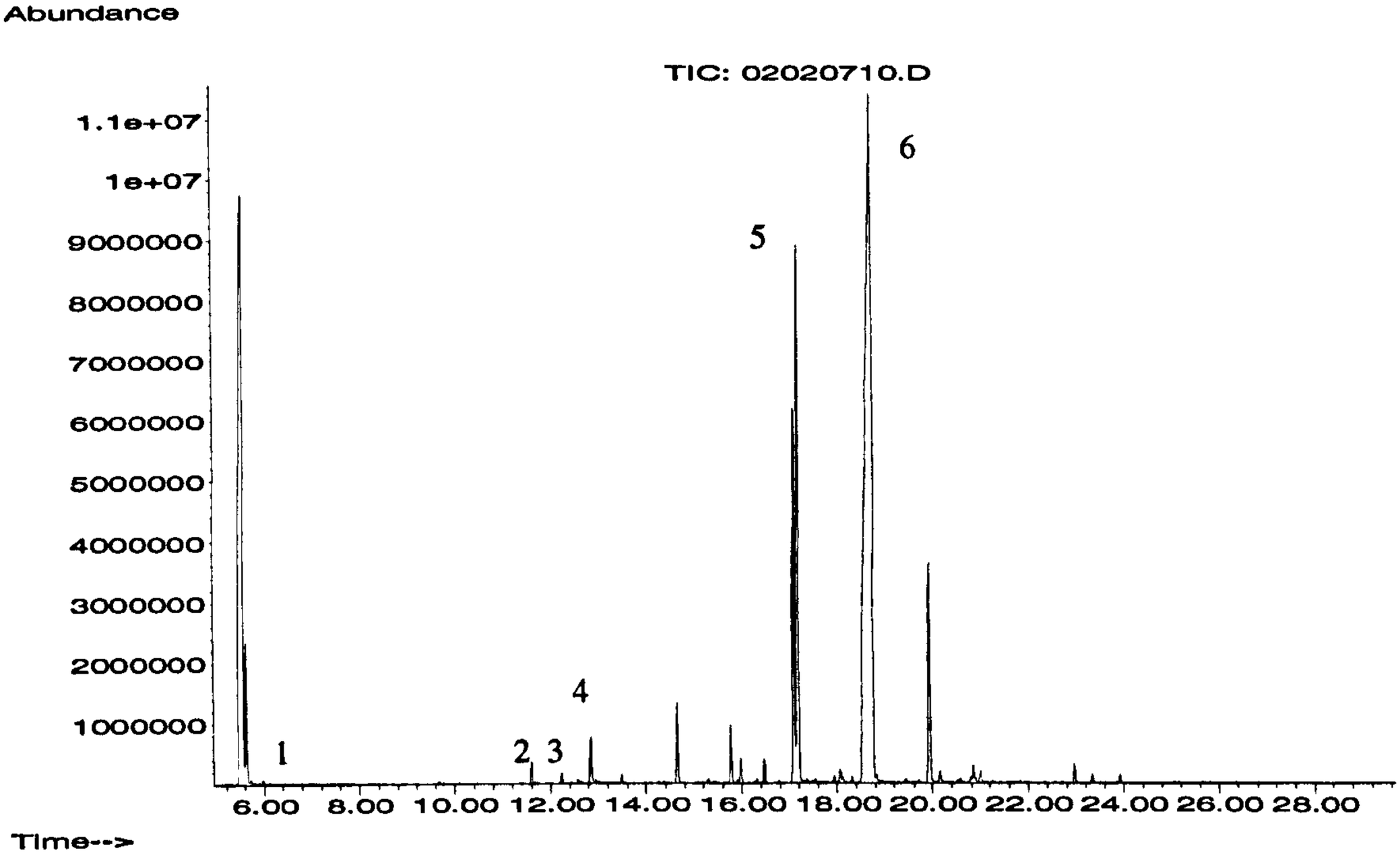


Figure 8.21 Impurity Profile of NND15

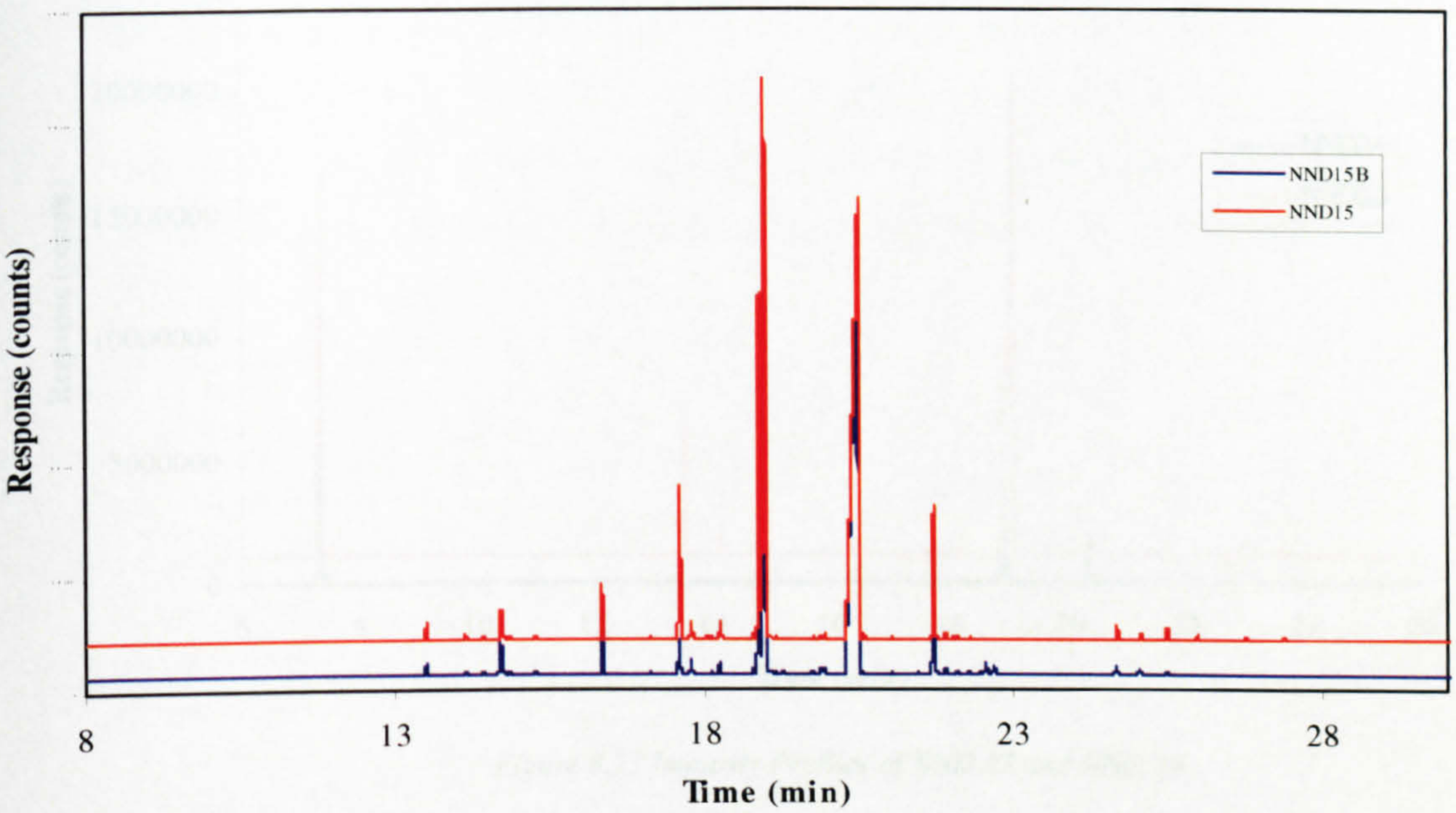


Figure 8.22 Impurity Profile of NND 15 and NND 15B

8.6.4. NND33 and 34

Samples NND 33 and NND 34 were not visually linked through impurity profiling since neither the amphetamine content nor the levels of impurities were similar. However, NND 33 and NND 34 were apparently linked by seizure as related samples from a single case. The amphetamine content may be different if one sample is more concentrated than the other (for example if one sample had been seized before diluents had been added) however, the impurity profiles should be the same for those impurities resulting from the synthesis. As may be seen from the profiles generated from the extraction and analysis of both samples in the FID profiles in Figure 8.23, sample NND 33 has almost twice the concentration of active drug (at 7.3min) as NND 34. NND 33 contains both pyrimidines seen here eluting at around 13-14 minutes, which are absent in NND 34. In addition, DPIA seen at 18.9 minutes in both samples has a higher concentration than amphetamine in NND 33 but much lower than the active drug in NND 34. NND 33 has also been shown to contain very low levels of the pyridine group.

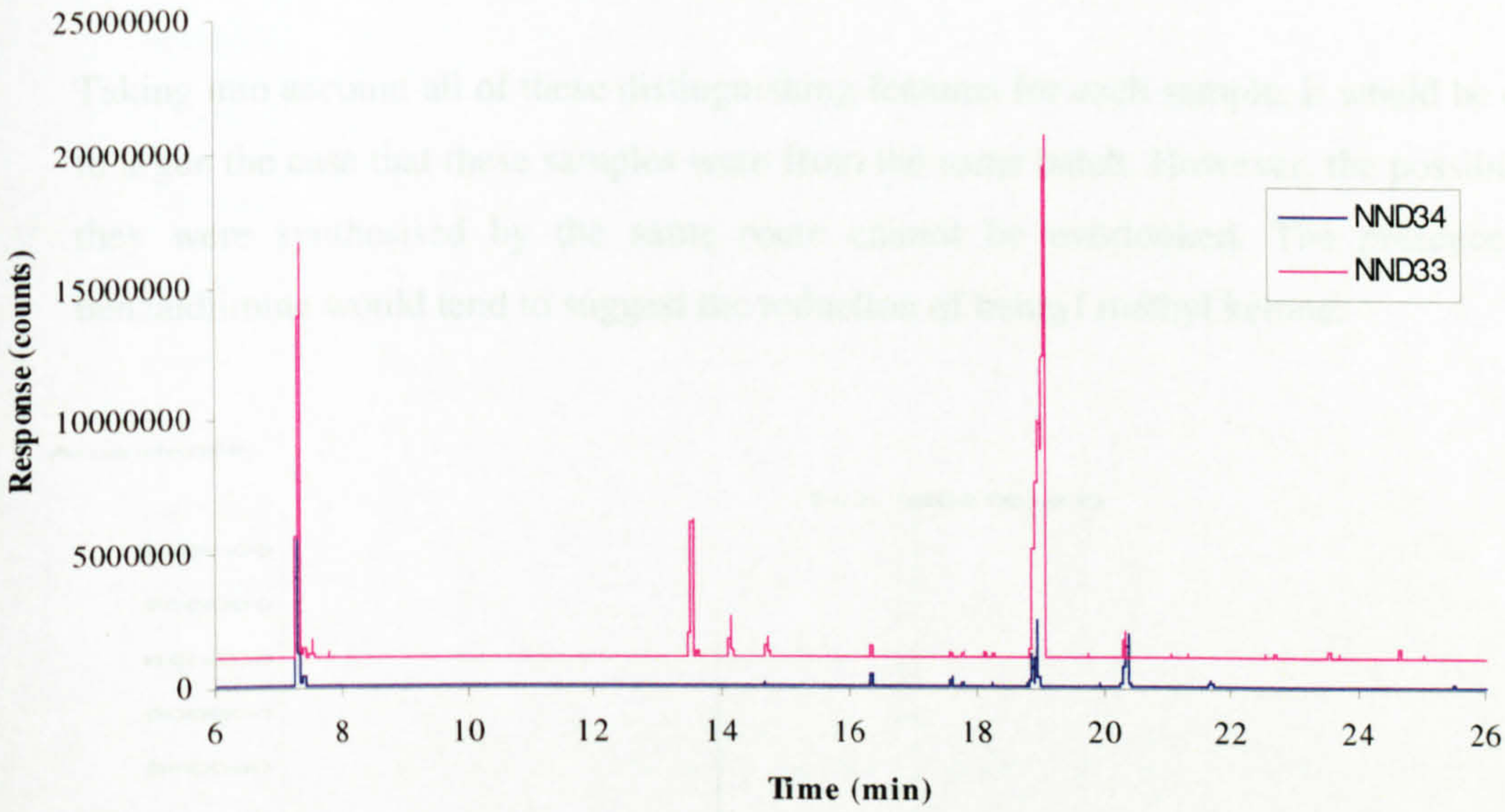


Figure 8.23 Impurity Profiles of NND 33 and NND 34

These samples would not be linked by impurity profiling and therefore, although they have been seized together, cannot be chemically related. Whether this is a failure on the part of the method, or whether the samples are actually not related by batch is unclear since the real detail of the case or the source of the samples is unknown. Having seen how similar chromatograms from a single source are (in the experiments detailed in Chapter 7 and in other samples in this chapter) I would conclude that although these samples have been seized together and relate to the same case, they do not come from a single batch of amphetamine.

8.6.5. NND 43 – NND 45

Although NND 43-45 have been suggested as having links by case, this would perhaps not have been picked up by impurity profiling. Although the profiles are similar (see Figures 8.24 to 8.26), in that all three contain Benzaldiimine (at 15.7 min) and the reduced form of Benzaldiimine (at 16.3 min), both pyrimidines (at 11.6 and 12.2 min) a high concentration of DPIA (at 17.3 min) and the group of pyridines (at 21-23 min) the relative responses of these compounds vary in all three samples. In addition to this, there are distinct peaks around 24 minutes in NND 44 and 45 (see Figure 8.27 for the mass spectra of the main peak at 23.9 min) which are absent in NND 43. Also, NND 43 and NND 44 have only a small peak at

around 20 minutes where NND 45 has a significant peak (see Figure 8.28 for the mass spectra of the peak at 19.8 min).

Taking into account all of these distinguishing features for each sample, it would be difficult to argue the case that these samples were from the same batch. However, the possibility that they were synthesised by the same route cannot be overlooked. The presence of the benzaldiimine would tend to suggest the reduction of benzyl methyl ketone.

