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Applications of extractive-derivatization sample preparation in a clinical toxicology laboratory setting

BY

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Summary

The metabolism of absorbed xenobiotic compounds in humans results in a mixture of target compounds applicable for analysis, trapped in complex biological matrices. Gas chromatography-mass spectrometry (GC-MS) is a powerful analytical technique that has been successfully applied in the analysis of volatile and semi-volatile compounds from complex biological samples. This is due to the ability of GC-MS to separate different sample constituents at trace levels while providing accurate molecular structural information for the resolved compounds. The complexity of biological specimens and their largely aqueous nature, combined with the physicochemical properties of target analytes resulting from metabolism, greatly precludes direct analysis of biosamples by GC-MS. Traditionally, highly laborious and time consuming sample preparation procedures are performed to isolate and chemically alter target analytes to attain suitable amenity for the detection system. Furthermore, routine analytical procedures in clinical toxicology laboratories are signified by short specimen turn-around times. The commonplace use of GC-MS in modern-day laboratories still suffer from prolonged turn-around times that result from both sample preparation steps and lengthy instrumental analysis. Simplified and cost-effective analytical procedures capable of extracting multiple analytes, with divergent functional groups, from biological matrices in a timely manner are therefore required.

To address this issue, this work describes the development of validated extractive-derivatization methods combined with fast GC-MS analysis for expedient and accurate quantitation of different analytes in occupational monitoring and workplace drug testing. Extractive alkylation of acidic analytes phenol, *o*-cresol, mandelic acid, hippuric acid, and (*o*-, *m*-, *p*-) methylhippuric acid for simultaneous urinary bio-monitoring of occupational exposure to benzene, toluene, ethylbenzene, and xylene, respectively, is performed. Extractive acylation for simultaneous urinary confirmation of basic analytes amphetamine, methamphetamine, norephedrine, methcathinone, ephedrine, methylenedioxyamphetamine (MDA), methylenedioxymethamphetamine (MDMA), methylenedioxyethylamphetamine (MDEA) and *N*-methyl-1-(3,4 methylenedioxyphenyl)-2-butanamine (MBDB) in

workplace drug testing is performed. The successful combination of abovementioned techniques alongside fast GC-MS allows increased sample throughput and decreased turn-around time for routine analysis while maintaining bioanalytical quantitative criteria, as required in a clinical toxicology laboratory setting.

Opsomming

Die metabolisme van geabsorbeerde xenobiotiese verbindings in die mens produseer 'n mengsel van teiken-komponente geskik vir analise, maar wat vasgevang is in komplekse biologiese matrikse. Gas chromatografie-massa spektrometrie (GC-MS) is 'n kragtige analitiese tegniek wat reeds suksevol toegepas word vir die analise van vlugtige en semi-vlugtige verbindings vanuit komplekse biologiese monsters. Dit is te danke aan die vermoë van GC-MS om die komponente van komplekse mengsels teen spoorvlak-konsentrasies te skei, met die behoud van akkurate molekulêre strukturele informasie vir geskeide komponente. Die kompleksiteit van biologiese monsters en hul inherente waterige natuur, tesame met die fisies-chemiese eienskappe van die teiken analiete wat uit metabolisme voortspruit, verhoed grootliks die direkte analise van bio-monsters deur GC-MS. Tradisioneel is arbeidsintensief en tydsame monster voorbereidings prosedures gevolg om teiken-verbindings te isoleer van die matriks en chemies te verander, om geskik te wees vir die analitiese deteksie sisteem. Verder word die roetine analitiese prosedures in klinies toksikologiese laboratoriums gekenmerk deur kort analise omdraai-tye. Die alledaagse gebruik van GC-MS in hedendaagse laboratoriums gaan steeds gebuk onder verlengde omdraai-tye as gevolg van uitgerekte monster voorbereidings prosedures asook lang instrumentele analises. Vereenvoudigde en koste-effektiewe analitiese metodes met die vermoë om menigte chemies-diverse analiete te isoleer vanuit biologiese matrikse, word dus benodig.

Hierdie werk beskryf dus die ontwikkeling van valideerde ekstraksie-derivatiserings tegnieke in kombinasie met vinnige GC-MS analise vir 'n klinies toksikologiese laboratorium, met betrekking tot die akkurate kwantifisering van verskeie teiken-verbindings soos genoodsaak deur beroeps gesondheids- en dwelmmonitering. Gelyktydige ekstraksie en alkilering (ekstraktiewe-alkilering) van die suur-funksionele verbindings fenol, *o*-kresol, amandelsuur, hippuursuur, en (*o*-, *m*-, *p*-) metielhippuursuur vir gesamentlike urinêre bio-monitering van beroeps-blootstelling aan benseen, tolueen, etielbenseen en xileen, respektiewelik, is ontwikkel. Gelyktydige ekstraksie en asilering (ekstraktiewe-asilering) vir gesamentlike urinêre bevestiging van die basis-funksionele verbindings amfetamien, metamfetamien, norefedrien, metkatinoon, efedrien, metileendioksie-

amfetamien (MDA), metileendioksie-metamfetamien (MDMA), metileendioksie-etielamfetamien (MDEA), en *N*-metiel-1-(3,4 metileendioksiefeniel)-2-butanamien (MBDB) met betrekking tot beroeps-dwelmmonitering, is ontwikkel. Die suksesvolle toepassing van bogenoemde tegnieke tesame met vinnige GC-MS lei tot verhoogde monster-omset sowel as verkorte analise-omdraaitye, terwyl die gevestigde kriteria vir kwantitatiewe bioanalitiese metodes streng nagevolg word. Die metodes is voldoende vir roetine analitiese doeleindes in 'n moderne klinies toksikologiese laboratorium.

Publications

This dissertation is based on the two publications listed below:

- A.A.S Marais, J.B. Laurens, *Analysis of urinary biomarkers for exposure to alkyl benzenes by isotope dilution gas chromatography-mass spectrometry*, Journal of Separation Science 28 (2005) 2526-2533.

- A.A.S. Marais, J.B. Laurens, *Rapid GC-MS confirmation of amphetamines in urine by extractive acylation*, Forensic Science International 183 (2009) 78-86.

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"The most valuable of all education is the ability to make yourself do the thing you have to do, when it has to be done, whether you like it or not."

(Aldous Huxley, 1894-1963)

Chapter 1

INTRODUCTION: CLINICAL TOXICOLOGY LABORATORY SETTING AND ANALYTICAL REQUIREMENTS

1.1. Introduction

"Do not talk a little on many subjects, but much on a few." (Pythagoras, 582 – 497 BC)

Mass spectrometry (MS) was developed from 1907 to 1919 by Thomson and Aston to identify isotopes [1]. Beginning in the 1950's, MS instruments became more commercially available and found application in the determination of accurate molecular mass and subsequent calculation of elemental composition of a wide variety of organic compounds. Hyphenation of MS as detection system with a powerful analytical technique such as gas chromatography (GC), which was able to separate individual components of complex mixtures, was further propagated during the following decades. The high sensitivity, specificity, and near-universal detection offered by GC-MS for analyzing complex compositions of organic compounds such as those found in bio-samples, was highly sought after. The great disadvantage leading up to the 1980's was however the high costs and complexity of the systems offered commercially. The viable use of GC-MS instrumentation for routine analysis in a clinical laboratory was realised with the eventual availability of inexpensive and robust quadrupole mass selective detectors that could be maintained and easily handled by trained operators. The advent of high performance fused-silica capillary GC columns allowed GC-MS to become the most powerful method for separation, identification and quantification of relatively apolar organic molecules isolated from complex matrices such as biological samples. However, the single most important prerequisite for successful GC-MS analysis remained sample preparation, as compounds of interest had to be isolated from the matrix and presented in amenable form to the detection system [2].

Nearly all substances that enter a living organism are biologically transformed to some degree, according to the genetic make-up and physiological nature of the specific organism [3]. This process of bio-transformation is known as metabolism and results in compounds that the organism may readily absorb or excrete. Metabolism of foreign compounds in the human body usually results in the conversion of a lipophilic compound into a more polar, hydrophilic compound that is easier to excrete. The excreted compound is termed a metabolite, and the structure is usually typified by addition or creation of a functional chemical group compared to the initial absorbed (parent) compound. Common metabolite functional groups are carboxyls (-COOH), hydroxyls (-OH) and amines (-NH).

The wide variety of analytes with divergent functional groups and chemical properties that arise from metabolism pose a great challenge in biological sample preparation steps for GC-MS. Grouping metabolites with acidic (eg. -COOH, -OH), neutral (eg. –OH) and basic (eg. –NH) functional moieties respectively, and applying a suitable sample preparation procedure for GC-MS analysis, is a logical approach [4]. Cost-effective procedures capable of extracting multiple analytes in a timely manner are therefore required to meet the analytical needs of a clinical toxicology laboratory that largely employs GC-MS for quantitation of metabolites in biological matrices.

1.2. Scope of the work

In a clinical toxicology laboratory setting which endeavours to perform routine analysis for industrial purposes, the highest sample load is expected to be received from industries involved in occupational biomonitoring. Occupational exposure to volatile organic compounds in industry is ubiquitous. Authorities thus enforce biomonitoring of exposed individuals to ensure that the level of exposure does not exceed acceptable levels above which health would be affected negatively. An extension of occupational monitoring is so-called workplace drug testing. The same risks to individual health, co-worker safety, as well as increased employer expenditure due to employee absenteeism, traditionally associated with occupational exposure to harmful substances, is also linked to illicit substance abuse [5]. Occupational monitoring is largely performed to protect the individual employee’s health status, but also to assist employers in preventing unnecessary costs associated with absenteeism and gross medical expenditure in cases

of work-related disability. The selection of analytes to serve as example for multi-analyte method development was therefore determined by the most common analysis requests from various industries, combined with selection of analytes that represent the general scope of metabolite formation (i.e. acidic to basic).

Routine analytical procedures in clinical toxicology laboratories are signified by short specimen turn-around times. The commonplace use of GC-MS in modern-day laboratories still suffer from prolonged turn-around times that result from both sample preparation steps and lengthy instrumental analysis [6]. Batch-analysis alleviates instrumental difficulties to a certain degree, as the analytical system can be kept stable under specific conditions for a specific analysis type. Optimisation of GC instrumental conditions are still required to achieve expedient analysis times. Multi-analyte extraction and processing reduces time and labour involved in biological sample preparation for GC-MS analysis. The present research involved the development of validated extractive-derivatization methods combined with fast GC-MS analysis for rapid and accurate quantitation of metabolites in urine as required in occupational monitoring and workplace drug testing. Extractive alkylation of the acidic analytes phenol, *o*-cresol, hippuric acid, and (*o*-, *m*-, *p*-) methylhippuric acid for simultaneous urinary bio-monitoring of occupational exposure to benzene, toluene, ethylbenzene, and xylene, respectively, was studied. Extractive acylation for simultaneous urinary confirmation of basic analytes amphetamine, methamphetamine, norephedrine, methcathinone, ephedrine, methylenedioxyamphetamine (MDA), methylenedioxymethamphetamine (MDMA), methylenedioxyethylamphetamine (MDEA) and *N*-methyl-1-(3,4 methylenedioxyphenyl)-2-butanamine (MBDB) in workplace drug testing was studied.

The analytical approach to expedient quantification of compounds in a routine clinical toxicology setting, exemplified by the aforementioned analytes, is detailed in the current work as follow:

1. General theory and practise in analytical toxicology:

    a. Chapter 2 - General principles of toxicology (metabolism, sampling etc.)

    b. Chapter 3 - Analytical measurements (instrumentation, quantitative requirements etc.)

    c. Chapter 4 – Sample preparation (extraction methods and technical considerations)

2. Specific applications in routine laboratory settings

a. Chapter 5 – Extractive alkylation of acidic urinary analytes

b. Chapter 6 – Extractive acylation of basic urinary analytes

c. Chapter 7 - Conclusions and further considerations for routine clinical toxicology laboratories

1.3. References

[1] J.T. Watson, Introduction to Mass Spectrometry, 3rd ed., Lippincott-Raven, New York, 1997.

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[6] K. Maštovská, S.J. Lehotay, J. Chromatogr. A 1000 (2003) 153.

Chapter 2

TOXICOLOGICAL PRINCIPLES: METABOLISM AND OCCUPATIONAL BIOMONITORING

2.1. Introduction

"All substances are poisons: there is none which is not a poison. The right dose differentiates a poison and a remedy." (Paracelsus, 1493-1541)

This premise forms the central theme to the subject of toxicology. Xenobiotics (Greek, xenos "foreign"; bios "life") can be defined as chemical substances that are foreign to a biological system or specific living organism. The broad scope of xenobiotics thus covers nearly all substances or compounds that a living organism comes into contact with. The focus of toxicology is to seek deeper understanding of the underlying mechanisms involved in the interaction between the biological system and the xenobiotic: absorption characteristics, whether its effect is therapeutic or pathologic, why this effect can be noted and to what extent (i.e. dose-response relationship). To gain a clearer understanding of why "all substances are poisons", the concept of homeostasis must be grasped.

The concept, first put forth by French physician and physiologist Claude Bernard, of an organism requiring a constant internal *milieu* for sustainment of life amidst an ever changing external environment, was later refined by American physiologist Walter Cannon, coining the term homeostasis [1]. Homeostasis is the need of an organism to remain in a certain baseline, or steady-state, regarding specific physiological parameters. Simply put, it is the organism's reaction to a stimulus to remain in equilibrium. This can be equated with Le Chatelier's principle in chemistry: a system at equilibrium subjected to a stress will adjust to relieve the stress and restore equilibrium. Failure to maintain homeostasis over a certain period of time disrupts normal function, which may result in a diseased state or pathological condition. Pathological reactions can generally be divided into two groups: those that originate from failure of some internal physiological process (e.g. autoimmune disease) and those

that originate from an outside source (e.g. physical trauma). Xenobiotics and their possibly harmful effects to health fall in the latter category. It is clear to see why all substances can be considered poisons, as all substances that enter the human body alter homeostasis. A substance at a specific concentration that helps maintain homeostasis or assists in returning to homeostasis from a pre-existing disrupted state may be considered therapeutic. A substance at a specific concentration that only acts to disrupt homeostasis and eventually elucidates a specific pathology is considered to be toxic.

Nearly all substances that enter a living organism are biologically transformed to some degree, according to the genetic make-up and physiological nature of the specific organism. A simple example is the digestion of foods, where complex macromolecules are biologically transformed through specific physico-chemical processes into monomeric constituents that are readily absorbed by the organism to facilitate the continuation of its own life processes. Xenobiotics are also transformed in the same manner to yield compounds that the organism may readily absorb or excrete. This process of biotransformation is known as metabolism. In the present chapter, the general metabolism of xenobiotics in the human body is discussed. Subsequently, the means of how xenobiotics may enter the body, the basic toxicokinetic principles of absorption and elimination, as well as theoretical methods to ascertain quantitative values that reflect interaction between humans and a xenobiotic are also considered. These broader principles are highlighted to facilitate understanding of the pre-analytical variables and challenges faced in toxicology and related sub-fields such as occupational health monitoring. The latter is of special significance, as the present study is chiefly concerned with analytical approaches for occupational monitoring of:

1. Human exposure to specific chemical substances that fall in the larger class of xenobiotic compounds known as aromatic hydrocarbons. Aromatic hydrocarbons may also be grouped under the term volatile organic solvents, which for the purposes of the present study are defined as “generic name for a group of chemical compounds or mixtures which are liquid in the temperature range of approximately 0-250 °C. They are volatile and relatively chemically inert. Solvents are used industrially to extract, dissolve or suspend materials not soluble in water (e.g. fats, lipids, resins and polymers)” [2]. Specifically, this work deals with exposure to benzene and certain alkylated derivatives thereof: toluene (methylbenzene), xylene

(dimethylbenzene) and ethylbenzene. Chapter 5 examines these substances in greater detail.

2. Human ingestion of illicit substances that alter performance and /or pose a significant health risk to the individual themselves or co-workers in the performance of their daily tasks. Specifically, this work deals with abuse of amphetamine and related analogs that are discussed in further detail in Chapter 6.

2.2. Metabolism of xenobiotics

The first macromolecules that organized themselves into forms with the basic attributes of life were faced with disruptive forces from the environment that threatened their integrity. Compounds that interacted with these organisms and acted to disrupt the delicate homeostatic balance that existed, forced the organism to develop a means of dealing with this disruption if it were to stay viable. This probably occurred through creation of a physical barrier such as a cell membrane, and manufacturing of molecules capable of reacting with the disruptive compound in order to decrease its biological activity. Glutathione is an example of such a molecule found in most mammals. It is a potent nucleophile that reacts with numerous electrophilic compounds to reduce their pharmacologic activity. This reduction in pharmacologic or biological activity is commonly referred to as detoxication. A likely evolutionary step in forming protective mechanisms may have been the development of macromolecular catalysts that were able to chemically alter disruptive compounds. These catalysts may have been the first detoxication enzymes, as evidence points to the occurrence of specific enzymes in both animal and plant species ranging from simple, unicellular organisms to complex, multicellular ones [3].

Many of the enzymes involved in xenobiotic metabolism are also involved in metabolism of endogenous biochemical compounds. This has lead to the hypothesis that these enzymes merely represent enzymes of normal anabolic and catabolic activity and it is purely fortuitous that they also metabolise xenobiotics. A greater affinity for endogenous substrate seemingly supports this. Factors that diminish confidence in this hypothesis are the fact that numerous xenobiotic metabolising enzymes rapidly respond

with increased activity to the presence of xenobiotics as well as environmental change [1, 3, 4]. It also seems unlikely that enzymes involved in normal cellular metabolism would evolve to metabolise the thousands of naturally occurring and synthetic compounds that are foreign to organisms. The enzymes with greater affinity for endogenous substrate are usually isozymes of xenobiotic enzymes, and it is thus a possibility that cells utilized pre-existing xenobiotic metabolising enzymes to perform cellular metabolism. An alternative hypothesis is that enzymes underwent parallel evolution. In humans xenobiotic metabolising enzymes are largely found in the liver, however, they are also found in nervous tissue, kidney, lung, plasma, and the gastrointestinal tract (bacterial flora, digestive secretions and the intestinal wall). On a sub-cellular level, they are located mainly in the microsomes, but some are in free solution in the cytoplasm [3].

Xenobiotic metabolism does not always end in detoxication of the initial absorbed compound. In many cases the product of metabolism (metabolite) is more biologically active than the parent compound. Conversion of a xenobiotic to a more biologically active and possibly toxic metabolite is termed metabolic activation. Enzymes that perform a protective function with regards to certain chemical compounds may be responsible for the toxicity of others. There thus exists a delicate balance between detoxication and metabolic activation during exposure to xenobiotics, a balance that is subject to both endogenous (genetics, physiology, toxicodynamics) and exogenous (environment, diet) factors [4]. These factors may merely change the kinetics of the enzyme reaction or completely alter the pattern of metabolism, thereby changing the toxicity of a certain compound.

Genetic variation on both inter-and intra-species levels exists. There is diversity in the occurrence, function and rates of specific enzymes. Bacteria contain less highly developed systems and lack certain pathways altogether; between male and female, as well as between persons of different ethnicity, there are also predispositions for specific metabolic pathways as a function of genotype. Age is the main physiological factor, as the very young and elderly have impaired metabolism. In the elderly this is largely due to impaired liver volume and hepatic blood flow, while in the young enzyme kinetic rates are reduced. Hormones, disease, pregnancy and nutritional status also play a role. Toxicodynamic factors include dose, frequency, route of exposure and tissue distribution [1].

Dietary lipid and protein deficiencies diminish microsomal xenobiotic metabolising activity. Deficiencies in antioxidants such as ascorbic acid, riboflavin and α-tocopherol also result in reduced microsomal enzyme activity. Induction or inhibition of enzymes due to the presence of other compounds in the environment is also an important consideration [4]. Metabolism or biotransformation in the human body has been divided into two distinct phases.

Phase 1 reactions are functionalization reactions, i.e. the addition or creation of a functional group occurs. This usually results in the conversion of a lipophilic compound into a more polar, hydrophilic compound that is easier to excrete. Phase 2 reactions are biosynthetic or conjugative reactions, where a functional group is masked by the addition of a new group that will increase the polarity and water solubility in order to facilitate ease of excretion further. Most xenobiotics undergo both phase 1 and phase 2 metabolism. Table 2.1 indicates reactions classed as phase 1 or phase 2 metabolisms.

Phase 1	Phase 2
Oxidation	Glucuronidation/glucosidation
Reduction	Sulfation
Hydrolysis	Methylation
Hydration	Acetylation
Dethioacytelation	Amino acid conjugation
Isomerisation	Glutathione conjugation
	Fatty acid conjugation
	Condensation

Table 2.1 Reactions classed as phase 1 or phase 2 metabolism [1].

2.2.1. Phase 1 metabolism

Biotransformation in phase 1 metabolism is predominated by oxidation reactions. Most compounds are oxidized by a group of non-specific enzymes in liver microsomes known as either cytochrome P-450-monooxygenase, mixed-function oxidase or microsomal hydroxylase. They are membrane bound to the endoplastic reticulum and function as a multi-component electron transport system responsible for the metabolism of a variety of endogenous substrates as well as xenobiotic compounds.

The most important function of this enzyme system is its ability to incorporate a single oxygen atom into an organic molecule concomitant with the reduction of the other atom of oxygen to water. The introduction of a hydroxyl group into the lipophilic compound provides a site for subsequent conjugation with hydrophilic compounds, thus increasing the solubility of the metabolite for its transport and excretion. The principal liver microsomal drug-metabolising enzyme system consists of at least two protein components: a heme protein called cytochrome P-450 and a flavoprotein called NADPH-cytochrome P-450 reductase, which contains both FMN and FAD. Cytochrome P-450 is the substrate- and oxygen-binding site of the enzyme system, while the reductase functions as an electron carrier, shuttling electrons from NADPH to cytochrome P-450. Cytochrome b_5 reductase has also been implicated in the function of the enzyme system. Figure 1.1 illustrates the catalytic cycle of cytochrome P-450-monooxygenase. Table 2.2 indicates the various reactions performed by the cytochrome P-450-monooxygenase system.

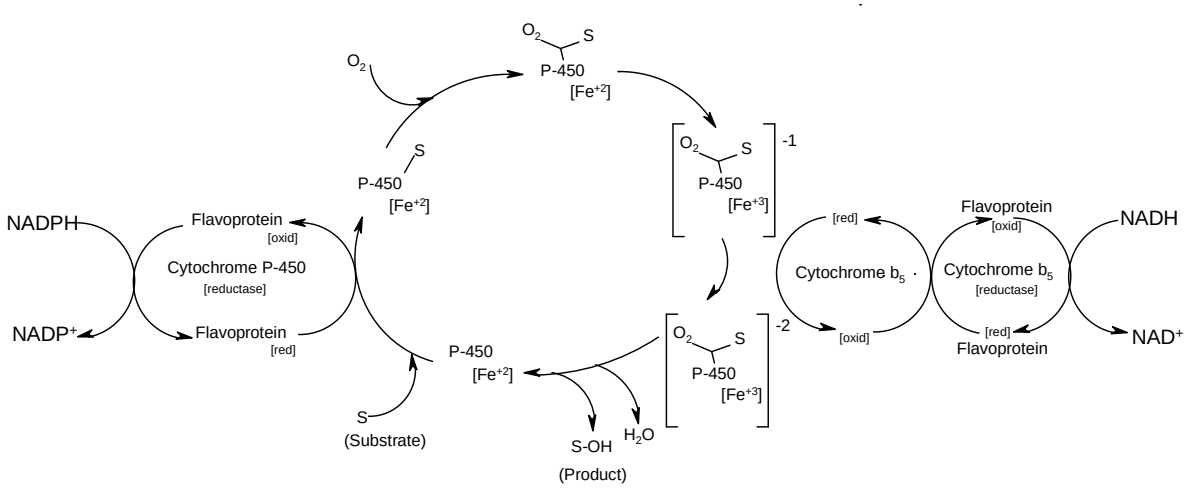


Figure 2.1 Catalytic cycle of the cytochrome P-450-dependent monooxygenase [3].

Reaction	
Aromatic hydroxylation	Oxidative deamination
Aliphatic hydroxylation	*N*-oxidation
Epoxidation	*S*-oxidation
N-Dealkylation	Phosphothionate oxidation
O-Dealkylation	Dehalogenation
S-Dealkylation	Alcohol oxidation

Table 2.2 Reactions performed by cytochrome P-450 monooxygenase system [1].

For the purposes of the present study aromatic and aliphatic hydroxylation as well as *N*-dealkylation is discussed in more detail.

2.2.1.1 Aromatic hydroxylation

The metabolic oxidation of aromatic compounds yields phenolic products. For mono-substituted benzene derivatives, *para* hydroxylation predominates with some *ortho* product formed as well. The position of hydroxylation is influenced by the type of substituents on the ring according to the theories of aromatic electrophilic substitution [5]. Hydroxylation is enhanced by electron-donating substituents and diminished by electron-withdrawing substituents. Steric factors are also considered, as oxidation occurs at the least hindered position. Hydroxylation of aromatic compounds has been considered to be mediated by an arene oxide (epoxide) intermediate. Figure 2.2 shows the general reaction scheme.