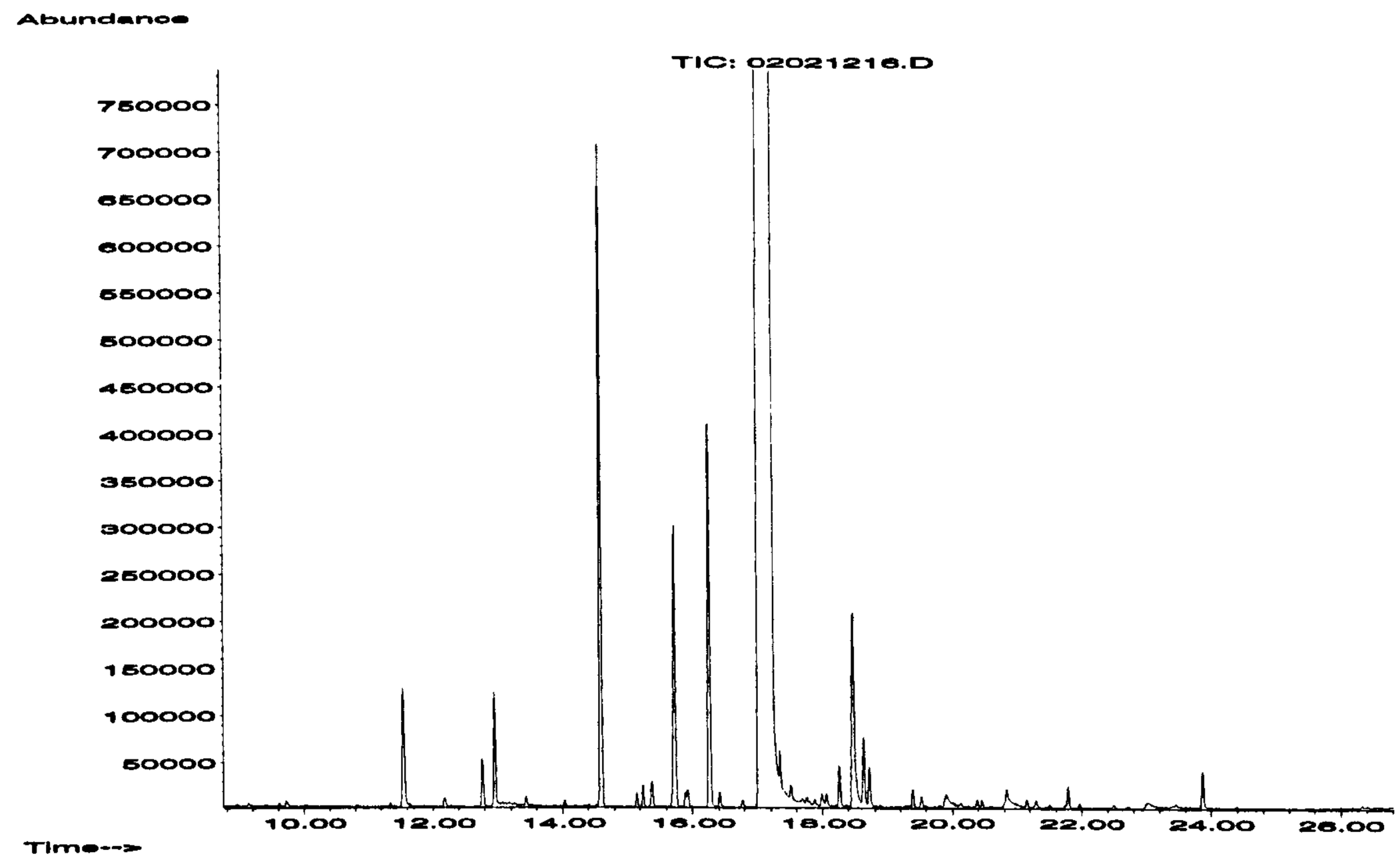


Figure 8.24 Impurity Profile of NND43

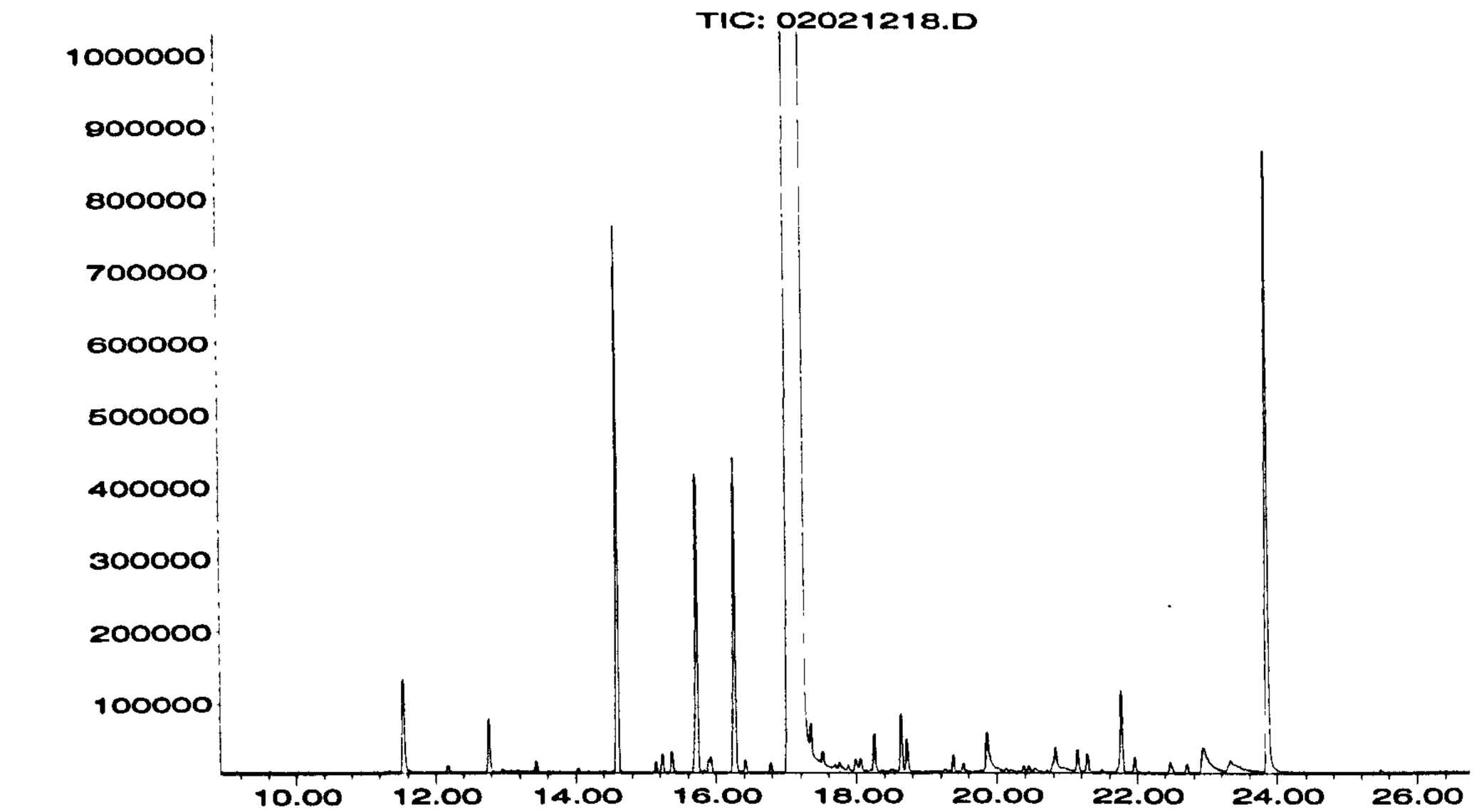


Figure 8.25 Impurity Profile of NND44

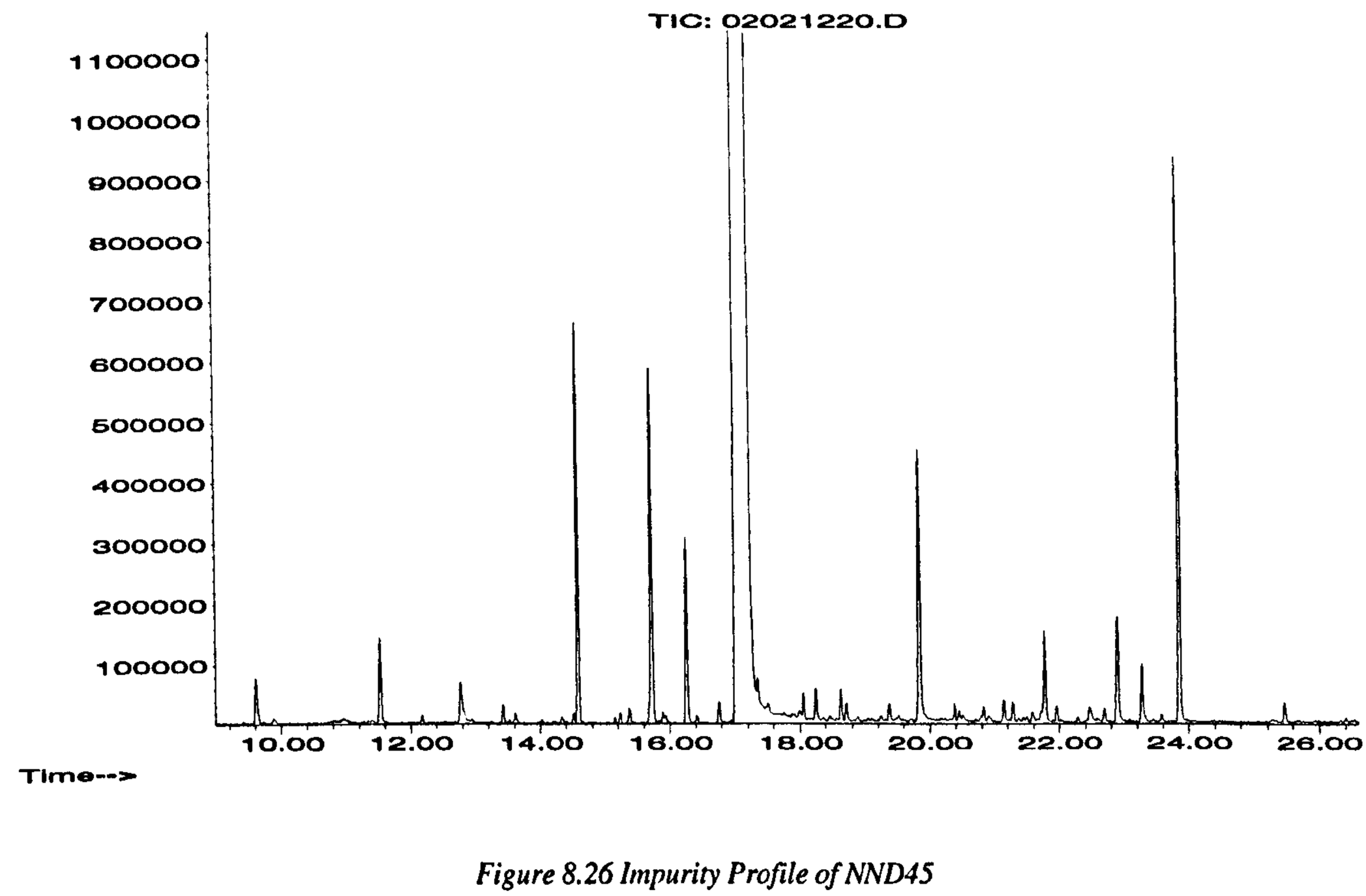


Figure 8.26 Impurity Profile of NND45

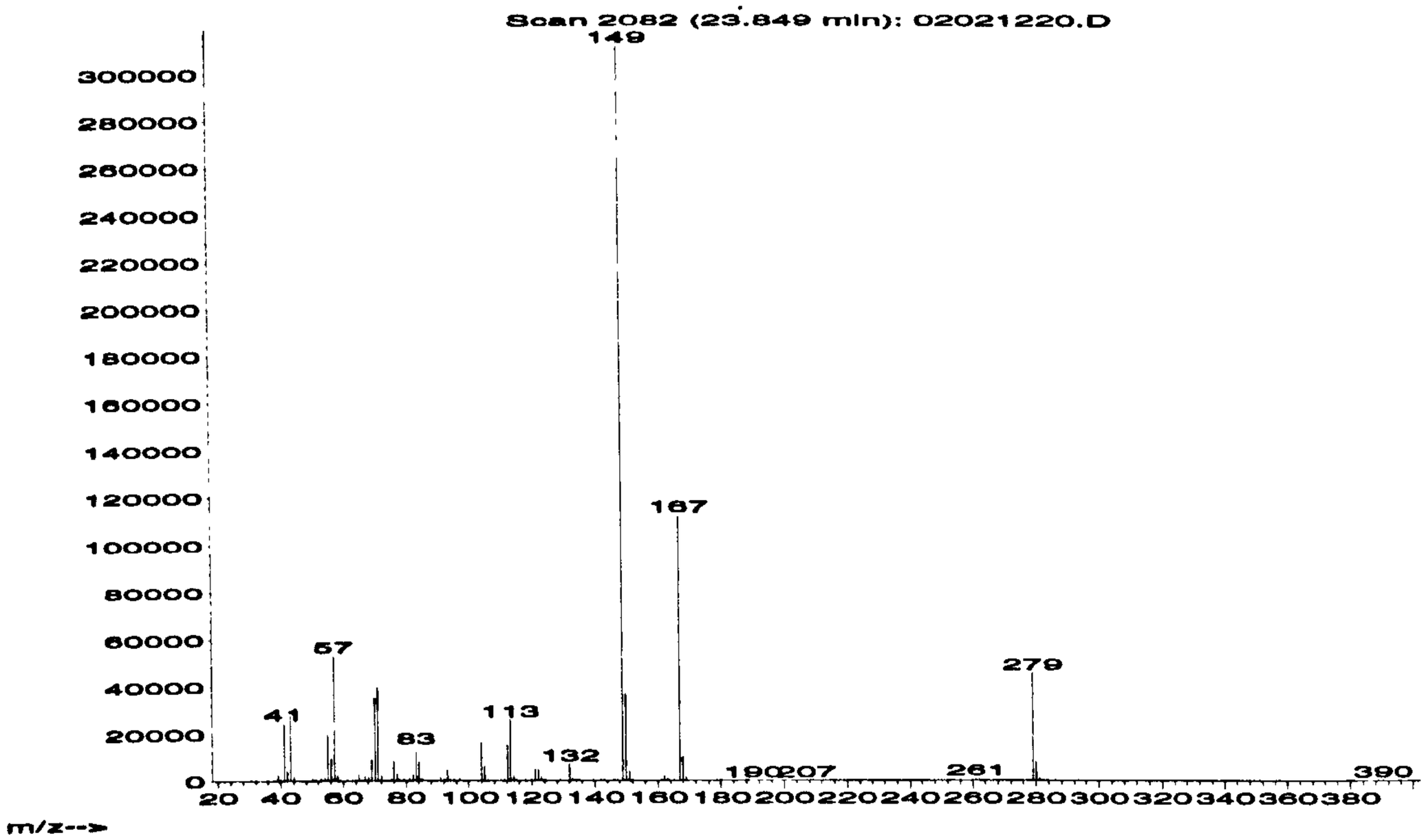


Figure 8.27 Unknown Impurity at 23.9 min

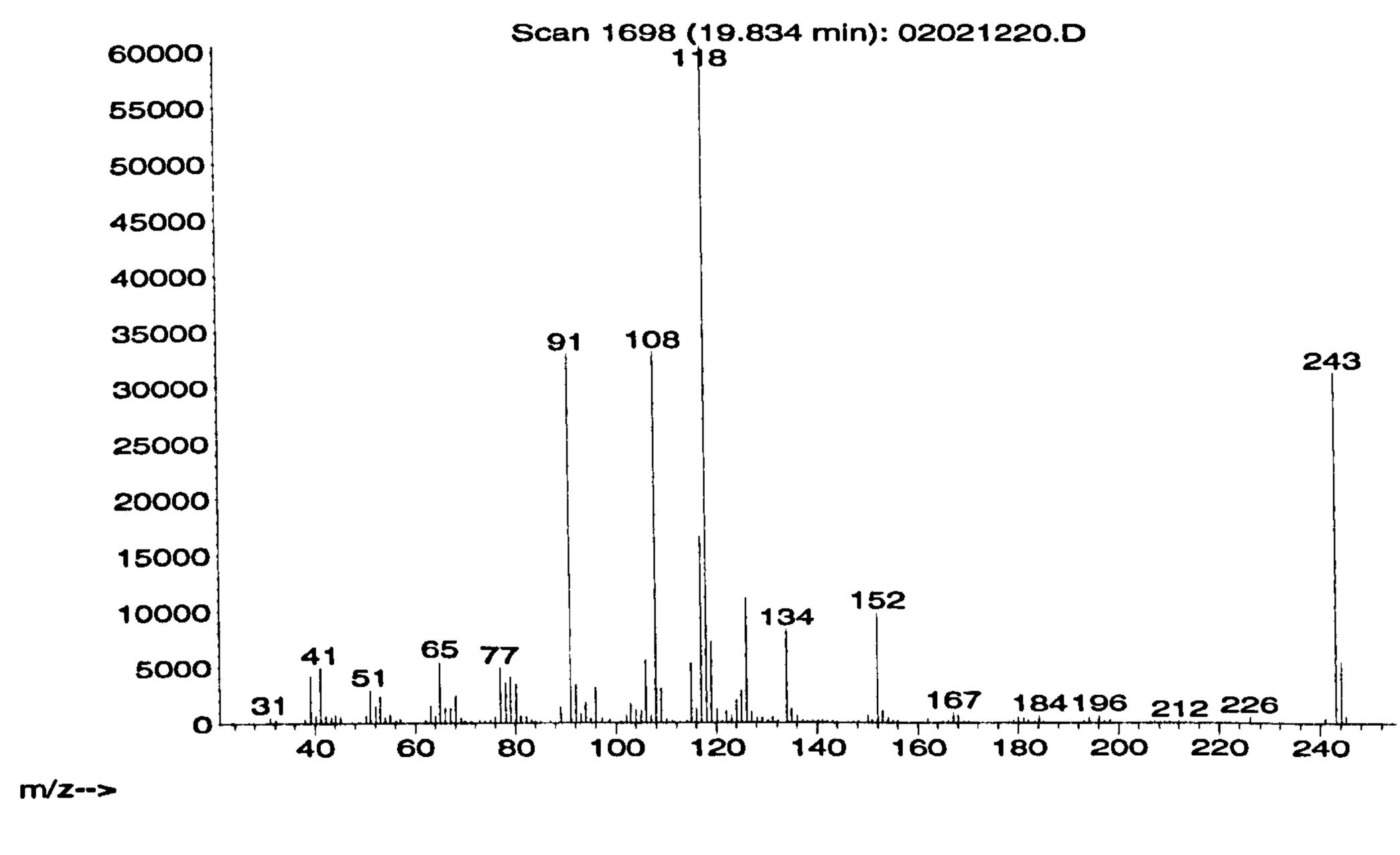


Figure 8.28 Unknown Impurity at 19.8 min

8.6.6. NND 51-NND 76

Although by seizure, NND 51 through NND 76 are connected, the entire seizure appears to be made up of several discrete batches of sample with some batches producing almost identical impurity profiles. However, the whole seizure has a very similar appearance – white crystalline powder containing small particles of yellow solid. The homogeneity of the batches looked to be poor with what appeared to be yellow crystalline amphetamine salt grains mixed into white powdered diluent. It appeared that no effort had been made, on the part of the individual involved in bulking the sample for sale, to ensure that each individual sample contains the same levels of active drug. The mass spectra shown in Figure 8.29 and 8.30 are from NND 52 as examples of Acetylamphetamine and Benzaldiimine. Figures 8.31 and 8.32 show the impurity profiles of NND 52 and NND 58 to show the distinctly different profiles generated by samples which have been linked by case but clearly do not come from a single source.