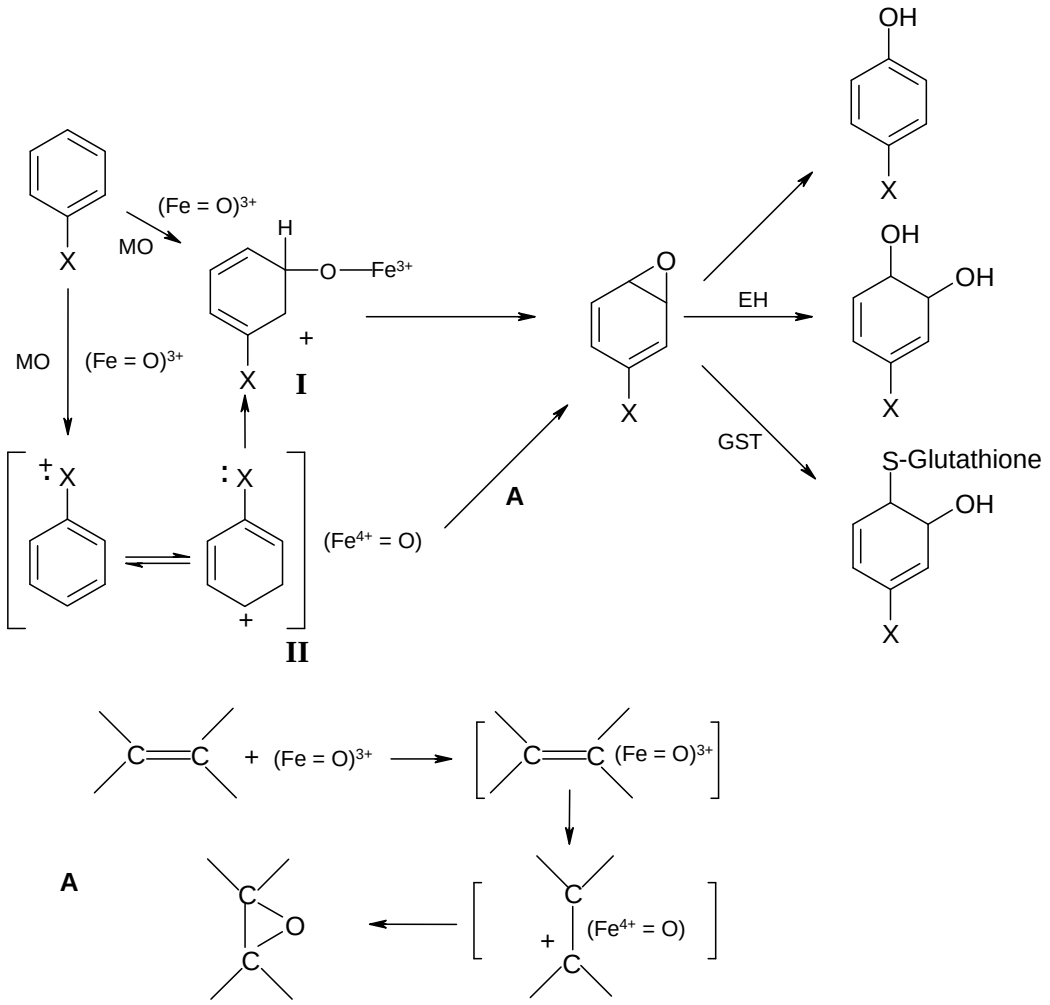


Figure 2.2 Proposed mechanism for hepatic oxidation of aromatic compounds and alkenes to their corresponding epoxides [4]. MO: cytochrome P-450 monooxygenase; EH: epoxide hydrase; GST: glutathione *S*-transferase. **A** represents the reaction mechanism for pathway **II.**

The proposed mechanism is electrophilic aromatic substitution by perferryl-oxygen to a cation intermediate (I) that could close to give an epoxide, depending on the enzyme active site characteristics. Single electron oxidation of the substituent attached to the ring may also occur to yield a radical cation intermediate (II) that collapses to a meta-substituted intermediate. This intermediate can then close to an epoxide analogous to that of the electrophilic aromatic substitution pathway [4]. The epoxides are generally unstable entities that rearrange non-enzymatically to phenolic derivatives, or enzymatically to 1,2-dihydrodiols that are dehydrogenated to 1,2-diphenols. As described by Figure 2.2, epoxides are also conjugated with glutathione to form hydroxymercapturic acids. Formation of the three different end-metabolites is also influenced by the stability of the epoxide itself. Electron-withdrawing groups stabilize

arene oxides while electron-donating groups destabilizes them. This determines whether the epoxide spontaneously rearranges or enters subsequent metabolic pathways.

2.2.1.2 Aliphatic hydroxylation

Alkyl groups of various carbon-chain lengths are hydroxylated by the cytochrome P-450-monooxygenase system. Methyl groups can be found adjacent to numerous structural moieties (e.g. phenyl, aromatic heterocycles), but its main metabolic pathway remains oxidation to the hydroxymethyl derivative. This is usually followed by further oxidation to the carboxylic acid (Figure 2.3), but not in all instances. In the case of several equivalent methyl groups only one is normally oxidized. For aromatic methyl groups oxidation occurs on the least hindered group. Hydroxylation of alkyl side chains attached to aromatic compounds is influenced by the aromatic ring structure. Oxidation occurs preferentially on the methylene group adjacent to the aromatic ring but may also occur at other positions on the side chain, following the same pattern as hydroxylation of methyl groups.

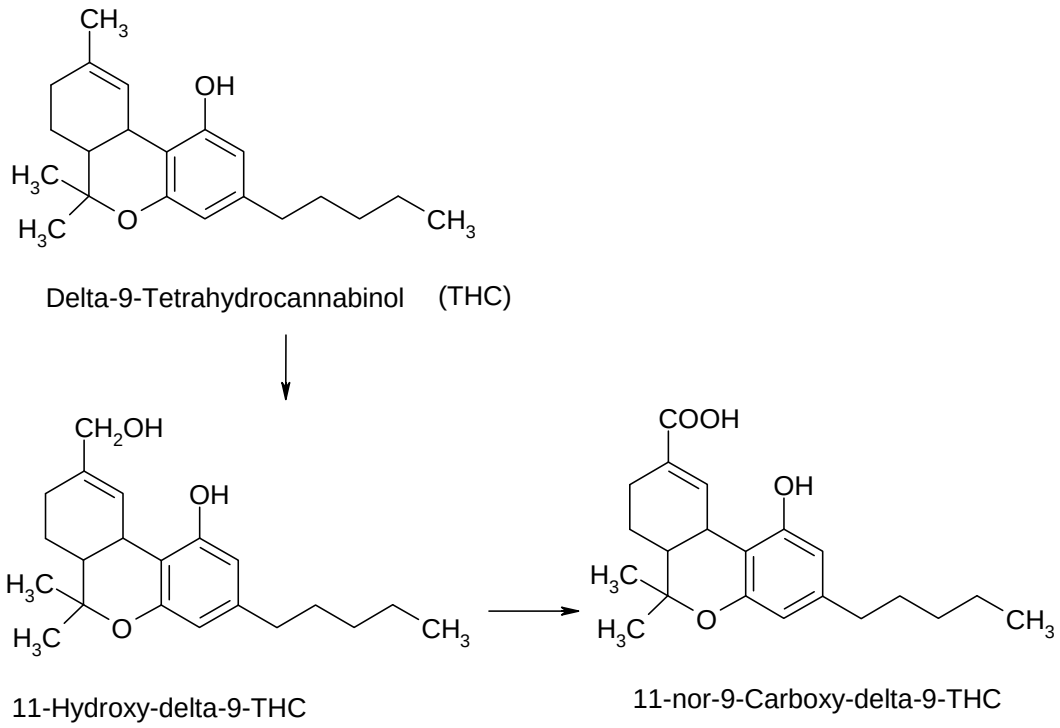
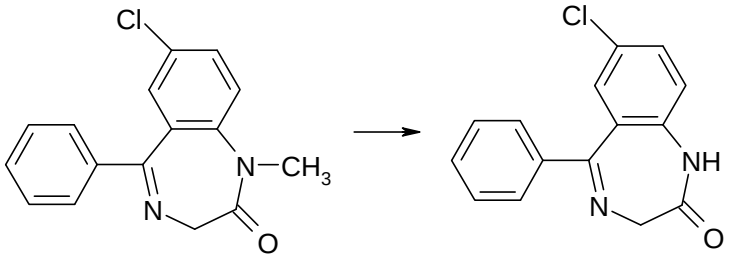


Figure 2.3 Aliphatic hydroxylation in the metabolism of Δ^9-Tetrahydrocannibinol.

2.2.1.3 *N*-dealkylation

The dealkylation of secondary and tertiary amines to their respective primary and secondary amines are some of the most common phase 1 reactions to occur via the cytochrome P-450-monooxygenase system. Tertiary amines require a hydrogen atom on the adjacent carbon for this process to transpire. The general *N*-substituents removed in this process include methyl, ethyl, n-propyl, n-butyl, allyl, benzyl, and other s that contain a α-hydrogen. Tertiary amines are dealkylated faster to secondary amines than secondary amines are to primary amines. This occurrence seems to be related to lipid solubility and accumulation of metabolites that are more polar than the parent molecule can therefore pose a risk of toxicity if they are also pharmacologically active (Figure 2.4).



Diazepam Nordiazepam

Figure 2.4 *N*-dealkylation of diazepam to form pharmacologically active nordiazepam.

2.2.2. Phase 2 metabolism

Two major reactants are required for conjugate synthesis: a xenobiotic with an appropriate functional group and a co-substrate that can be conjugated with it. The conjugation or biosynthetic reactions offer a mechanism by which a functional group of a xenobiotic or its phase 1 metabolite is blocked through the addition of a conjugate moiety derived from carbohydrates, lipids or proteins. The conjugate intermediate does not react directly with the xenobiotic, but rather in an activated form itself or with the activated form of the xenobiotic. The activated intermediates are usually nucleotides and the reaction is catalyzed by specific transferases. The major conjugation reactions of phase 2 metabolism are outlined in Table 2.3.

Reaction	Enzyme	Functional group
Glucuronidation	UDP- Glucuronyltransferase	-OH
		-COOH
		$-NH_2$
		-SH
Glycosidation	UDP- Glycosyltransferase	-OH
		-COOH
		-SH
Sulfation	Sulfotransferase	$-NH_2$
		$-SO_2NH_2$
		-OH
Methylation	Methyltransferase	-OH
		$-NH_2$
Acetylation	Acyltransferase	$-NH_2$
		$-SO_2NH_2$
		-OH
Amino acid conjugation	Co-enzyme A	-COOH
Glutathione conjugation	Glutathione-S-transferase	Epoxide
		Organic halide
Fatty acid conjugation		-OH
Condensation		Various

Table 2.3 Conjugation reactions [2].

Glucuronide formation is one of the principle phase 2 metabolism pathways of xenobiotics and accounts for the largest share of conjugated metabolites. Glucuronidation, sulfation and reactions with glutathione and amino acids are discussed in more detail.

2.2.2.1 Glucuronic acid Conjugation

The abundant supply of glucuronic acid in the liver combined with the large number of functional groups that form glucuronide conjugates indicates the significance of this metabolic pathway. Similar to cytochrome P-450 monooxygenase being the main phase 1 enzyme, uridine diphospho-glucuronosyltransferase (UDP-glucuronosyltransferase) is the principle phase 2 enzyme. Glucuronidation requires the

availability of three reactants: UDP-α-D-glucuronic acid (UDPGA) generated in the cytoplasm from glucose-1-phosphate, UDP-glucuronosyltransferase bound to the membranes of the endoplastic reticulum, and a suitable substrate with the correct functional group. Figure 2.5 presents the general reaction scheme for glucuronidation.

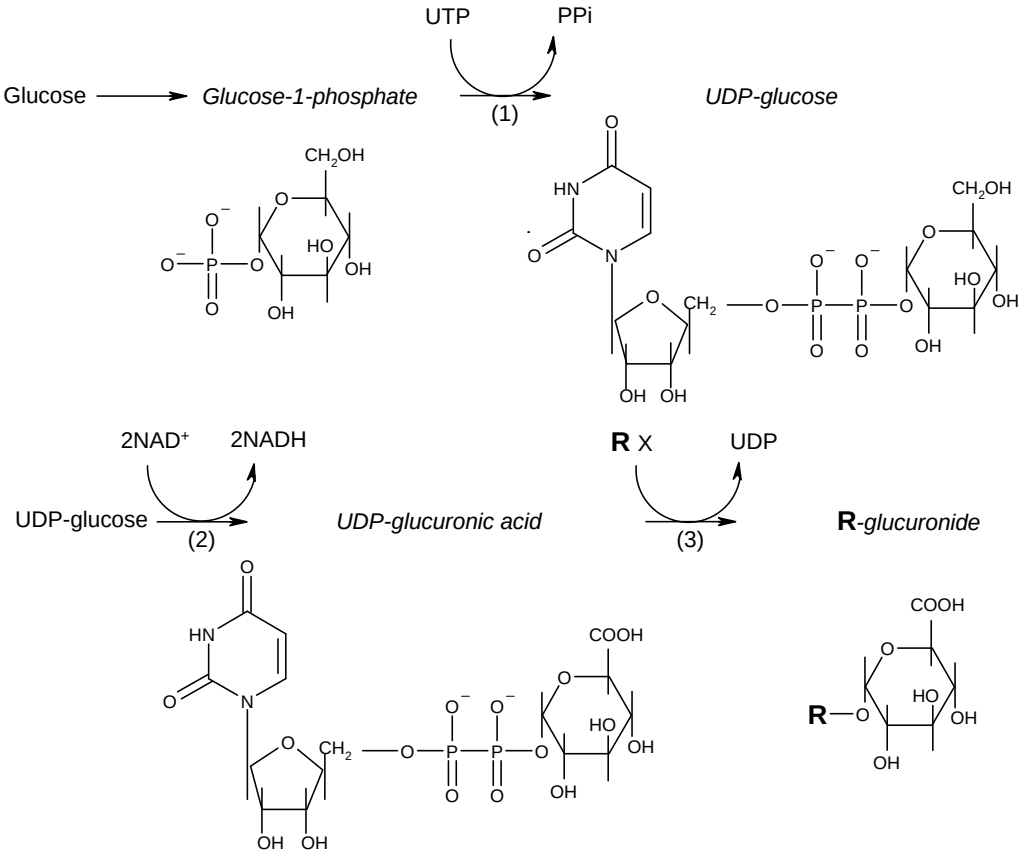


Figure 2.5 Glucuronidation reaction scheme: (1) Pyrophosphorylase; UTP, uridine triphosphate; PPi, pyrophosphate; (2) UDP-glucose dehydrogenase; (3) UDP-glucuronosyl transferase; UDP, uridine diphosphate; R, activated metabolite where X is OH, COOH, SH or NH_2.

Glucuronic acid conjugation is thus responsible for the deactivation and enhanced excretion of numerous xenobiotics that would otherwise remain in the body and exert prolonged toxicity. One of the most important aspects to detoxication is stability of the final metabolic derivative. Breakdown of the glucuronide may lead to reformation of reactive electrophilic species, either the parent compound or the phase 1 metabolite. The stability of the conjugate is thus a function of the linkage to the functional group. Most commonly encountered glucuronides involves linkage of glucuronic acid and the xenobiotic through the oxygen atom. Typical *O*-glucuronides include aryl, alkyl and acyl compounds. Aryl-*O*-glucuronides are formed through an

ether bond and are highly stable in alkaline and acidic pH. Alkyl-*O*-glucuronides are ether-linked and are formed from a range of primary, secondary and tertiary alcohols. They are stable at physiological pH but may be hydrolyzed under acidic conditions. Acyl compounds form ester glucuronides from a variety of carboxylic acids including primary, secondary and tertiary aliphatic acids alongside both aryl and heterocyclic compounds. They are stable under acidic conditions but may be hydrolyzed in an alkaline environment. A severe lack of stability exists with *N*-glucuronides, especially at low pH and can thus be easily hydrolyzed. Uncommon group are the *S*-glucuronides, but they show similar stability as *O*-glucuronides [1, 4].

2.2.2.2 Sulfate Conjugation

Sulfate conjugates are sulphuric acid esters but have historically been named ethereal sulfates, as the belief was that these conjugates were ethers formed between aryl compounds and inorganic sulfate. The formation of sulfate conjugates is a common reaction to increase hydrophilicity for both endogenous compounds and xenobiotics in the human body. Sulfotransferase enzymes facilitate the conjugation through transfer of an active sulfate from 3'-phosphoadenosine –5'-phosphosulfate (PAPS) to a xenobiotic acceptor. These enzymes are found in not only the liver, but also in the kidneys, lungs and intestines and thus represent an important extra-hepatic detoxication mechanism. Conjugation with sulfate is a less predominant pathway due to the limited pool of sulfate that is readily exhausted. Figure 2.6 shows the general reaction scheme for sulfate conjugation [1].

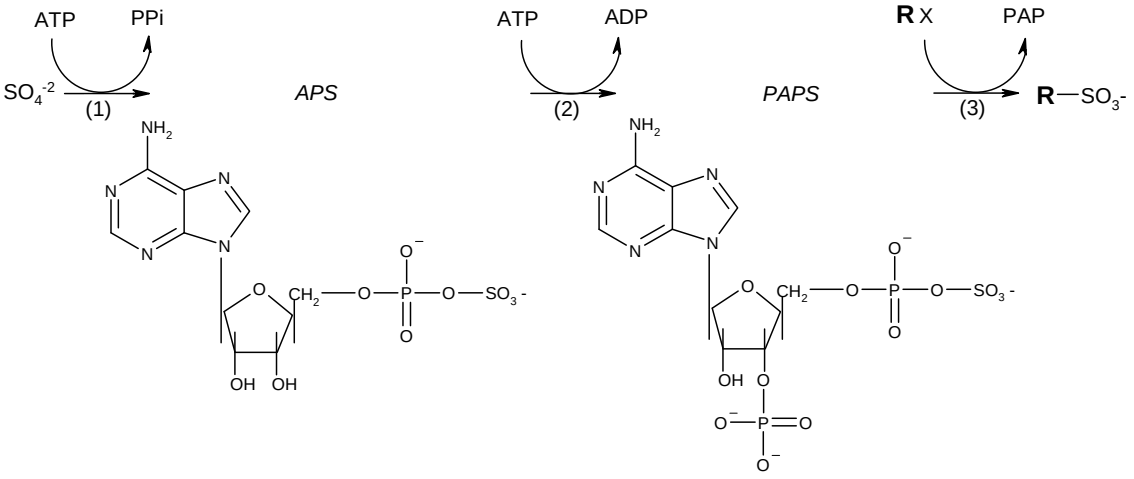


Figure 2.6 Sulfation reaction scheme: (1) ATP-sulfurylase; ATP, adenosine triphosphate; PPi, pyrophosphate; APS, adenosine 5'-phosphosulfate; (2) APS-phosphokinase; ADP, adenosine diphosphate; PAPS, 3'-phosphoadenosine 5'-phosphosulfate; (3) Sulfotransferase; PAP, 3'-phosphoadenosine 5'-phosphate; R, activated metabolite where X is OH, SO_2NH_2 or NH_2.

2.2.2.3 Amino acid and glutathione reactions

The reaction of an amino acid with a xenobiotic typically results in the synthesis of an amide conjugation product, either through the conjugation of a carboxylic acid-containing compound with the free amino group of an amino acid, or by the acytelation of a xenobiotic containing a primary amine. Amide synthesis for both pathways requires the participation of the active form of acetyl co-enzyme A (CoASH). Glycine is the most common amino acid and is able to form hydrophilic conjugates with aromatic, arylaliphatic and heterocyclic carboxylic acids that are readily excreted into urine or bile. Figure 2.7 outlines the general reaction scheme for amino acid reactions.

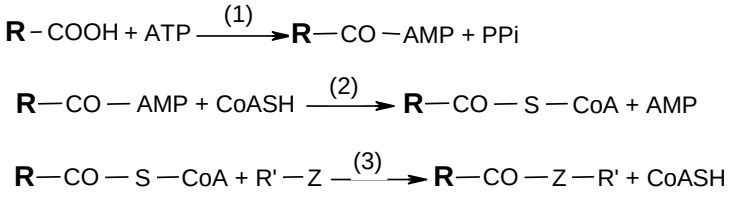


Figure 2.7 Amide synthesis reaction scheme: (1) Acyl-synthetase or thiokinase; (2) Acyl-thiokinase; (3) Transacylase; CoASH, acetyl co-enzyme A; R, activated metabolite with carboxylic functional group; R', amino acid with Z the NH_2 functional group.

Glutathione is synthesized in the cytosol of most cells through the γ-glutamyl cycle and comprises of three amino acids that enter the cycle from several biochemical pathways: cystine, glycine and glutamic acid. Glutathione *S*-transferases are enzymes located in the cytosol that catalyze the conjugation of glutathione with a substrate bearing an electrophilic atom. It is hypothesized that the enzyme increases the ionisation of the thiol group of glutathione, thereby increasing its nucleophilicity towards electrophiles [4]. This action protects other vital nucleophilic centers in the cell such as nucleic acids and proteins by creating a conjugate that is less lipophilic and likely to be excreted in urine or bile. Glutathione is also capable of reacting non-enzymatically with electrophilic sites on neighbouring macromolecules. Glutathione *S*-transferases also contribute to protection of critical cellular sites in two other ways. Firstly, it is capable of binding a xenobiotic to the enzyme surface. This binding may or may not inhibit the catalytic role of the enzyme, but it prevents the xenobiotic from interacting with any other molecules. Secondly, the enzymes can form covalent bonds between reactive xenobiotics and the active catalytic site. The binding inactivates the enzyme, but also inactivates the reactive xenobiotic. This is referred to as suicide inactivation.

Electrophilic carbon, nitrogen, sulfur and oxygen act as substrate for glutathione *S*-transferases. Reactions with electrophilic carbon include displacement of leaving groups (e.g. halides, sulfates, phosphates and nitro groups), opening of strained epoxide rings and addition to activated double bonds. Conjugation of epoxides to glutathione results in formation of hydroxymercapturic- or premercapturic acids that undergo dehydration to mercapturic acids. Mercapturic acids are thus *S*-derivatives of *N*-acetylcysteine. The general reaction scheme indicating the formation of mercapturic acids is shown in Figure 2.8. Mercapturic acid synthesis represents an important pathway in reducing toxicity from metabolically activated compounds that result from the phase 1 metabolism by cytochrome P-450 monooxygenase system. The more reactive epoxides appear to be better substrates for synthesis of mercapturic acid derivatives [1].

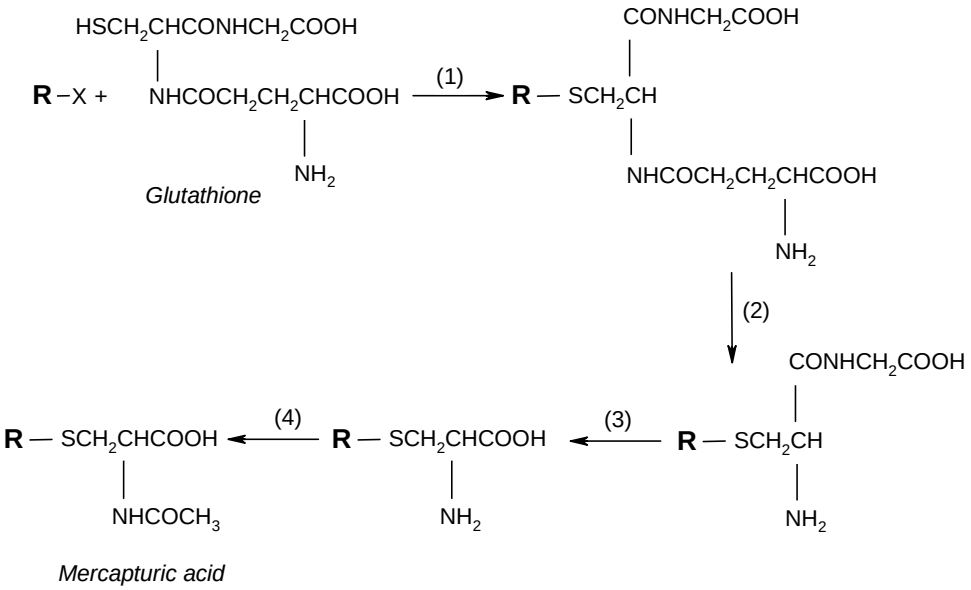


Figure 2.8 Mercapturic acid synthesis reaction scheme: (1) Glutathione *S*-transferase; (2) γ-Glutamyl transferase; (3) Cysteinyl glycinase; (4) Acetylase; R, activated metabolite where X is a strong electrophile.

2.2.3. Toxicity from metabolism

The complete mechanism of action of only a few toxic xenobiotics has been fully elucidated, yet the general consensus is that numerous toxic compounds have to be metabolized to reactive intermediates before their toxicity manifests. These reactive intermediates are mostly electrophiles such as epoxides or free radicals that are formed by the cytochrome P-450-monooxygenase system. Reactive intermediates tend to be oxygenated in sterically hindered positions that make them unacceptable substrates for subsequent detoxicating enzymes and this allows covalent interaction with intracellular macromolecules including DNA. This may lead to a number of toxicities such as carcinogenesis, mutagenesis, tissue necrosis and hypersensitivity reactions. Xenobiotic metabolism is a complex system involving multiple pathways for metabolic activation. Transport of inert metabolites to sites where innate instability of the derivative, enzyme action or microfloral metabolism causes reforming of the reactive intermediate is also a strong possibility. At these sites in different organ systems the necessary detoxication enzymes are not always present to remove the toxic compound.

Exposure to multiple xenobiotics is also possible, with subsequent competition for detoxication enzymes or depletion of co-substrates. Induction of enzymes in multiple exposure cases is another factor to consider. Numerous xenobiotics stimulate the activity of microsomal detoxication enzymes, thus altering the metabolism of other compounds. This may occur through increasing the kinetic rate of the enzymes, causing proliferation of the enzymes through *de novo* protein synthesis, or increasing the total amount of endoplasmic reticulum in the cell. Induction of enzymes may also lead to stimulation and enhanced production of endogenous compounds that compete for xenobiotic metabolising enzymes (e.g. steroids), thereby allowing toxic compounds to remain active for longer. The absolute increase in detoxication ability is also not necessarily a positive aspect, as this may lead to an absolute increase in reactive and possibly toxic intermediates. Both of the principle enzymes involved in phase 1 and phase 2 metabolism, cytochrome P-450-monooxygenase and UDP-glucuronosyltransferase respectively, are subject to induction by numerous xenobiotics. The complexities of metabolically induced toxicity are illustrated in Figure 2.9.