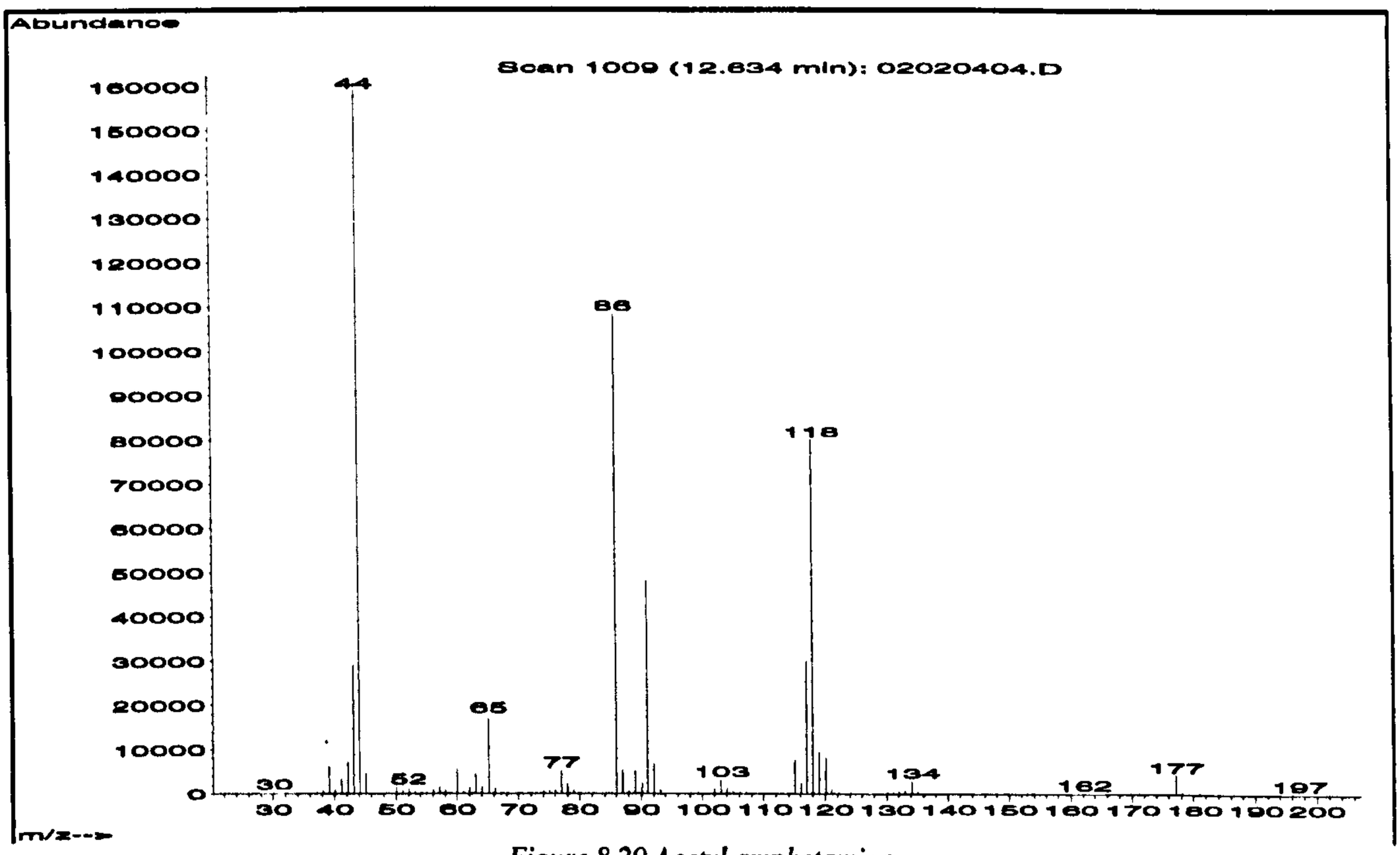


Figure 8.29 Acetyl amphetamine

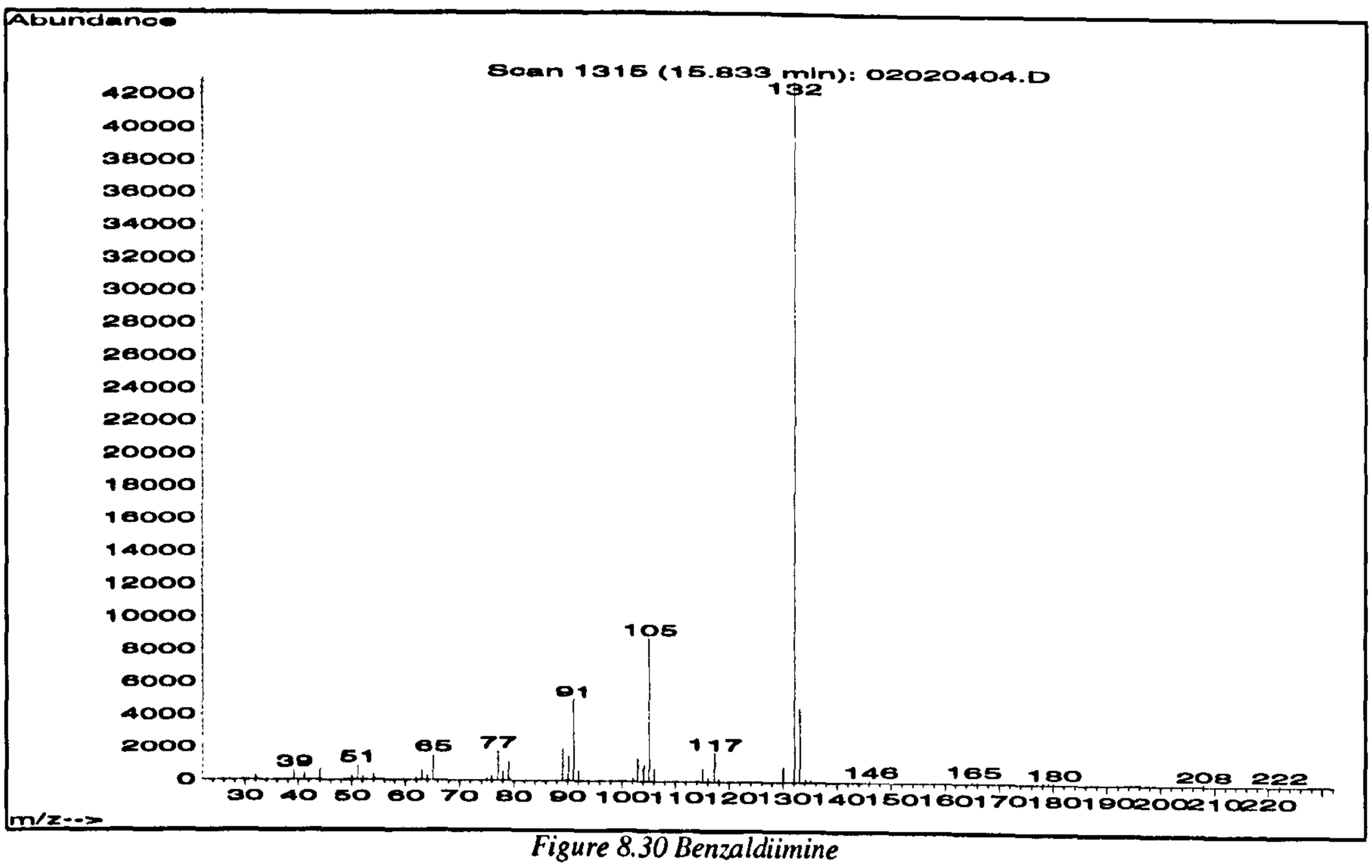


Figure 8.30 Benzaldiimine

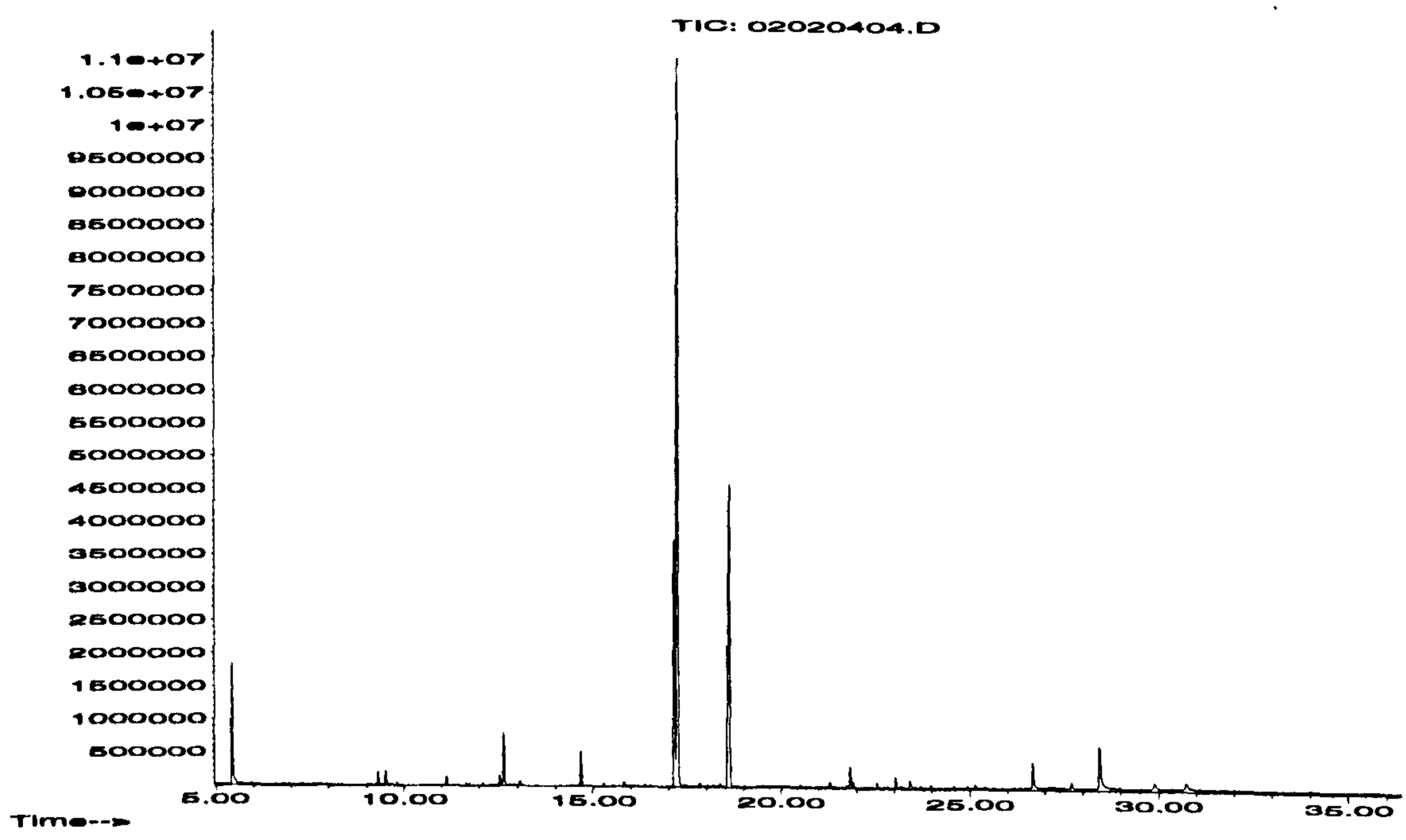


Figure 8.31 NND 52

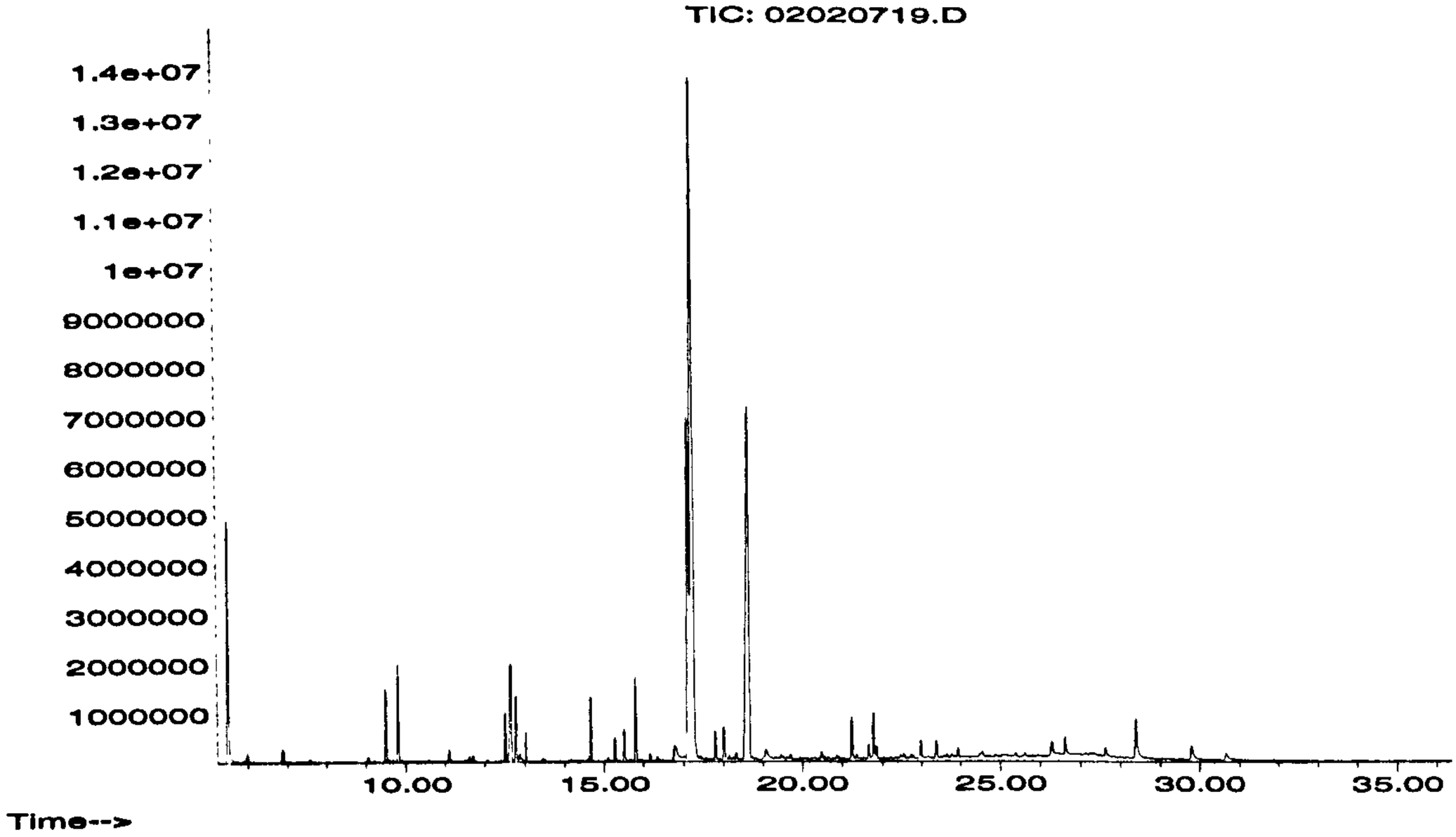


Figure 8.32 NND 58

The impurity profiles of NND 52 and NND 53 shown in Figure 8.33 highlight that the impurity levels are relatively small but cover a wide range of retention times. The highest level impurity peak, DPIA has been removed to allow the baseline to be seen more clearly. It may be seen that again the peaks eluting after 23 minutes show significant differences in profile – since there are no known synthesis impurities eluting at this time these impurities may be from another source.

The impurity profiles of NND 55, NND 63 and NND 70 are shown in Figure 8.34 with higher levels of impurities and a more complex impurity profile. The impurity profiles of these samples, however, clearly link these samples together and distinguish them from, for example, NND 52 and NND 53. NND 65 and NND 73 also form another distinct group as may be seen in Figure 8.35.

These sub-groups of the main group do not differ in the impurities present, simply in the relative levels at which they are found. Figure 8.36 shows the variation in the profile of samples NND 51 – NND 60 for a specific retention window.

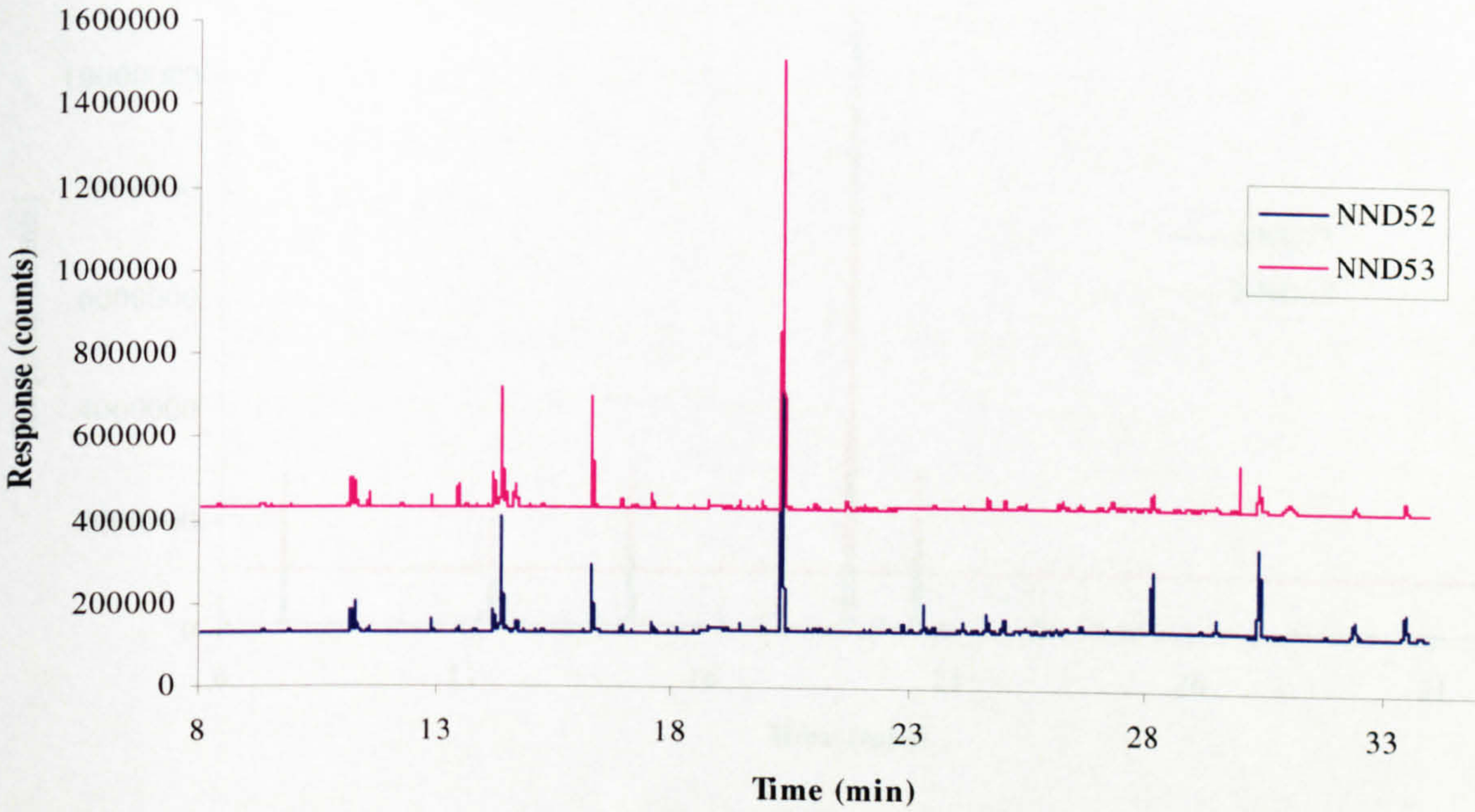


Figure 8.33 NND 52 and NND 53

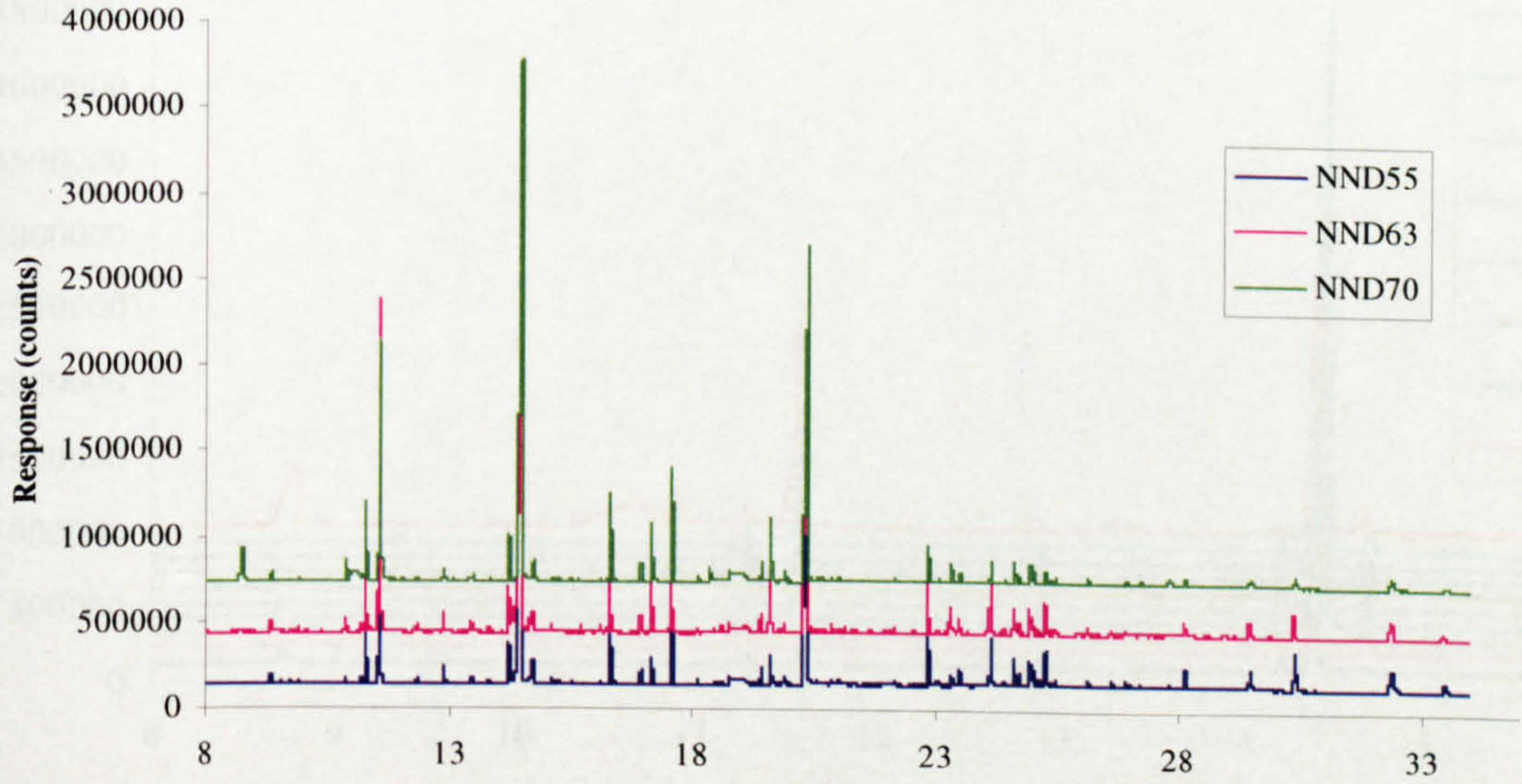


Figure 8.34 NND 55, NND 63 and NND 70

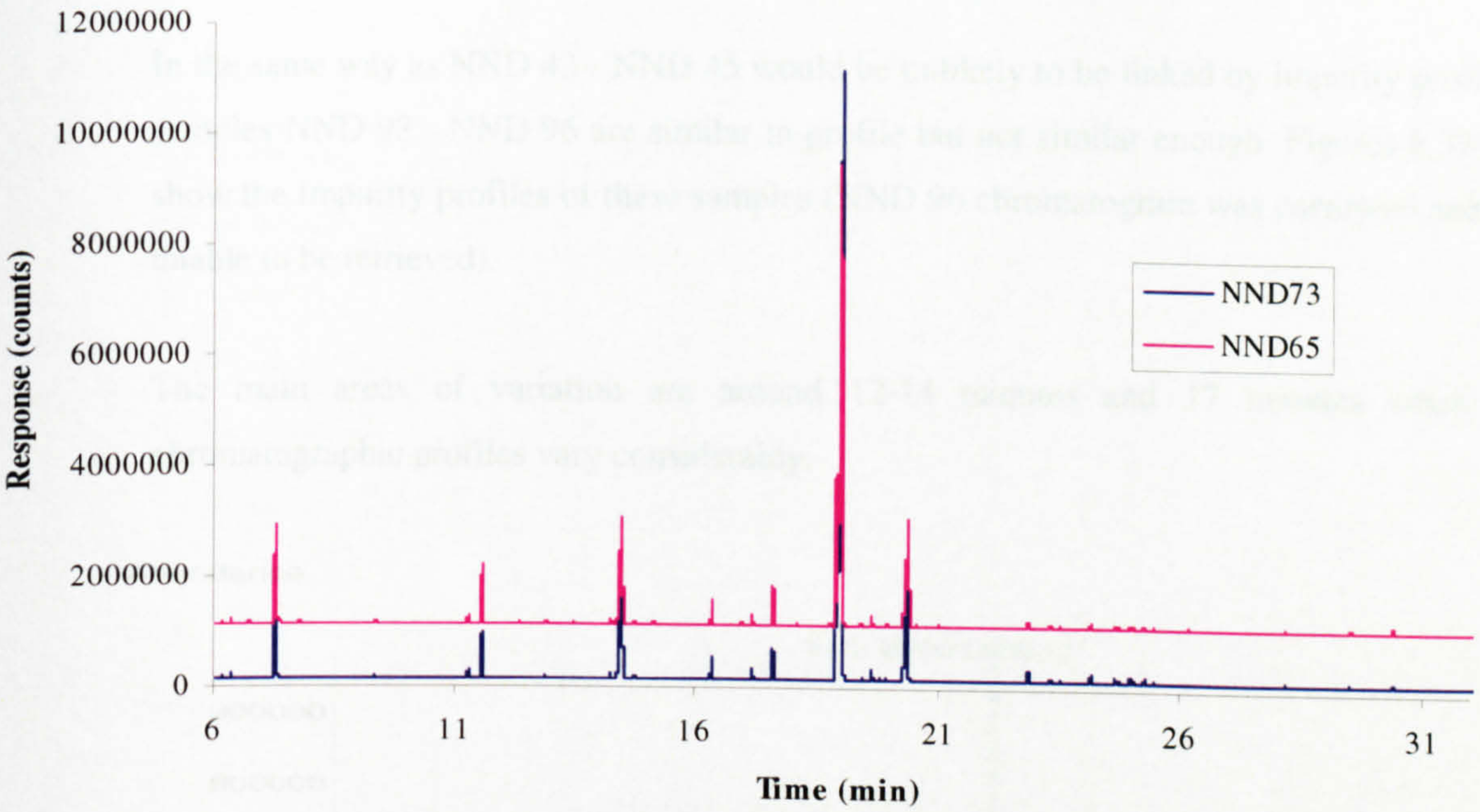


Figure 8.35 NND 65 and NND 73

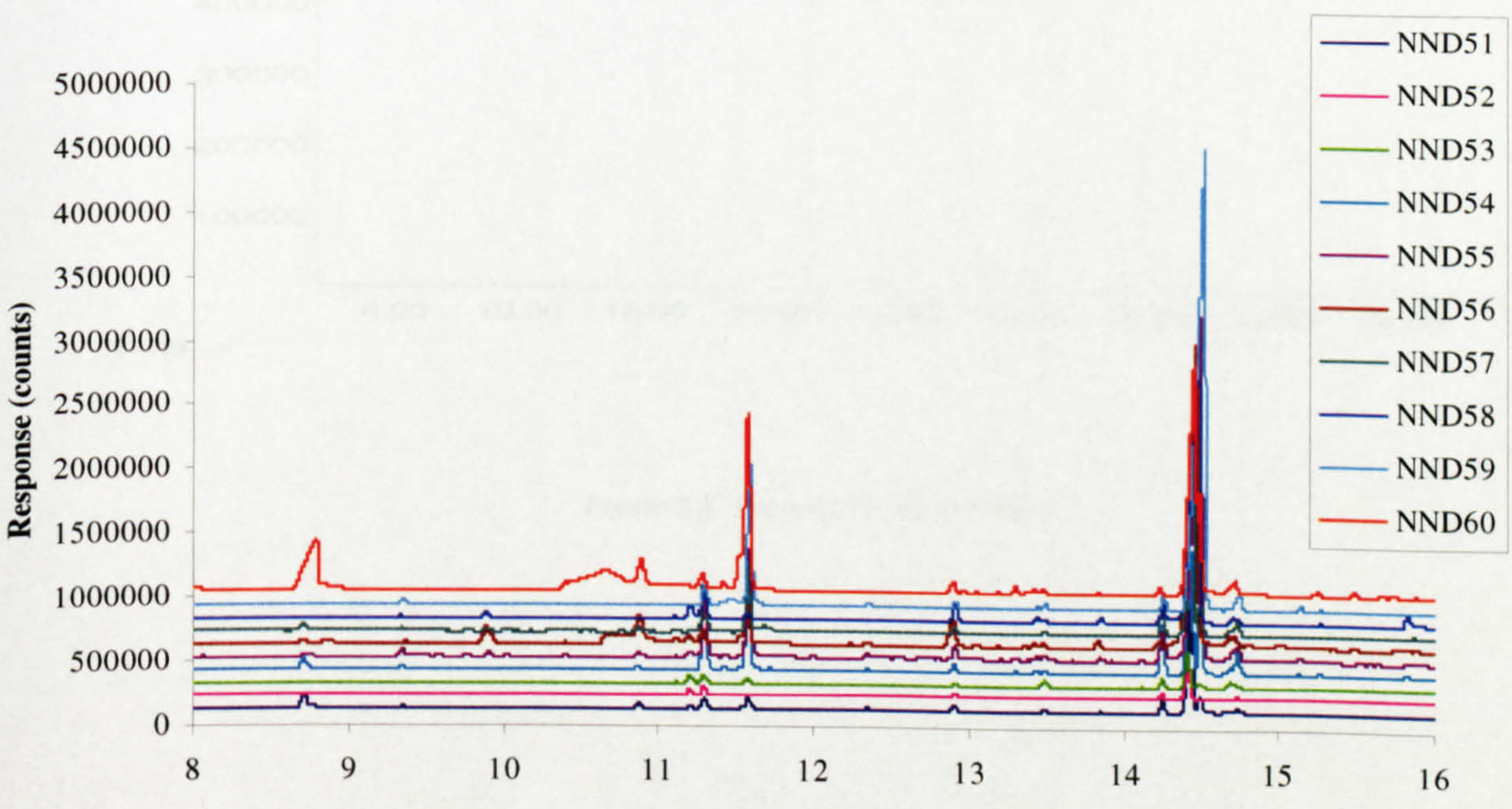


Figure 8.36 NND 51 – NND 60 (8-16 min chromatography window)

8.6.7. NND 93-NND 96

In the same way as NND 43 - NND 45 would be unlikely to be linked by impurity profiling, samples NND 93 - NND 96 are similar in profile but not similar enough. Figures 8.37-8.39 show the impurity profiles of these samples (NND 96 chromatogram was corrupted and was unable to be retrieved).

The main areas of variation are around 12-14 minutes and 17 minutes where the chromatographic profiles vary considerably.

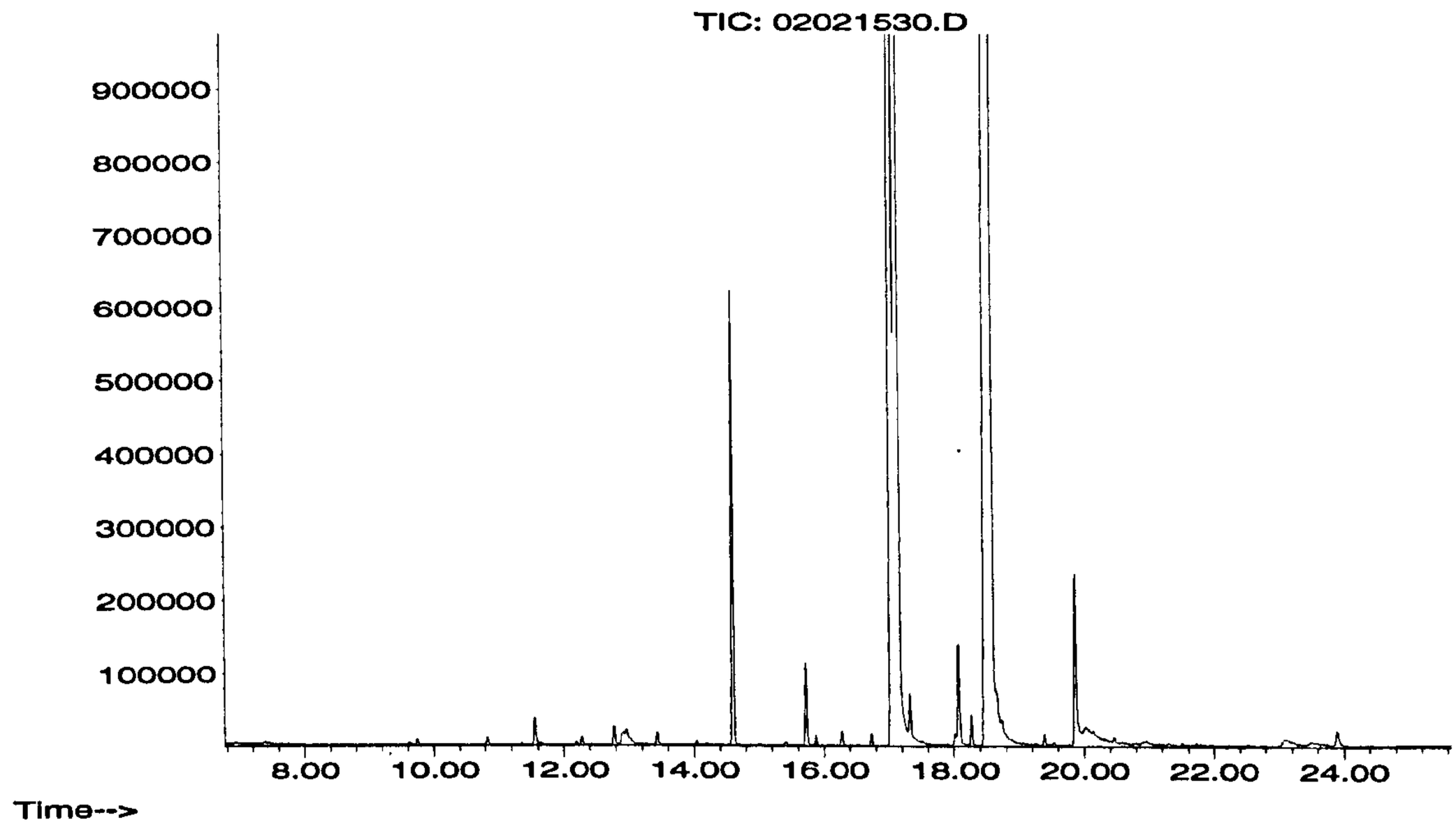


Figure 8.37 Impurity Profile of NND93

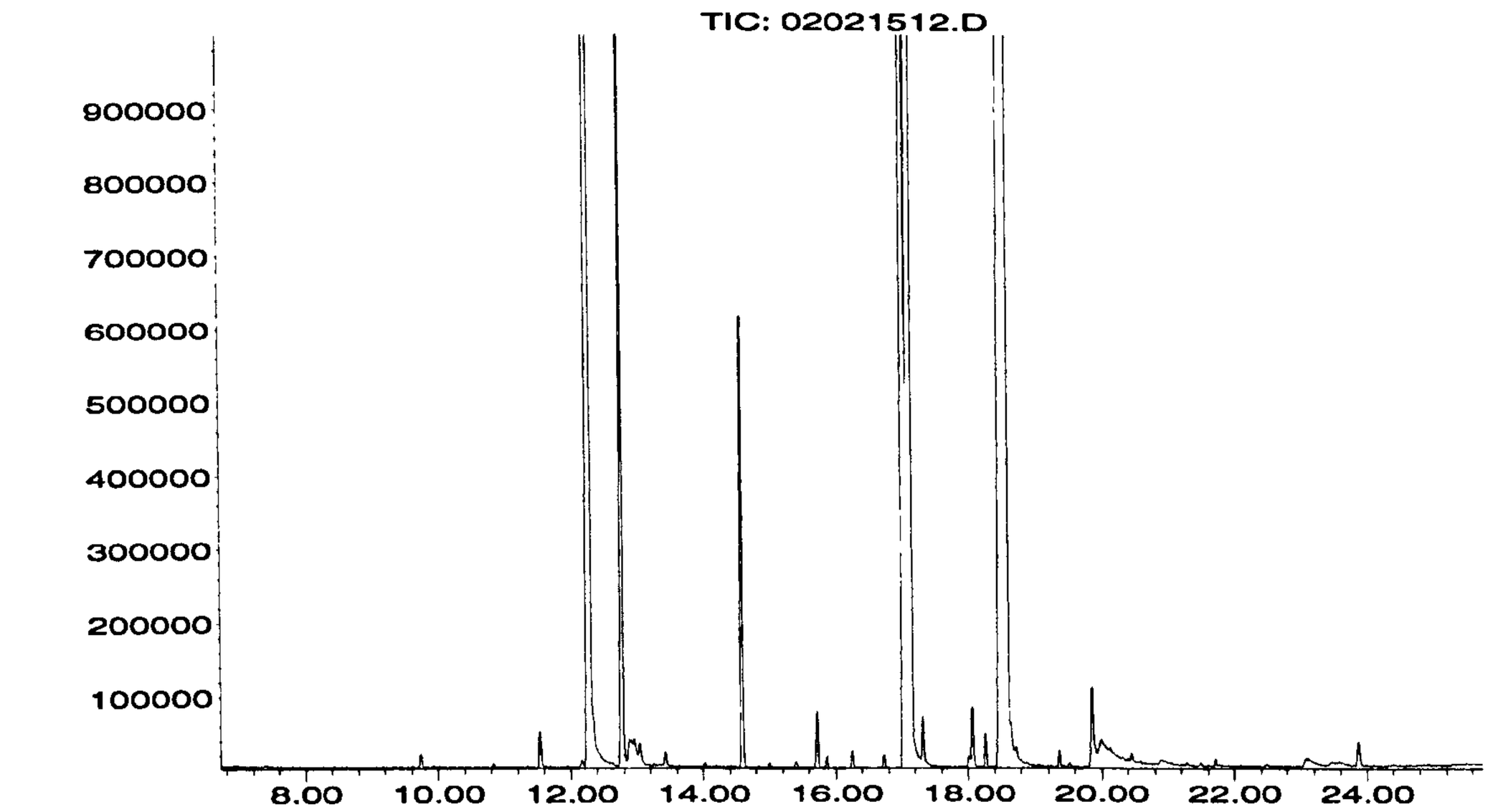


Figure 8.38 Impurity Profile of NND94

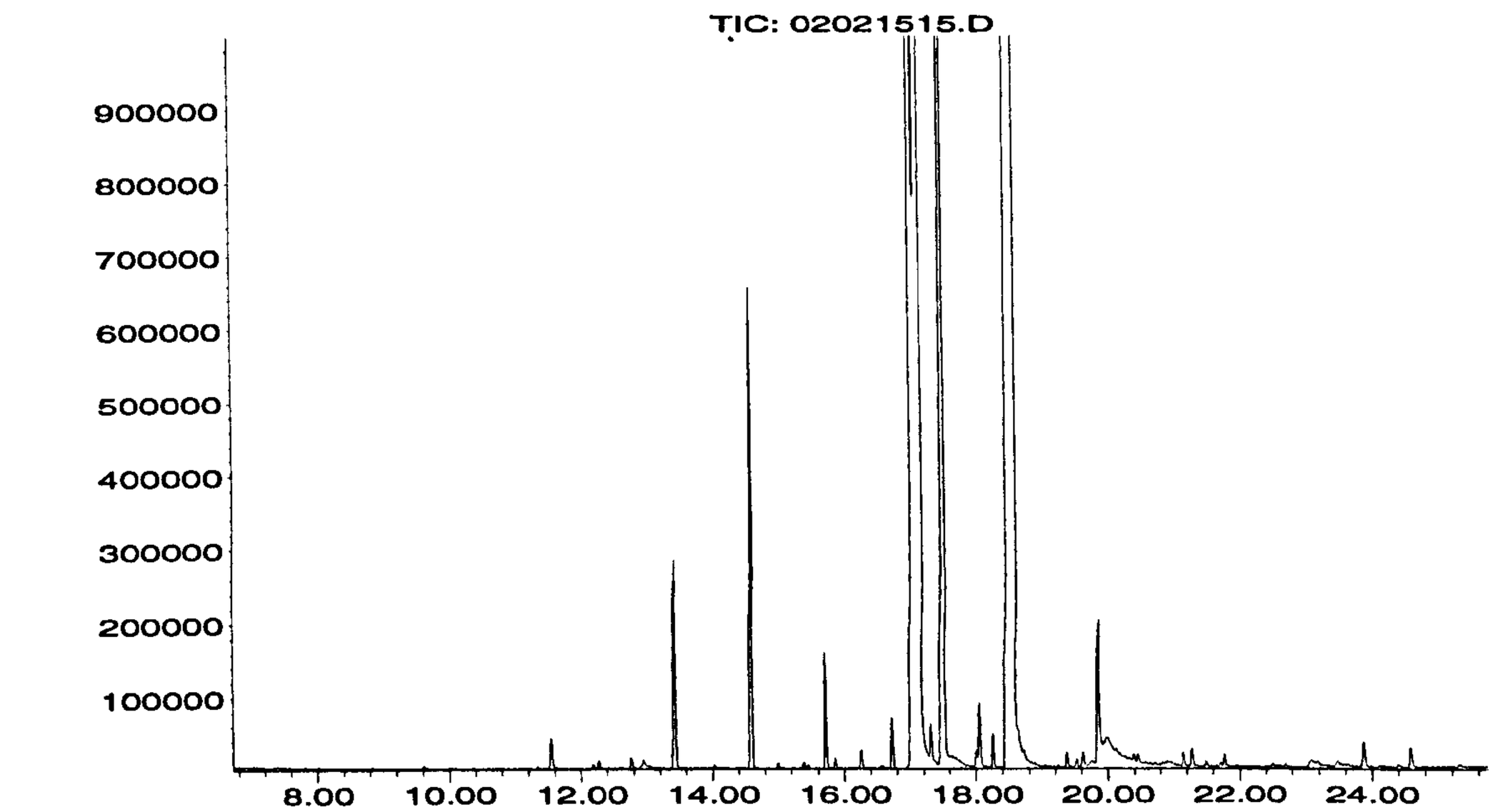


Figure 8.39 Impurity Profile of NND 95

8.6.8 NND 98 and NND 99

Visually, the impurity profiles from NND 98 and NND 99 were almost identical. Both with high amphetamine content and low levels of impurities except DPIA and caffeine, the profile was so similar that in overlaying chromatograms, the profiles superimposed almost completely. With the exception of one peak – the peak at around 14.5 min on the FID chromatograms is slightly larger in NND 99 than NND 98 (see Figure 8.40). Even with this slight difference in profile it was thought that the samples were related with over 30 peaks in common at similar relative concentrations. A link between these samples was confirmed by subsequent information received regarding the samples.

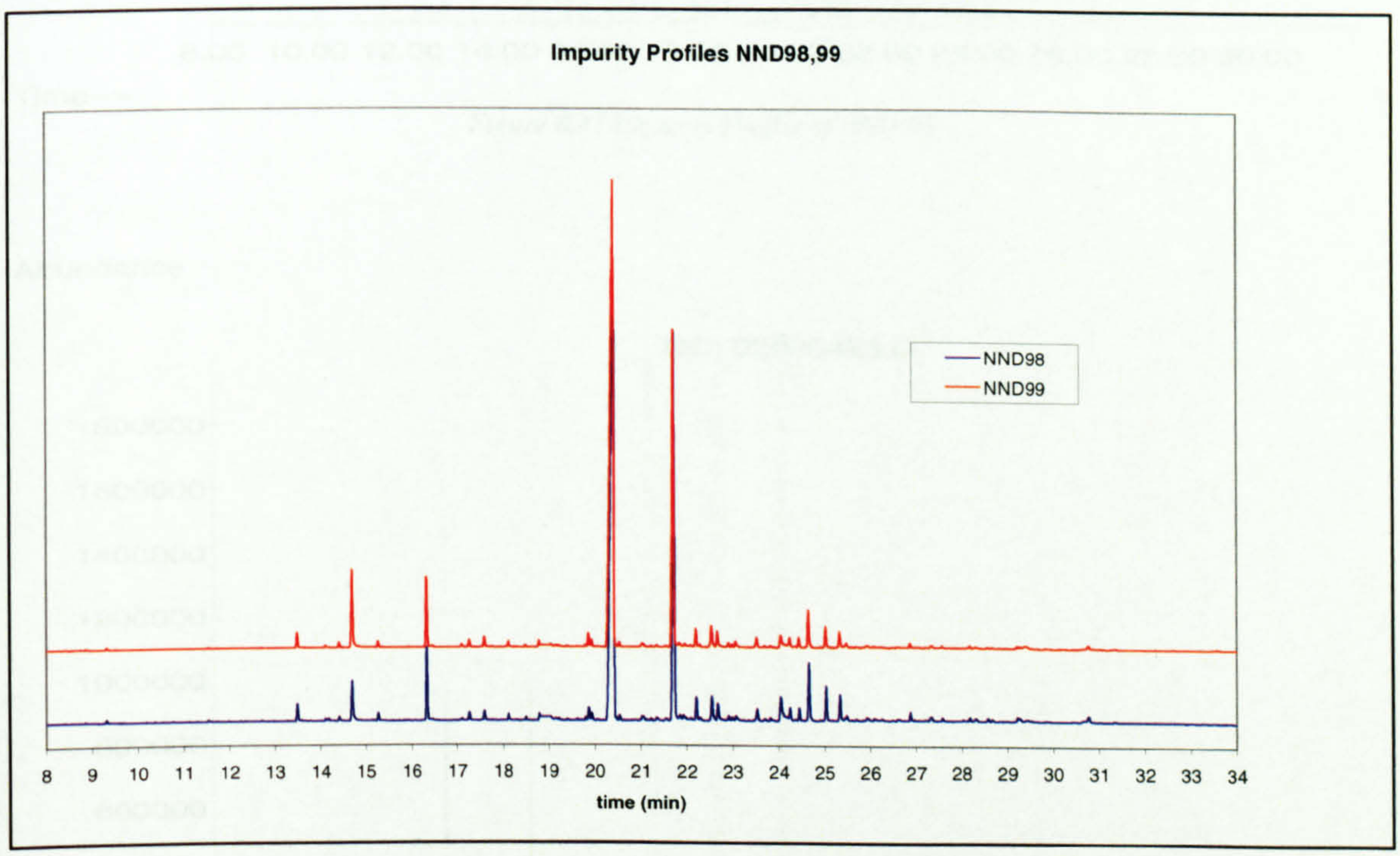


Figure 8.40 Impurity Profiles of NND 98 and 99

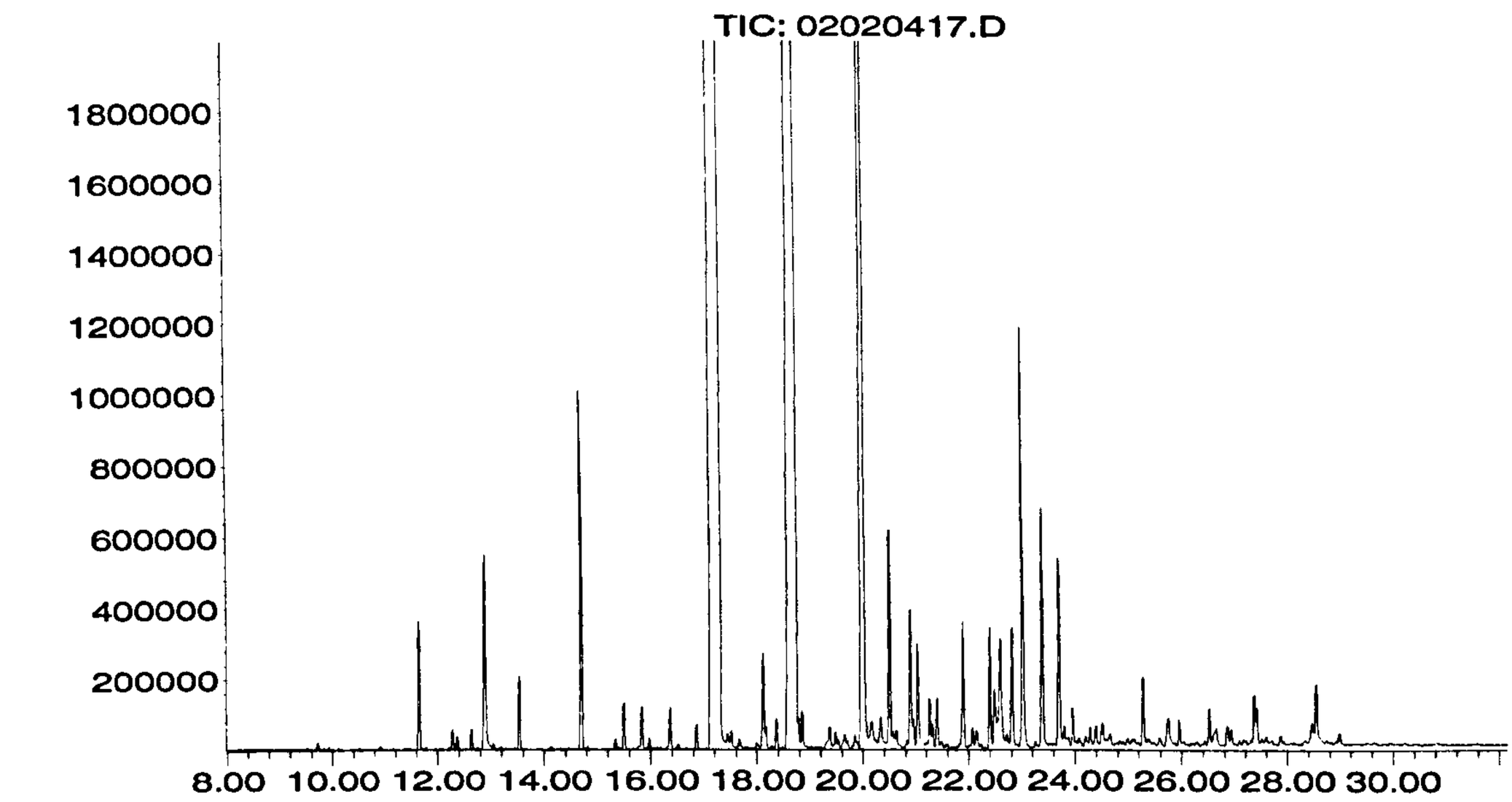


Figure 8.41 Impurity Profile of NND 98

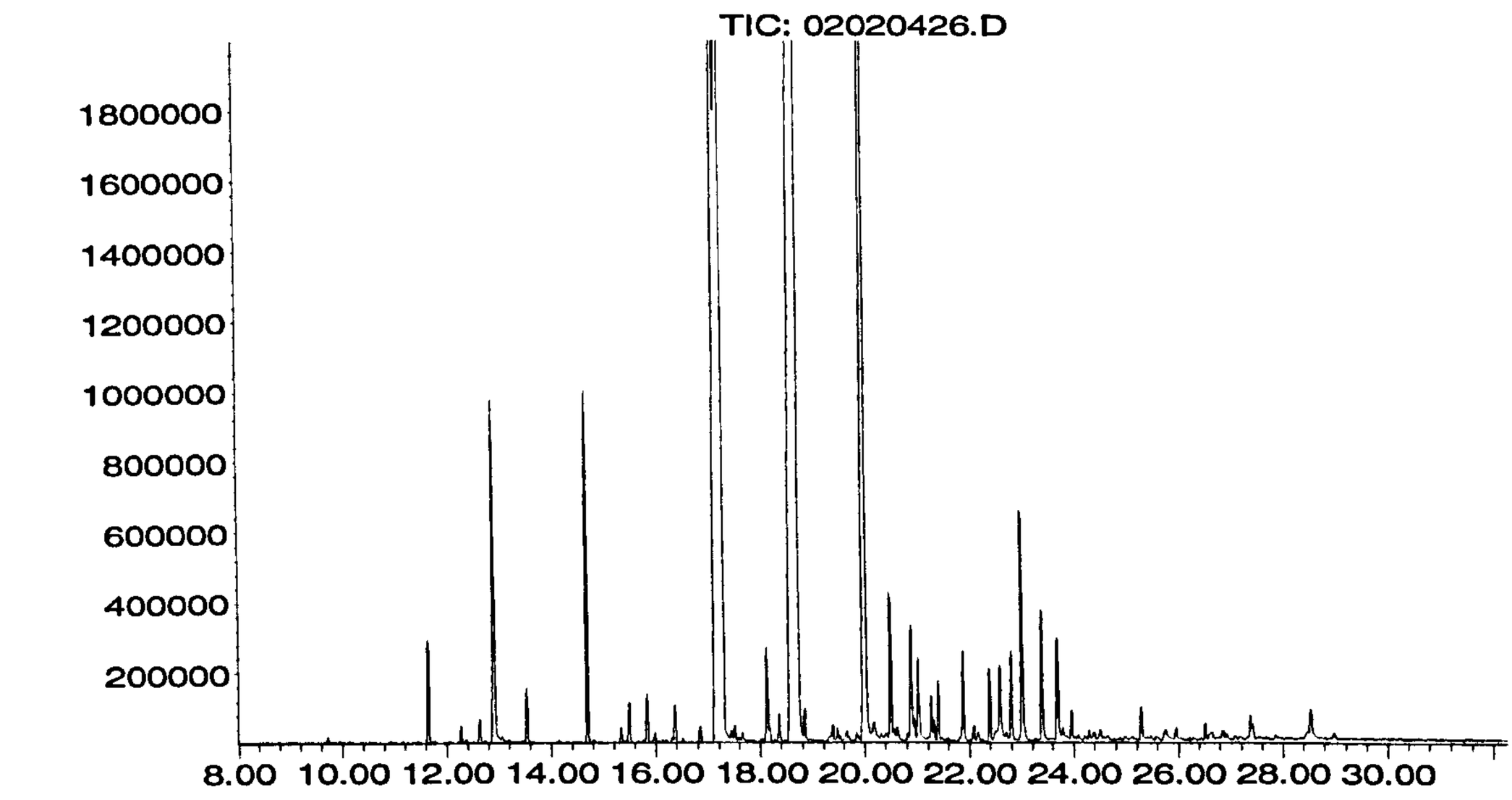


Figure 8.42 Impurity Profile of NND 99

8.6.7. NND103-110

Samples from seizures NND 103- NND 110 have similar impurities present but the relative levels of impurities within the samples individual profiles can only be matched between two sets of samples. NND 103 and NND 104 appear to be related as do NND 109 and NND 110. Both sets of samples appear to have the same set of impurities but at differing relative concentrations leading to an overall different profile. Looking at Figures 8.42 and 8.43 it may be seen that, while the lower level impurities produce a very similar pattern in all four samples, the two sub-sections can be distinguished by the levels of impurities eluting at 14.8, 20.2, 21.8 and 22.2min, which are markedly different between the sub-groups but similar within them.