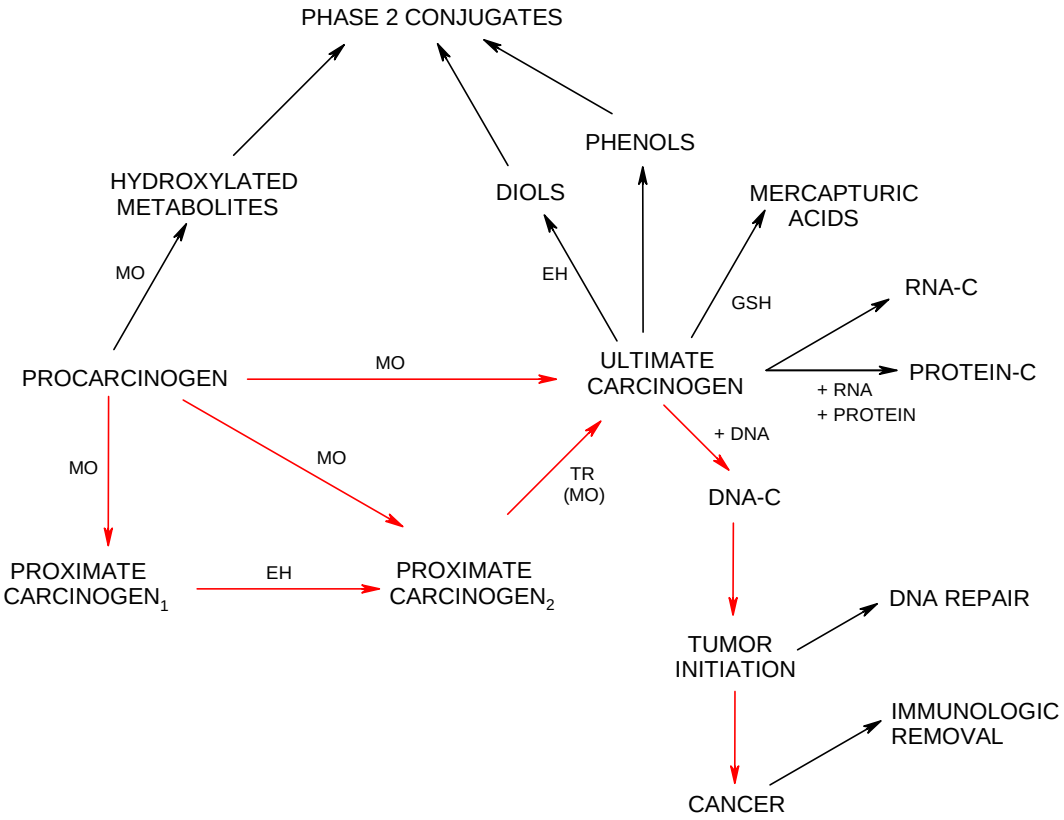


Figure 2.9. Metabolism of carcinogenic intermediates [4]. MO: cytochrome P-450 monooxygenase; EH: epoxide hydrase; GSH: glutathione; TR: transferases; DNA-C: DNA-carcinogen adduct.

The figure indicates possible chemical carcinogen metabolism, but the general scheme is applicable to other toxicities as well. The red arrows indicate reactions that would produce neoplasias. The black arrows show detoxication mechanisms that may occur on various levels. First of these is the creation of an inactive metabolite; second, the ultimate carcinogen may rearrange to be diverted from the critical macromolecule; third, the covalently bound molecule may be repaired; fourth, there may be immunologic removal of carcinoid cells.

2.3. Biological Monitoring

The metabolic fate of xenobiotics and the numerous possibilities for exerting toxic effects on the human body has been discussed in the previous sections. It is now necessary to understand more about how certain compounds can enter an individual's system and its disposition within that system, as this would affect the metabolism of that specific compound. Also, it is required that one is able to recognize the best approach in visualizing the biological activity exerted by the absorbed xenobiotic, because if one cannot visualize it through some analytical means then no discrimination of its effect is possible. Various techniques to achieve this are discussed in the following sections.

Homeostasis and the metabolism of xenobiotics establish the broad framework within which the process of monitoring human health takes places. Viewing specific chemical compounds, their interaction and effect, and perhaps most importantly how we can analytically ascertain their presence and quantities is key to our understanding and preservation of the health of an individual. The assessment of individual health status through a variety of analytical means is known as biological monitoring. The application of biological monitoring to industry for the benefit of both the employee and employer is known as occupational health monitoring.

2.3.1. Biomarkers

Exposure to a chemical compound may be defined as the concentration or amount of a chemical that reaches the organism [6]. Assessment of exposure is only

achieved through monitoring the subject that is being exposed. Monitoring is a complex activity that can occur at numerous levels. Measurement of the amount of a compound or pollutant in the general environment termed environmental monitoring. Contact with subject's physiological barriers is measured through personal monitoring (usually done by means of portable air sampling devices) and evaluation of the amount of a compound penetrating the body (bioavailability) is accomplished through biological monitoring. The International Union of Pure and Applied Chemistry (IUPAC) defines biological monitoring as follows: "Continuous or repeated measurement of potentially toxic substances or their metabolites or biochemical effects in tissues, secreta, excreta and expired air or any combination of these in order to evaluate occupational or environmental exposure and health risk by comparison with appropriate reference values based on knowledge of the probable relationship between ambient exposure and resultant adverse health effects" [7].

Indicators of exposure utilised in biological monitoring are referred to as biomarkers and are classified in three main groups. Biomarkers of exposure are concentration of substances, or their metabolites, in biological specimens (blood, urine, hair) or in an organ, cell or molecule. This type of biomarker indicates the occurrence and extent of exposure of a subject or a specific target structure. Biomarkers of effect reflect substance-subject interaction leading to biochemical alterations, with or without clinical expression. Biomarkers of susceptibility are biochemical indicators specific to an individual, reflecting sensitivity to an external challenge (e.g. enzyme activity), thus explaining variable inter-individual responses to exposure of similar substance levels [8]. Figure 2.10 summarizes the application of the various biomarkers in occupational health monitoring. Ambient monitoring includes personal and environmental monitoring. Health surveillance refers to both biomarkers of susceptibility and effect.

Biological monitoring of effect is complementary, even superior, to ambient monitoring as it is more directly related to the adverse health effects one attempts to prevent. It also takes into account uptake routes additional to inhalation, differences in workload and personal habits as well as use and efficiency of personal protective devices. Therefore, the individual uptake and its possible impact on a person's health can be estimated.

Various elements come into play in regards to the choice of a biomarker: specimen type and collection, specificity, inter- and intra-individual variability, stability

and presence of confounding factors, to name a few. Biomarkers of effect are also required to have biological significance; it must represent underlying biological interactions in a dose-dependent manner. To understand how the dose-response relationship occurs, the toxicokinetics of compounds with special reference to organic solvents are examined. The underlying principles are however similar when applied to pharmacology and may be interpreted with reference to ingested pharmacologically active substances as well.

2.3.2. Toxicokinetics

The relationship between exposure, uptake and the levels of the substance in blood, urine or tissues is determined by the kinetic behaviour of the particular organic solvent. The time course of the concentration of a substance in body fluids is a result of the dose and the basic kinetic process of absorption, distribution, and elimination, which may be via biotransformation (metabolism) and/or excretion (including exhalation). These processes are influenced by endogenous (e.g. genetic, phenotype, body mass, age, gender) and exogenous (e.g. workload, dietary habits, smoking) factors [9]. The rate at which compounds enter and leave the body is mainly a function of the partitioning of the compound between lipid and aqueous compartments in the body, which is in turn a function of the nature of the compound itself (degree of lipophilicity). Low concentration exposure and absorption follows first-order kinetics, an initial rapid increase that levels off gradually. The elimination shows a similar tendency, rapid initial decrease in concentration that slows down with time. When exposure is high and an eliminating process is saturated, the kinetics are independent of the degree of exposure and follow zero-order kinetics. Figure 2.11 illustrates both processes.

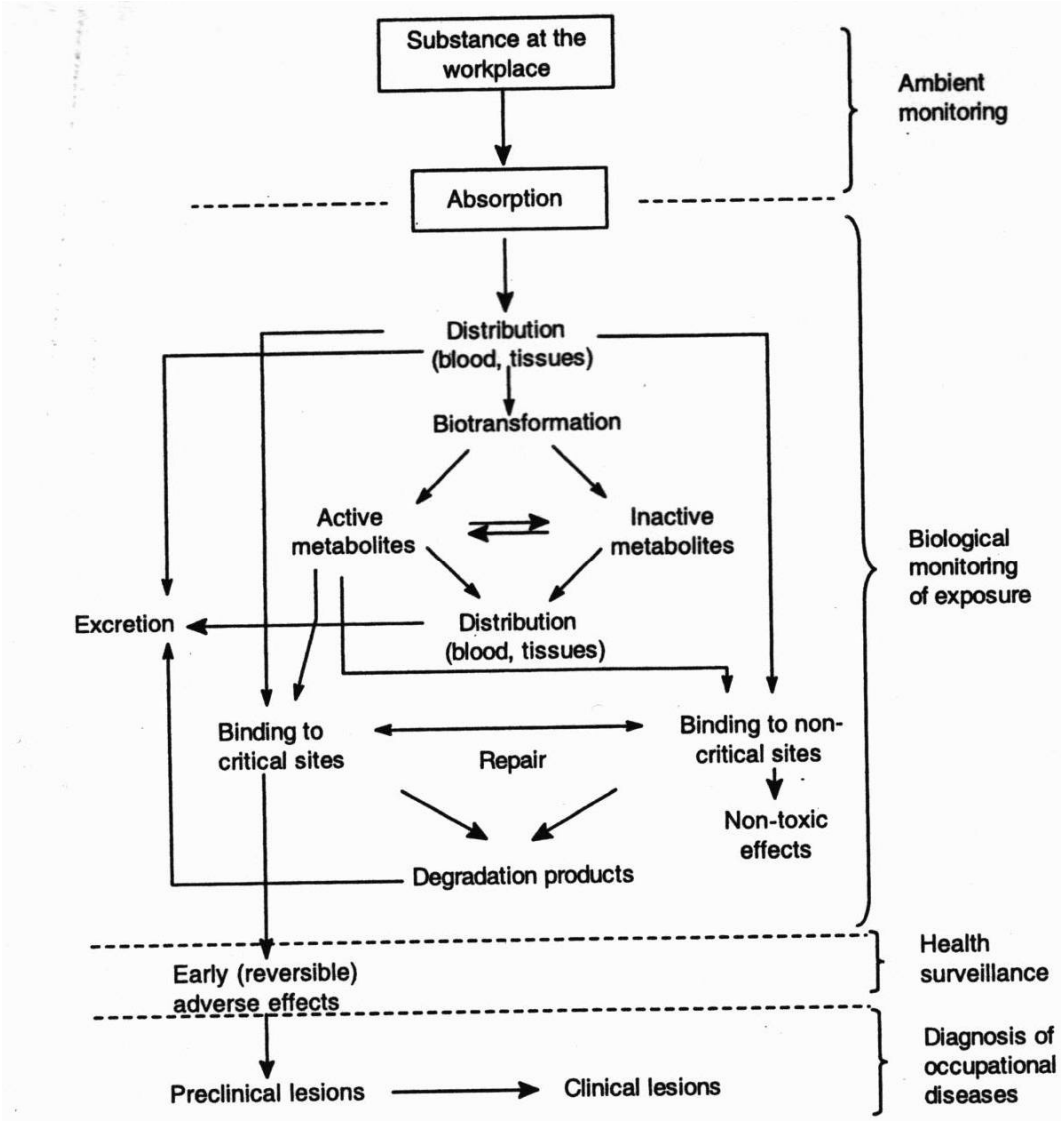


Figure 2.10 Biomarkers in biological monitoring [9].

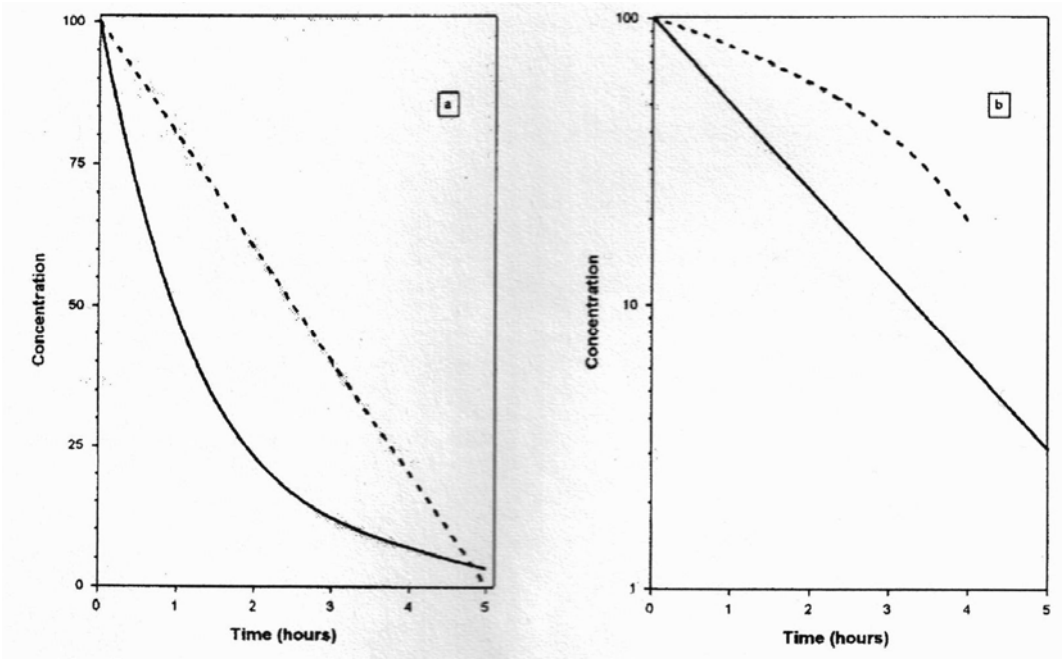


Figure 2.11 Elimination curves according to type of elimination kinetics. First-order kinetics is indicated by the unbroken line and zero-order kinetics by the broken line in (a) normal and (b) logarithmic scale.

In first-order kinetics the concentration decline is described by an exponential function with the rate constant, k_e, indicating the fraction of the compound in the body removed per unit of time. The biological half-life, $t_{½}$, of a substance in an organ, tissue or body fluid describes the time required to reduce the biological concentration by half. Half-life is inversely proportional to the elimination constant:

$$k_e = \ln 2 / t_{½} \qquad \text{(Eq. 2.1)}$$

The overall time a compound remains in the body is thus the result of several half-lives corresponding to the elimination from different compartments in the body. Half-life is an important factor in determining the sampling time. Table 2.4 indicates the effect of half-life on sampling time. Half-life is the most useful parameter to describe the elimination process. As indicated by the decay curve in Figure 2.11 different half lives can be observed, reflecting fractions from different body stores: an initial fast release from blood (minutes), an intermediate phase in muscle (minutes to hours) and a longer phase from fat (hours to days). Extent and length of exposure affect elimination kinetics, a longer more intense exposure will deposit more compound in the adipose compartment [10].

Half-life	Reflection of sampling time
< 2 hours	Recent rate of absorption
2 – 10 hours	Exposure during the day- sampling end of shift/beginning of next shift
10 – 100 hours	Exposure during week- sampling end of shift /work week

Table 2.4 Reflection of sampling time as a function of half-life of compounds [11].

2.3.2.1 Absorption

Absorption is the process of transfer of a compound from the site of exposure into the blood, most commonly through the airways or across the skin. Lipophilicity, water solubility and protein binding influence the absorption of a compound taking into account the body's composition of water, lipids and proteins. Absorption from the lungs is usually fast because of the large surface area, thin alveolar membrane that needs to be crossed and rich blood vessel perfusion. Absorption is much slower across the skin, having to cross numerous dermal layers. The kinetic parameter that defines the absorption rate, k_a, is the absorption half-life and the parameter that describes the amount absorbed is called bioavailability, F.

The introduction of organic solvent vapour into the lungs results in a rise in alveolar concentration of the substance, with a subsequent rise in its partial pressure. The balance between the ventilatory input and uptake in pulmonary blood governs the alveolar concentration of a substance. The uptake into pulmonary blood is a function of the compound characteristics (blood/air partition coefficient), the individual (workload) and environment (ventilation and circulation). Gas uptake increases in relation to the magnitude of the gas partial pressure difference between alveoli and pulmonary venous blood. When the alveolar-venous gas partial pressure difference equals zero, gas uptake from the lungs ceases. The uptake after reaching the steady state for benzene, toluene and xylene is between 50-70% of the total amount available [12].

Absorption across the skin occurs in mainly two ways: transepidermal, which is through the epidermal cells, and appendageal, which is through the hair follicles and sebaceous glands. The appendageal route offers greater permeability and plays an important role in the diffusion of ions and polar non-electrolytes, however the

transepidermal route, which occurs by passive diffusion, is more prominent [13]. Percutaneous absorption results from skin contact with a solvent, with the amount being absorbed dependent on the surface area of skin, properties of the compound and the constant of permeability, K_p. The rate of absorption in mg $cm^{-2}h^{-1}$ for benzene, xylene and toluene is 0.4, 0.12 and 0.6 respectively [14, 15].

2.3.2.2 Distribution

Distribution is the process of transfer of compounds from the blood into body tissues or body compartments. The processes involved are described by distribution rate constants and volume of distribution, V_d. V_d refers to the total amount of substance in the body relative to the concentration in blood, derived from dividing the dose by the determined concentration in blood or plasma. It can also be defined as the capacity of a compartment to retain a compound. Once in blood, volatile substance is lost primarily by distribution into tissues and metabolism.

2.3.2.3 Elimination

Metabolism and excretion are the two main routes of elimination in the human body. As described in section 2.2, metabolism eliminates a substance through biotransformation into a different chemical compound, the metabolite. Metabolites themselves may undergo further biotransformation or be excreted unchanged. Excretion of parent compounds or metabolites occurs through body fluids, mainly urine but also faeces and expired air. The elimination is described by the rate constant, k_e. Lipid-soluble compounds are reabsorbed in the renal tubules and are poorly excreted. Only 1% of volatile aromatic compounds are found unchanged in urine [16]. The main function of metabolism is to transform lipophilic compounds into more polar forms that may be readily excreted by the kidneys, through mechanisms described previously. Depending on the mechanism of excretion (glomerular filtration, tubular excretion or diffusion), the elimination of urinary metabolites are related to excretion of solids as a function of normal catabolic metabolism, and may be adjusted to creatinine excretion, urine density or urine volume.

2.3.3. Specimen type

The typical exposure profile of an individual occupationally exposed to organic solvents is described as an initial increase in the absorbed parent compound, followed by a steady state of that unchanged compound in the various body fluids. After the end of exposure the concentration of the parent compound decreases due to elimination by excretion and metabolism according to the various toxicokinetic parameters mentioned earlier. The decrease of the parent compound causes an increase in the appearance of the metabolite. It is thus key to determine which biological fluid and which compound in a specific fluid, the unchanged parent compound or biotransformed metabolite, to utilize for the purposes of biomonitoring. In the application of monitoring for illicit substances, additional forensic considerations have to be considered. These considerations are detailed in Chapter 6.

2.3.3.1 Parent compound in blood

Determination of unchanged organic compounds in blood is very specific due to the fact that the majority are not physiologically present in any of the body fluids. The central nervous system (CNS) consists of a large amount of lipids and lipoproteins. This fact causes the CNS to be an initial target organ for absorbed organic solvents that have a high degree of lipophilicity. Concentration of the parent compound in blood indicates individual internal dose and potential CNS toxicity. A great advantage of utilizing parent compounds in blood for biomonitoring is that nearly all absorbed organic solvent compounds can be analysed by the same method, due to their natural volatility (head-space analysis combined with gas chromatography-mass spectrometry). This allows simultaneous detection of all volatile organic solvents of interest in a single blood specimen. The main drawback however lies in the dependence on the fast elimination kinetics of most organic compounds. For elimination half-lives of minutes to a few hours it is essential that specimen collection occurs in a highly specific time period. Three general approaches are adhered to: collection during exposure, collection at the end of exposure, and collection at a defined time period after end of exposure. The first approach is susceptible to a high degree of fluctuation due to a host of practical problems such as hygienic and work disruption considerations. Collection at a defined

period after exposure is largely influenced by factors such as body fat, since these specimens represent release from the fat compartment. Collection at the end of exposure (end of shift) is normally used for compounds such as benzene, toluene and xylene, possessing half-lives of a few hours.

2.3.3.2 Parent compound in urine

Kinetic influences for unchanged organic solvent excretion in urine are distinctly lower than that of blood. Excretion of organic parent compounds is related to the urine/blood partition coefficient as well as urinary volume. Urinary concentrations of compounds are intrinsically linked to kidney function, drinking habits, sweating and so forth. To compensate for this, adjustment to urine density or creatinine content is recommended [17, 18]. It is suggested that urine specimens with extreme relative densities (higher than 1.030 or less than 1.002) or creatinine concentration less than 0.5 g/L or higher than 2.5 g/L should be excluded from analysis in order to prevent misinterpretation of results (e.g. in the case of impaired renal function). Positive analytical aspects for the use of urine specimens are: non-invasive specimen collection (compared to venipuncture in blood collection), minor kinetic influences and simultaneous quantification of mixture of compounds in a single urine sample. Negative aspects include the following: small percentage of lipophilic compounds excreted in urine, increased analytical requirements to detect these low levels, high probability of sample loss during pre-analytical phase and more complex sample handling [19].

2.3.3.3 Metabolites in blood

Metabolites formed in blood are not generally used for biological monitoring. The concentrations of the compounds to be measured are usually very low and other options such as measuring the parent compound in urine or blood, or measuring the metabolite in urine are usually more attractive. In a few cases metabolite levels in blood may be more relevant for biological monitoring than those in urine. An example of this is dichloromethane exposure, where carbon monoxide is formed as a metabolite in blood. The amount of carboxyhemoglobin is then measured to indicate the internal dose of dichloromethane in the body.

2.3.3.4 Metabolites in urine

Urinary excretion of metabolites may be a superior parameter of biological monitoring, especially if relevant toxicity of exposure to a compound is caused by the metabolite or its precursor and not by the unchanged parent compound itself (this is the case for benzene exposure, as will be discussed in Chapter 5). It may also be considered a more integrative approach for exposure to organic compounds compared to measurement of the unchanged compound in blood or urine, due to the longer half-lives of many of the principle metabolites as well as the urine sampling period. Concentrations of compounds in blood only reflect exposure level shortly before specimen collection. Requirements for use of metabolites in urine are that they must be in sufficient quantities to be detected and must be strongly correlated to exposure of different levels of the parent compound, thus significantly exceeding background levels in a non-exposed population. Metabolite levels are also less susceptible to analyte loss and contamination during pre-analytical phase and clean-up than unchanged compounds. Kidney function and creatinine adjustment are also applicable in the case of urinary metabolites. All the positive analytical aspects discussed in section 2.3.3.2 for collection of urine specimens as opposed to blood are also valid for measurement of metabolites in urine. From a purely analytical standpoint, urine also presents a much simpler matrix from which target compounds can be isolated. There are no proteins and other blood constituents that may interfere in the analysis of a target analyte. A major drawback is possible unspecificity of a metabolite, due to an observed high background level in non-exposed individuals or because the metabolite is formed by exposure to several independent compounds [20]. Table 2.5 indicates the specificity of some urinary metabolites for exposure to aromatic hydrocarbons.

Compound	Urinary metabolite	Metabolite specificity	Remark
Benzene	phenol	low	Diet, metabolite of Aromatic amino acids
	trans, *trans* muconic acid	medium	Metabolite of sorbic acid
	S-phenylmercapturic acid	high	
Toluene	hippuric acid	low	Diet, sodium benzoate
	ortho-cresol	high	
Xylene	methylhippuric acids	high	
Ethylbenzene	mandelic acid	medium	Exposure to styrene

Table 2.5 Specificity of urinary metabolites of selected aromatic hydrocarbons applied for biological monitoring [20].

2.3.3.5 Other

Measurement of unchanged organic solvents in expired air is another analytical strategy that is sometimes followed [21]. Similar to urine specimen collection, it too is a non-invasive procedure. It has the specificity of measuring parent compounds in a very clean matrix, air. Similar to parent compounds in blood, simultaneous detection of all volatile organic solvents of interest in a single specimen can occur. A number of drawbacks exist, the largest of which is practical pre-analytical difficulties. Sampling, shipping and storage of alveolar air all pose a problem, which may give rise to significant error in analysis. Altered pulmonary function in individuals can lead to systematic errors in the specimen collection process that cannot be eliminated through improved analytics. Excretion in expired air is as highly affected by toxicokinetic influences as blood specimens, thus sampling time is of the essence and protocol must be strictly adhered to. Measurement of parent compounds in blood is preferred to measurement of parent compounds in expired air due to the aforementioned reasons.

The use of organic solvent adducts to macromolecules, such as proteins, are also used in some instances for biological monitoring purposes. This is a fairly new approach that has been traditionally limited by the type of instrumentation utilized in analysis (typically gas chromatography-mass spectrometry). Progress in this field (liquid chromatography - tandem mass spectrometry) will probably ease the analytical requirements for quantifying adducts and in all likelihood assist in establishing links

between protein adduct level and subsequent health risk, especially for compounds suspected of carcinogenicity.

2.4. References

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Chapter 3

QUANTITATIVE ANALYTICAL METHODOLOGIES AND INSTRUMENTATION

3.1. Introduction

"Give me a lever long enough and a fulcrum on which to place it, and I shall move the world." (Archimedes, 287 - 212 BC)

The ability of humans to negotiate their way in the world is ultimately dependent on the information taken up by the various senses, and the processing of that information to form a concrete concept of the objects they come into contact with. Identification proceeds through recognition of colour, shape, scent, sound and so forth. In the same manner it is necessary for the analytical chemist to be able to visualize compounds in some way in order to identify and quantify them. This may be done through a direct process, e.g a precipitation reaction followed by weighing the precipitate, if the amount of substance to be identified is adequately large, or through certain indirect means, where a generated signal or other type of indicator that is somehow related to the appearance of an amount of target compound, is monitored. In order to achieve such visualisation it is obviously necessary to know what methods are available and more importantly, which ones are conducive to gaining an accurate and reliable answer with regards to the specific compound one is attempting to analyze. The old vantage of 'the right tool for the right job', as illustrated by the abovementioned quote, applies here. In fact, the quote illustrates three of the most important aspects applicable to any analysis: knowledge of the goal that needs to be achieved, knowledge of what techniques are available, and most importantly knowledge of how to correctly apply them.