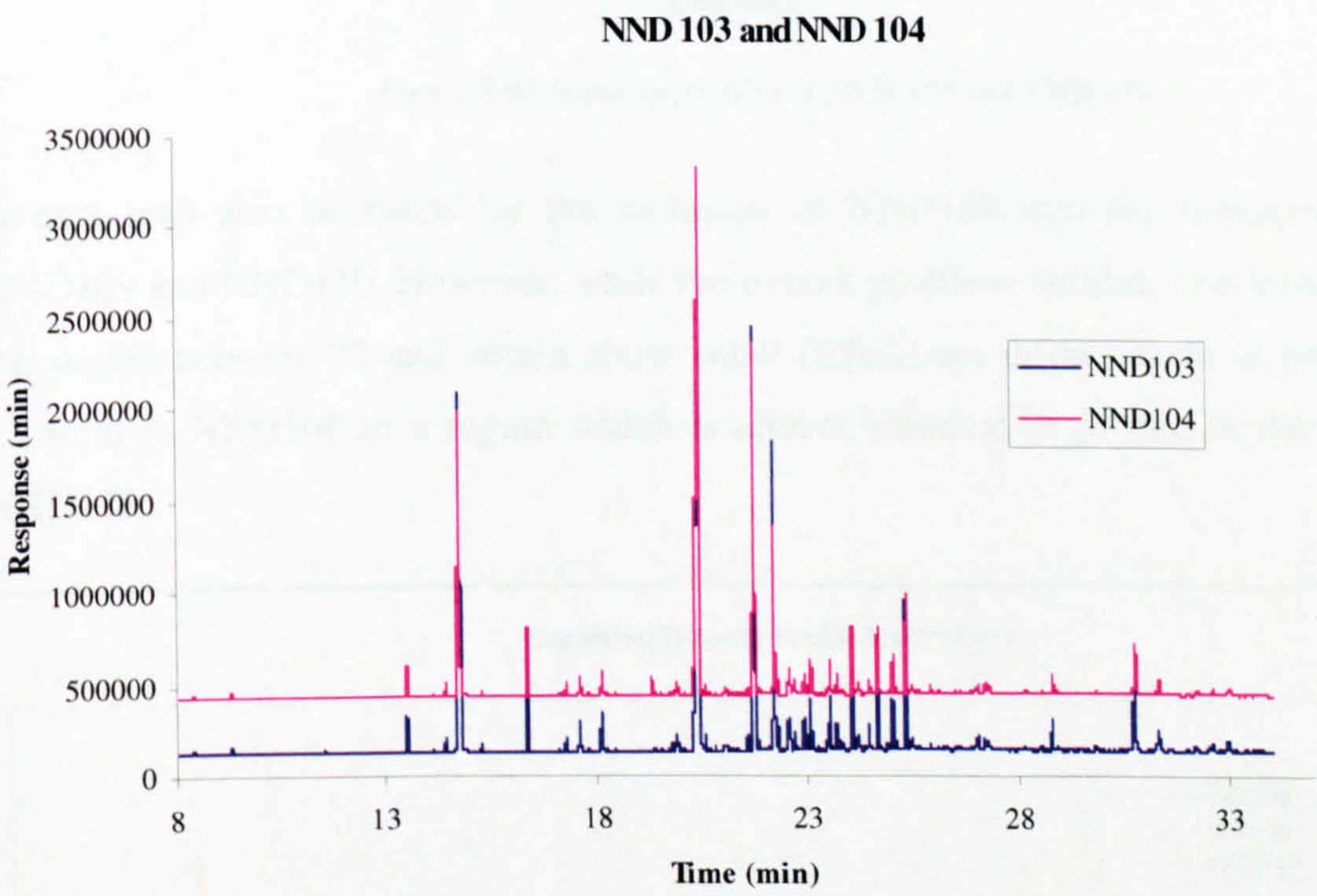


Figure 8.42 Impurity Profiles of NND 103 and NND 104

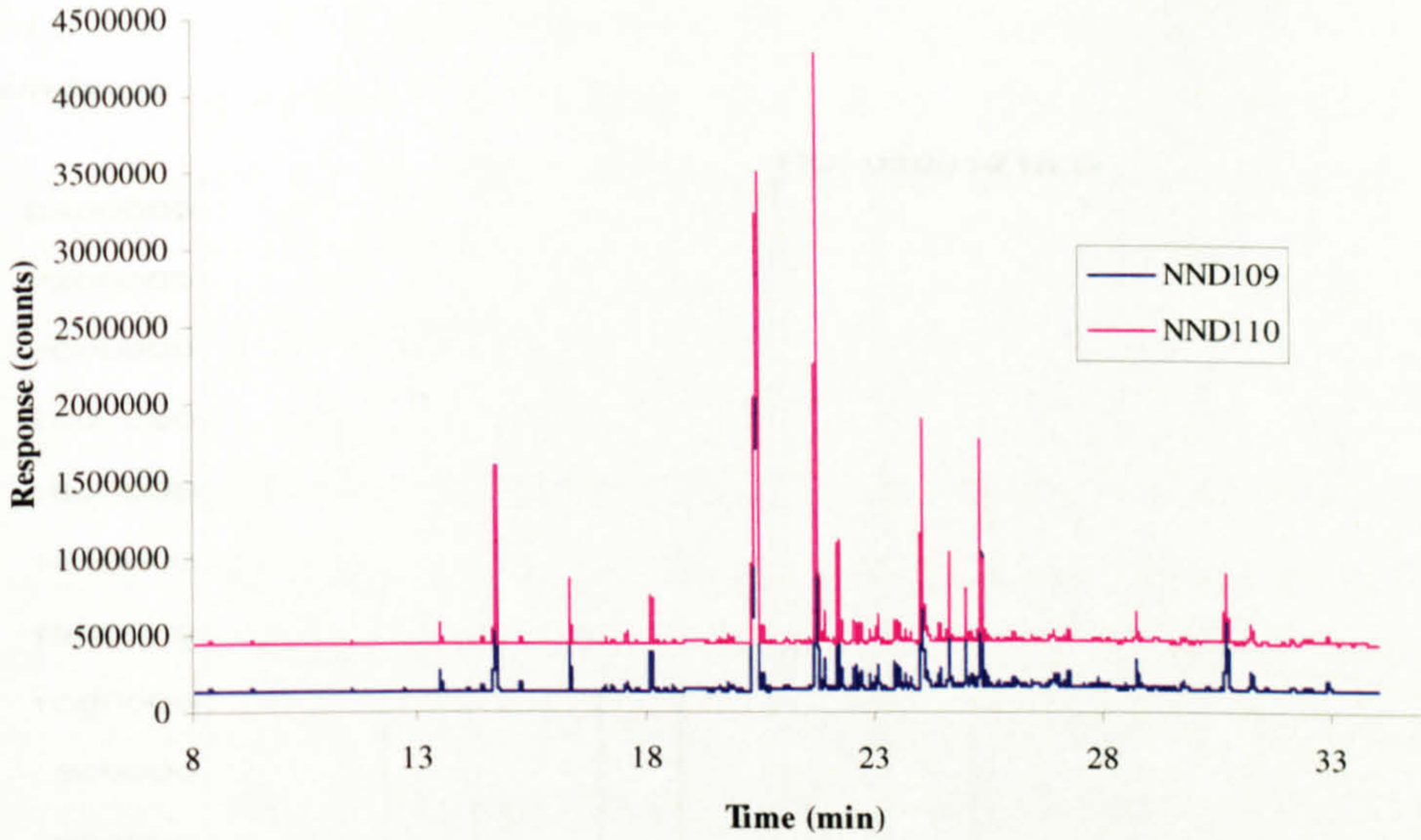


Figure 8.43 Impurity profiles of NND 109 and NND 110

A case may also be made for the inclusion of NND108 into the subsection containing NND109 and NND110. However, while the overall profile is similar, low-level impurities in the region between 22 and 26min show small differences in the levels of peaks at 23 and 23.5min in NND108 in a region which is almost identical in profile in the NND109 and NND110.

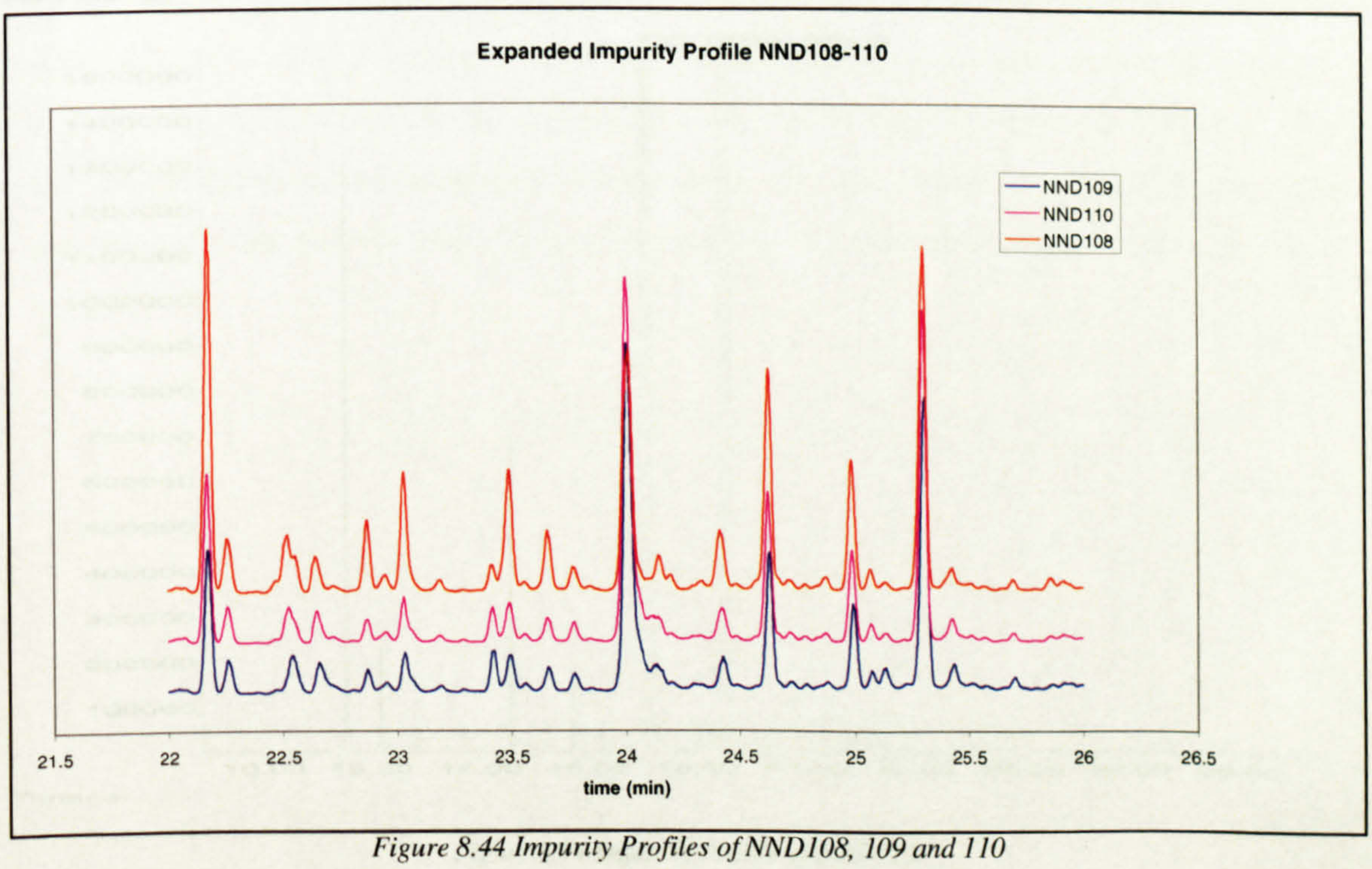


Figure 8.44 Impurity Profiles of NND108, 109 and 110

8.3.10. NND112 and NND113

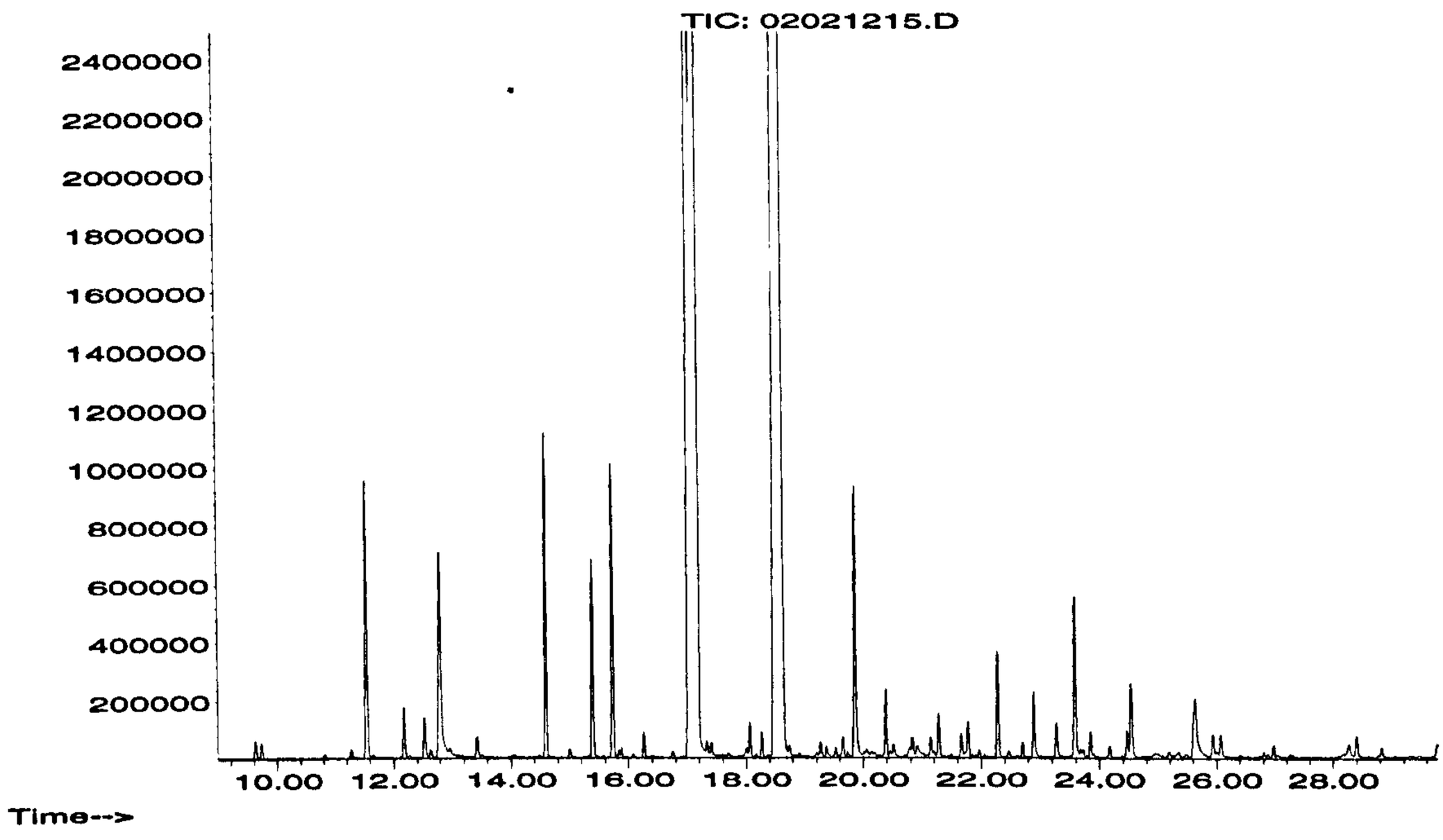


Figure 8.43 Impurity Profile of NND112

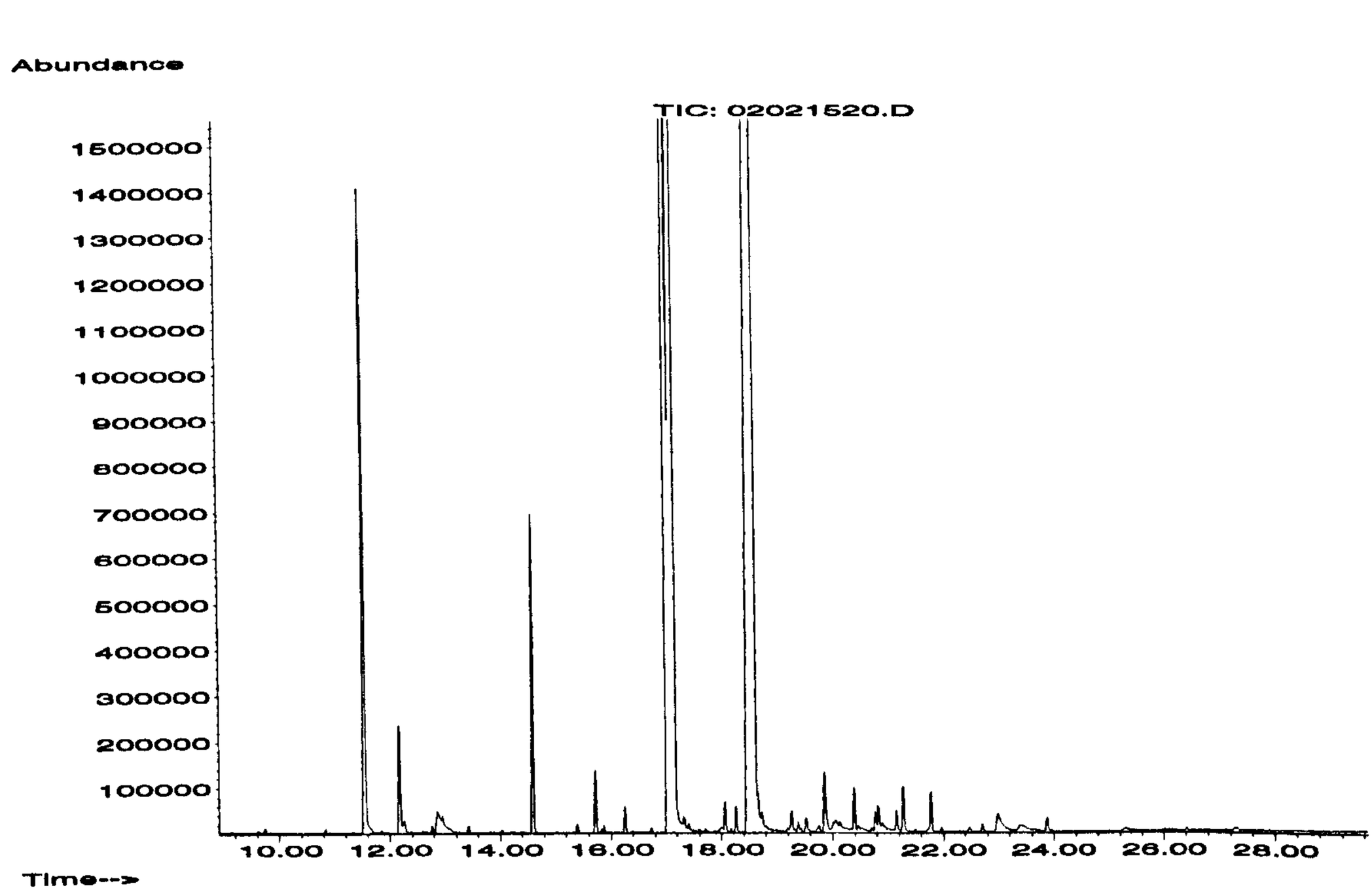


Figure 8.44 Impurity Profile of NND113

Although linked by case NND 112 and NND 113 would not be linked by impurity profiling. Both have similar levels of amphetamine, DPIA and caffeine and both contain the same impurities but at distinctly different levels in both samples. Both samples contain BMK (5.9 min), pyrimidines (11.5 and 12.2 min), N-formylamphetamine (12.8 min), benzaldiimine (15.7 min), DPIA (17.1 min), caffeine (18.5 min) and pyridines (21-23 min).

8.6.11. NND 119 - NND 125

The samples labelled NND 119 to NND 125 were similar in appearance although, having previously been removed from their original packing, a link between the individual samples could not be established visually. All samples were white crystalline powders and had no other distinguishing features. However, on extraction and analysis, the impurity profiles of the samples NND 119 through NND 125 showed many similarities. The FID profiles shown below in Figure 8.45 have had the largest impurity peaks (DPIA and amphetamine) peaks removed to enable the lower level impurities to be seen clearly. It is apparent that, while the amphetamine content may differ between seizures, the profiles are very similar and that samples NND 119 to NND 125 may be linked to a single source. In fact, around 30 impurity peaks in each chromatogram are present which may be used for profiling purposes.

Figure 8.46 shows the profile for NND 119 and NND 125 with amphetamine peak and the larger impurity peaks present. Here, although the percentage amphetamine content of each separate sample may be nominally different, the overall profile remains comparable. This is a good indication that, while the amphetamine content of batches might be indicative of a link where samples are thoroughly homogenised, in cases where the samples are very roughly cut with diluents impurity profiles may be a better sign of association between samples.