Analytical methodologies vary in the amount of dimensions applied to detect and then quantify a compound. The first dimension may be defined as detecting or quantifying a compound as a result of some unique characteristic of that specific compound, which is then amplified and exploited through some means, e.g. a visible colour reaction in the presence of another chemical.

The second dimension may include another, perhaps different, property of the compound that further separates it from other similar compounds, followed by detection in the same manner as the first dimension. These dimensions may increase stepwise for as many properties of a compound one can ascertain that would exploit its own uniqueness. The more dimensions added to a technique, the greater the certainty of the answer from the sum of various measurements. This gives rise to the measurement characteristic known as the selectivity of an analysis: the degree to which an analytical method is able to determine exclusively the analyte it claims to measure in the presence of other related substances. Another important consideration in analysis is the ability of the detection system to reflect various degrees of analyte abundance as determined by the exploitation of a characteristic of a compound. This is termed the sensitivity of an analysis and is defined by IUPAC as "the slope of the calibration curve and the ability of an analytical procedure to produce a change in the signal for a defined change of the quantity" [1]. Simply put, sensitivity is the change in detector response, relative to a change in quantity, concentration or property of the analyte. It must be stated that although selectivity and sensitivity are largely a function of the type of measuring instrument used and detection system selected, that the whole process of sample preparation (discussed in Chapter 4) must also be taken into account to determine the validity of the entire quantitative method. The third consideration that determines the appropriateness of a quantitative methodology is that of detection limit, defined by IUPAC as "the smallest concentration of an analyte that can be detected with reasonable certainty for a given analytical procedure" [1]. Detection limit is dependent on sensitivity and selectivity of the complete analytical method.

In this chapter, different analytical methodologies utilizing a multitude of detection systems are discussed alongside the requirements for a method to be considered quantitative in analytical toxicology.

3.2. Non-chromatographic methods

Detection systems that may be utilized as first dimension systems i.e. utilizing only a single characteristic of a compound for identification and quantification, are discussed in this section. As will be indicated, certain types of these systems may also

be combined with separation techniques for additional selectivity, so called hyphenated methods such as gas chromatography-mass spectrometry.

3.2.1. Optical spectroscopy

Optical spectroscopic devices are based on the absorption of electromagnetic radiation (ER). The simplest of these instruments is the colorimeter, where the human eye serves as a detector through comparison with known standards. A spectrophotometer is another widely used instrument that measures absorption, usually in the ultra-violet (UV) range [2].

Absorption spectroscopy has been used due to the wide application of species that absorb UV light and the ease and convenience of modern instruments. Sources of inaccuracy is amongst others the scattering of light in the transmittance pathway and the limitation of Beer's law at higher concentrations, where absorbing species interact with each other and thus affect each others energy states and subsequently absorbance at a specific wavelength.

Infrared (IR) spectrophotometry (absorbance in the infrared region of ER) may be considered the most powerful spectrophotometric technique, as the absorbance spectra obtained through this method may be used to deduce the actual structure of an unknown compound. Samples must not contain a lot of other compounds in this methodology though, because of the greater complexity of the spectra obtained and the possibility of contamination.

3.2.2. Electrochemistry

Electroanalytical techniques are quantitative analytical methods, based upon the electrical properties of a solution of the analyte, when it is made part of an electrochemical cell. The two main techniques utilized are coulometry and voltammetry [2].

Coulometry requires that a current be developed in a cell that contains the analyte, and the analyte is quantitatively converted through electrolysis to a new oxidation state. The amount of charge required to achieve this new oxidation state is measured and thus related to the amount of analyte that was converted.

Voltammetry is an electrochemical method based upon the measurement of current as a function of the potential applied to a microelectrode. This normally occurs under conditions that encourage the polarization of and indicator electrode.

Electroanalytical methods are fairly widely applicable in terms of the compounds one is able to analyze. It also has the advantage of being exceptionally sensitive and moderately selective, which translates into possibly very low detection limits. The biggest disadvantage is the fact that the sample compound is in direct contact with the detector surface, which may give rise to various adsorption phenomena and memory effect. Both the methods described in this section may be combined with certain chromatographic and non-chromatographic separation techniques.

3.2.3. Mass spectrometry

Mass spectrometry may be considered the most widely applicable of all the detection systems in analytical chemistry [3]. It is capable of providing information about the structure of inorganic, organic and biological molecules similar to IR-spectroscopy, but has the main advantage of analysing complex mixtures. A mass spectrometer is an instrument that separates rapidly moving ions on the basis of their mass-to-charge ratios, *m/z*. Molecular weights are expressed as atomic mass units, *amu*, and the mass-to-charge ratio of a compound is calculated by dividing the mass, *m*, by the number of charges it gains when ionised, *z*. Thus, for a compound such as benzene carrying a single charge the equation is: $^{12}C_6{}^{1}H_6{}^{+}$, 78.112/1 = 78.112 *m/z*. Most ions are singly charged, and the mass-to-charge ratio is simply referred to as mass, while the *m/z* value is usually rounded to a whole number precision, termed the nominal mass. For benzene, $^{12}C_6{}^{1}H_6{}^{+}$, the nominal mass would thus be 78 *amu*, or more correctly 78 *m/z*. Another powerful characteristic of mass spectrometry is the ability to resolve isotopes even in low resolution instruments. Benzene, with a single ^{13}C substitute, would thus be 1 *amu* higher and have a mass-to-charge ratio of 79. This is sufficient to allow for very precise quantitative determinations of target analytes utilizing their stable isotope substituted analogs in a technique known as isotope dilution mass spectrometry. The modern mass spectrometer consists of five main components: a vacuum system to maintain low pressure environments required for ion movement and integrity; an inlet system to allow compounds into the system directly or to act as interface in hyphenated

instrumentation; an ion source to generate electrons and ionise analytes; a mass analyzer to separate ions according to *m/z* values; and a detector to visualize generated signals. In this section, the fundamentals of mass spectrometry together with the main types of mass spectrometers routinely utilized, is discussed with reference to their main differences and similarities in ion source and mass analyzer components.

3.2.3.1 Ionisation techniques

Mass spectrometers are maintained at a high vacuum (10^{-5} to 10^{-8} torr) to allow ions to move predictively through a pathway without interaction with atmospheric compounds. The high vacuum thus determines that analytes that enter the source must be in a gaseous state to allow ionisation. This requirement in turn determines the two main categories within which various ionisation sources may be classified. Gas-phase sources are those that first vaporize and then ionise analytes, while desorption sources convert analytes in a solid or liquid state directly into gaseous ions. Gas-phase sources are restricted to thermally stable compounds and molecular weights in the order of 10^3 *amu*, while desorption sources, requiring no sample volatilization, are applicable to thermally labile compounds and molecular weights of up to 10^5 *amu*. Furthermore, ionisation techniques are also grouped as hard or soft. Hard ionization techniques pass on enough energy to leave analyte molecules in highly excited energy conformations, subsequently followed by relaxation that involves bond breaking which then produces abundant fragment ions. In contrast, soft techniques impart little energy to an analyte and yield a low degree of fragmentation. Hard ionization result in decreased sensitivity, but the mass spectrum contains a large amount of structural information useful for the identification of unknowns. Soft ionization normally only provides information about the molecular ion, but results in better sensitivity than hard ionization. Table 3.1 shows the most common ion sources utilized in mass spectrometry [4].

Basic category	Name	Ionising agent
Gas-phase	Electron impact (EI)	Energetic electrons
	Chemical ionisation (CI)	Reagent gas ions
Desorption	Electrospray ionisation (ESI)	High electric field
	Matrix assisted desorption/ ionisation (MALDI)	Laser beam
	Atmospheric pressure chemical ionisation (APCI)	Corona discharge

Table 3.1 Common ion sources for mass spectrometry.

Electron impact (EI) ionisation is currently still the most widely utilized ion source. Samples are brought to a temperature high enough to produce a vapour that is ionised by a beam of energetic electrons. The electrons are emitted form a heated tungsten filament and accelerated by a potential between the filament and an anode. The potential is usually 70 eV but may be adjusted (usually not lower than 50 eV) depending on the requirements of the analyte, i.e. the energy can be decreased as to have less fragmentation occur. The path of the electrons and the molecules perpendicular and intersect in the centre of the source where collision and ionisation occur. The primary reaction in EI is thus : $M + e^- \longrightarrow M^{\bullet +} + 2e^-$

where M = target molecule

$M^{\bullet +}$ = molecular ion

The molecular ion is a radical ion with the same molecular weight as the original molecule. Ions produced in EI are both positive and negative, but only positive ions are allowed to be transmitted. Table 3.2 indicates the typical reactions that occur in EI ionisation.

Reaction type	Reaction mechanism
Molecular ion formation	$ABCD + e^- \rightarrow ABCD^{\bullet+} + 2e^-$
Fragmentation	$ABCD^{\bullet+} \rightarrow A^+ + BCD^\bullet$
	$ABCD^{\bullet+} \rightarrow A^\bullet + BCD^+ \rightarrow BC^+ + D$
	$ABCD^{\bullet+} \rightarrow AB^+ + CD^\bullet \rightarrow A^+ + B$
	$ABCD^{\bullet+} \rightarrow AB^+ + CD^\bullet \rightarrow A + B^+$
	$ABCD^{\bullet+} \rightarrow AB^\bullet + CD^+ \rightarrow C + D^+$
	$ABCD^{\bullet+} \rightarrow AB^\bullet + CD^+ \rightarrow D + C^+$
Rearrangement/Fragmentation	$ABCD^{\bullet+} \rightarrow ADBC^{\bullet+} \rightarrow AD^+ + BC^\bullet$
Collision/Fragmentation	$ABCD^{\bullet+} + ABCD \rightarrow (ABCD)^{\bullet 2+} \rightarrow BCD^\bullet + ABCDA^+$

Table 3.2 General reaction mechanisms in EI ionisation [3].

Ionisation as a result of ion-molecule collisions (last equation in Table 3.2) may produce fragments at higher nominal mass numbers than that of the molecular ion. The most significant of these reactions is when a hydrogen atom is transferred to the ion to yield a protonated molecular ion $(M + 1)^+$. This is commonly known as a quasi-molecular ion. Although one of the greatest advantages of an EI ion source is the extensive fragmentation that occurs, allowing unambiguous identification of analytes, the ionisation efficiency is generally low with approximately one molecule in a million being ionised. Samples are also required to be thermally stable and sufficiently volatile to allow ionisation to occur [3, 4].

Chemical ionisation is likely to be the second most common procedure for producing ions in mass spectrometry. A reagent gas is introduced into the ionisation region where the ratio of reagent to analyte is in the order of 10^4, to allow near exclusive reaction of the ion beam with reagent molecules. Subsequent collisions between analyte molecules and high-energy reagent molecules yield analyte ions through mainly proton transfer or proton abstraction, or hydride transfer. Negative and positive ions are produced, and depending on the requirements of the analysis and the nature of the instrument, both positive-ion chemical ionisation (PI-CI) or negative-ion chemical ionisation (NI-CI) mass spectra may be obtained. This type of ion source is much softer than traditional EI and normally yields strong molecular or quasi-molecular ions in relative high abundance, but not extensive fragmentation. It thus allows for generally more sensitive analysis with lower detection limits, but not unambiguous identification [5].

The aforementioned gas-phase ion sources are well suited for volatile compounds, or hyphenated techniques where compounds that pass through the separation phase are not in a liquid or solid state (e.g. gas chromatography-mass spectrometry).

The most common desorption ion source, utilized for thermally labile or non-volatile compounds as well as in hyphenated techniques where compounds are in a liquid state, is undoubtedly electrospray ionisation (ESI). Shortly, a solution of analytes is delivered through a capillary needle at low flow rates, which is maintained at several kilovolts by a cylindrical electrode that surrounds the capillary. The result is a fine spray of charged analyte-solvent droplets. Evaporation of the solvent occurs to an extent where the charge density becomes so great that a coulomb explosion occurs and ions enter the rest of the pathway. This is also a soft ionisation technique that results mostly in quasi-molecular ions, but may also form further adducts with e.g. metal ions present in the solvents [6].

3.2.3.2 Mass analyzers

The output of any ion source is a stream of gaseous ions that are subsequently accelerated into a mass analyzer by applying lens voltages. Requirements for the mass analyzer include the ability to distinguish between minute mass-to-charge differences (resolution, *R*) and allow passage of sufficient numbers of ions to yield measurable ion currents (sensitivity). The most common mass analyzer in mass spectrometry is the quadrupole mass spectrometer. Figure 3.1 illustrates the basic components and function of a quadrupole mass spectrometer, as employed in the study.

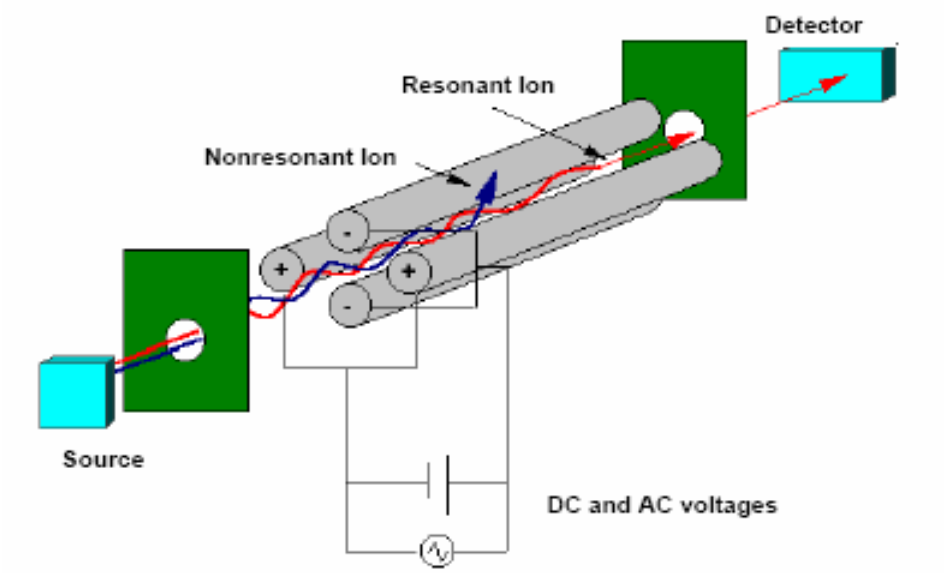

Figure 3.1 Basic components and function of a quadrupole mass spectrometer.

Opposite rods are connected electrically, one pair attached to the positive side of a variable direct current (DC) source and the other pair to the negative. The polarity of each rod pair is constantly alternated by variable high radio-frequency (RF) alternating current (AC) potentials that are 180 degree out of phase with respect to each other. Ions are accelerated into the space between the rods by an applied potential (5-10 V) while the AC and DC voltages on the rods are increased simultaneously at a constant ratio. All ions apart from those with specific mass-to-charge ratios strike the rods and are converted to neutral molecules, thus becoming non-resonant and exiting the pathway [3-6]. Resonant ions with a limited range of *m/z* values reach the detector. As indicated by Figure 3.2, ions move through the quadrupole pathway in what is generally accepted as an oscillating movement according to the alternating RF values. Continuous variation in AC and DC voltages at a constant ratio allows for full scan mass spectra to be obtained, i.e. a range of ions with increasing m/z values is allowed to fall onto the detector. The detector in most quadrupole mass analyzers consists of a electron multiplier tube that amplifies the signal to a digital system connected to the instrument.

Application of preset voltages for a set period of time allows for monitoring of only resonant ions for those preset values, i.e. only specific ions fall onto the detector. This selected ion-monitoring mode (SIM) is therefore a more sensitive instrument detection mode suitable for precise quantification.

Quadrupole mass analyzers easily resolve ions that differ by 1 *amu*, and also have the advantage of high scan rates (entire mass spectrum obtained in less than 100 ms) as well as robustness [3-6].

Time-of-flight, magnetic sector and ion trap mass analyzers are not as common as quadrupole instruments, each one having its own advantages and disadvantages. In time-of-flight instruments positive ions are produced periodically by bombardment of the analytes with brief electron pulses. Ions produced in this way are then accelerated by a high voltage electric field pulse that has the same frequency of the ionization pulse, but lags behind it. All ions ideally have the same kinetic energy and pass into a field-free drift tube, and their respective velocities in the tube are inversely related to their masses. Time-of-flight analyzers have greater resolution than quadrupole instruments and an almost limitless mass range [3-6].

Magnetic sector instruments are largely utilized for exact mass analysis, but are fairly costly and difficult to maintain. Ions are accelerated through a slit into a curved electrostatic field that focuses the beam of ions onto another slit containing a curved magnetic field. In this field the ions with the lowest *m/z* values are deflected most and the heaviest the least. The dispersed ions are usually detected by a photomultiplier tube or electrooptical linear array detector in these types of instruments [3-6].

Ion trap analyzers are less costly than both magnetic sector and quadrupole types, but have limited mass range. Ions are confined by electric or magnetic fields and further collisions may be induced to create unique fragments from the parent ion. Consecutive fragmentation filters out all ions that are not being scanned for.

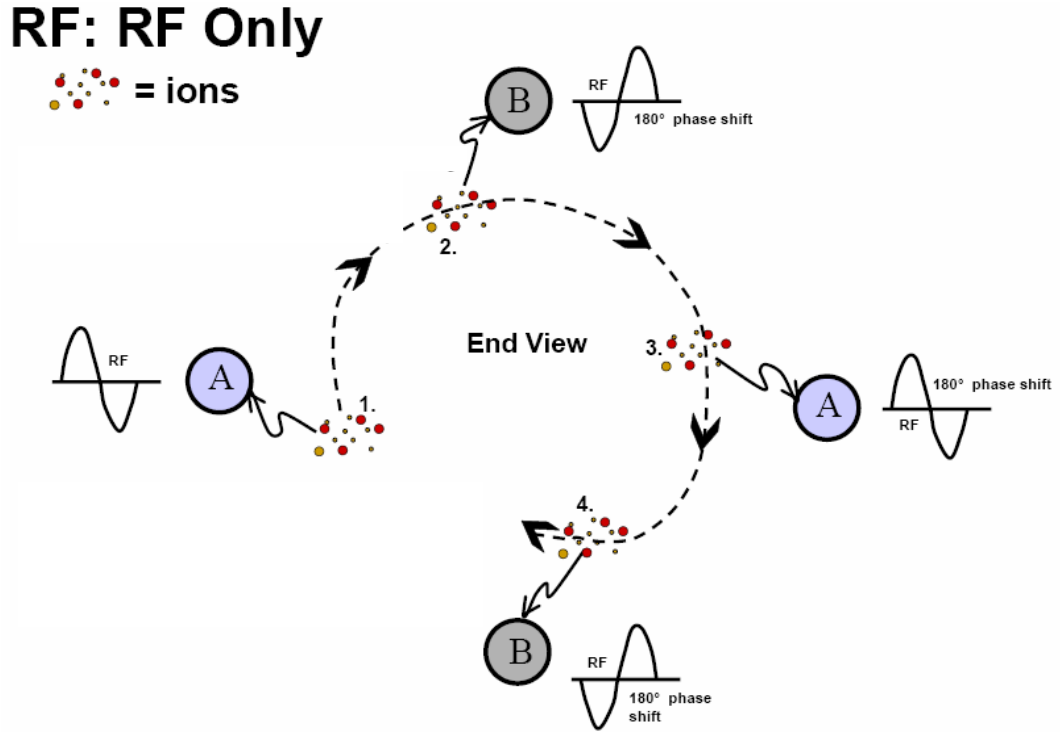

Figure 3.2 Movement of ions through quadrupole. 1.All ions attracted to A pole; 2.RF voltage reversed, all ions attracted to B pole; 3. RF voltage reversed, all ions attracted to A pole; 4. RF voltage reversed, all ions attracted to B pole.

Any of the aforementioned mass analyzers may be coupled to separation techniques described in the following sections to create the so-called hyphenated methods. Although separation of compounds in space and time, as is the case for chromatography, before detection by mass is a powerful analytical tool, separation in only the mass dimension may sometimes be better. This may be achieved by coupling mass analyzers in series, resulting in tandem mass spectrometers. Similar to ion trap analyzers, where ions are put through a continuing process of mass filtration and fragmentation, tandem mass spectrometers may filter out a range of *m/z* values in the first mass analyzer, fragment those ions again and detect the daughter ions from the original parent ion. Conventional chromatographic separation in space and time may take from minutes to hours, whereas separation in only mass takes a few microseconds. Tandem mass spectrometers are fairly costly however, and when a complex matrix is involved, as is the case with biological samples, it is still preferred to employ a good separation method such as chromatography [4].

3.3. Chromatographic methods

Chromatogrophy (Greek, chroma "colour"; graphein "write") is a powerful separation method invented and named by the Russian botanist Mikhail Tswett (1872-1919), employing the technique to separate various plant pigments such as chlorophylls and xanthophylls by passing solutions through a glass column packed with fine calcium carbonate [7]. The separated analytes appeared as discreet coloured bands on the column, thus accounting for the chosen name of the method. Current chromatography encompasses a host of diverse analytical techniques to selectively separate closely related components of complex mixtures that cannot be achieved through classical methods such as precipitation, distillation and extraction. In all chromatographic methods there are two phases involved. Analytes are transported by a mobile phase, which may be a gas, fluid or supercritical fluid, while interacting with a stationary phase. The stationary phase usually has a different polarity or is immiscible with the mobile phase and is fixed in a column or on a solid surface. The two phases are chosen specifically to allow analytes to distribute themselves between them to variable degrees, depending on the chemistry of the analytes themselves. Components with more interaction with the stationary phase are termed to be retained by the stationary phase, while compounds that spend all their time in the mobile phase are said to be unretained. Column elution chromatography involves continuous addition of mobile phase to allow all retained compounds to elute from the stationary phase and be detected or collected at some predefined endpoint. Based upon the types of mobile and stationary phase involved and the nature of the equilibria involved, the current section focuses on two distinct categories of column elution chromatography: gas chromatography (GC) and high performance liquid chromatography (HPLC or LC). General chromatographic theory and practise of both methods are discussed alongside the common detection systems that are normally linked to these techniques.

3.3.1. Applied chromatography theory

A chromatogram is the visual record of the detector response as a function of elution time or volume. A series of peaks is plotted where the positions of the peaks on the time axis serves to identify the individual components of a mixture, and the areas

beneath the peaks provide a quantitative measure of the amount of each component [8, 9, 10]. An important parameter in all forms of chromatography is the equilibrium constant, K_p, also known as the distribution or partition coefficient and defined as

$$K_p = C_s / C_m \qquad \text{(Eq. 3.1)}$$

C_s= molar concentration in stationary phase

C_m= molar concentration in mobile phase

Conditions where the above equation applies results in Gaussian type peaks and is called linear chromatography. Conditions that lead to non-linear behaviour and consequently distorted peak shape will be considered further on in this chapter. The time taken for a peak to reach the detector after application onto the column is called the retention time, t_R. The time taken for elution of a peak that is not retained by the stationary phase at all, is termed the dead time, t_M. The dead time is similar for all analytes and is related to the average rate of movement of the mobile phase or linear flow rate, u. The rate of solute migration is not only dependent on the linear flow rate of the mobile phase, but also the retention factor. The measure of interaction with the stationary phase is termed the capacity factor. The capacity factor, k', is defined as follows:

$$k' = t_R - t_M / t_M \qquad \text{(Eq. 3.2)}$$

$t_R - t_M = t'_R$, the adjusted retention time

$$k' = t'_R / t_M \qquad \text{(Eq. 3.3)}$$

If the capacity factor equals four, it implies that an analyte will spend four times longer in the stationary phase than in the mobile phase. It follows that at any given stage, four times the amount of analyte is present in the stationary phase opposed to the mobile phase. Capacity factor, k', can be related to the distribution constant, K_p, since the amount of analyte in any given phase and the concentration in that phase are related through volume. The equation for capacity factor may therefore be written as

$$k' = K_p V_s / V_m \qquad \text{(Eq. 3.4)}$$

K_p= distribution coefficient

V_s= Volume stationary phase

V_m= Volume mobile phase

Another important parameter when multiple components are separated is the selectivity factor of a column, α. The selectivity factor refers to the inherent ability of a chromatographic column to discriminate between two analytes and is related to the capacity factor for two analytes A and B as follows [8, 9, 10]:

$$\alpha = k'_B/k'_A \quad \text{(Eq. 3.5)}$$

or $$\alpha = t'_{RB}/t'_{RA} \quad \text{(Eq. 3.6)}$$

B= strongly retained species

A= weakly retained species

Chromatography columns are considered to consist of a number of theoretical adjacent zones in which there is sufficient space for complete equilibration of an analyte between the two phases, each zone termed a theoretical plate, *N*. The length of column containing one theoretical plate is called the height equivalent to theoretical plate or plate height, *H*. The two terms are both a function of the total column length, *L*, and are related to each other through the equation [8, 9, 10]:

$$N=L/H \quad \text{(Eq. 3.7)}$$

Efficiency of chromatographic columns will increase as the theoretical plate count becomes greater, achieved by either increasing the total length of the column or by decreasing the height equivalent to one theoretical plate.

The success of any chromatographic separation is defined by its ability to resolve one analyte from another in a complex mixture. Resolution, R_s, is a function of column efficiency, selectivity factor and capacity factor according to the equation:

$$R_s=(\sqrt{N}/4)(\alpha-1/\alpha)(k'/1+k') \quad \text{(Eq. 3.8)}$$

or $$N=16R_s^2(\alpha-1/\alpha)^2(k'/1+k')^2 \quad \text{(Eq. 3.9)}$$

To describe the contribution of each of the aforementioned parameters to a successful analysis, the specific chromatographic technique utilised, i.e. gas chromatography or high performance liquid chromatography needs to be viewed individually. Gas chromatography will mainly be discussed in this work and it is out of scope to enter in-depth into the theory of high performance liquid chromatography [11].

3.3.2. Gas Chromatography

As the name suggests, a gaseous mobile phase is employed in gas chromatography to separate sample components introduced into the chromatographic system. Due to the inert character of the gas used as mobile phase (helium, hydrogen or nitrogen), there is no interaction between analytes and the mobile phase, it only acts as transport medium through the column. Only compounds in the vapour phase can be

separated by GC and usually under elevated temperatures to ensure a sufficiently high vapour pressure of each sample constituent. Based upon the character of the stationary phase, GC can be subdivided into gas-solid chromatography (GSC) and gas-liquid chromatography (GLC).