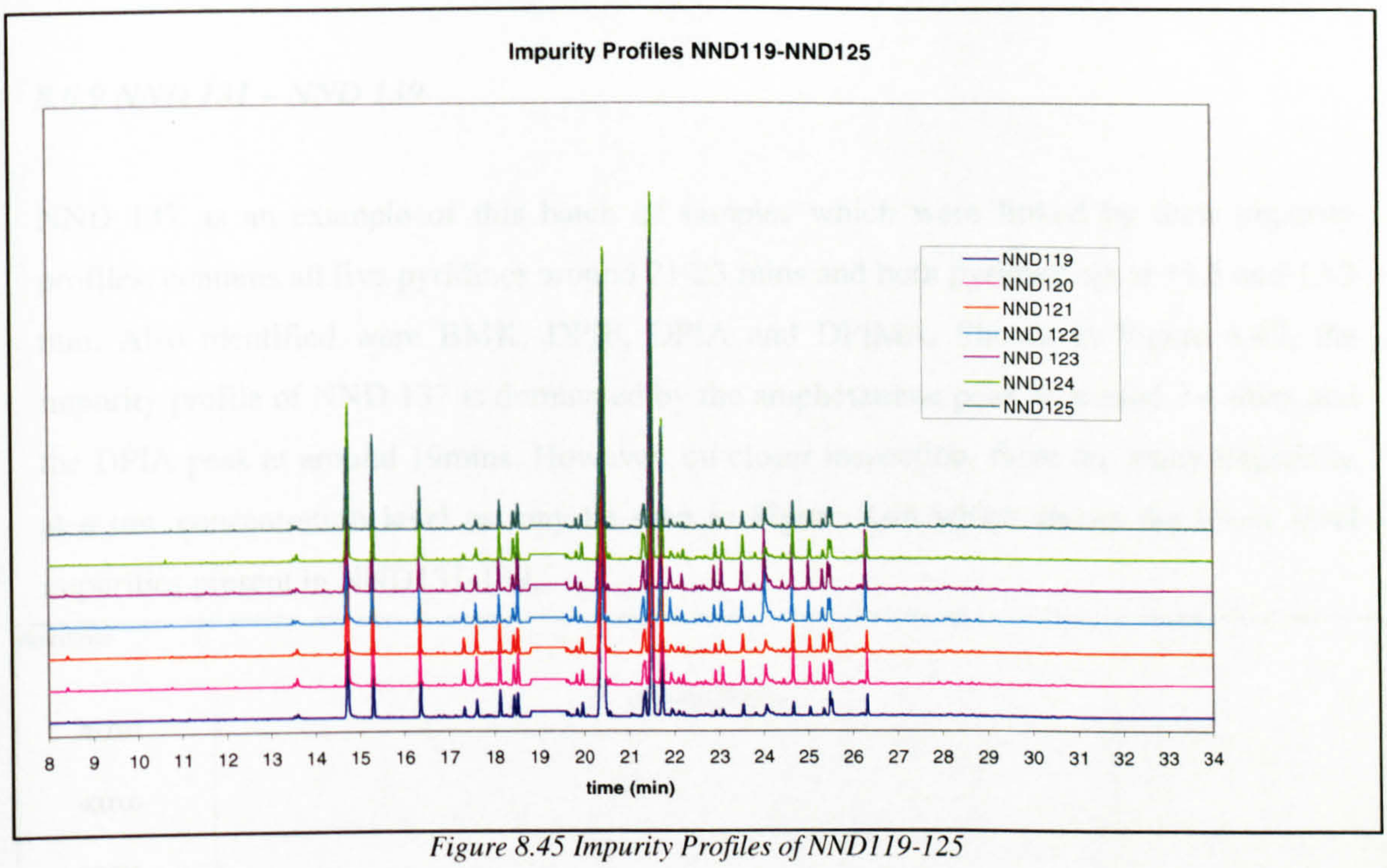


Figure 8.45 Impurity Profiles of NND119-125

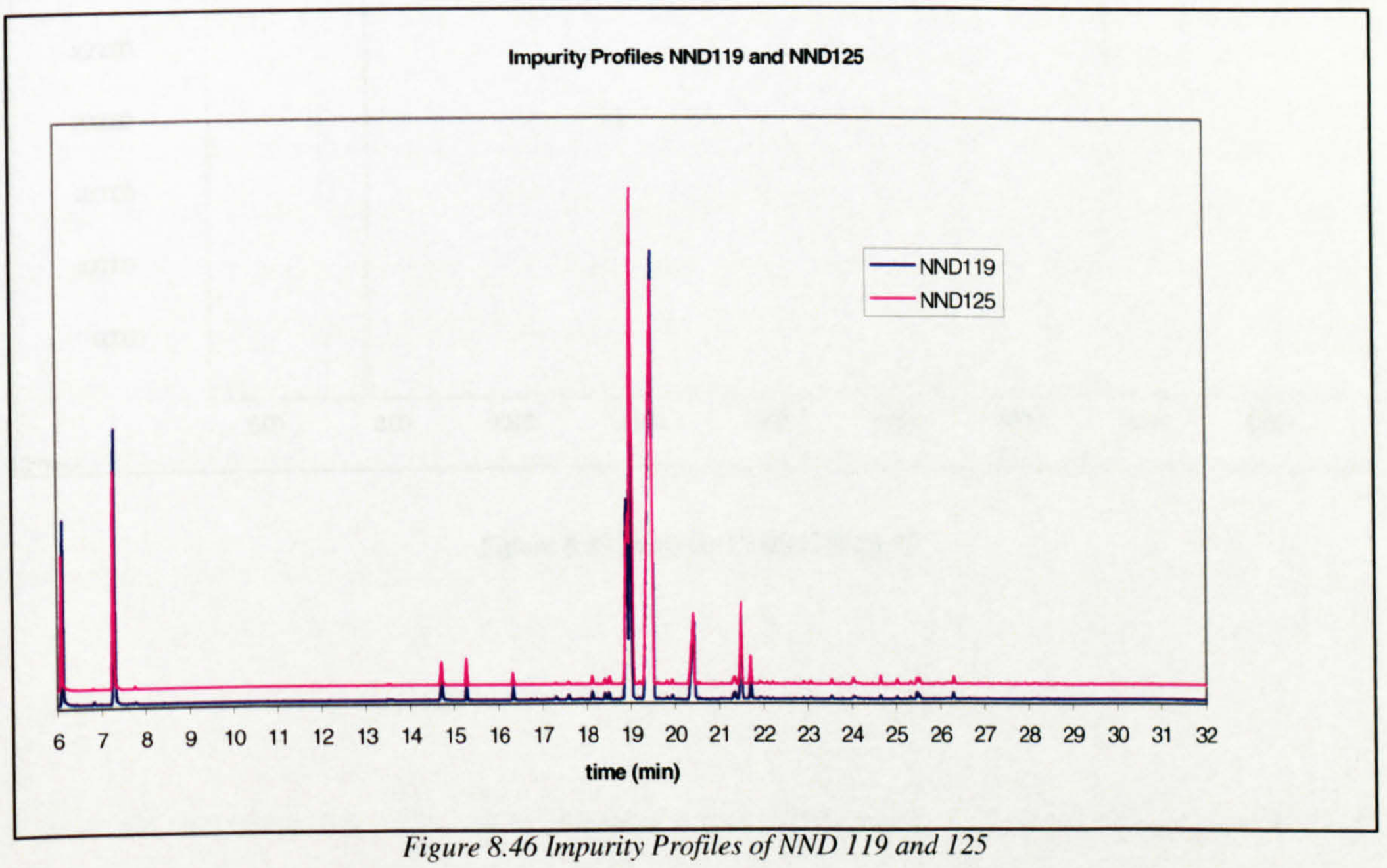


Figure 8.46 Impurity Profiles of NND 119 and 125

8.6.9 NND 131 – NND 139

NND 137 as an example of this batch of samples which were linked by their impurity profiles, contains all five pyridines around 21-23 mins and both pyrimidines at 11.6 and 12.3 min. Also identified were BMK, DPIF, DPIA and DPIMA. Shown in Figure 8.47, the impurity profile of NND 137 is dominated by the amphetamine peak at around 7.4 mins and the DPIA peak at around 19mins. However, on closer inspection, there are many impurities at a low concentration level as may be seen in Figure 8.48 which shows the lower level impurities present in NND131-139.

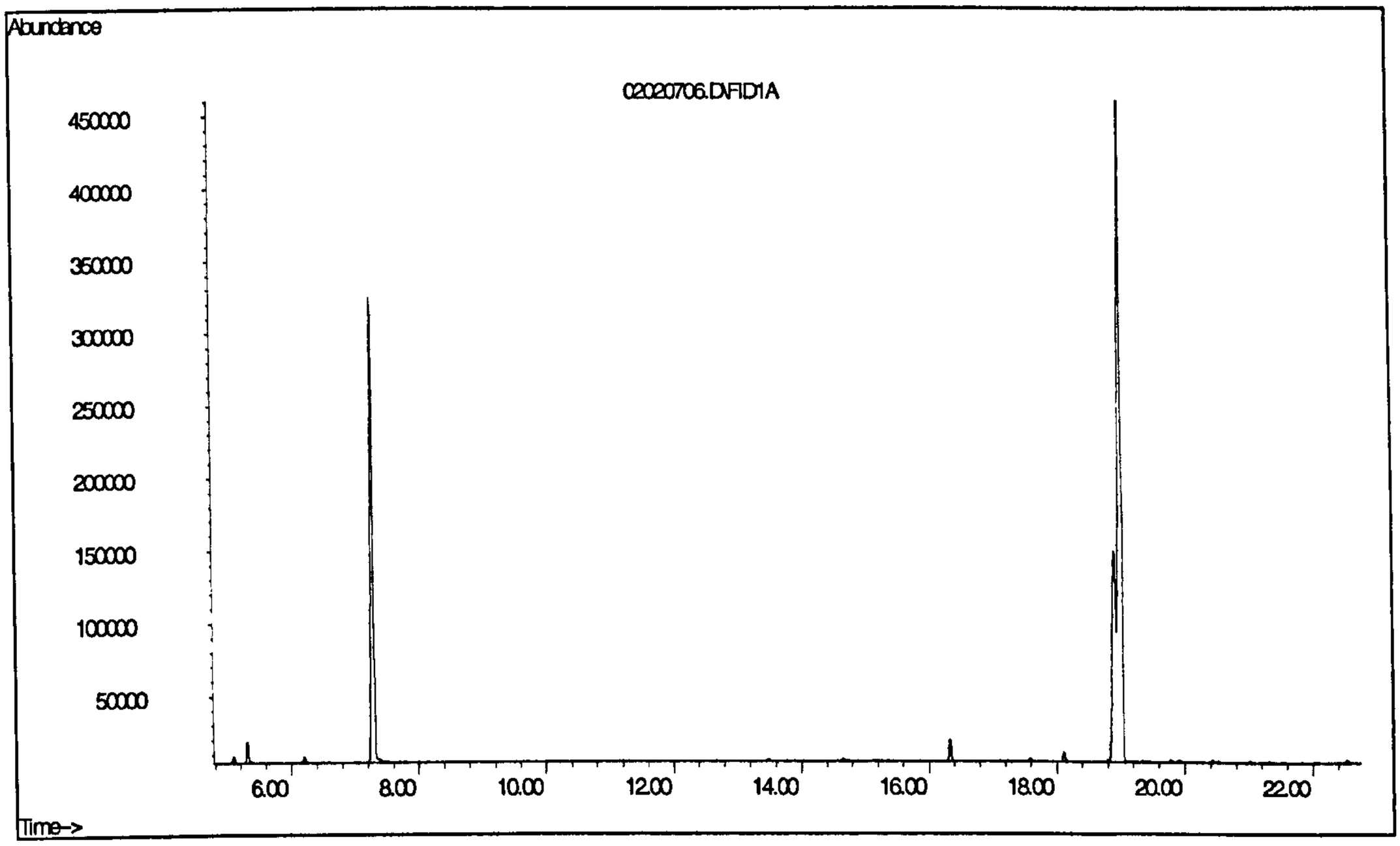


Figure 8.49 Impurity Profile NND137

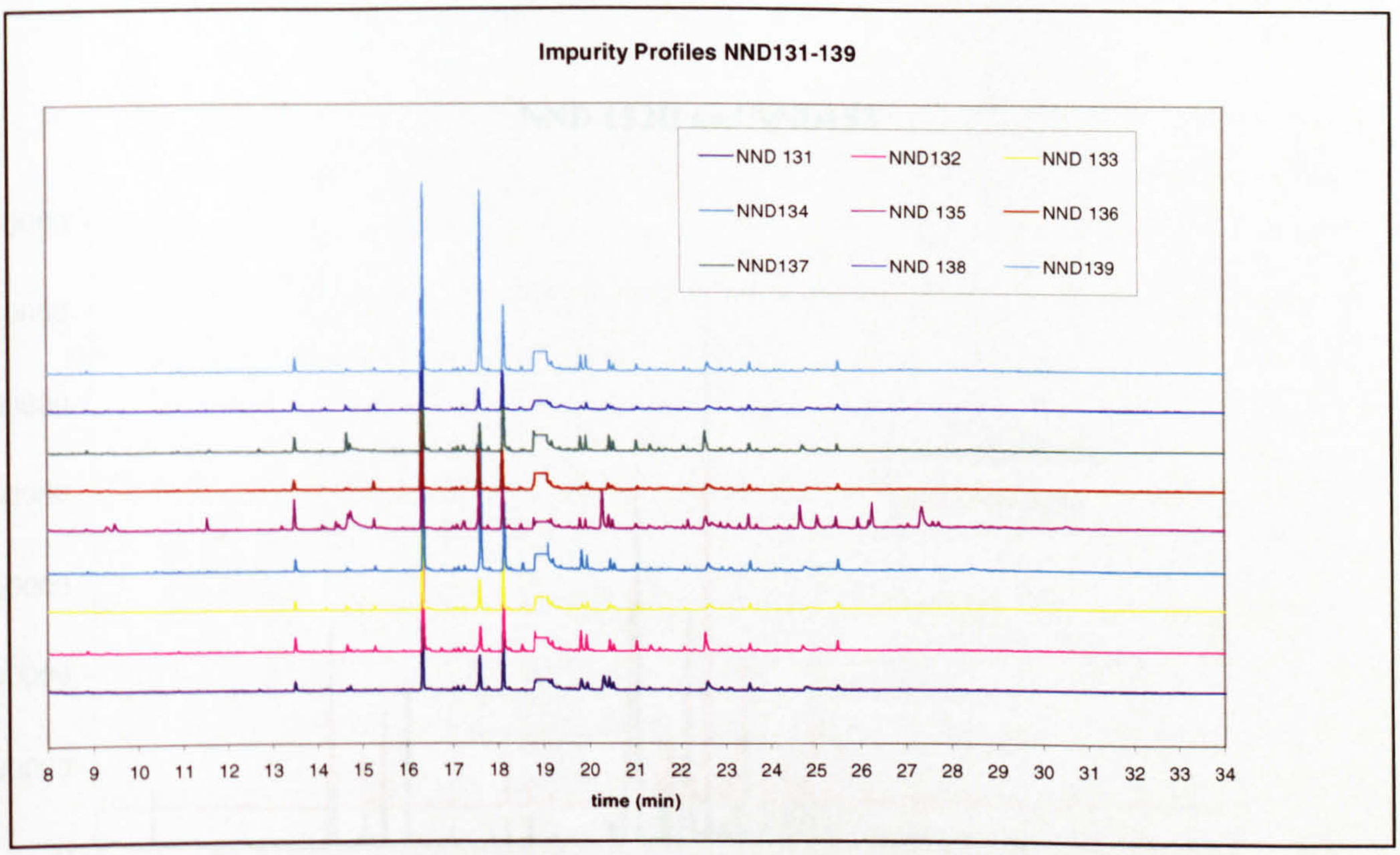


Figure 8.48 Impurity Profiles of NND131-139

8.6.10. NND 150 – NND 153

From the data received after extraction and analysis with regard to which samples and seizures are related, samples NND 150 through NND 153 are grouped together by case. However, two distinct subsections are clear when the impurity profiles are examined. Samples NND 150 and NND152 A appear to be linked. Samples NND152 B and NND 153 seem to be related and sample NND 151 bears no relation to either subsection and may be regarded as separate.

Samples NND 152 and NND 153 have a high concentration of active drug, around 8 times as much as samples NND 150 and NND 152A. Sample NND 151 has an extremely low level of active drug but relatively high impurity content.

The profiles in Figure 8.49 show the impurity levels of NND 152B and NND 153 with the amphetamine peak and the largest impurity peak (DPIA) having been removed. With over 30 peaks present in common in both profiles there is good ground for establishing a relationship between the samples. In NND152 B, amphetamine, BMK, all pyridines, benzaldiimine, DPIA, both pyrimidines and caffeine have been identified

NND 152B and NND153

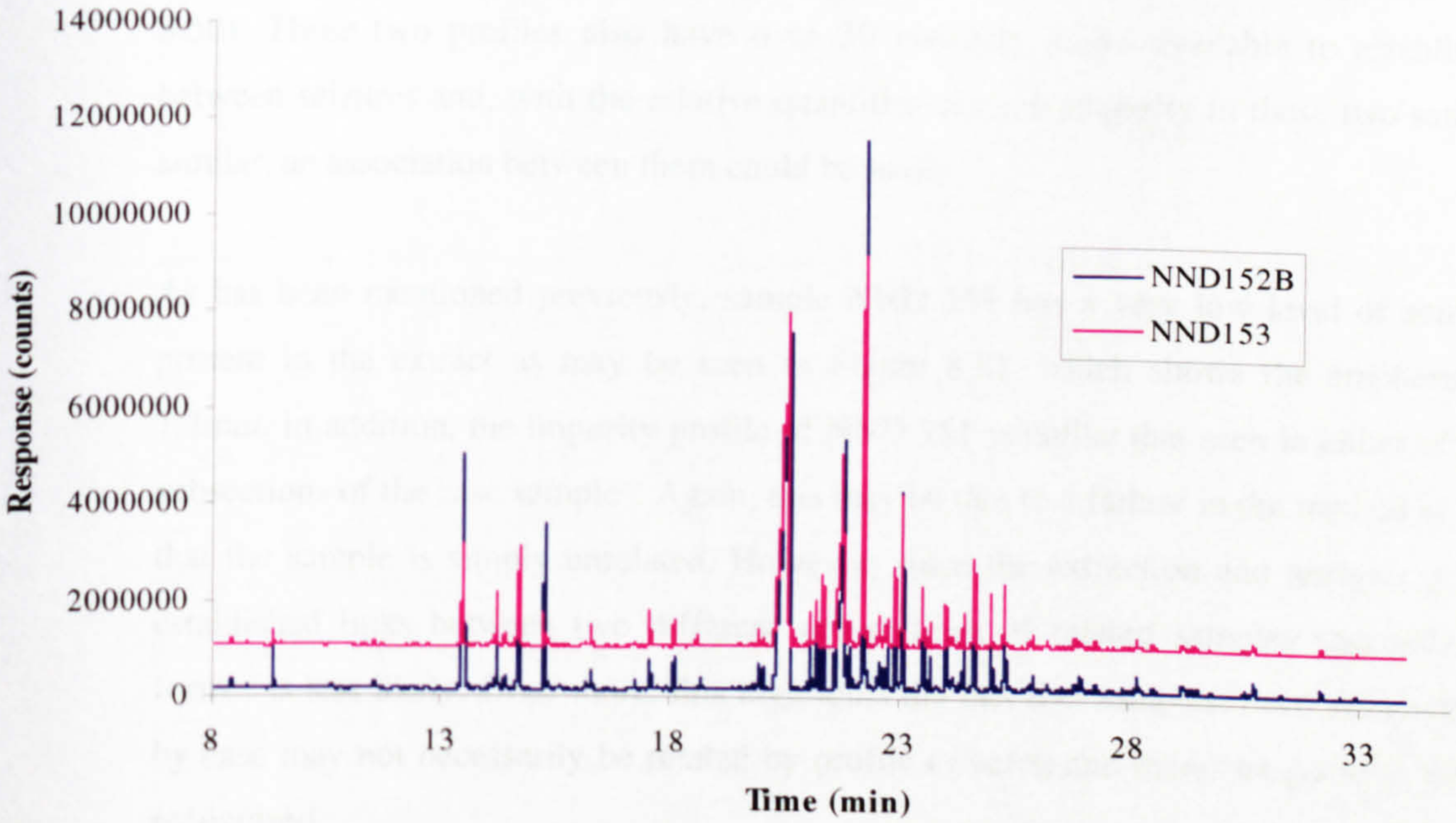


Figure 8.49 Impurity Profiles of NND 152B and 153

NND150 and NND152A

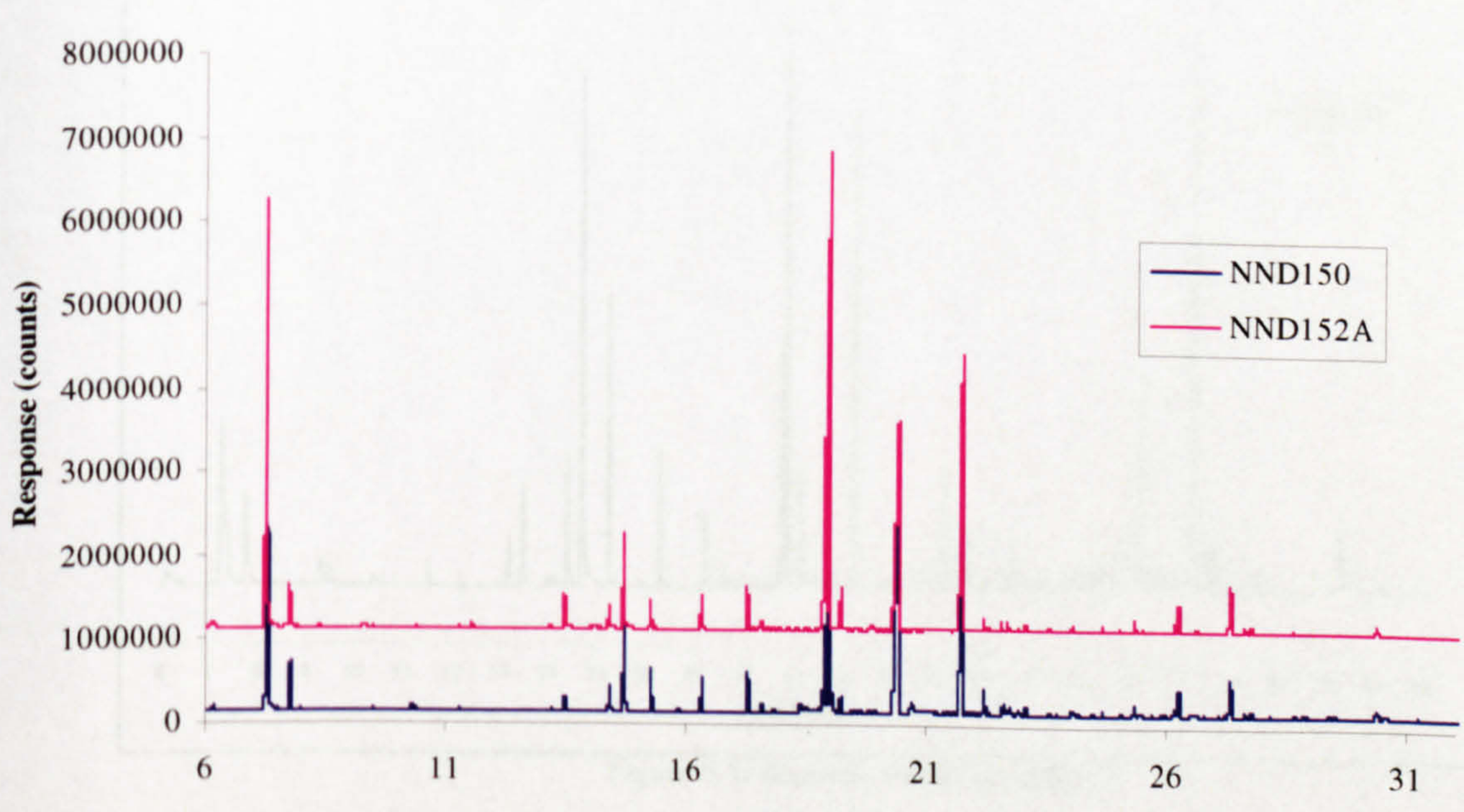


Figure 8.50 Impurity Profiles of NND 150 and 152A

Again having removed the amphetamine peak to enlarge the view of low level impurities, the resultant chromatograms of NND 150 and NND 152A show very similar profiles (Figure 8.50). These two profiles also have over 30 common peaks available to establish links between seizures and, with the relative quantities of each impurity in these two samples so similar, an association between them could be made.

As has been mentioned previously, sample NND 151 has a very low level of active drug present in the extract as may be seen in Figure 8.51, which shows the amphetamine at 7.3min. In addition, the impurity profile of NND 151 is unlike that seen in either of the two subsections of the case samples. Again, this may be due to a failure in the method or the fact that the sample is simply unrelated. However, since the extraction and analysis procedure established links between two different sub-sections of related samples successfully, the former is less likely. Once more, this highlights the fact that samples, even although related by case may not necessarily be related by profile or batch and therefore possible links may recognised.