Gas-solid chromatography is based upon a solid stationary phase where adsorption of analytes induces separation of sample components. The most common use of GSC is with porous layer open tubular (PLOT) columns for determination of low molecular weight compounds such as air components and other gasses.

Gas-liquid chromatography is currently the most commonly used technique and is based upon the partition of analytes between the gaseous mobile phase and a liquid phase that has been immobilized on the surface of an inert solid. Reference to gas chromatography usually refers to gas-liquid chromatography (GLC is simply known as GC).

Traditionally packed columns with internal diameters greater than 2 mm and containing stationary-coated particles of 100 to 250 µm in diameter have been used, but have recently been largely replaced by more efficient and faster open tubular columns. More specifically, fused silica capillary columns have come into use, for most applications, due to their ability to reduce analysis time. Figure 3.3 indicates the structure of a common fused silica capillary column.

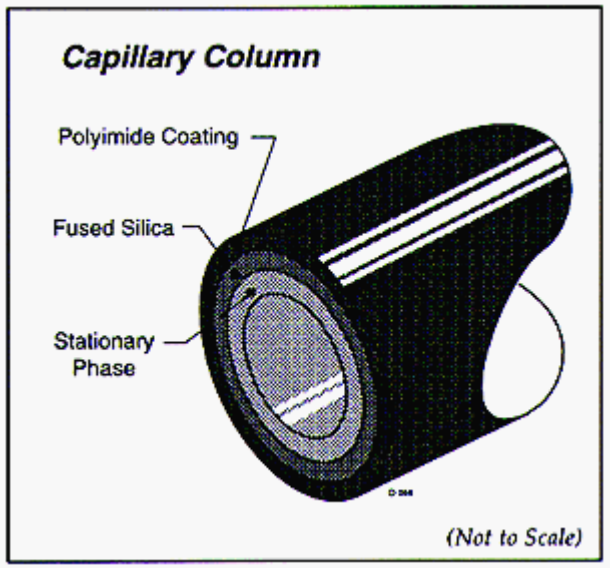

Figure 3.3 Basic components of a fused silica capillary column.

Capillary columns can facilitate faster analysis times without loss of resolution. To illustrate how this is achieved the theory of band or zone broadening must be explored. This also assists in understanding conditions that lead to non-linear chromatography, which is unfavourable regarding resolution and quantitation of compounds using GC.

3.3.2.1 Kinetic theory of chromatography

A series of replicate measurements, whose values are plotted against their frequency of occurrence, give rise to a normal error or Gaussian curve. The Gaussian shape is a result of an amount of uncertainty equal to the total amount of infinite small, random and undetectable uncertainties connected with any single measurement. These uncertainties have an equal likelihood of being positive or negative, and by chance largely cancel each other out across the spectrum of measurements, thus giving rise to a mean value. The final result is a series of measurements that are slightly more/slightly less than the mean value, but with a symmetrical spread around the mean value.

A single solute molecule travelling through a column undergoes many thousands of transfers between the mobile phase and stationary phase. The time spent in either phase is unequal for each transfer and depends on the molecules gaining or losing sufficient thermal energy to complete a reverse transfer. The variance in resident time in any given phase at any given point in time affects the average rate at which an individual molecule migrates down the column, relative to the average rate of movement of the mobile phase. In other words, certain molecules travel rapidly due to chance inclusion in the mobile phase for longer periods of time, while others seem to lag behind due to incorporation into the stationary phase for longer periods [10].

Due to the variance created by these random individual processes for each molecule, a symmetric spread of velocities around the mean value is obtained for a specific analyte migrating through a column. This symmetrical spread is similar to that of a Gaussian curve and an analyte is thus eluted and passed through a detector in a discreet band or zone with distinct Gaussian characteristics. Figure 3.4 illustrates this process.

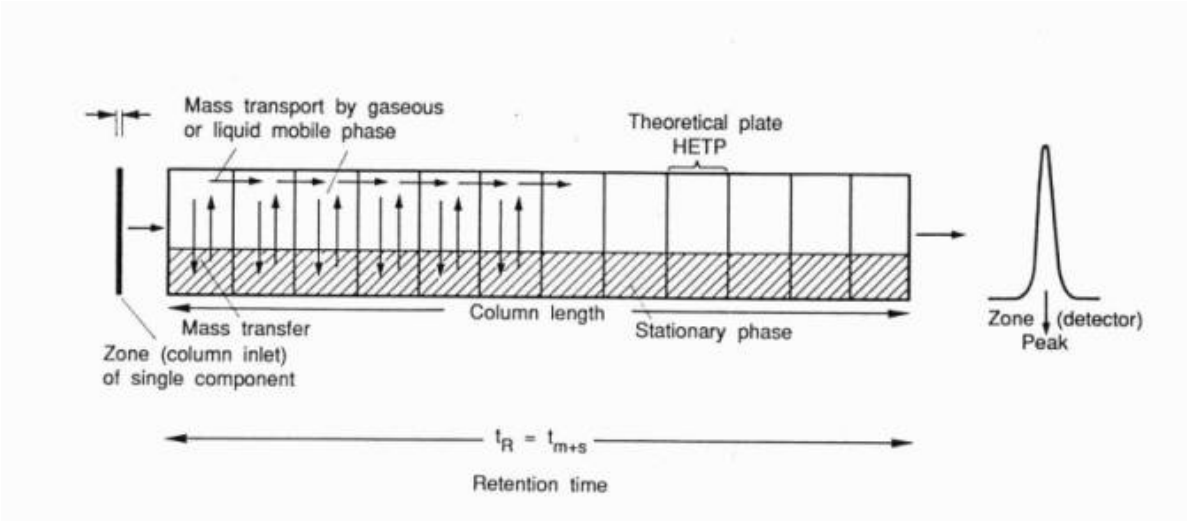

Figure 3.4. Mass transfer and transport in column elution chromatography.

The rate at which the several mass-transfer processes can occur while migrating down a column is limited, thus the narrow band that initially enters the chromatographic system is broadened since more time is allowed for spreading to occur with each mass transfer.

The rates that govern the mass-transfer processes can be adjusted by altering certain experimental variables, which in turn influences the degree of zone broadening and the speed of separation. An efficient separation results in narrow bands passing the detector. From Equation 3.7 in section 3.3.1, $N=L/H$, it is noted that increase in N results in increased efficiency. Thus, a decrease in the plate height, H, will yield an increase in plate count and result in more efficient chromatography. Due to the fact that the band breadth is directly related to column residence time and inversely related to mobile phase velocity, the H-value as a function of the carrier gas velocity, u, gives a measure of the zone spreading. The mathematical relation H-u was derived for packed columns by Van Deemter, Zuiderberg and Klinkenberg [12] in the 1950's and is known as the van Deemter equation:

$$H = A + B/u + (C_S + C_M)u \qquad \text{(Eq. 3.10)}$$

The terms are defined in Table 2.3. The equation was further simplified and adapted by Golay [12] for open tubular capillary columns:

$$H = B/u + (C_S + C_M)u \qquad \text{(Eq. 3.11)}$$

Table 3.3 Kinetic processes contributing to zone broadening [2].

Process	Equation term	Relation to column property
Multiple flow paths	A	$A = 2\lambda d_P$
Longitudinal diffusion	B/u	$B/u = 2\gamma D_M/u$
Mass transfer to and from stationary phase	$C_S u$	$C_S u = (f_S(k')d^2_f / D_S)u$
Mass transfer in mobile phase	$C_M u$	$C_M u = (f_M(k')d^2_c / D_M)u$

u: Linear velocity of mobile phase

D_M, D_S: Diffusion coefficient in mobile and stationary phase, respectively

k': Retention factor (section 3.3.1)

d_c: Diameter of column

d_f: Film thickness of stationary phase

λ, γ: constants dependent on packing quality

$C_S u$, $C_M u$: Mass transfer coefficients in stationary and mobile phase, respectively

It is beyond this scope of the current work to elaborate on all the terms in the van Deemter equation, but in short it is as follow.

A is the coefficient of zone broadening related to multiple pathways by which a molecule can migrate through a packed column. It is referred to as eddy diffusion and is directly proportional to the particle diameter the packing consists of. It is absent in capillary columns.

B is the coefficient of zone broadening due to longitudinal diffusion of molecules from the concentrated centre of a band to the more dilute regions ahead and behind the zone centre. This diffusion occurs parallel to the mobile phase movement and is inversely proportional to the mobile phase velocity. From Equation 3.11 and Table 3.3,

$$B = 2\,D_m \qquad \text{(Eq. 3.12)}$$

C is the mass transfer coefficients for the mobile and stationary phase, respectively, which is related to zone broadening due to resistance to mass transfer from on phase to another. In practise the equilibrium between the mobile phase and stationary phase for any analyte molecule happens so slowly that a column is always operated under non-equilibrium conditions. Molecules at the front of a band are swept ahead before they have time to equilibrate with the stationary phase, while molecules at the trailing edge are left behind by the fast moving mobile phase. Thus, the faster the mobile phase moves the less time exists for equilibrium to be approached, and so mass-

transfer broadening effect is then directly proportional to mobile phase velocity. From Equation 3.11 and Table 3.3,

$$C = (1/8)(d_c^2/D_m) \qquad \text{(Eq. 3.13)}$$

where $k' \geq 3$ and C_S is negligible (thin film column).

All these terms can be illustrated by the so-called van Deemter plot, from which the optimum velocity for greatest efficiency for a specific chromatographic column can be derived. Figure 3.5 illustrates van Deemter plots for a packed and a capillary GC column. The deflection of the $C.u$ line, i.e. the $(C_S + C_M)u$ term in the equation, is much less for the capillary column than the packed column. This is partly because it is an example of a van Deemter plot for a thin film column, and so the C_S term becomes negligible [12]. Correct selection of film thickness, d_f, and column diameter, d_c, for capillary columns allows one to keep the ratio of mobile gas phase to liquid stationary phase, simply termed the phase ratio, at a point where resistance to mass–transfer in the stationary phase is infinitely small and thus negligible, as is the case with thin film columns. From the graphs it can be noted that for the same separation the analysis time with a capillary column could be approximately three times faster (greater u_{opt}) with better efficiency (lower H_{min}) than a packed column.

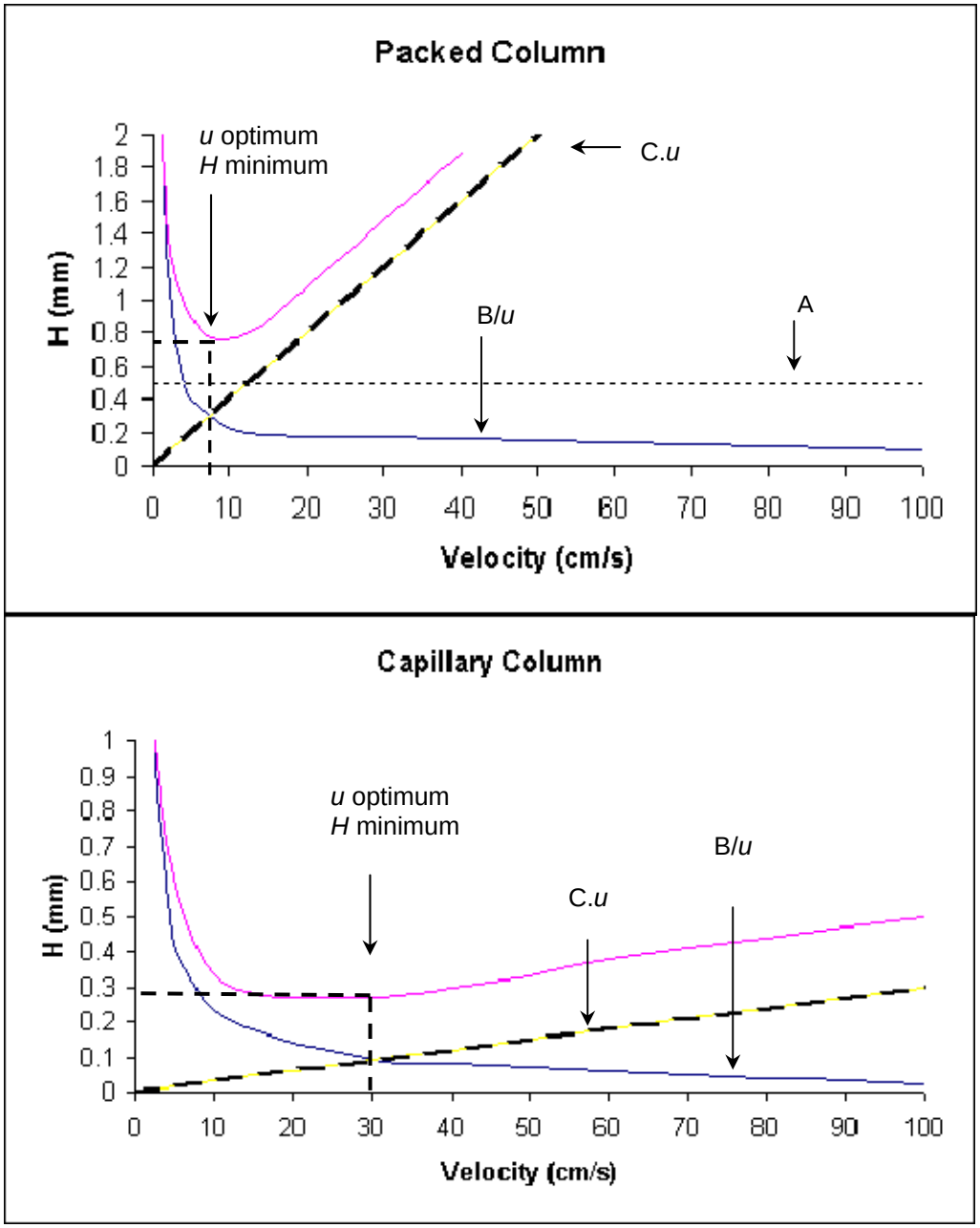

Figure 3.5. Van Deemter plots for packed and thin film capillary GC columns. C.*u* refers to the term (C_S + C_M)u in the van Deemter/Golay equation.

3.3.2.2 Fast chromatography

The practical selection of a column for fast and efficient GC separation may be performed considering the information contained in the van Deemter/Golay equation. The greatest efficiency (H_{min}) and fastest elution time with minimal loss of efficiency (u_{opt}) may be viewed by factoring Equation 3.12/3.13 into Equation 3.11. The following relationships are therefore obtained:

$$u_{opt} = \sqrt{B/C}$$

therefore $$u_{opt} \approx 4(D_m/d_c)$$ (Eq. 3.14)

$$H_{min} = 2\sqrt{B/C}$$

therefore $$H_{min} \approx d_c$$ (Eq. 3.15)

Taking a closer look at Equation 3.14, it is noted that the velocity may be increased while maintaining a fairly constant column efficiency with the selection of carrier gas such as He or H_2 (faster diffusion in mobile phase, D_m increase) and reduction in column diameter (u_{opt} inversely proportional to d_c). With a given retention factor for a stationary phase ($k' \geq 3$) the efficiency is also proportional to column diameter (Equation 3.15, H_{min} decreases as d_c decreases). Taking all of these relations into account the use of thin-film, small diameter, open tubular capillary columns would seem a good option for the fastest and most efficient chromatographic separation. So-called microbore capillary columns ($d_c < 0.2$ mm) may be utilised for this purpose [13].

A quantitative chromatographic separation for a multi-analyte mixture will usually have a critical separation pair, i.e. two compounds that elute close together but requires sufficient resolution for accurate quantitation. Resolution and analysis speed are usually opposing considerations, thus a sufficient resolution may be defined that allows the fastest possible chromatographic separation to occur. An *Rs* value of 1 or greater is considered acceptable for quantitative work [14, 15], however *Rs* values above 1.5 are considered over-resolved and may unnecessarily increase elution time. The following equation can be used to identify all chromatographic parameters that may be utilised to increase analysis speed at a fixed resolution [15]:

$$t_R = L/u(1+k')$$ (Eq. 3.16)

t_R = Retention time

L = Column length

u = Carrier gas velocity

k' = Capacity/retention factor

The retention time of a compound is directly proportional to column length, L. The reduction in column length is therefore the simplest way to increase analysis speed (halving length halves analysis time). Resolution may be slightly affected by reduction in column length as plate number, N, is also reduced by shortening a column. This is usually negligible in microbore columns as halving the diameter easily doubles the plate

number per column length unit. Also, as indicated by Equation 3.8, the R_s is only reduced by $\sqrt{N}$.

The retention time of a compound is indirectly proportional to the carrier gas flow rate, u. Increasing the flow rate past u_{opt} reduces analysis time but decreases column efficiency as the theoretical plate height is increased beyond H_{min}. However, as indicated by the van Deemter plot for a capillary column in Figure 3.5, an increase of u=2 u_{opt} (i.e. 30cm/s to 60 cm/s) will cause a marginal decrease in column efficiency in the case of microbore capillaries. The use of a carrier gas (He, H_2) with high diffusivity for the solute in vapour phase will further aid in maintaining good column efficiency.

Lastly, the reduction of retention factor, k', will reduce elution time for a given analyte. This can be done by selecting a different type of stationary phase (solute interacts less), a thinner column film (reduce d_f), or by rapid temperature programming. Increased temperature translates to increased kinetic energy for solutes, and thus less retention time in the solid phase.

3.3.2.3 Non-linear chromatography

The kinetic theory of chromatography assumes that a linear relation exists for partition of molecules between the mobile phase and stationary phase. This linear partition isotherm, illustrated in Figure 3.6(A), results in symmetrical Gaussian type peaks. Deviation form linearity has a major influence on peak shape [9].

A convex isotherm is known as the Langmuir adsorption isotherm and causes characteristic tailing of a band, shown by Figure 3.6 (B). This may be due to the stationary phase surface being covered in a monolayer of analyte molecules and subsequent interaction with the stationary phase is less strong, resulting in break through. As the total quantity of analyte molecules increase the fraction in the mobile phase increases and thus the areas of highest concentration migrate with the greatest velocity. The fast moving, high concentration centre catches up with the slow moving front and moves far ahead of the slow moving tail section of the band. The higher the concentration the easier one moves into the non-linear region of the isotherm.

A concave isotherm, also known as anti-Langmuir isotherm, gives rise to a band with a leading edge or fronting peak, indicated in Figure 3.6 (C). This is normally due to concentration overload or if the analyte is very different in terms of polarity regarding the stationary phase.

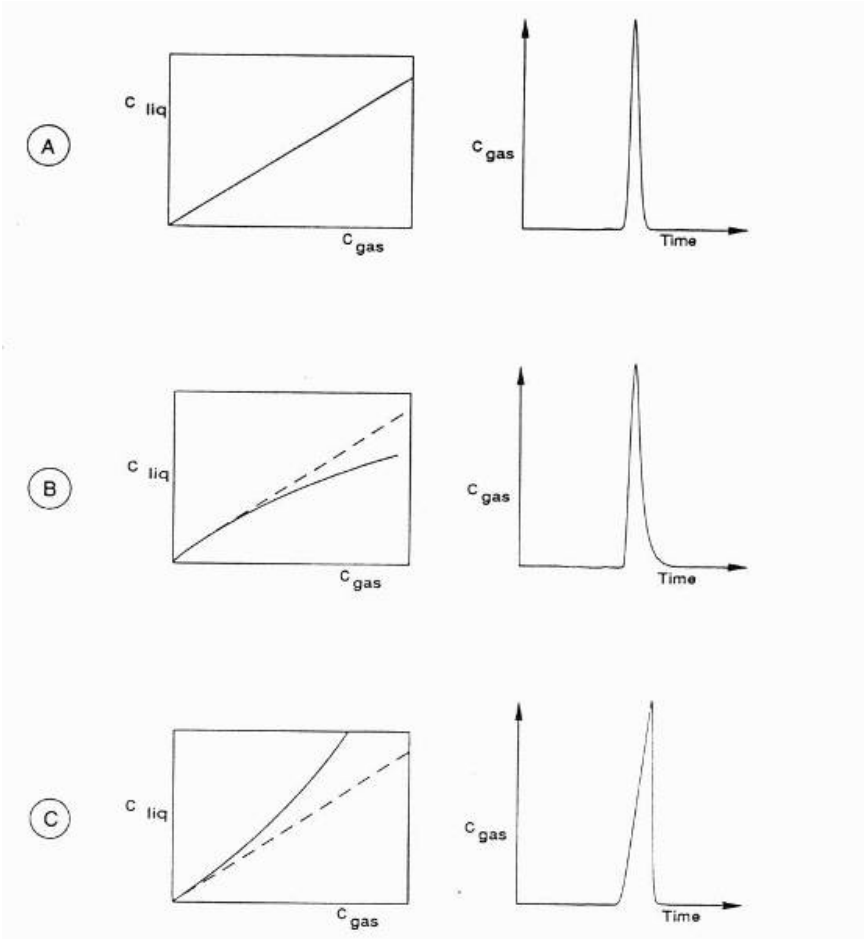

Figure 3.6. Influence of partition isotherm shapes on band symmetry.

The reduced capacity of microbore thin film capillary columns (e.g. dc < 0.2 mm; d_f < 0.25 µm) easily results in non linear chromatography when the volume applied to the column is too great (anti-Langmuir). The available stationary phase active sites are reduced in thin film columns and secondary retention mechanisms within complex mixtures applied onto the column may also arise (Langmuir).

3.3.2.4 Sample introduction in Gas Chromatography

The introduction of a sample onto the chromatographic column as a discreet band is required to further ensure that the minimum broadening of symmetrical Gaussian type peaks occur upon elution. Modern GC instruments are equipped with automatic injectors that introduce samples directly into a heated injection port (vaporization chamber), where vaporization occurs and the flow of carrier gas pushes the vaporized molecules onto the stationary phase of the column. See Figure 3.7.

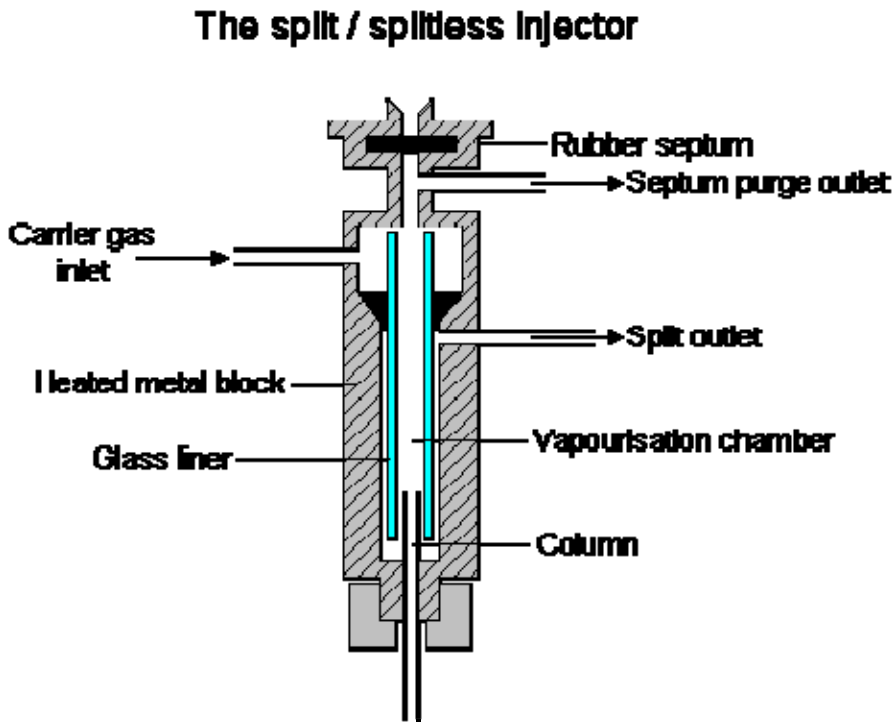

Figure 3.7. Simplified diagram of a modern split/splitless injector.

The total volume of carrier gas flowing through the injection port at the time of sample vaporization may be channelled onto the column for a set period of time (after which it is purged), resulting in splitless injection. Since the column opening is the only exit of the vaporizing chamber during the set time period, virtually all sample material is introduced onto the stationary phase, therefore resulting in greater analytical sensitivity. However, parameters such as column temperature, purge time, and solvent type/volume (for liquid sample introduction) need to be carefully selected to reduce band broadening effects [9].

During split injection the vaporized sample is divided into two parts that may be adjusted by differing split ratios. One portion is pushed onto the stationary phase by carrier gas flow (as in splitless injection), whereas the other portion is concurrently diverted and vented through a split outlet located in the injection chamber. The proportion of vaporized sample which enters the column usually lies between 0.3% - 50% of the total introduced sample volume, depending on the selected split ratio. It is therefore not recommended for very sensitive trace analysis. Narrow initial bands are ensured in split injection through the small volume of vapour introduced on-column and may largely be utilised regardless of parameters such as column temperature and solvent type [9].

In routine analysis split injection is the simplest and most common sample introduction technique. Where increased sensitivity is required, splitless injection is most often utilised. There is however a wider variety of sample introduction techniques available, with cold on-column injection and programmed temperature vaporizing injection (PTV) becoming increasingly popular for selected applications. Considering factors such as physicochemical properties of analytes under investigation (e.g. thermal stability), sample matrix (e.g. headspace or liquid), solvent systems, analytical sensitivity, instrument costs and so forth, any of the sample introduction techniques may be applicable.

3.3.2.5 Detectors in Gas Chromatography

Many detectors utilised in gas chromatography are merely class-specific with regards to chemistry. Only the hyphenated techniques, gas chromatography-mass spectrometry (GC-MS) and gas chromatography-infra red spectrometry (GC-IR), have the power to elucidate structure of a compound without use of a pure standard. All other detection systems can only produce the retention time of a compound, which then is compared to that of an injected pure standard that may identify the compound. The ideal detector in gas chromatography must display certain characteristics: adequate sensitivity, stability and reproducibility, good dynamic range (linear response over several orders of magnitude), high reliability and ease of use and maintenance, non-destructive of sample. No single detector can fulfil all of the above requirements and depending on the analytical demands of a certain analyte, the correct detector must be selected. Mass spectrometry is a near universal detector and due to the different types commercially available fulfils most of the requirements of an ideal detector, even though it is destructive toward the sample [7]. It is also completely amenable to fast chromatography, as compounds that might be poorly resolved chromatographically may be resolved in mass. Other common GC detectors are the flame ionization detector (FID), electron capture detector (ECD) and nitrogen-phosphorus detector (NPD).

3.4. Quantitative analysis in clinical toxicology

The information age has ushered in the expectation of accurate and immediate results at the snap of a finger or press of a button. The requirements for clinical laboratories has accordingly shifted in recent years to increased sample throughput and decreased turn-around time. Although the appearance of sophisticated hyphenated instrumentation in clinical laboratories has increased (particularly GC-MS) to accommodate the need for complex analyses, the routine application is still limited in most instances. Fully automated electrochemical, immunochemical and spectrophotometric instrumentation is available for the most commonly requested analyses for example serum electrolytes, cholesterol, glucose etc. Instrumentation such as GC-MS cannot compete in these cases due to the manual nature of sample preparation (Chapter 4), data analysis and interpretation.

In a clinical toxicology setting, however, the myriad of active xenobiotics and their metabolites that have to be isolated from complex biological matrices make it impossible to have separate instrumentation for each analysis. The only viable option is therefore the aforementioned hyphenated techniques. The lower cost and robust nature of quadrupole GC-MS instruments have made them the most popular choice in recent years. Optimisation of all parameters for a specific analysis (extraction, instrumental analysis, data analysis, reporting) is essential to meet the requirements of increased sample throughput and decreased turn-around time similar to automated techniques.

Development of new analytical methods from existing scientific literature or even novel concepts is commonplace in the clinical toxicology setting. The data generated by any method must have the highest degree of certainty. This is especially true in analytical toxicology, where important legal and clinical decisions are often based solely on a single analysis. Correct decisions can only be made from reliable data which in turn stems from reliable analytical methods. Validation is the only objective measure available to demonstrate the quality of analytical methods by fulfilment of established minimum acceptance criteria. This serves as proof that a newly developed method is fit for its intended purpose, and that data generated from that method may be used with the greatest amount of certainty available.

Analytical methods in clinical toxicology are aimed towards screening and identification of drugs, poisons, and their metabolites in biological fluids and tissues and

their quantification in these matrices. Quantitative bioanalytical procedures require evaluation of the following parameters: selectivity, calibration model (linearity), stability (processed, freeze-thaw), accuracy (bias), precision (repeatability, intermediate), and the lower limit of quantification (LLQ). Additional parameters that may be considered include recovery, reproducibility, ruggedness, and limit of detection (LOD) [16,17].

3.4.1 Selectivity

Selectivity has been defined as "the ability of the bioanalyitcal method to measure unequivocally and to differentiate the analyte(s) in the presence of components which may be expected to be present. Typically these might include metabolites, impurities, degradants, matrix components etc." [17]. Specificity is sometimes used interchangeably with selectivity, although the former refers to a response generated for a single analyte, whereas selectivity refers to a response for a number of distinguishable compounds, as is the case for multi-analyte methodologies.

The absence of response in blank matrix for monitored signals of the analytes of interest and internal standards is regarded as the best approach to prove selectivity in analytical toxicology. This is achieved through analysis of blank matrix from at least six different sources, although recently it has been suggested that ten to twenty sources may be required [18]. The blank specimens may not be pooled immediately, as the dilution effect might hide possible interferences. Spiking blank samples with the highest expected concentrations of suspected interferences is further recommended.

Analysis of authentic specimens that contain suspected interfering compounds should be performed where possible, as the interfering component and its metabolites, that may not be commercially available for spiking purposes, are present. Care should be taken in the use of stable isotope-substituted internal standards in isotope dilution strategies. These analogues ideally compensate for variation in sample preparation and analysis, and are easily separated by mass from the analyte of interest, however they may contain the very same analyte as the unsubstituted impurity or contribute to the analyte signal by having similar fragmentation patterns.

To ascertain the absence of such interference, blank matrix may be spiked with only the internal standard. If there is a percentage contribution it can be calculated to

what degree the internal standard must be diluted to approach non-significance in the selected calibration range (Section 3.4.2). It is also possible that the analyte may contribute to the signal for the internal standard. This is especially the case for deuterium substituted internal standards where it is only mono-,di- or tri-deuterated. If an alternative *m/z* cannot be utilised for the internal standard a replacement will be required, either a heavier substituted analogue or another compound with similar chemical properties that may not be a common dietary constituent or therapeutic drug.

3.4.2 Calibration model

The calibration model is a mathematical description of the detector response to increasing concentrations of a monitored compound. In analytical toxicology this response is usually measured as the peak area ratio of analyte vs. internal standard to compensate for variation in sample preparation steps, and ideally is described by a linear relationship $y = mx + c$.

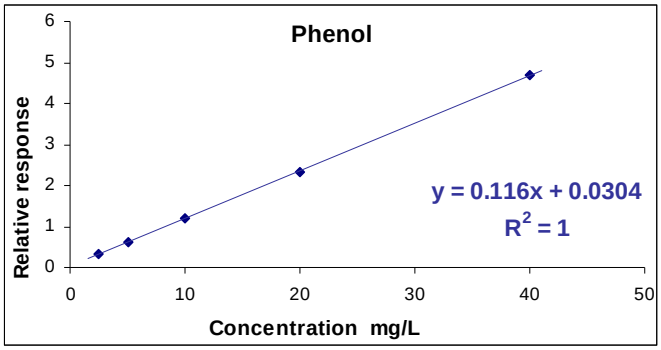

Figure 3.8 Example of a constructed standard curve with a linear response $y = mx + c$.

Contribution to signals for either the analyte or internal standard, as described in Section 3.4.1, may lead to non-linear responses (changes in m) and subsequent over/underestimation of concentration values.

Calibrators should be matrix-based (spiked blank matrix) and equally spaced over the required concentration range. A minimum of five to eight concentration levels and two to six replicates at each level should be analysed to determine linearity. The use of unweighted, least squares regression models are commonly used but may not be appropriate in some cases. Significant heteroscedasticity in data sets spanning a few orders of magnitudes may require transformed or weighted regression models (1/x,

$1/x^2$). This may be assessed by statistical tests for model fit. Traditional use of correlation coefficients to evaluate calibration models is not correct from a statistical point of view. After a suitable calibration model has been established fewer calibrators may be used for subsequent routine analyses [17, 19].

3.4.3 Accuracy

Accuracy is determined by systematic (bias) and random (precision) error components. Generally any reference to accuracy only refers to bias, which is simply the difference between the expected result and an accepted reference value. It is usually indicated as a percent deviation from the reference value. Certified external quality control (EQC) samples are ideal to assess accuracy, but are not always available. Internal quality control (IQC) samples prepared from different lot numbers of pure standard other than lots used for calibrators, and preferably prepared by a separate analyst, is advised [19, 20]. Calculation of accuracy is as follow:

$$\text{Accuracy (\%)} = (^{\text{Amount expected}}/_{\text{Amount measured}}) * 100$$

Acceptable criteria for accuracy are ±15% from reference value and ±20% at the LLQ [17-22].

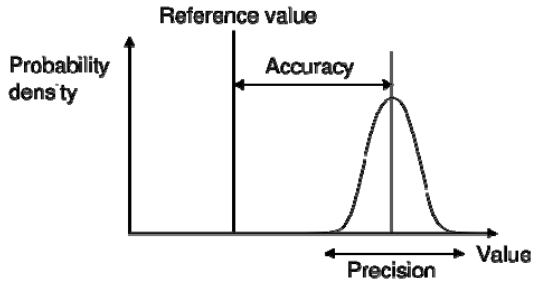

Figure 3.9 Influence of systematic (bias) and random (precision) error in analytical measurements.

3.4.4 Precision

Precision describes the degree of scatter between a series of measurements obtained from multiple sampling of the same homogenous sample under prescribed conditions. It is considered at three levels: repeatability (within-run/day), intermediate precision, and reproducibility. It is usually measured in terms of imprecision and expressed as relative standard deviation (% RSD) [17-22].

$$\% \text{ RSD} = \left(^{\text{standard deviation } (\sigma)} / _{\text{mean concentration value } (x)}\right) * 100$$

Repeatability refers to precision over a short time period with the same operating conditions, sometimes referred to as intra-assay precision. Intermediate precision refers to total precision under varied conditions (different days, different analysts, different instruments etc.) Reproducibility describes the precision for methods that are to be carried out in different laboratories (comparative studies) and is mostly reserved for standardization of methods [21]. Precision is usually assessed by analysis of IQC samples that cover the extremes of the calibration range and one or more QC near the middle of the range. The recommended approach is that replicate QC samples at the selected concentration levels are analysed in successive runs, e.g. triplicate analysis of each QC specimen for 6 days. One way analysis of variance (ANOVA) with the varying factor (day, run etc.) as the grouping variable is performed. This allows the assessment of repeatability and intermediate precision simultaneously, with a better evaluation of the true repeatability of the method due to data acquired on several occasions rather than once (within-day). Acceptable criteria for precision are ±15% from reference value and ±20% at the LLQ [17-19, 22].

3.4.5 Stability

Stability is defined as "the chemical stability of an analyte in a given matrix under specific conditions for given time intervals". Stability of the sample during the whole analytical procedure is a prerequisite for accurate quantification and therefore short-term and long-term stability assessment is recommended [17-19, 21]. Short term stability includes in-process stability (bench-top stability) and processed sample stability. Long term stability is assessed as freeze/thaw stability, as this is how samples are generally stored. The stability in the sample matrix should be established under storage conditions, i.e. in the same vessel, at the same temperature and over a storage period at least as long as the one expected for authentic samples.

In-process stability is the stability of analytes under conditions of sample preparation, e.g. ambient temperature or pH. This is usually only assessed if evidence of degradation is observed in early method development phase.

Processed sample stability is determined under conditions of analysis e.g. maximum time and temperature on the autosampler tray. It is sometimes also assessed

under storage conditions for cases where prepared samples have to be stored prior to analysis.

Freeze/thaw stability is assessed by analysing replicate QC samples after various freeze/thaw cycles. A minimum of three cycles of two concentration levels in triplicate are analysed and compared to QC values obtained before cycling. Accuracy of 80-120% of the mean for comparison QC samples indicates sufficient stability under storage conditions.

3.4.6 Lower limit of quantification

The LLQ is the lowest amount of analyte in a sample that can be measured with acceptable precision (±20% RSD) and accuracy (±20% bias). It is recommended that these validations occur in samples separate from the calibration curve. Care should be taken to try and establish the LLQ as a concentration value close or slightly above the lowest calibrator (25% above). As concentrations may not be quantified below the lowest calibrator, the inherent analytical imprecision could cause a variation in measured concentration values for LLQ samples that may fall below this value and therefore deemed unquantifiable. The traditional approach estimating LLQ was based on signal-to-noise (S/N) ratio, defined as the height of the analyte peak (signal) and the amplitude between the highest and lowest point of the baseline (noise) in a certain area around the peak. In bioanalytical chemistry a S/N = 10 was associated with the LLQ, however it is no longer acceptable if it is not supported by validation data indicating the relevant analyte concentration at S/N = 10 is within aformentioned precision and accuracy criteria [21, 22]. It is entirely possible for a large S/N value to be unreliably quantified due to inherent variation in the complete analytical methodology. The opposite is also true, a low S/N value may still be accurately quantifiable with a given methodology. Utilising S/N values alone are largely indicative of instrumental signal strength and it is an incorrect assumption that a high signal strength automatically translates to precise and accurate quantification. It is also acceptable to establish a combination of criteria, e.g. S/N = 5 and precision and accuracy within 20% of reference values.

3.4.7 Additional parameters

Recovery, limit of detection (LOD) and robustness are additional validation parameters that may be addressed but are not considered essential. Recovery is calculated as the percent response of the analyte after sample preparation steps, compared to the response of the pure analyte at a theoretical 100% recovery. Absolute recoveries are not always possible, as is the case where derivatization is involved. Derivatives are usually not available as reference standards and the recovery can only be assessed after chemical treatment of the pure compound, which is in itself subject to a variation in completeness of reaction. Considering data for accuracy, precision and LLQ are acceptable the recovery of analytes is therefore unimportant. A possible exception is validation for LC-MS/MS methods where recovery can be seen as part of the experimental design to assess matrix effects in ion suppression/enhancement studies.

Concentration values below the LLQ are considered as semi-quantitative. The LOD is therefore defined as the lowest concentration of analyte in a sample that can be reliably differentiated from background noise and still fulfil established identification criteria [21, 22]. Dilution of spiked samples until the signal approaches a S/N = 3 is usually employed to estimate the LOD.

Robustness is the measure of susceptibility of a method to small changes that may occur in routine analysis such as changes in pH, temperature etc. This parameter is usually only assessed for inter-laboratory techniques as is the case for reproducibility.

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Chapter 4

SAMPLE PREPARATION TECHNIQUES

4.1. Introduction

"Divide each difficulty into as many parts as is feasible and necessary to resolve it."
(Rene Descartes, 1596-1650)

A chef has all the culinary knowledge and correct equipment at his disposal that enables him to prepare a variety of dishes, but he cannot proceed to do so if he struggles to get the ingredients out of the package. This exceedingly simple argument illustrates the dilemma faced in analytical chemistry: where one has technologically advanced and highly sensitive instrumentation able to detect trace amounts of a compound, but what may be considered a fairly low-tech (perhaps non-selective and insensitive) method of getting the analyte of interest out of the sample matrix and into the detection system.

The intricate nature of analyte-matrix-extraction system is less understood than physichochemically simpler and confined systems such as separation in chromatography and detection in mass spectrometry. This has led to the idea that sample preparation is more of an art than a science. The introductory quote should serve as inspiration for the analytical chemist where sample preparation is concerned, compounded by the knowledge that a complex system can be reduced into simpler sub-systems from which the solution of interaction in that system becomes a rule, so that in all probability it suffices to aid in elucidating the interaction in following sub-systems.

It is somewhat ironic that large amounts of research and funding has gone into the creation of instruments with highly sensitive and selective detection, but comparatively very little in the accompanying sample preparation techniques to complement these instruments. This consequently undermines the potential power of an analysis. Sampling and sample preparation are the first steps in any analysis and it is a fact that due to inherent analytical necessity of one step being within accuracy limits before the next step may proceed, great attention must be paid to these first steps to ensure the end-result is acceptable. Biological sampling has largely been discussed in Chapter 2; this chapter will deal with sample preparation and focus on a few common

techniques that are widely applicable such as liquid-liquid extraction (LLE), solid-phase extraction (SPE), derivatization and extractive derivatization.

It is beyond the scope of the current work to enter in-depth into the thermodynamics and kinetics of the array of extraction methods. Focus will rather be on similarities and differences between the various techniques and practical considerations for the use of each. The fundamental similarity between extraction techniques is the contact between the extraction phase and the sample matrix, with mass transfer of analytes between the phases. The objective of all sample preparation techniques may be described as the isolation of components of interest from the sample matrix, and presenting these components in suitable form for a specific detection system.

4.2. Liquid-Liquid Extraction

Liquid-liquid extraction is generally considered the most basic extraction method as the essential underlying mechanism is simply the degree of solubility of the analyte within a selected organic phase. It involves the distribution of sample components between two immiscible liquid phases, a polar or aqueous phase and a non-polar or organic phase [1]. Agitation of the two-phase mixture creates arbitrary dispersion of either phase into the other as drops, termed the dispersed phase, while the other is termed the continuous phase. Convection in both phases is a secondary result of agitation. Movement of a chemical species from one phase to another is a consequence of a thermodynamic driving force in the form of a difference in chemical potential for a neutral species, or in electrochemical potential for an ionic species. Movement continues until phase distribution equilibrium is reached. The mechanism by which a species moves from one phase to another is thus convective-diffusive mass transfer that will increase for increasing rates of convection.

This type of LLE is also defined as an exhaustive extraction technique, i.e. analytes are completely removed from a sample matrix and transferred to an extraction phase. Traditionally these separations have been accomplished by agitation of a mixture to disperse drops of one phase in the other, thereby facilitating mass transfer of analytes, followed by discontinuation of agitation to allow drop coalescence and then separating the phases by means of a separatory funnel. Large quantitities of solvent and sample are

associated with this method and for the most part it is time-consuming and laborious. This has caused a more favourable disposition toward other types of batch-phase distribution techniques such as SPE. Biological samples however are severely limited in terms of sample volume available, and LLE performed on such samples accordingly have reduced usage of solvents compared to LLE in an industry setting. The arguments against LLE due to enhanced cost for large volume solvent waste disposal are hence not always completely applicable in bio-analysis [1].

As for any extraction technique the requirements in LLE are analyte isolation and preconcentration. The first requires a high selectivity for partitioning of the analyte component into the extractant over potential interferents,. The second is favoured by a high distribution ratio in order for the component to be extracted into a small volume of organic phase. The partition coefficient, K_p, for a species B is given by the following:

$$K_p = (C_{org})B/(C_{aq})B \qquad \text{(Eq. 4.1)}$$

$(C_{org})B$ = Concentration of B in organic phase

$(C_{aq})B$= Concentration of B in aqueous phase

This highlights one of the advantages of LLE, the organic extract may be directly introduced into an analytical system such as a gas chromatograph without any further preparation steps required. If initially a large volume of organic phase was employed, preconcentration is easily facilitated by evaporation of excess organic phase under a stream of dry nitrogen or air. Problems such as carry-over and memory effect can also be negated because a fresh portion of extracting solvent is used for each individual sample.

Within the sample matrix analytes are also involved in secondary chemical equilibria, where a species can exist in for example its free base form, B, along with its conjugate acid, BH^+. Generally, quantitative interest would be in determination of the total concentration of the analyte, thus $B + BH^+$. Only species B will distribute itself into the organic extraction phase and when extraction proceeds to equilibrium, the secondary acid-base equilibrium of $BH^+ \leftrightarrow B$ in the aqueous phase will be shifted completely to the right. The acid-base equilibrium is termed to have been perturbed by the extraction. In this case, the extraction would be quantitative for the analyte. Figure 4.1 illustrates the equilibria in LLE.

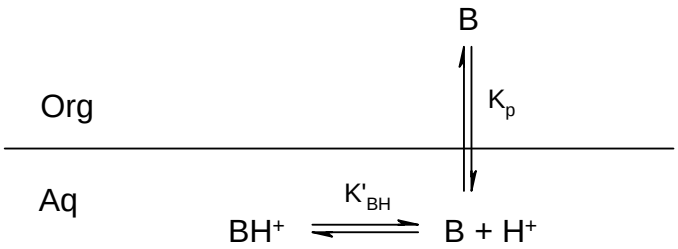


Figure 4.1 Simplified illustration of the essential equilibria in a two-phase LLE system. BH = Acid/Base pair; B = Analyte; K'_{BH} = Acid dissociation constant of BH; Kp = Distribution constant of BH.

Extraction rarely continues until phase distribution equilibrium is reached due to time constraints, thus in practise most LLE is non-equilibrium extraction and the acid-base equilibrium may be left unperturbed, resulting in non-quantitative extraction yield. From the above example we would only be determining the concentration of the free base, *B*, of the analyte in the sample. In order for the extraction to still be quantitative under such conditions, the acid-base equilibrium that exists in the sample matrix is pre-emptively shifted in the direction required (to the right in Figure 4.1) by adjusting pH with dilute acids and bases.

Selectivity in LLE is largely dependent on the chemical nature of the analyte of interest. Solubility characteristics and partitioning coefficients (K_p) determine the choice of organic extraction phase. By in large most solvents will extract a host of compounds alongside the analyte of interest, which of course makes LLE a good general extraction technique but prone to enhanced noise for low concentration analysis, especially in biological matrices. The only strategy to reduce noise and adding a degree of selectivity is through manipulation of the speciation nature of compounds as indicated above. By altering pH conditions to 2 units above or below the pK_a value of an analyte of interest much less additional compounds may be extracted than when pH adjustment is at extremes.

Furthermore, if a forward extraction into an organic solvent is followed by a back extraction with weak/strong acids and bases to remove excess strong/weak acidic or basic compounds, a much cleaner extract is obtained. Small, highly polar compounds present a particular challenge for LLE and conventionally the best method of extraction involve lowering its partition coefficient in the aqueous phase by addition of a salt that competes for solubility. This is known as the "salting-out" effect and can lead to significant increases in extraction efficiency for a specific analyte.

A modern approach for liquid-liquid extraction is so-called liquid phase microextraction (LPME). This is a non-exhaustive (does not extract entire burden of analyte) extraction technique based on equilibrium with the surrounding matrix [2]. A microlitre drop of organic phase is suspended in a large volume of aqueous phase containing the analytes of interest. The aqueous or continuous phase is made to flow by simple means such as a magnetic stirrer bar. Alternatively, the drop is contained within a microlitre syringe and the plunger is moved in and out, thereby repeatedly sucking in a plug of aqueous phase into the syringe tip where analyte transfer into the organic phase takes place. This method is termed dynamic-LPME. The greatest advantage of these methods is the high levels of sample enrichment in a small volume of organic extractant that may then be directly analysed in a gas chromatograph, allowing for a very sensitive analysis.

4.3. Solid-Phase Extraction

Solid-phase extraction effectively isolates and concentrates analytes from typically a flowing gas or liquid by their transfer to, and subsequent retention or adsorption on, a solid phase consisting of a specific sorbent. The isolated analytes are recovered by elution using a liquid with favourable solubility for the compounds of interest, or by thermal desorption into the gas phase. This form of SPE is an exhaustive extraction technique (similar to LLE), whereas the suspension of an SPE device in a liquid or gas phase until equilibrium is reached is a non-exhaustive process (similar to LPME) termed solid-phase microextraction (SPME). SPE may be considered a miniaturized form of column chromatography due to the physicochemical considerations with regards to analytes of interest, the solid adsorbent and the solvents passed through the sorbent bed [3].

Modern day SPE devices mainly consist of disposable cartridges containing chemically bonded sorbents of selectable particle size as well as stationary phase volume and phase type, that is packed between porous plastic or metal frits in an open syringe barrel. These cartridges, or packed columns, may be fitted to extraction manifolds and gentle vacuum or positive pressure applied to force liquid solutions through the sorbent bed at faster rates and therefore shorten extraction times.

SPE discs are also utilized, where particle loaded membranes contain sorbent particles immobilized in a web of short polytetrafluoroethylene (PTFE) fibrils formed into typically 0.5 -1 mm discs of various diameters. They contain about 90% by weight of sorbent and provide higher sample processing rates for large sample volumes. Reduced solvent volume extraction columns are so-called microbed packed and offer the advantages of SPE discs combined with the robustness of cartridges.

A very large selection of sorbent phases are available commercially, with silica-based chemically bonded sorbents being most widely utilised. Figure 4.2 summarizes the most commonly used silica-based sorbents [4].

Structures of silica-based chemically bonded sorbents

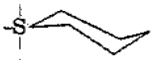
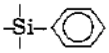
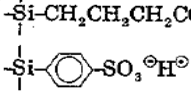
Type	Functional group	Structure
C18	Octadecyl	$-Si-C_{18}H_{37}$
C8	Octyl	$-Si-C_8H_{17}$
C2	Ethyl	$-Si-C_2H_5$
CH	Cyclohexyl	
PH	Phenyl	
CN	Cyanopropyl	$-Si-CH_2CH_2CH_2CN$
NH_2	Aminopropyl	$-Si-CH_2CH_2CH_2NH_2$
DIOL	2,3-Dihydroxypropoxypropyl	$-Si-CH_2CH_2CH_2OCH_2CH(OH)CH_2(OH)$
SAX	Trimethylaminopropyl (Quaternary amine)	$-Si-CH_2CH_2CH_2\overset{\oplus}{N}(CH_3)_3Cl^{\ominus}$
CBA	Carboxypropyl	$-Si-CH_2CH_2CH_2COOH$
SCX	Benzenesulfonic acid	
PRS	Propylsulfonic acid	$-Si-CH_2CH_2CH_2SO_3^{\ominus}H^{\oplus}$

Figure 4.2 Structures of commonly utilised silica-based chemically bonded sorbents in SPE [4].

Bonded phases are manufactured by the reaction of organosilanes with activated silica (SiO_2). Silicon and oxygen atoms form tetrahedral structures with the silicon atom at the centre, and four oxygen atoms on the corners of each tetrahedron. Each of these tetrahedra may be chemically attached to up to four neighbouring tetrahedra through silicon-oxygen (siloxane) bonds. Stable silyl ether linkages are formed during polymerization reactions of carbon chains to this silica backbone. Unendcapped phases allow hydroxyl sites to remain open, while endcapped phases have deactivated hydroxyl

sites as indicated in Figure 4.3. The latter is therefore slightly more hydrophobic and is largely preferred due to greater pH stability and simplified extraction mechanisms, as only non-polar interactions are present.

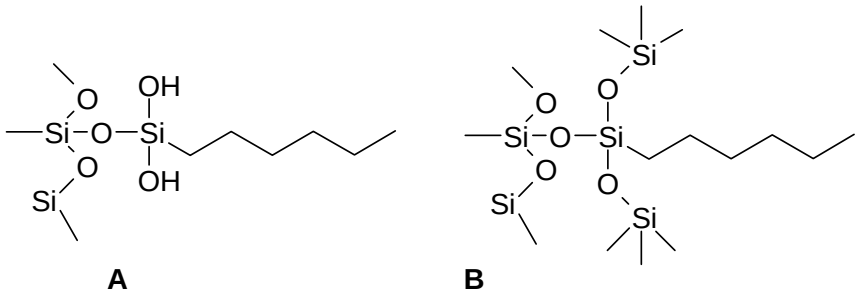


Figure 4.3 Structures of (A) unendcapped and (B) endcapped solid-phases.

The primary consideration in the choice of solid-phase is the physichochemical and structural characteristics of the analyte with regards to various molecular level interactions within its surrounding environment. The selected sorbent must facilitate these interactions between target molecules and environment to selectively dominate over any analogous interactions of analytes within the original matrix. In so doing, an enriched sample free from interferences present in the original liquid matrix may be obtained in the final eluate.