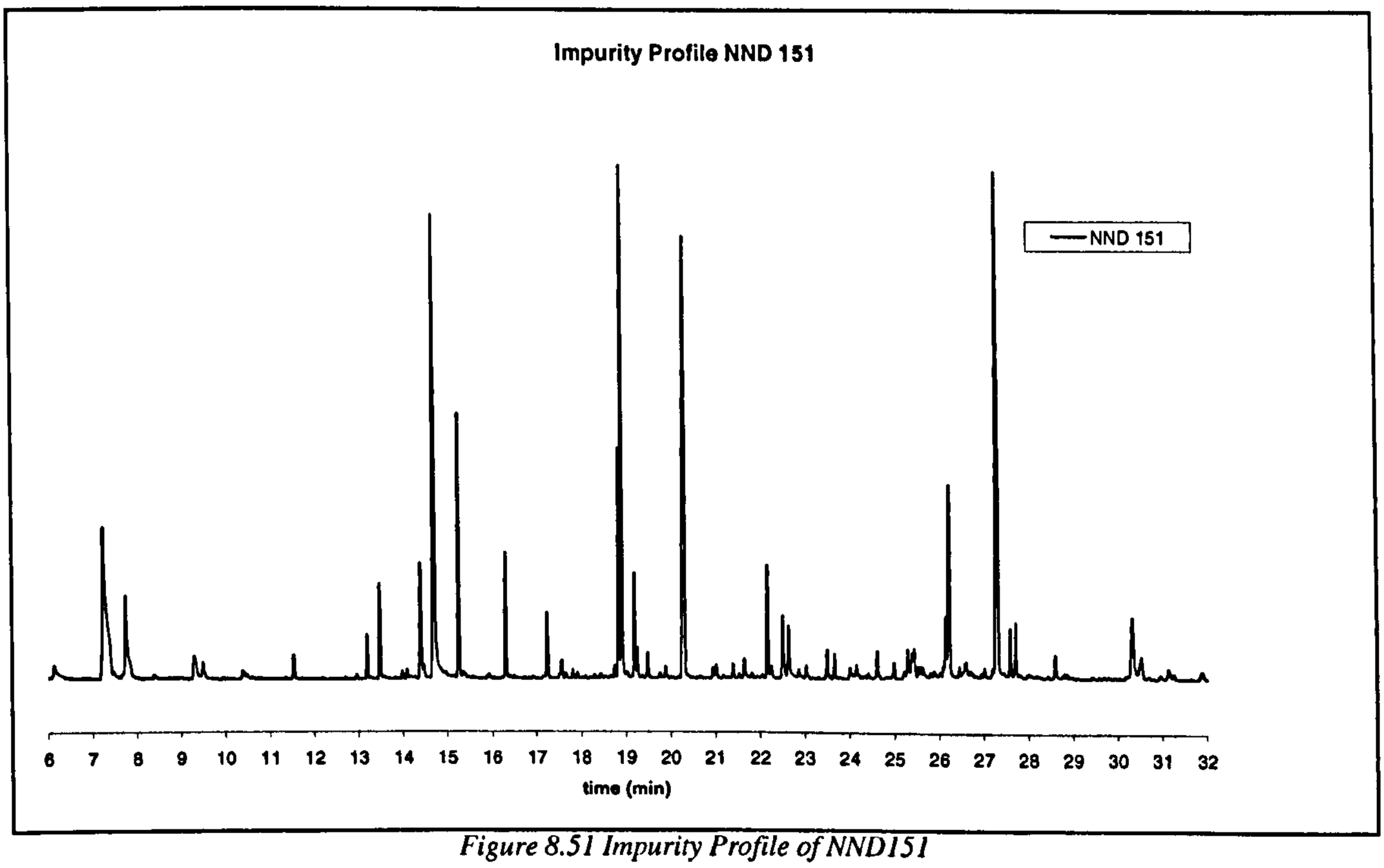


Figure 8.51 Impurity Profile of NND151

8.6.14 NND 155 and NND 156

Although linked by case, samples NNND 155 and NND 156 would not be linked by impurity profiling. With different relative levels of amphetamine and DPIA and different low level impurity profiles around 20-25 min, a link could not be established.

However, as a test, the ion chromatogram of ion 259 was extracted to view only the pyridines present in the sample. In looking only at these impurities, the profile for both was very similar (Figures 8.54 and 8.55) although the actual responses were different – this may simply be because one sample contained more amphetamine than the other. It was thought that this could be a way of distinguishing selected impurities from the main chromatogram to make comparison easier. This was never fully explored due to time constraints but these very limited tests show how samples that do not appear related on the surface may have very similar selected impurity patterns.

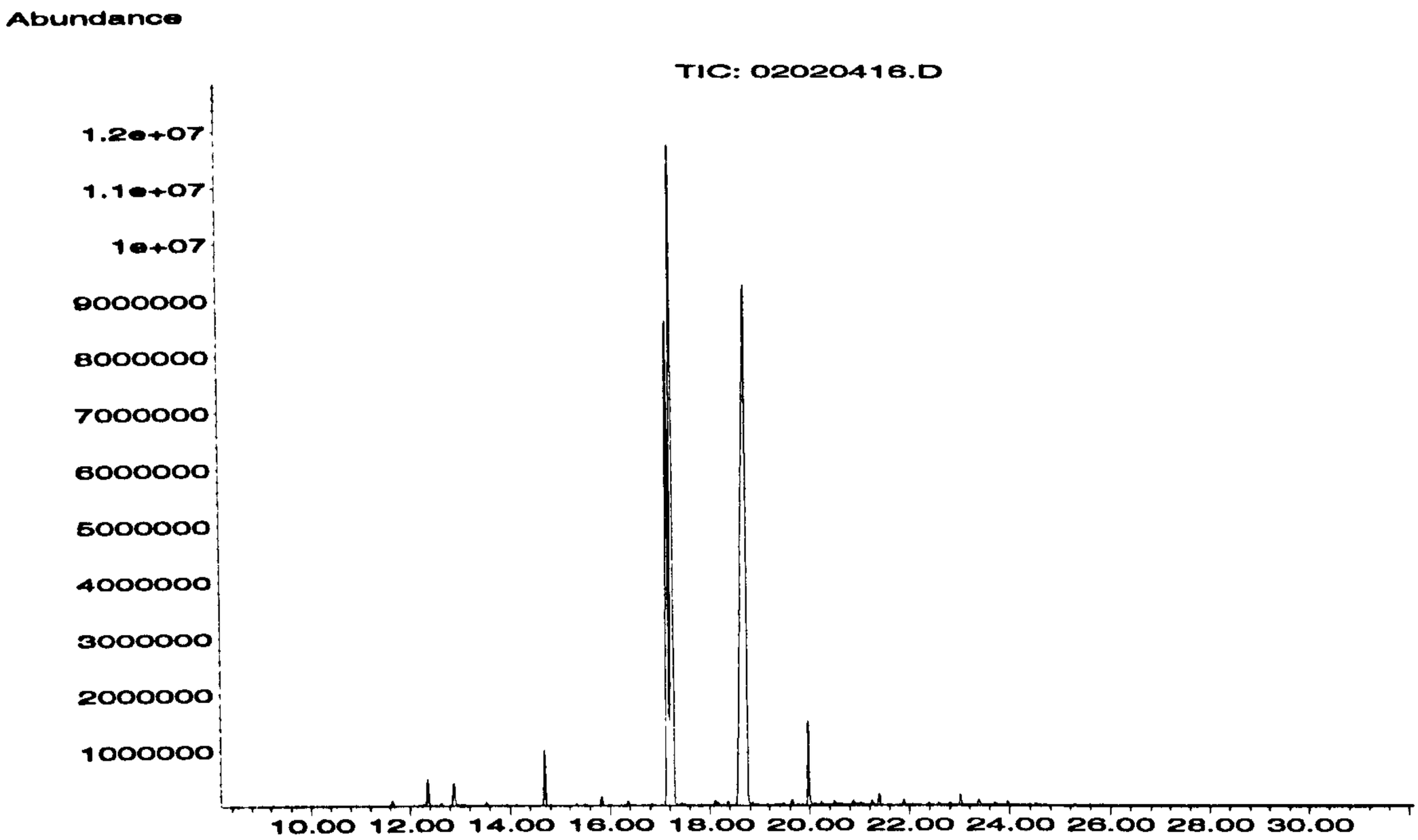


Figure 8.52 Impurity Profile of NND 155

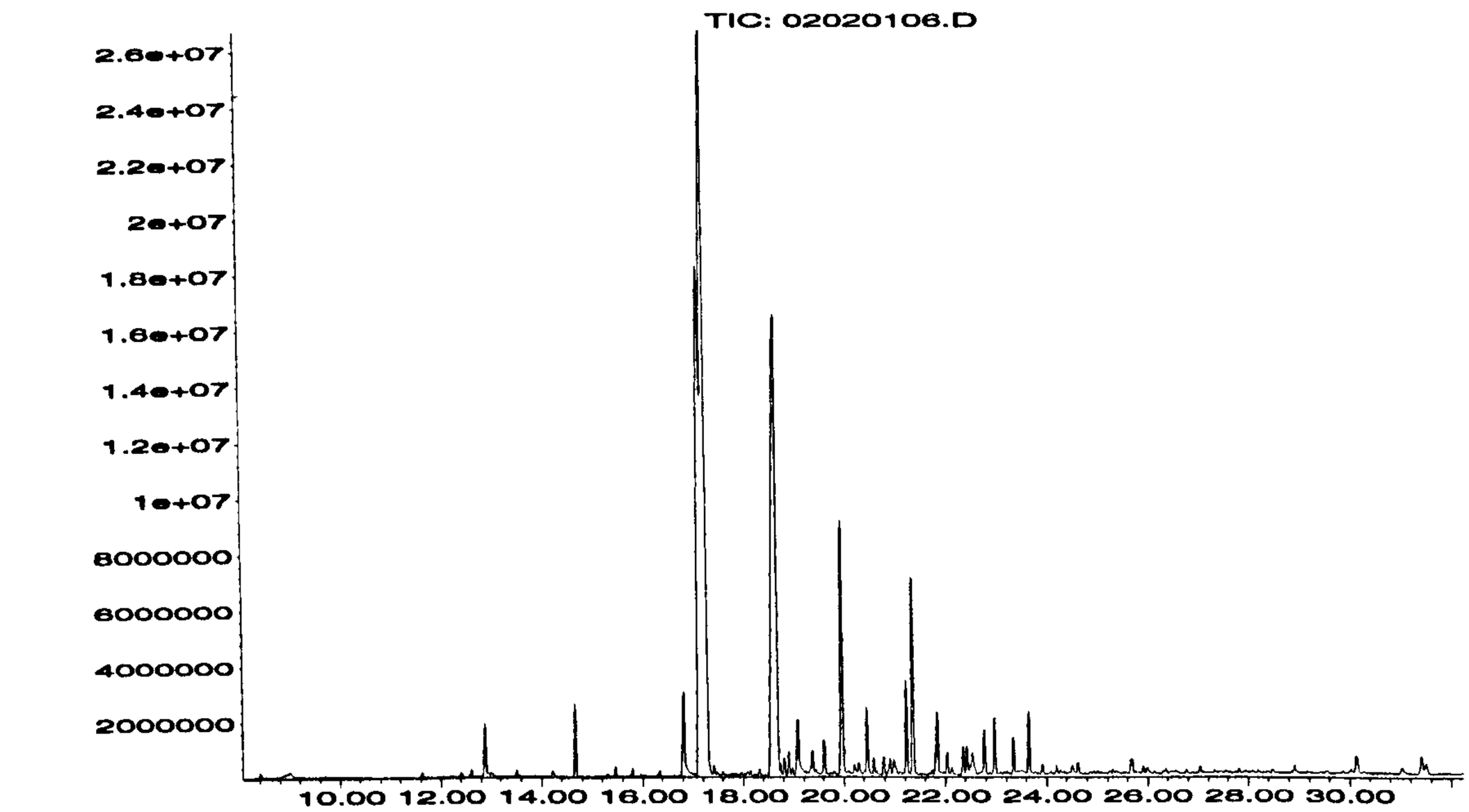


Figure 8.53 Impurity Profile of NND 156

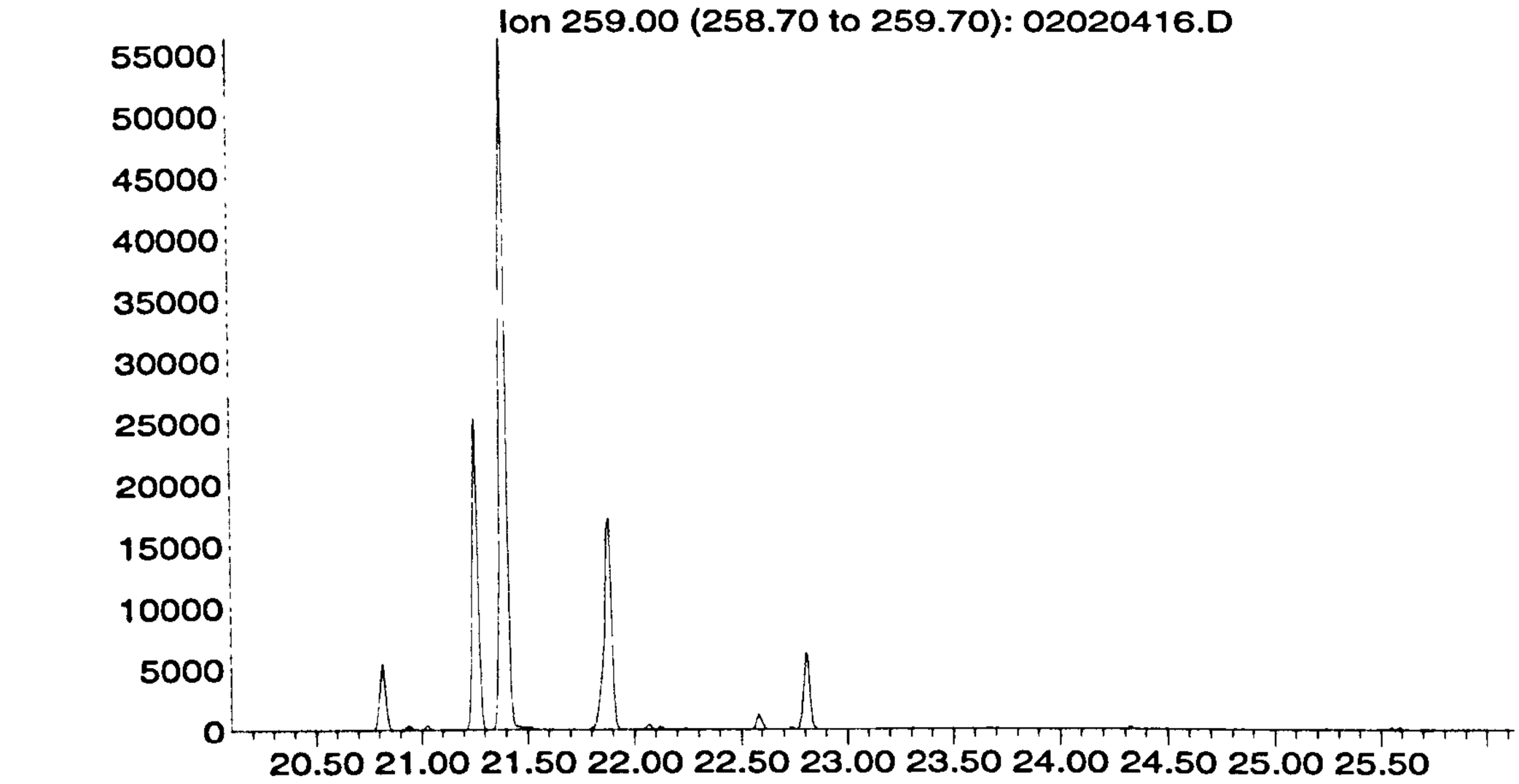


Figure 8.54 Selection of Ion 259 Impurity Profile of NND 155

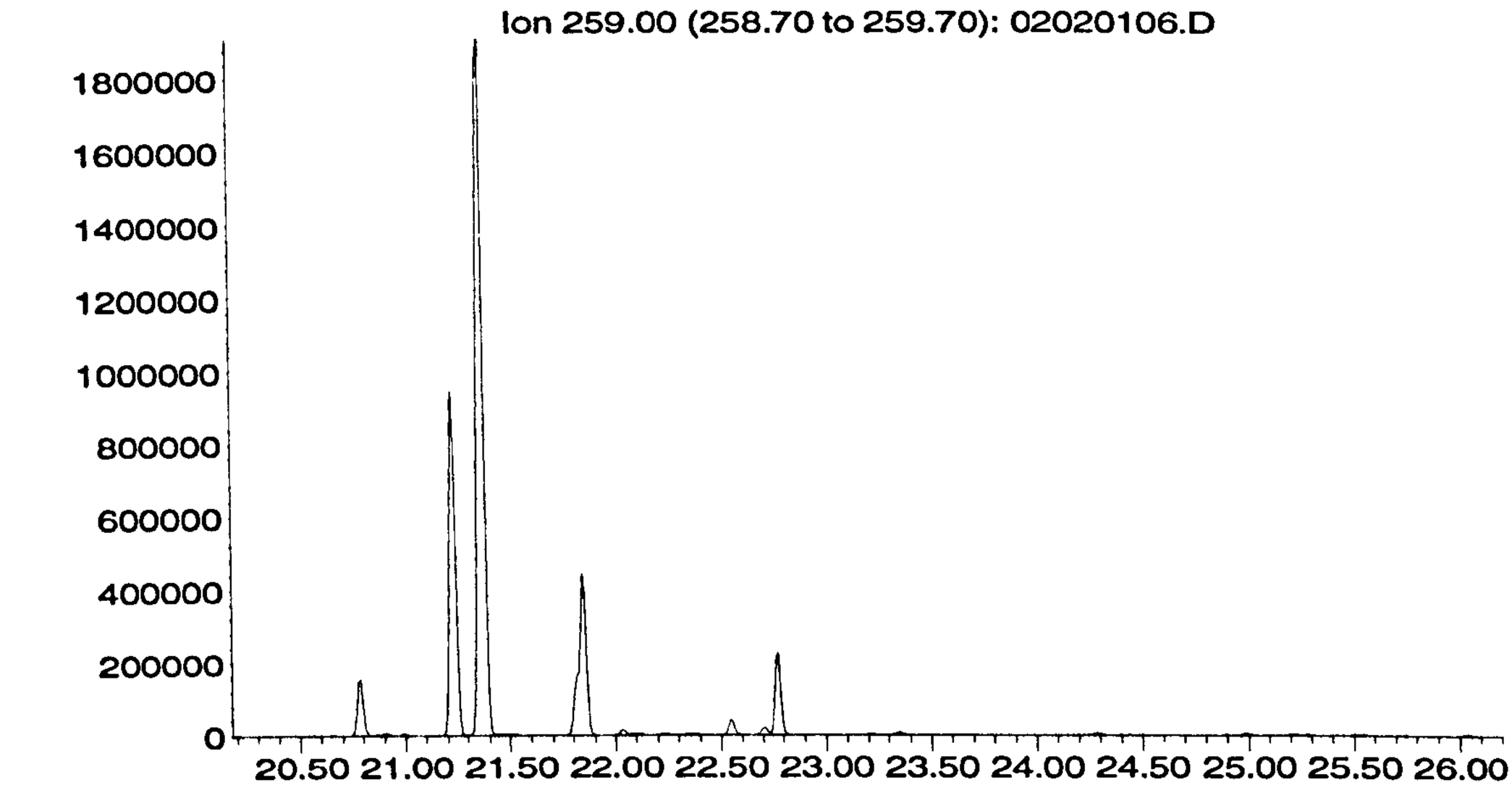


Figure 8.55 Selection of Ion 259 Impurity Profile of NND 156

8.6.15 NND 152B Extracted Ion Chromatograms

As the previous section involving NND 152B suggested, this sample had a very high number and concentration impurities as the impurity profile in Figure 8.56 shows. In order to simplify this complex chromatogram, the extracted ion chromatograms were extracted to show specific impurities to display how a complex mix of impurities may be broken down to show selected impurities only. This may in future work ease the comparison of data sets and allow data to be broken down into smaller sets for principal component analysis or numerical or statistical manipulation.