The octadecyl (C18) sorbent is the most popular solid phase as a large number of compounds with differing chemical properties may be extracted. Analytes must however exhibit some non-polar or neutral characteristics, as only hydrophobic or reversed phase mechanisms that partition a non-polar functional group from a polar matrix are present. The non-polar group is attached by Van der Waal's or dispersive forces to the sorbent. This mechanism is considered relatively non-selective and has similar considerations with regards to the underlying principles of LLE and the speciation of analytes. Speciation of compounds in the sample matrix as mentioned in Section 4.2 presents the same challenges in terms of non-quantitative extraction yield. Ionic species will show poor retention on C18 columns and acid-base shifts of the matrix to contain only free acid or free base forms of analytes is recommended. The ionization of compounds can however be otherwise exploited by simply selecting a solid-phase capable of doing so.

Ion-exchange sorbents are composed of a silica backbone bonded with a carbon chain that is terminated by a negatively or positively charged functional group.

Extraction proceeds through ion-exchange interactions between the fixed charge of the sorbent terminal site and a compound containing a functional group of opposite charge. Compounds are retained on the sorbent through ionic bonds, which are much stronger than non-polar dispersive forces in reversed phase mechanisms. There are three types of ion-exchange mechanisms: anion-exchange, cation-exchange, and copolymeric mixed-mode ion-exchange. Ion-exchange sorbents are traditionally classified as weak or strong depending on the identity of the ionic group and whether its charge is independent of the sample pH or manipulated by altered pH.

Strong cation-exchangers (SCX) such as benzenesulfonic acid is always negatively charged and utilised to extract positively charged compounds such as amine containing analytes. Strong anion-exchangers (SAX) such as quaternary amines are always positively charged and utilised to extract negatively charged acidic analytes. Mixed mode ion-exchange sorbents are copolymeric and have both hydrophobic and ionic retention mechanisms that may be applied to acidic, neutral and basic analytes. The four steps in sample processing using SPE (condition, load, rinse, elute) can be explained at the hands of a copolymeric cation-exchange sorbent [5].

The sorbent is initially conditioned with a solvent to remove impurities and facilitate effective sorption of analytes. The conditioning solvent is then replaced by the same solvent as the sample matrix, after which the sample is passed through the device at a controlled flow rate. Samples are usually dissolved in a buffer to ensure constant pH across the entire batch extraction. After sample loading, compounds are initially retained by hydrophobic interactions. A washing step is introduced utilising a neutral buffer or water to remove excess salts and polar-neutral molecules. An acid rinse is then used to favour protonation of basic compounds and thereby increase the ionic interaction between the analyte and the cation-exchanger. Elution with an organic solvent at this stage isolates neutral and acidic compounds that only have hydrophobic retention mechanisms. Subsequent elution with an aqueous-organic solution containing a base capable of displacing the positive charged analytes at the active sites effectively isolates the basic analyte fraction. Elution solvent volumes may be reduced as for LLE to be amenable for introduction into the specific detection system being utilized.

4.4. Choice of LLE versus SPE

SPE is considered a replacement for LLE, as the conventional application of the latter is considered too labour intensive, difficult to automate, and requiring large volumes of high purity solvents with costly disposal. The general consensus is that SPE benefits from lower intrinsic costs, shorter processing times, low solvent consumption, and is easier to automate.

The technique is also faced with problems however, the surface chemistry of sorbents are not as reproducible as solvent properties and also tend to have a higher level of contamination from manufacturing and packaging materials than solvents. Limitations in sorption capacity, analyte displacement or plugging of sorbent pores by matrix components result in changes in analyte recovery that sometimes go unnoticed.

Consider the following examples that may be encountered in a clinical laboratory that have both GC and HPLC instrumentation available:

(*a*) a high concentration-level neutral compound to be quantified in urine;

(*b*) a screen for traces of sedative-hypnotics in blood;

(*c*) forensic confirmation of a basic metabolite of an illicit substance in hair.

A simple LLE procedure using a small amount of solvent (< 1 mL) might be the best approach for (*a*). The sample matrix is not highly complex and doesn't contain proteins that may likely lead to gel-formation, the concentration levels are high so a fraction of the extract may be directly introduced into either a GC or HPLC system. Solvent selection may be geared toward being highly specific for the analyte of interest as to reduce co-extraction of background interferences. Unless extraction discs or reduced volume SPE cartridges are utilised, the final volume of SPE eluate and LLE solvent will likely be very comparable if assumed a 100 or 500 mg sorbent bed is used. The higher costs associated with SPE cartridges will favour the use of LLE, however the impact of a cleaner extract possibly obtained by SPE must also be taken into account. Less instrumental maintenance and reduced labour of laboratory personnel also translates to reduced costs.

High throughput requirements, as may be the case in (*b*), will favour SPE with the availability of HPLC instrumentation, as very robust on-line extraction and analysis is possible using SPE-LC hyphenated techniques. On-line coupling of SPE with GC is more difficult due to the inherent incompatibility of the aqueous nature of SPE and the

requirement of dry eluates to be amenable to GC analysis. Off-line SPE may need to be performed, where sample preparation is separated from direct instrumental analysis. In such cases LLE might also then be considered, particularly if GC is to be used as analytical instrument. A standard SPE manifold has typically 24 positions for cartridges to be attached. A large volume of samples can only be processed in batches of 24 at a time using offline SPE, while LLE can be performed simultaneously in tubes of up to 100 and agitated using a multishaker platform. The required detection levels however play a significant role, concerning both (*b*) and (*c*), as the ability of SPE to concentrate analytes and remove background interferences with robust rinsing procedures is unmatched in LLE. Tedious double and triple LLE with acidic/basic back extractions as clean-up step will likely have to be employed. Depending on the varied characteristics of all analytes that may need to be included in a screening procedure, LLE might be more inclusive than SPE.

In forensic analysis the highest possible assurance in the identification of the correct compound from the associated sample is required, due to the impact the result may have in legal terms. A highly specific target analysis as in (*c*) favours SPE as the reduction in background contribution and the more structure specific elution principles contained in the technique itself supports a high degree of confidence. The greatest drawback in using SPE in this event is the costs involved in single use of cartridges. The risk of carry-over and subsequent inclusion of blank analysis to prove the absence of this occurrence is highly laborious. There should also be evidence that no target compound appears in blank eluates from cartridges before the application of samples, which also prolongs sample preparation time further. Utilizing LLE it is much easier to simply check the solvents directly for traces of target compound. The use of a fresh extraction portion for each sample ensures no contamination will occur.

A large number of factors, economic and technical, have to be considered when selecting SPE or LLE for a particular analysis as indicated by the above examples. It is often the case that LLE and SPE methods may be developed for the same problem and final selection is based on a feasibility study where on technique emerges as preferred choice based upon comparison of positive and negative aspects of both.

4.5. Derivatization

As described in Chapter 2 (Table 2.2), all substances that enter the human body undergo biotransformation to some degree. The addition or uncovering of functional groups such as –COOH, -NH_2, and –OH in phase 1 metabolism result in a greater degree of hydrophilicity to ease excretion of absorbed compounds. Most biological samples are therefore not suitable for direct analysis by GC after analyte isolation from the matrix, due to the presence of one or more polar functional groups that decrease volatility and thermal stability. Direct introduction of such specimens into HPLC systems with typically UV or MS detectors are however possible. The presence of active hydrogens further complicates analysis by GC due to possible hydrogen bonding with active sites somewhere in the instrument system (e.g. inlet sleeve), which leads to non-linear chromatography (Chapter 3, Section 3.3.2.3) and a concomitant decrease in resolution and detectability of target analytes. One way of overcoming these difficulties is through the introduction of analytical derivatization reactions to the sample preparation procedures. In this respect, derivatizations are considered by many as a means of last resort, i.e. due to the demands of the instrumentation additional steps in the analytical process have to be included. Although it is true that the use of GC or GC-MS largely dictates the mandatory inclusion of analytical derivatives for successful analysis, additional advantages and disadvantages must also be considered [6-10].

The greatest advantage presented by derivatization reactions is the introduction of additional selectivity for target analytes. This may occur through a highly selective reaction mechanism that only derivatizes a small group of relevant compounds and only these are subsequently introduced into the detection system, or by the change in chemical structure a compound is left more detectable within a specific system even amongst a host of other similar compounds. In GC-MS many compounds show poor fragmentation with few diagnostic ions even when utilising electron ionisation. High-mass ions have greater diagnostic value than low-mass ions, as the latter are common background contributors from both biological matrix contaminants and instrumental interferences (e.g. column bleed). The chemical alteration of a molecule into a specific derivative radically alters the fragmentation pattern and ions of higher *m/z* values in greater abundance may be obtained.

Detectability may be further increased by matching the derivative with a specific detector. The common use of halogen containing derivatives that increase ionisation efficiency in NI-CI GC-MS is a typical example of this [11]. Here is not only the signal strength increased (sensitivity) but the background is also decreased (selectivity) as only compounds with high electron affinity are ionised and therefore detected. The use of fluorophores in HPLC also exploits this principle of derivative formation.

In the analysis of unknown compounds the preparation of more than one derivative presents additional information with regards to the molecular mass, and the use of isotopically substituted derivatives employed to study fragmentation patterns further assists in structure elucidation. Analytes that may be unstable in solution after extraction may also routinely be derivatized to increase stability.

Some of the greatest disadvantages that mitigate against the use of derivatives are the increase in reagent cost, labour and analysis time [6]. Additionally, incomplete derivatization that results in multiple derivatives or low derivative yield decreases the sensitivity of the analysis. Destruction or uncontrolled transformation of the analyte of interest due to side reactions may also occur. Instrument contamination, interference with detectors and false positives due to the formation of a related compound not originally present, e.g. acetylation of morphine to form diacetylmorphine (Heroin), may also occur as result of the derivatization reactions. The technical difficulties may largely be avoided by appropriate selection of derivative reagent and reaction conditions. The criteria for successful analytical derivatizations may be summarized as follow [10]:

- simple and rapid derivatization under mild reaction conditions
- reproducible formation of a high yield
- single derivative for each compound
- stable in reaction medium

Derivatizations in GC sample preparation are micro-scale synthetic reactions that commonly replace active hydrogens in functional groups with less polar and thermally stable groups. Chemical reactions that are frequently employed to achieve this include silylation, alkylation, acylation and various condensation reactions. Several functional groups may be affected by a single reaction, such as silylation occurring on carboxyl, hydroxyl and amine functionalities. Alkylation can form ethers, esters and enol ethers from hydroxyls, carboxyls and carbonyls respectively. The type of

derivatizing reagent utilised and the selection of reaction conditions determine the reactivity toward a specific functional group.

4.5.1 Silylation

Nearly all protic functional groups present in organic compounds may be converted to silyl esters or ethers, thus making silylation the most widely used derivatization procedure for GC-MS analysis. Silylation occurs through active proton displacement (e.g. –OH, -NH, -COOH, -SH) by an alkylsilyl group, most commonly forming trimethylsilyl (TMS) or *tert*-butyltrimethylsilyl (TBDMS) derivatives. The reactivity of functional groups depends on the specific derivatization agent employed, but generally follow the order (in decreasing reactivity): alcohols > phenols > carboxylic acids > amines > amides [10].

TMS derivatives combine thermal and chemical stability, high volatility, are easy to prepare, and show excellent chromatographic behaviour [12, 13, 14]. The most common agents currently utilised are: *N*,*O*-bis-trimethylsilyltrifluoroacetamide with 1% trimethylchlorosilane as catalyst (BSTFA + 1% TMCS); *N*-methyl-*N*-trimethylsilyl-trifluoroacetamide (MSTFA); and trimethylsilylimadozole (TMSI). All silylation reagents and derivatives are sensitive to moisture, and prolonged procedures to maintain anhydrous conditions are often required. TMS derivatives are more prone to hydrolysis than more sterically hindered derivatives such as TBDMS, and processed sample stability is often not ideal.

N-methyl-*N*-*tert*-butyldimethylsilyltrifluoroacetamide (MTBSTFA) is the most common reagent employed to donate a TBDMS group to molecules with active hydroxyl, carboxylic acid, thiol, and primary/secondary amines [15, 16]. The TBDMS derivative has greater hydrolytic stability than the corresponding TMS analogue, however there is greater difficulty in accessing sterically hindered groups. The higher masses donated to TBDMS derivatized compounds is also advantageous in many cases as higher mass fragments are obtained for quantification. The by-products of all the aforementioned reagents are highly volatile and largely neutral. Direct injection into the GC-MS of the derivatization mixture is possible with low risk for deterioration of the column stationary phase.

The mass spectra (EI) of silyl derivatives are characterized by weak or absent molecular ions, although very specific fragmentation patterns exist. TMS derivatives have a high abundance of the $(M-15)^+$ ion in their mass spectra, formed by the loss of a methyl group bonded to silicon. Similarly the TBDMS derivatives show strong signal at $(M-57)^+$ due to the loss of a *tert*-butyl group bonded to silicon. The ions formed by these neutral losses are structure specific, usually in high abundance and, depending on the derivatized compound structure, of sufficient high *m/z* value to allow quantitation in SIM mode. The MTBSTFA derivatization of oxazepam is indicated in Figure 4.4 as example of the general reaction scheme for silylating agents.

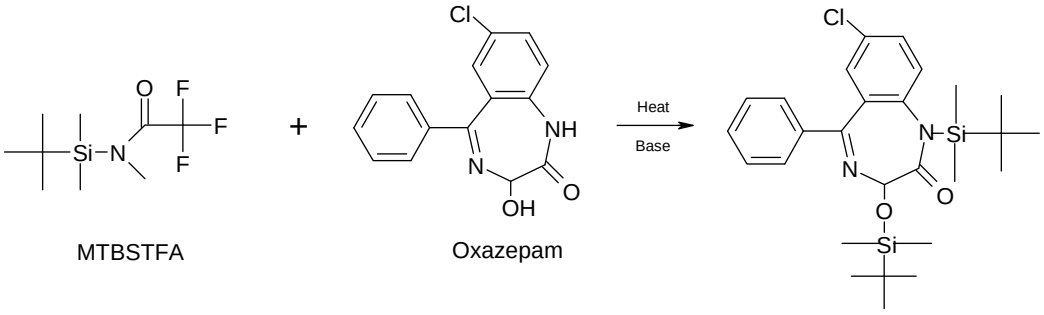


Figure 4.4 Simplified reaction scheme for silylation of oxazepam utilising MTBSTFA.

4.5.2 Alkylation

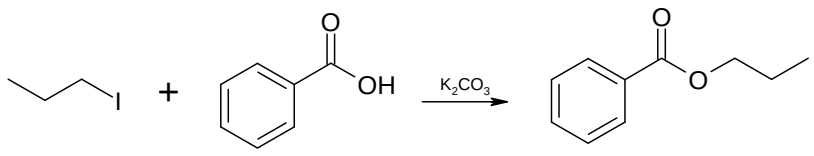
Alkyl derivatives are formed by the replacement of an active hydrogen with a alkyl or aryl group to form esters and ethers. The main functional groups subjected to alkylation reactions are carboxylic acids, phenols, alcohols and thiols. Amines, amides and sulfonylamides may also be alkylated, but are considered better targets for silylation or acylation reactions. The most common reagents in alkylation reactions are low molecular weight alkyl halides (e.g. iodomethane, iodoethane, iodopropane etc.), followed by benzyl- and substituted benzyl bromides (e.g. pentafluorobenzyl bromide, PFBBr). Most of the alkylation reactions using these reagents take place in the presence of a catalyst such as dry potassium carbonate, sodium hydride, silver oxide etc. Alkylations often do not require anhydrous conditions such as silylation and are relatively simple to perform. Alkyl derivatives show good chromatographic

performance and due to the possible selection of relatively small alkyl groups (e.g. methyl) can derivatize sterically hindered groups. In many cases the reaction mixture may be injected directly into the GC system [17-20].

Alkylation of carboxylic acids are commonly performed by esterification with alcohols. Methyl and ethyl esters have easily been formed by adding the relevant alcohol containing an acid catalyst such as hydrochloric acid, sulphuric acid or boron trifluoride, to a mixture of carboxylic acids [21]. Higher molecular weight derivatives may be formed by using higher mass alcohols or substituted alcohols (e.g. pentafluoropropanol).

Diazoalkanes have also been used to alkylate carboxylic acids and phenols. This has primarily been achieved by the use of diazomethane, however the exposure risks associated with this compound and its high reactivity in forming side reactions make it unfavourable to work with [22].

Flash (pyrolytic) alkylation is the formation of an alkyl derivative in the heated injector port of a GC system from the thermal decomposition of an acidic compound and the resultant quaternary ammonium salt of the acid. Methyl derivatives are most often prepared employing a mixture of the analytes and tetramethylammonium hydroxide at an injector port temperature of 250 – 300 ºC. This is a very rapid form of derivatization but increases the time for maintenance of the GC system. Unfavourable side reactions are also known to occur [23]. The propyl ester of benzoic acid after derivatization with 1-iodopropane is indicated in Figure 4.5 as example of the general reaction scheme for alkylating agents.



Iodopropane Benzoic acid

Figure 4.5 Simplified reaction scheme for alkylation of benzoic acid utilising 1-iodopropane.

4.5.3 Acylation

Acylation is performed using three main types of reagents: acyl halides, acid anhydrides or acylated imidazoles. Acylated derivatives are usually formed from amines, amides, phenols and alcohols before analysis by GC-MS. Acyl halides and anhydrides are highly reactive but can lead to undesirable side reactions (e.g. enolization, dehydration etc.) due to the strong acidic nature of the reaction medium. The presence of an acidic receptor such as pyridine is therefore often included. The acid anhydrides are further preferred above the acyl halides, as excess reagent that may cause chromatographic deterioration over prolonged injection cycles, are easier to remove.

The perfluoroacyl anhydrides such as trifluoroacetic anhydride (TFAA), pentafluoropropionic anhydride (PFPA) and heptafluorobutyirc anhydride (HFBA) are particularly widely utilised due to their volatile nature and ease of excess removal after derivatization. Molecular ions are usually identified in EI mass spectra alongside abundant ions of moderate to high *m/z* values suitable for quantitation in SIM mode [24-26]. The pentafluoropropionyl ether of codeine after derivatization with PFPA is indicated in Figure 4.6 as example of the general reaction scheme for acylating agents.

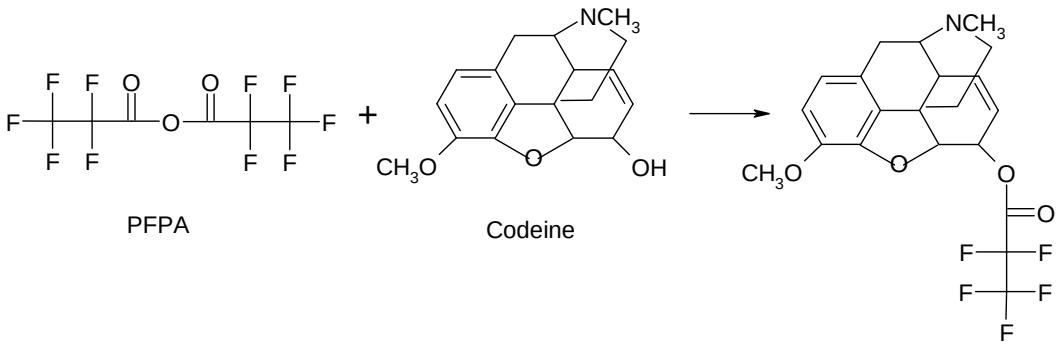


Figure 4.6 Simplified reaction scheme for acylation of codeine utilising PFPA.

4.6. Extractive derivatization

Reliability and accuracy of results are first and foremost the essential considerations in any analytical method development. Methods to be used in routine settings have additional requirements such as low reagent costs and instrument

demands, time efficiency, and ease of use. Combining separate steps in sample preparation procedures into a single one provides a means to expedite analytical results, in order to meet the demands of faster turn-around times and sample throughput in the modern clinical toxicology laboratory. As stated earlier in this chapter, the objective of all sample preparation techniques is the isolation of analytes of interest from the sample matrix, and presenting these compounds in suitable form for a specific detection system. Regarding GC-MS it has also been established that derivatization is largely required for successful quantification due to possible chromatographic problems (e.g. volatility, degradation, adsorption) or unfavourable mass spectra. The combination of extraction and derivatization as a single sample preparation process therefore holds the promise of shorter and less laborious work-up procedures.

4.6.1 Extractive acylation

Acylation of amines and alcohols in aqueous solution have been performed under so called Schotten-Baumann conditions [6]. Acyl chlorides and anhydrides, in the presence of a base such as sodium hydroxide or sodium bicarbonate ($NaHCO_3$), react readily in a biphasic aqueous basic solution to form the acyl derivatives of primary and secondary amines as well as phenolic hydroxyls. Although water hydrolyses the anhydrides, amines and phenolic hydroxyls react much faster with the anhydride and may therefore be acylated in aqueous solution. The general reaction scheme is indicated in Figure 4.7.

The acylation reaction produces one equivalent of acid from the anhydride that may diminish derivative yield by forming salts with unreacted amines. The presence of excess base optimises reaction conditions by removal of any acid by-products. The specific anhydride being used also may affect yield as reactivity differs, e.g. TFAA may react more readily than PFPA and HFBA. The addition of a solvent to the aqueous reaction mixture as the acylation takes place serves to extract all derivatized compounds. Solvent selection is based on final solubility of relevant derivatives.

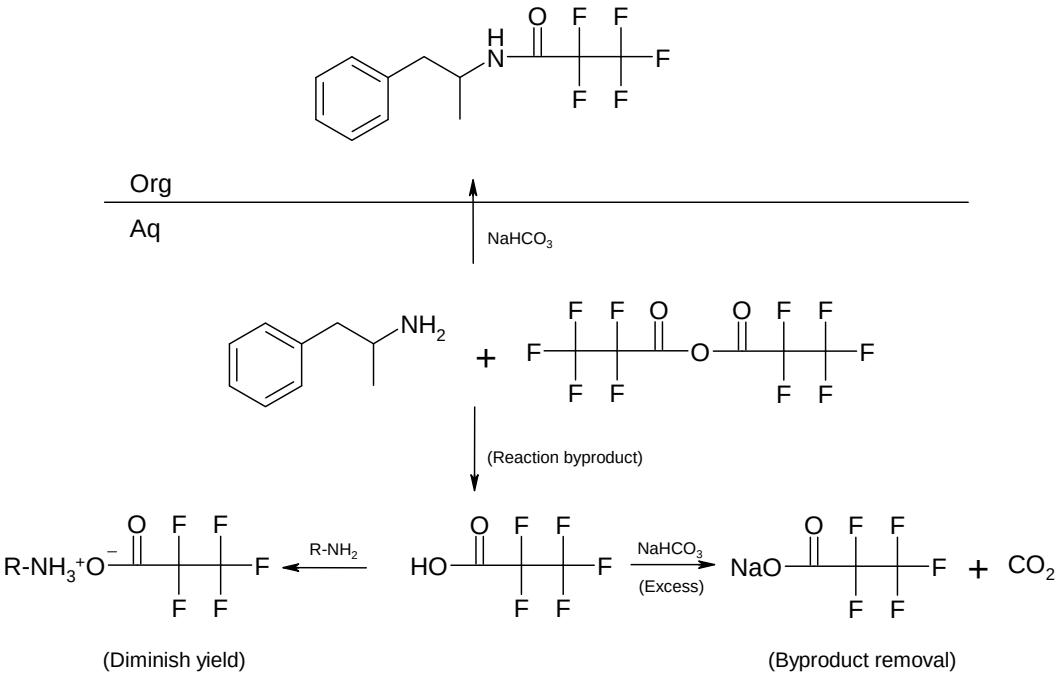


Figure 4.7 Schotten-Baumann reaction scheme for acylation of amphetamine with PFPA.

Extractive derivatization has historically been used in organic and preparative chemistry. The formation of the analgesic paracetamol (acetaminophen) from *para*-aminophenol by extractive acylation with molar equivalents of acetic anhydride is well known [6]. The amine group has greater reactivity than the phenolic hydroxyl group which remains underivatized due to the limiting reagent. Derivatization reactions for GC-MS, however, always employs an excess of reagent to ensure all active hydrogens have been replaced by more volatile groups.

The advantage of utilising this type of extractive derivatization is the combination in selectivity of the reaction mechanism coupled to the selectivity of the solvent chosen to perform the extraction. The reagent by-products are also completely removed (remain in aqueous phase) which allows direct analysis of the extract. Selecting a small volume of extraction solvent or evaporation of the solvent bulk also allows for enrichment of the derivatives if increased sensitivity is required.

4.6.2 Extractive alkylation

Alkylation reactions often include catalysts (Section 4.5.2) to increase reactivity and yield of the selected derivative. Acidic compounds in anionic form such as ionised

carboxylic acids and phenols are highly reactive species and may readily react with alkyl/benzyl halides, however the ionized form of the species only exist in aqueous solution. Extraction of ionic species from aqueous to organic phase is unfavourable due to poor solvation in non-polar solvents commonly employed (Section 4.2). Formation of a neutral entity by association of oppositely charged ions, as is the case for ion-pair extraction, presents an elegant solution that is well known to analytical chemists [27, 28]. Ion pairs are stable, thermodynamically distinct species coexisting in equilibrium with free ions:

$$(Q^+A^-) \rightleftharpoons Q^+ + A^- \qquad \text{(Eq. 4.2)}$$

(Q^+A^-) = Ion pair

Q^+ = Cation

A^- = Anion

Phase transfer catalysis takes advantage of the appearance of ion pairs by extracting ionic species into an organic phase where subsequent dissociation may occur and some type of chemical reaction proceed (alkylation in the present study). Alkylation by phase transfer catalysis, known as extractive alkylation, involves extraction of anions as neutral ion pairs into an aprotic solvent containing a reagent such as PFBBr, iodomethane etc. The ion pair dissociates leaving the anion poorly solvated and open to nucleophilic attack, such that S_N2 reactions may proceed readily [29-34].