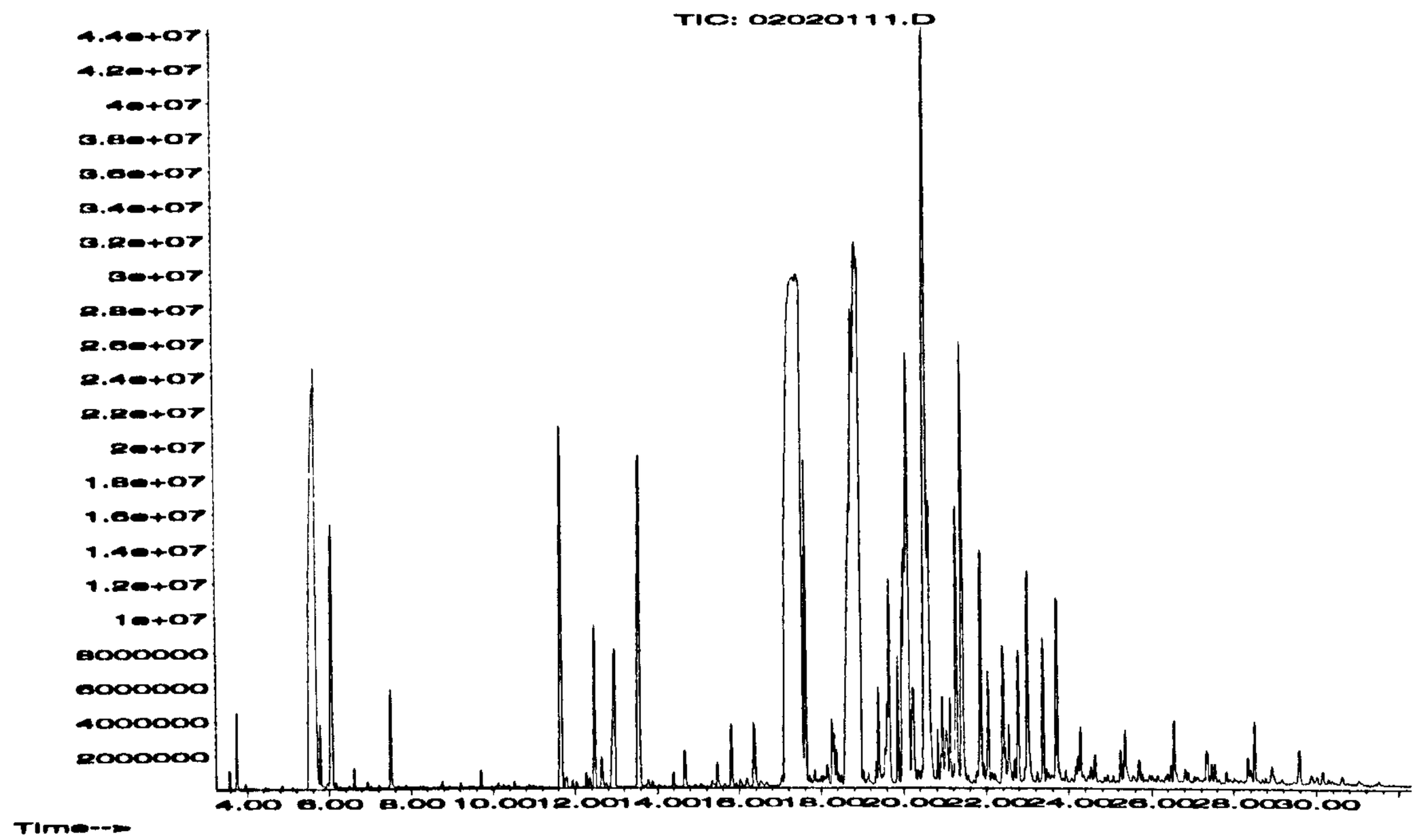


Figure 8.56 Impurity Profile of NND 152B

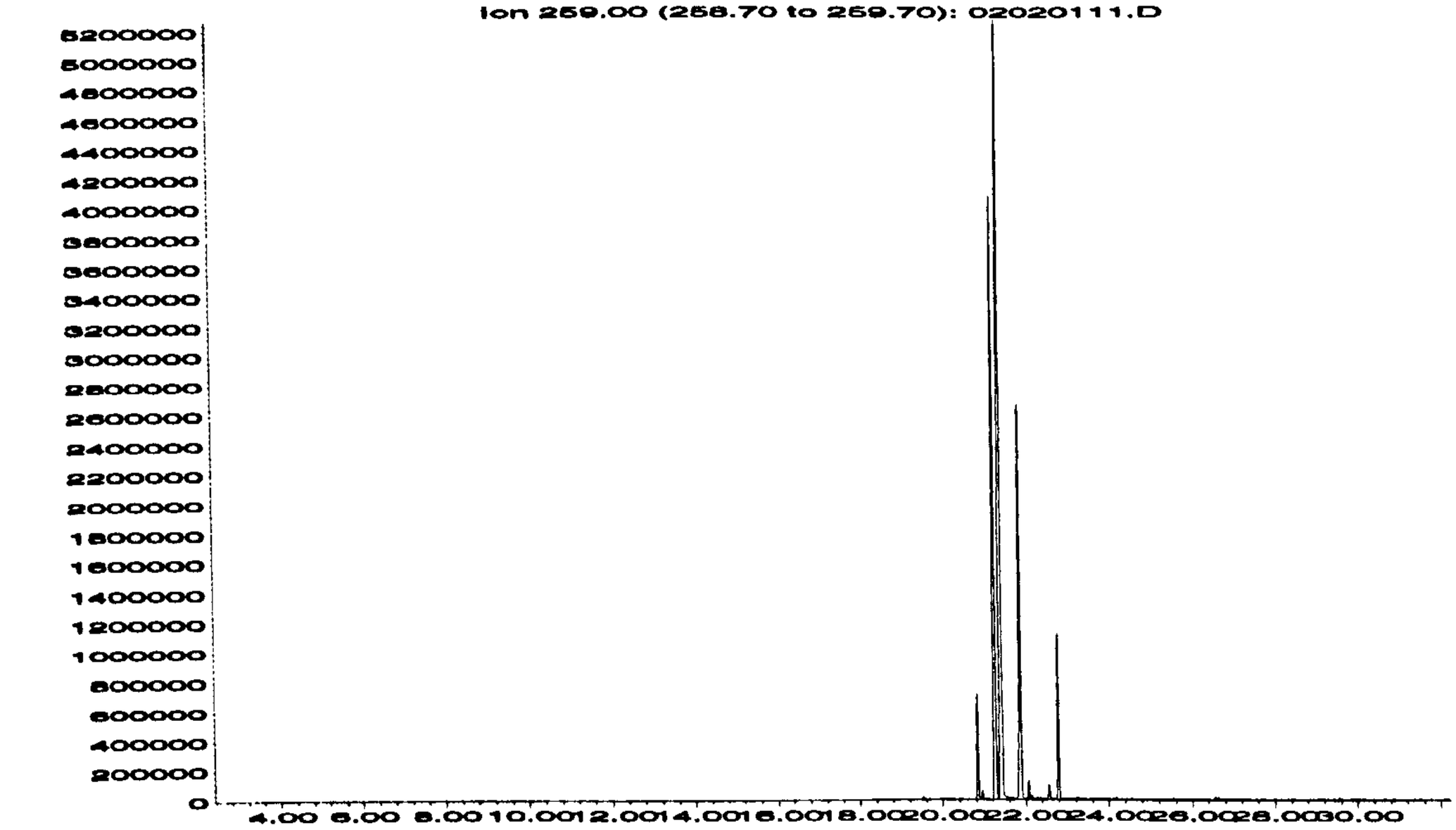


Figure 8.57 Ion 259 extracted to show pyridines

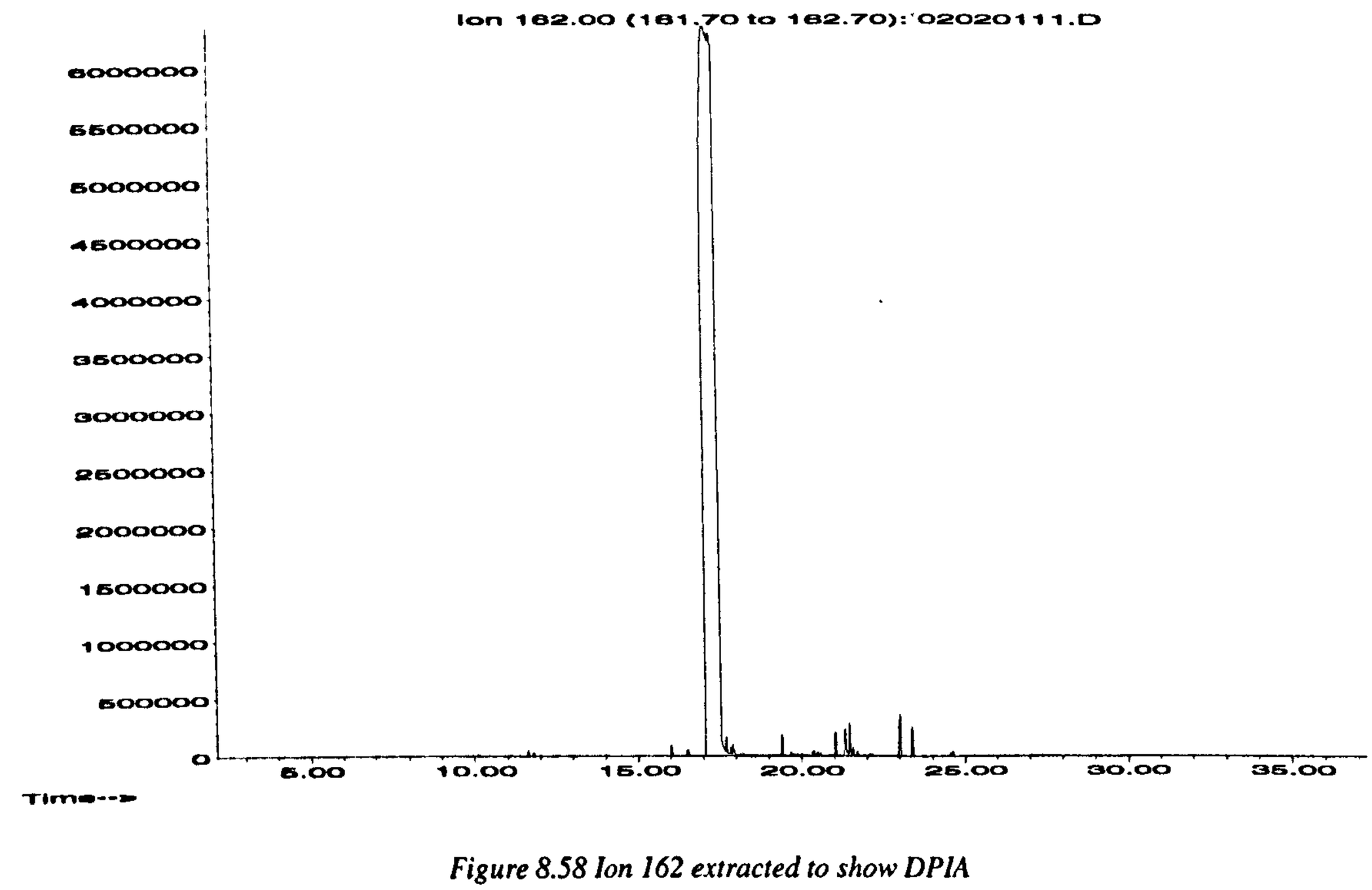


Figure 8.58 Ion 162 extracted to show DPIA

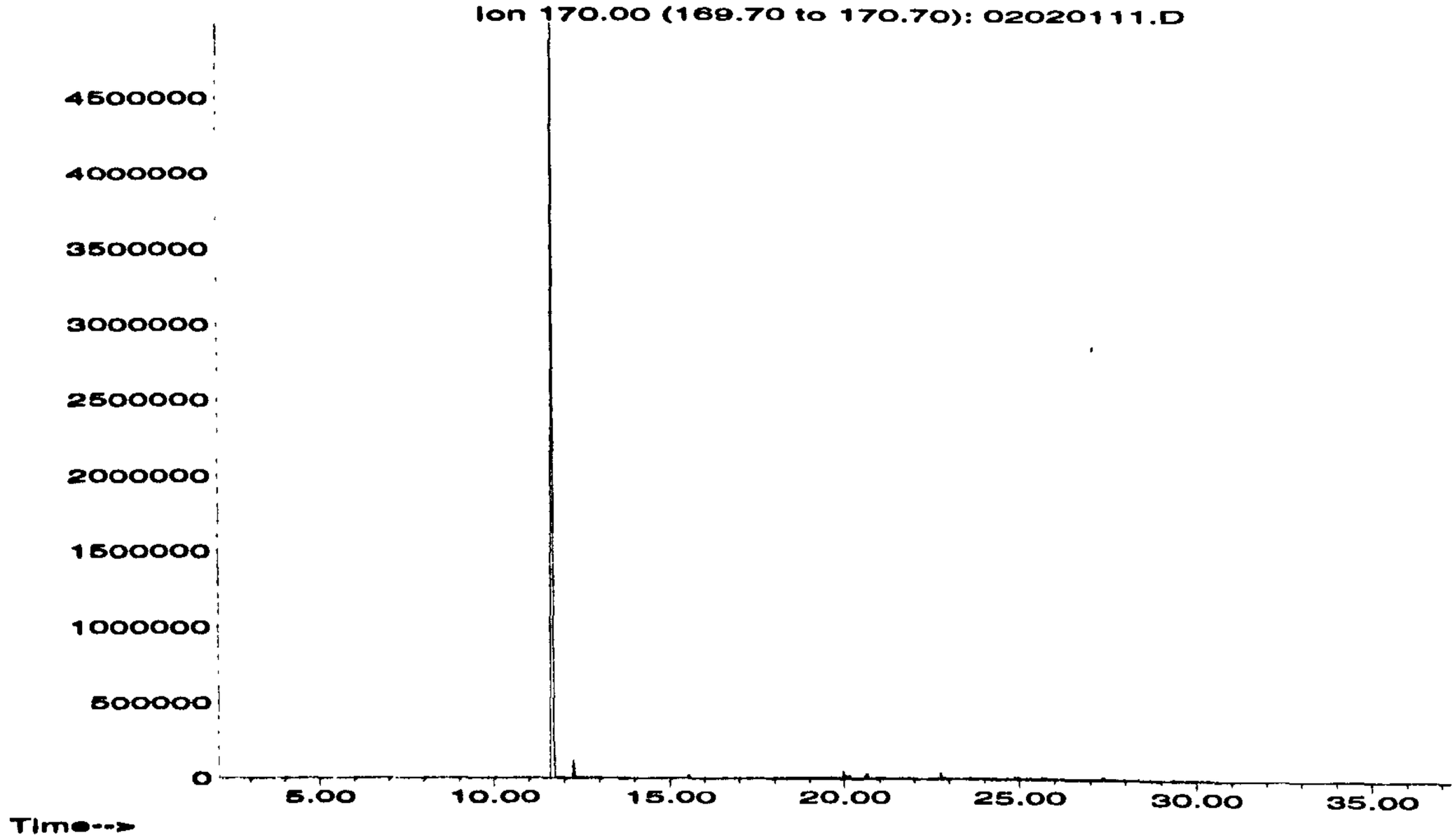


Figure 8.59 Ion 170 extracted to show pyrimidines

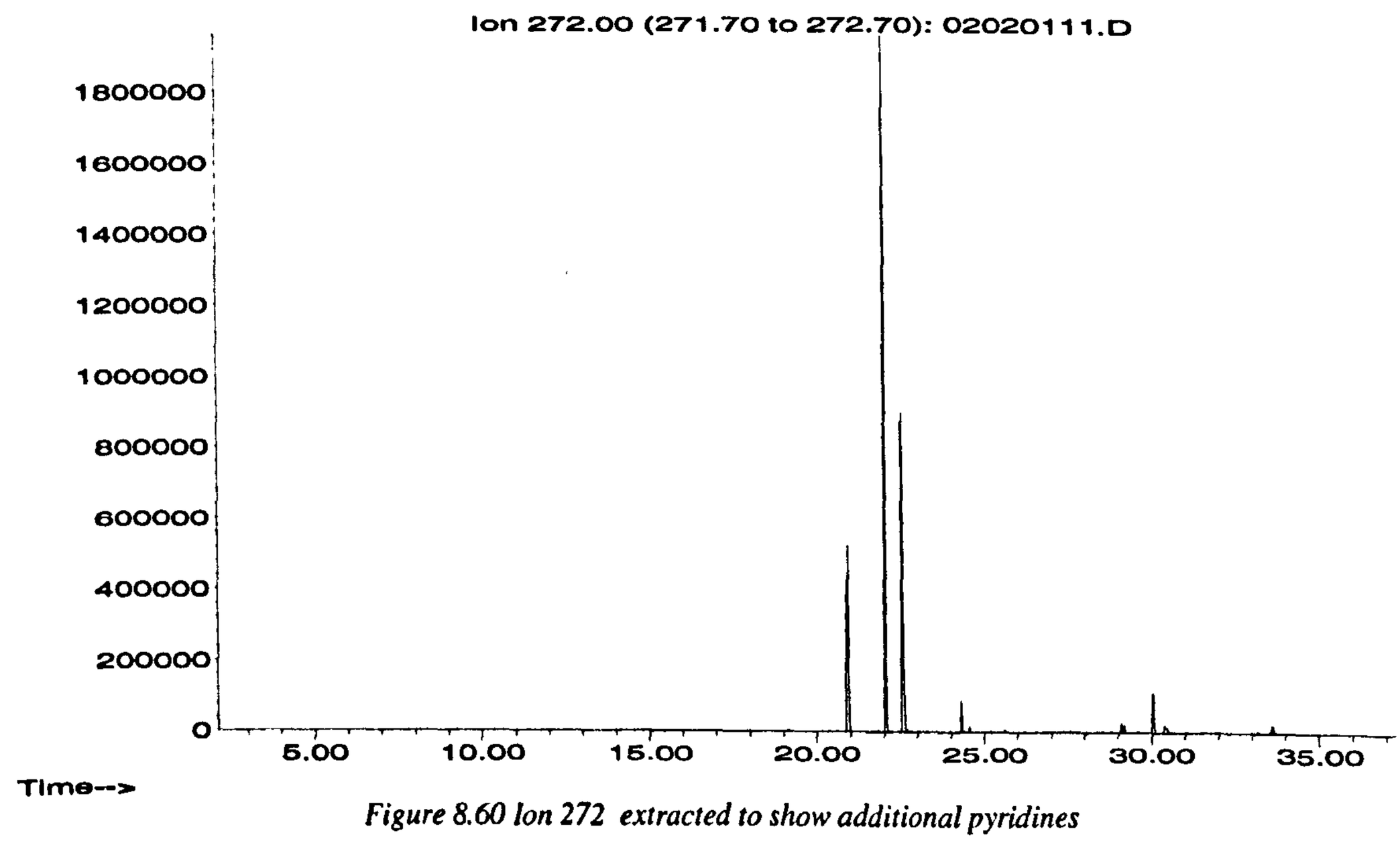


Figure 8.60 Ion 272 extracted to show additional pyridines

Chapter 9

Conclusions

In the course of the experimental work involved in this project, I was able to draw some conclusions based on my own experiences in some of the technical aspects of profiling amphetamines. In addition to this, I have also had the opportunity to think through some of the more practical and financial obstacles that lie in the path of creating a harmonised method for impurity profiling case samples.

The final method chosen for the harmonised method was based on a compromise between what were perceived to be the most important aspects of profiling. In this project, as a complete novice in this field, I was unable to fully direct any of the work schedules, as these were undertaken by members of the group with many years of experience in this area. In the end, the group agreed on the basis for a method, which, from the point at which I left the group, was still a work in progress.

However, I have come to some conclusions purely based on my results from the method development experiments and in the case sample study that was not part of the harmonisation itself.

On a practical level, I feel that I would have liked to explore further, the possibility of extracting from a larger volume of buffer into a larger volume of solvent and, to concentrate the sample, evaporating some solvent off under a stream on nitrogen. This would perhaps allow for a more consistent extraction process and lessen some of the solubility problems experienced throughout the project with a number of samples. It would also allow for extraction into a more manageable volume and reduce the effect of slight evaporation while using a sonic bath to dissolve the sample. It would also, I believe, allow for a more controlled evaporation of the solvent while maintaining a concentrated sample of impurities.

If, in the future, quantification of specific impurities becomes necessary, I would perhaps introduce an internal standard with chemical similarities to these compounds and spike the internal standard at the outset of the extraction. In this way, the internal standard 'recovery' would provide a measure of the success of the extraction and highlight possible problems

with the extraction process that would otherwise not be identified. In adding the internal standard as part of the extraction solvent, anomalies with the preparation of the buffer, preferential extraction of diluents and so on, is not compensated for by the internal standard.

I feel that it makes more sense to look at impurities that are consistently and reproducibly extracted rather than those which may be route-specific although their identification by mass spectrometry is crucial also. In reality, since the vast majority of samples are prepared either by the Leuckart route or by reductive amination, the presence of route-specific impurities does not give any real spectificity. What is more important, in my opinion, is the pattern of all impurities present and looking at the 'bigger picture' rather than focusing too closely on the route of synthesis.

I would argue that GC-FID alone is not specific enough for true impurity profiling. Retention time is not sufficient to unequivocally identify impurities. I believe that mass spectrometry is necessary for the identification of at least some amphetamine-based impurities in a sample. However, I do not believe that only impurities that can be identified should be used in profiling. Sometimes the most significant peaks are those that cannot be identified since these peaks may be the only way to distinguish one batch of amphetamine from another or samples which have come from a single source but have then been split and different diluents added.

If possible in the future, it would be preferable to build up a reference library of known impurities using professionally manufactured synthetic standards. It is essential to build this library for each individual system while using the same standard compounds as those other labs wishing to participate and share information. In this way, the variation in instruments is taken into account but possible variation in standard preparation is eliminated. I believe one area in which the project came to a hiatus was, specifically in my case, the expectation that an analytical chemist can automatically become responsible for organic synthesis of standard compounds. This is simply not the case. This task was one which I felt would have been better designated to a more experienced organic chemist since it was a frustrating time for me which could have been better spent in familiarising myself with the instrumentation.

The use of the GC-MS in SIM was never fully considered in this project. If the decision was made to focus only identified impurities, then selecting specific ions to monitor would 'clean up' the profile and provide a clearer picture of a real amphetamine-impurity profile as opposed to an organic-impurity profile which would include environmental contaminants, impurities in the solvent and so on. Or alternatively, using the extraction of ion chromatograms to limit the data to specific ions while still having the complete data set if

necessary is another option. If the peaks of interest are known, this can reduce the data from hundreds of peaks in a complex chromatogram to perhaps 20 peaks of interest which would be easier for PCA or statistical packages to cope with. In addition, if impurity profiling progressed in such a way that only the identification and quantification of specific impurities was necessary, the use of LC-MS/MS should be considered. This would lessen the importance of the total chromatographic separation of compounds while providing more unambiguous identification of impurities through careful selection of parent and daughter ions. It would also lessen the amount of sample preparation required for injection.

As for thoughts I have formed about the possible use of profiling on a wider scale, I simply cannot see that impurity profiling of amphetamine samples will become commonplace in operational forensic laboratories in the near future. This is partly because of the fact that agreement on a single method of extraction and analysis will take time and partly because impurity profiling will not (and in my opinion, should not) be a priority for operational forensic laboratories with caseloads already taking up all of their time and finances.

Far from being secondary to the profiling itself, I feel that the identification and quantification of amphetamine content by HPLC should remain the primary goal in the analytical process. This seemed to somehow have been lost in the project. The presence or otherwise of a significant quantity of amphetamine should not be overlooked in the pursuit of a possible link between samples. In any case, if only a small amount of sample is available, the priority must surely be identification and quantification of amphetamine leaving a limited amount of sample available for supplementary analyses.

Since the profiling of samples is in itself a time consuming, and therefore expensive process, I feel that this step in the analytical process should be limited to those samples already determined to have a high level of amphetamine. In this way, there should be (hopefully) a high level of impurities present thus ensuring a strong profile. This also focuses the criminal process (which is fundamentally what profiling is intended to aid) on those in the higher echelons of the drug-dealers, those higher up the production and distribution chain with access to the uncut drugs.

It must also be accepted that amphetamine in clandestine labs may be manufactured to such a high purity that the levels of impurities present will be minimal. This is perhaps the biggest problem facing authorities hoping to eliminate the clandestine production of the drug. The most professional criminal manufacturers are likely to produce purer drugs in bulk quantities and have a wider distribution network. The smaller-scale producers may produce batches with

more impurities but only distribute their produce across a small area. This hampers the intelligence work in that, those larger networks and distribution rings which the authorities want to infiltrate become more 'invisible', simply by virtue of the geographically scattered nature of seizures and the fact that they are virtually impossible to link by their limited levels of impurities. Additionally, those seizures made which have a higher level of impurities are likely to have been produced in makeshift kitchen labs on a small scale. By linking these batches, the authorities may eliminate a local source of the drug but will be unlikely to break an intercontinental chain.

In addition, it is my opinion that the real emphasis on research into the possible profiling of drugs should be on Class A drugs. Recent research into drug misuse clearly states that the number of people abusing amphetamines is decreasing while those abusing cocaine is on the increase. The number of heroin-related deaths as well as the extent of cocaine and crack cocaine abuse must surely remain at the forefront of forensic researchers minds. Although amphetamine can cause death and is undoubtedly a dangerous drug when misused, I feel that the most pressing task facing law enforcement agencies is to reduce the availability of heroin and crack cocaine and this is where the majority of funding for profiling research should be streamed.

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