Even the most simple two-phase (water/organic) substitution reaction between the anion of a salt and an organic substrate involves a number of equilibria:

a) Overall reaction:

$$B_{aq}^+A_{aq}^- + RX_{org} \xrightarrow{(Q^+A^-)\,/\,(Q^+X^-)} B_{aq}^+X_{aq}^- + RA_{org} \qquad \text{(Eq. 4.3)}$$

Aq = Aqueous phase

Org = Organic phase

RX = Alkylating reagent

X^- = Halide ion

(Q^+A^-) = Ion pair (analyte)

(Q^+X^-) = Ion pair (halide)

Q^+ = Cation (catalyst)

A^- = Anion (analyte)

B^+ = Cation (matrix)

(b) Organic phase chemical reaction (K_{diss})

$$RX_{org} + (Q^+A^-)_{org} \rightleftharpoons RA_{org} + (Q^+X^-)_{org} \quad \text{(Eq. 4.4)}$$

(c) Extraction equilibrium

i) $Q_{aq}^+ + A_{aq}^- \rightleftharpoons (Q^+A^-)_{org}$ (K_{ex}) (Eq. 4.5)

ii) $Q_{aq}^+ + X_{aq}^- \rightleftharpoons (Q^+X^-)_{org}$ (K_{ass}) (Eq. 4.6)

(d) Extraction constant of equation (c) i)

$$K_{ex} = (Q^+A^-)_{org} \,/\, (Q_{aq}^+)\times(A_{aq}^-) \quad \text{(Eq. 4.7)}$$

The above reactions and equilibria involved in phase transfer catalysis are illustrated in Figure 4.8. utilising the extractive alkylation of phenol.

It should be noted that the extraction equilibrium in equation (d), K_{ex}, is influenced by both the anion and cation concentrations in the aqueous phase since the denominator consists of a product. Due to the complex nature of the extraction system a number of factors other than the extraction equilibrium also play a role in extraction rate. These include, but are not limited to:

1. Association or dissociation of ion pairs in the organic phase, which lowers the concentration of $(Q^+A^-)_{org}$ and thus aids in the extraction.
2. Association effects of the cation or ion pair in the aqueous phase that results in decreased extraction.
3. pH-dependent equilibria in the aqueous phase that influence the effective anion concentration,

   $$A_{aq}^- + H_3O^+ \rightleftharpoons HA + H_2O \quad (K'_{BA}) \quad \text{(Eq. 4.8)}$$

4. Extraction of uncharged species such as HA (K_D) into the organic phase together with the possible formation of ion associates and aggregates (e.g. $Q^+HA_2^-$).

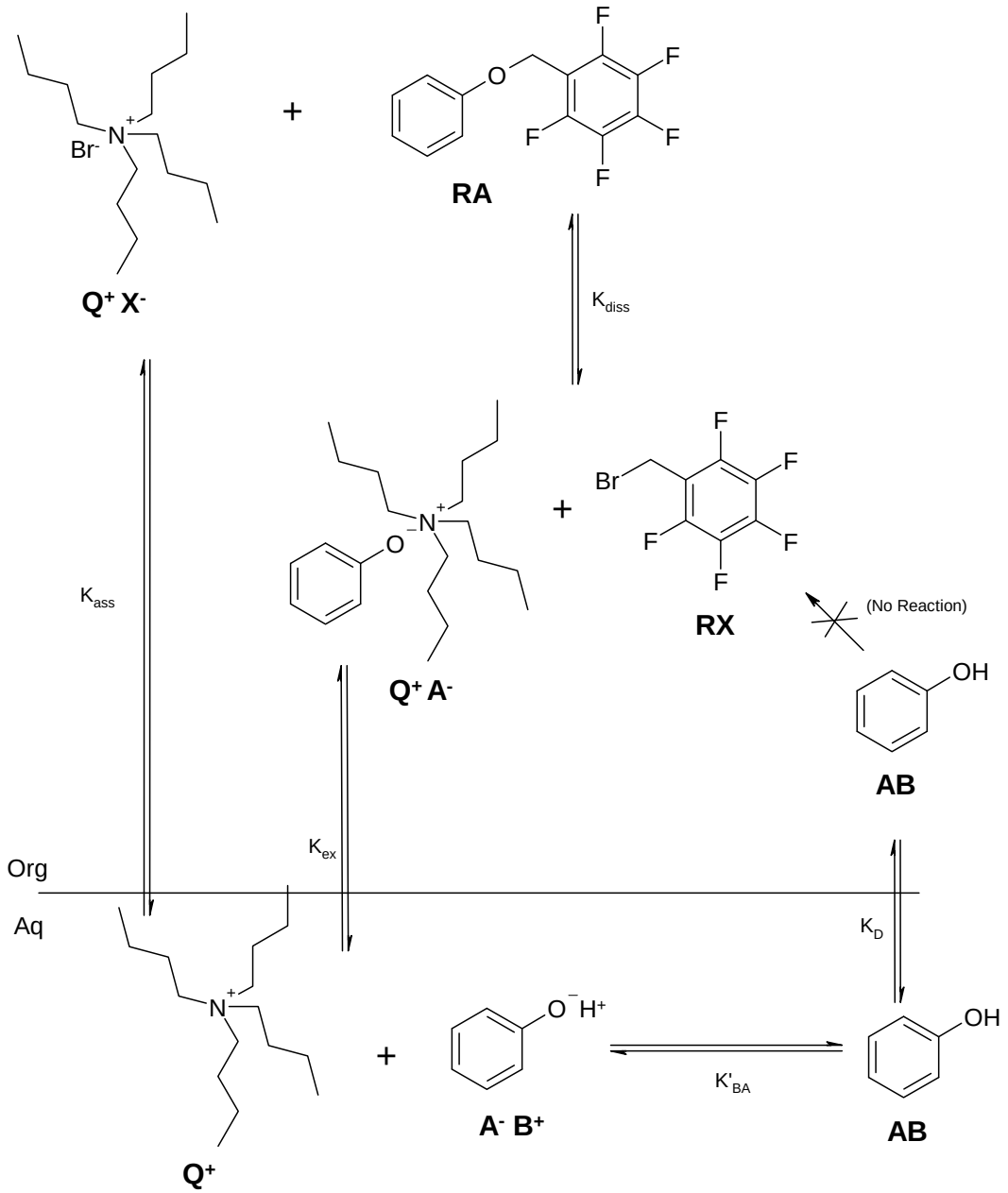


Figure 4.8 Phase transfer catalysis reaction scheme for phenol-anion using tetrabutylammonium as phase transfer-cation and PFBBr as alkylating agent. K'_{BA} = Acid dissociation constant of AB; K_D = Distribution constant of AB; K_{ex} = Extraction constant of ion pair QA; K_{diss} = Dissociation constant of ion pair QA; K_{ass} = Dissociation constant of ion pair QX. Equations are indicated in the text (Eq. 4.3 – Eq. 4.8).

Phase transfer catalysis is usually carried out in aprotic solvents of low polarity. In these reactions solvents cannot simply be regarded as continuous, structureless substances in phase transfer catalysis. To exemplify, ion-dipole interactions occur on contact between solvent and solute molecules. Dipole, dispersion, induction, and

hydrogen bonding combine with Coulomb forces to determine the stability of an ion pair, therefore the natures of the phase transfer catalyst cation, analyte anion and solvent are equally important. The solvating power of an aprotic solvent depends not only on its dielectric constant but also on its ability to donate or accept electron density. Common aprotic solvents and their dielectric constants are shown in Table 4.1 [35]

Solvent	Dielectric constant
Dichloromethane	8.9
Chloroform	4.7
Diethyl ether	4.2
Benzene	2.3
n-Hexane	1.9

Table 4.1 Common aprotic solvents and their dielectric constants [35].

The solubility of inorganic salts in these solvents are negligible but quaternary ammonium, phosphonium and other –onium salts are highly soluble, especially in dichloromethane and chloroform. As a rule of thumb solvents with a dielectric constant lower than 10 have almost no free ions in solution, ion pairs being the dominant species. Since the interactions between the ion pairs and solvent molecules are weak, reaction with nucleophiles in the organic medium is fast. An ideal solvent in phase transfer catalysis should therefore be aprotic, highly immiscible with water, and non-reactive toward the ion pair.

Quaternary ammonium salts are of special interest for phase transfer catalysis purposes since they are least likely to interfere in chemical reactions and have been well studied [35, 36]. Figure 4.9 indicates the structure of different sized quaternary ammonium salts alongside the one used in this study, namely tetrabutylammonium hydrogensulphate (TBA).

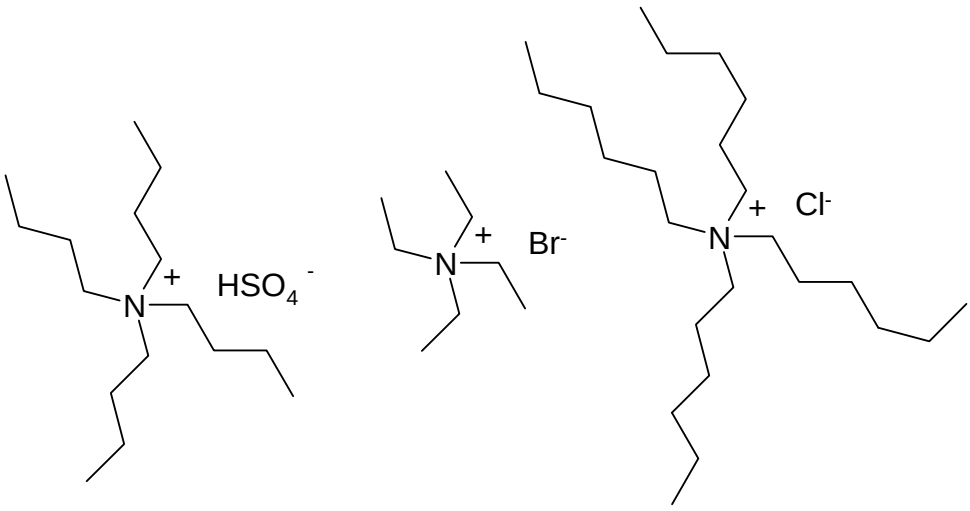


Figure 4.9 Structure of tetrabutylammonium hydrogensulphate – N(*n*-C_4H_9)$_4$, tetraethylammonium bromide – N(*n*-C_2H_5)$_4$, and tetrahexylammonium chloride – N(*n*-C_6H_{13})$_4$.

The relationship between cation size and the extraction constant (K_{ex}) is an important parameter to consider and should be viewed with respects to the physicochemical properties (e.g. hydrophobicity, pK_a) of the anions that need to be extracted. Extraction constants for a series quaternary ammonium ion-pairs (increasing in size) of picric acid is shown in Table 4.2 [37]. It can be seen that the extraction constants increase with increasing cation size irrespective of the solvent system used.

Solvent	Ammonium Cation			
	$N(CH_3)_4$	$N(C_2H5)_4$	$N(C_3H_7)_4$	$N(C_4H_9)_4$
CH_2Cl_2	1.5	220	2.9×10^4	4.8×10^6
$CHCl_3$	0.22	21	4.4×10^3	8.1×10^5
C_6H_6	ND	0.22	35	3.9×10^3
CCl_4	ND	ND	ND	87

Table 4.2 Extraction constants {$K_{ex} = (NR_4^+Pic^-)_{org} / (NR_4^+)_{aq} \times (Pic^-)_{aq}$} of a series of quaternary ammonium picrates extracted from water into an organic solvent. Adapted from [35]. ND = Not detected. [37]

The extraction constants for the homologous series of quaternary ammonium salts indicate a definite increase per added carbon irrespective of the solvent choice. The following order of lipophilicities has been established [35]:

Tetrahexylammonium > Tetrapentylammonium > Tetraisopentylammonium > Tetrabutylammonium

It is clear to see that even if a solvent performs poorly (low extraction constant with medium sized catalysts) the choice of cation may largely compensate for retarded reaction rates. Dichloromethane however does seem to be the best solvent for this particular extraction. The number of carbon atoms is not the only factor to be taken into account. Additional groups (e.g. benzyl) may be added to alter the lipophilicity of the catalyst cation for greater solubility in a different solvent system.

Larger catalyst cations are able to extract more hydrophilic anions, however employing the same cation size there is an established order to the extraction of simple anions for a variety of aprotic solvent systems (in decreasing order of lipophilicity) [35]:

Picrate >> MnO_4^- > ClO_4^- > SCN^- > I^- > (ClO_3^-, toluenesulfonate) > NO_3^- > Br^- > (CN^-, benzoate) > (NO_2^-, Cl^-) > HSO_4^- > (HCO_3^-, acetate) > F^- > OH^- > SO_4^{2-} > CO_3^{2-} > PO_4^{3-}

The importance of the anion paired with the catalyst cation as –onium salt lies in its function to dissociate in the aqueous phase, allowing ion-pairing of the target analyte anion to take place. From the above order one can note that for medium sized catalysts such as TBA, hydrogen sulphates are excellent salts to use as catalysts. They are very stable and in the presence of a base the hydrogen sulphate anion will be transformed into neutral sulphate, which cannot interfere because it is less easily extractable than almost any other inorganic or organic anion.

The production of halide anions is the second consideration as alkyl/benzyl halides are typically used as alkylating reagents in extractive alkylation procedures. Excessive amounts of reagents containing iodide may be detrimental to reaction rates as it is clearly preferentially extracted (it lies fifth in the above series). So called “catalyst poisoning” may occur as iodide is liberated during the course of the reaction, bringing the phase transfer of target analyte anions to a halt [35]. Alkylating reagents containing bromide or chloride may be preferable (lies respectively eighth and twelfth in the above series).

Compounds from xenobiotic metabolism that may form organic analyte anions in aqueous matrices such as urine are much more complex than those listed above. Acetate and formate are the simplest and most hydrophilic organic anions. Addition of alkyl groups and other lipophilic substituents will increase reaction rates for organic anions, however side reactions such as mentioned previously and competitive anion

extraction from non-targeted matrix components are more likely to occur due to the complex nature of the matrix itself. Sterically hindered groups might not form suitable ion-pairs or have difficulty in alkylating once in the organic phase and thereby slow the reaction rate. Careful selection of parameters such as aqueous phase pH, phase transfer catalyst type and concentration, alkylating agent type and concentration, solvent and phase ratio must be performed. The multivariate nature of this type of extraction methodology allows for good selectivity that may be considered similar to SPE.

The greatest advantage is that extraction, derivatization and concentration may be achieved in a single procedure followed by direct analysis of the extract without further derivative enrichment or other processing required.

4.7. References

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Chapter 5

EXTRACTIVE ALKYLATION OF URINARY METABOLITES OF BENZENE AND ALKYLATED DERIVATIVES

5.1 Introduction

"Measure what is measurable, and make measurable what is not so." (Galileo Galilei, 1564-1642)

Occupational monitoring is largely performed to protect the individual employee's health status, but also to assist employers in preventing unnecessary costs associated with absenteeism and gross medical expenditure in cases of work-related disability. Occupational exposure to volatile organic compounds in industry is ubiquitous. Authorities thus enforce biomonitoring (Chapter 2) of exposed individuals to ensure that the level of exposure does not exceed acceptable levels above which health would be affected negatively. Unsubstituted aromatic hydrocarbons such as benzene and alkylated homologs (toluene, xylene, ethylbenzene) are exhaustively utilized in industry and occupational exposure to these compounds are widespread. They mainly originate from processes such as petroleum distillation and coke manufacturing. Benzene, toluene and xylene are often used as constituents of gasoline, and solvents in various applications such as paints, lacquers and adhesives. Ethylbenzene is employed as solvent, fuel additive and chemical intermediate in the production of styrene.

5.1.1. Exposure and metabolism

Acute exposure to high levels of benzene can produce death by fatal cardiac arrhythmias [1]. The major toxic effect of benzene however is its haematopoietic toxicity. Bone marrow depression induced by chronic exposure may initially manifest as anaemia, leukopenia or thrombocytopenia [2]. Continued exposure may lead to myelodysplasia and ultimately progress to acute myeloid leukaemia [1, 3, 4]. Acute exposure to alkylated benzenes are characterized by central nervous system depression

with symptoms such as dizziness, drowsiness and nausea. Chronic exposure to high levels may result in cerebellar damage as encountered in glue sniffers [2]. The toxicity of many compounds is caused by metabolic activation resulting in reactive species that have genotoxic effects. A prime example of this is benzene, which is converted to benzene oxide by the microsomal mixed function oxidase (CYP2E1) as part of the body's detoxification processes of xenobiotics [2]. Alkyl benzenes however seem to follow a metabolic pathway which creates less reactive species that are readily excreted. A series of oxidation reactions is followed to form benzoic acid derivatives that may or may not be conjugated with glycine before excretion in urine. Evidence suggests that only 1% of inhaled toluene may follow the same route as benzene and thus have similar genotoxic results. Subsequently the reduced genotoxicity suggests that alkyl benzenes are not carcinogenic. Figure 5.1 illustrates the detailed metabolism of benzene [5] and Figure 5.2 indicates the general metabolism of the alkylated benzenes [6].

The metabolites of interest for quantitation of exposure to benzene are urinary phenol, *S*-phenylmercapturic acid (*S*-PMA) and *trans*, *trans*-muconic acid (*t*,*t*-MA). Phenol is the main metabolite of benzene but is inadequate for exposures at low levels due to its background presence in non-exposed individuals. Sorbic acid, which is present in certain food as a preservative, is a possible confounding factor for the use of *t*,*t*-MA as biomarker for benzene at very low levels. Previous studies have indicated that *S*-PMA is a superior marker for low-level exposure to benzene [7]. The metabolites of interest for the assessment of exposure to alkyl benzenes are: *o*-cresol and hippuric acid (HA) for toluene exposure, total methylhippuric acid (*o*-MHA, *m*-MHA, *p*-MHA) for xylene exposure and mandelic acid (MA) for ethylbenzene exposure.

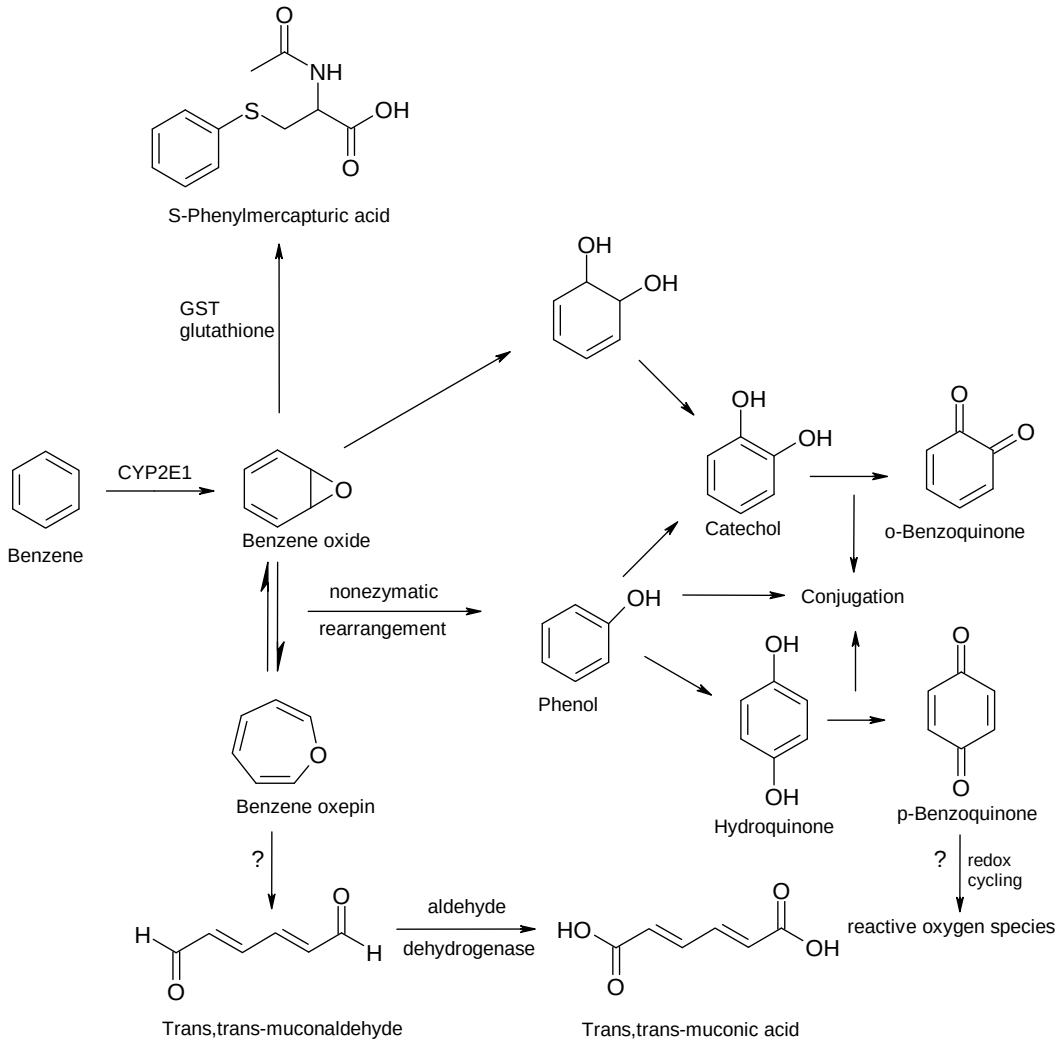

Figure 5.1 Metabolism of benzene [5].

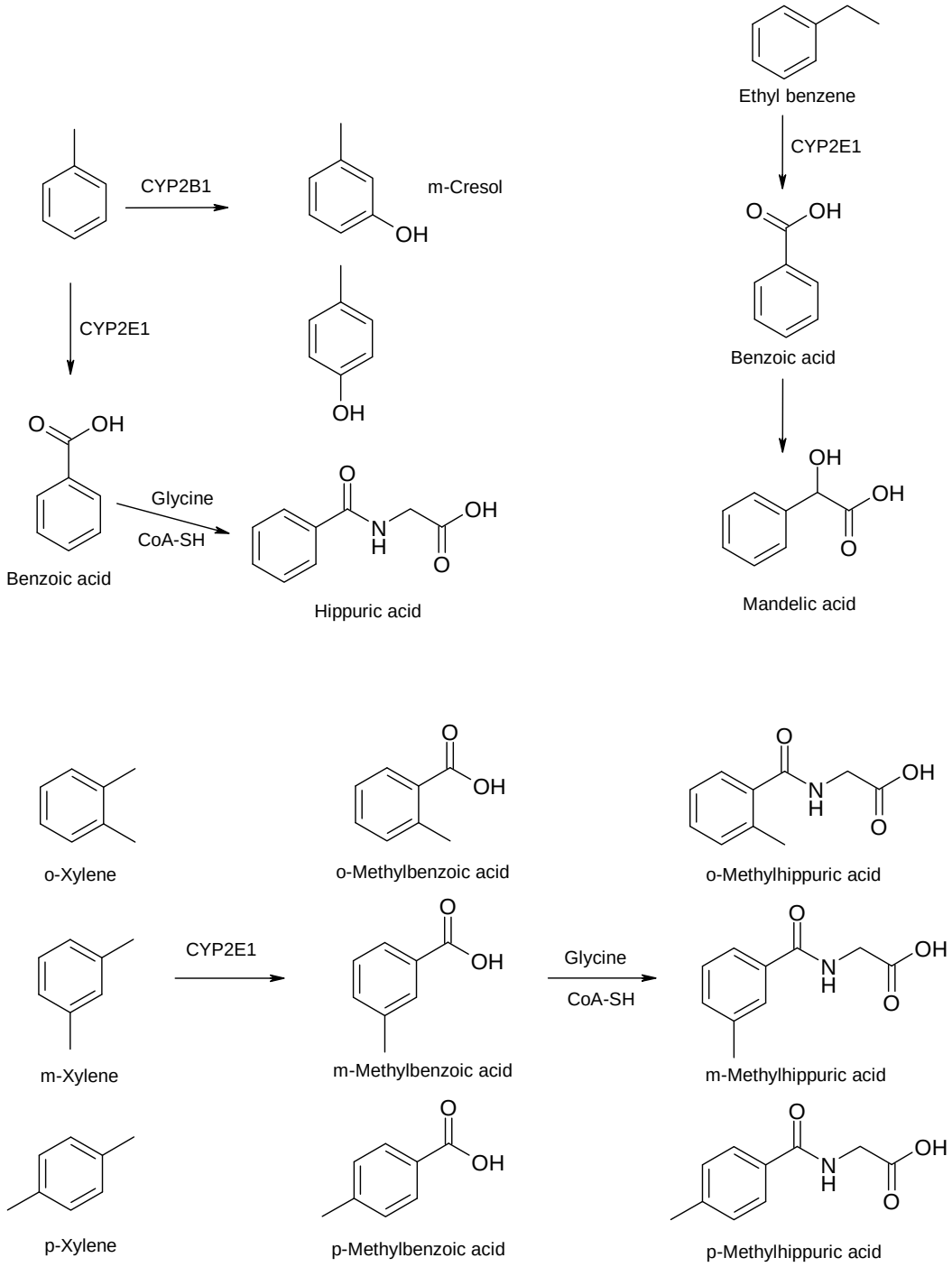

Figure 5.2 Metabolism of alkylated benzenes [6].

5.1.2 Analytical approach

As discussed in Chapter 4, numerous factors have to be taken into consideration before development of an analytical methodology may proceed. In a routine clinical toxicology laboratory issues such as instrument availability, cost of analysis, turn-around time, sample throughput and labour of personnel are essential to take into account. Analytical criteria are those discussed in Chapter 3 (Section 3.4) and should not be compromised to simply accommodate such workplace requirements, but optimum conditions for both should be approached. Consider the following:

A GC-MS system fitted with a mid-polar, narrowbore DB-5 column is utilised each day, due to the wide applicability for non-polar to mid-polar analytes. Batch analysis of a wide variety of diverse analytes and derivatives, isolated from different biological matrices, is performed on a 24 hour cycle. It is therefore prudent for any newly developed method to be geared toward accommodating these instrument limitations if it is to be successful. Ideally, injected extracts will be clean (reduce instrument maintenance), derivatives stable (prevent repeat sample preparation), chromatographic elution short (reduce total analysis time), and mass spectra specific (reduced data handling). Instrument capabilities should match sample requirements. This means that assessment of the available matrix, the type of analytes and required detection limits should be performed and compared to the available analytical system.

Quantitation of the various biomarkers of exposure to alkyl benzenes has been achieved by high performance liquid chromatography (HPLC) [8, 9], gas chromatography (GC) [10, 11] and gas chromatography-mass spectrometry (GC-MS) [6, 12, 13]. The aforementioned metabolites require derivatization for successful gas chromatographic analysis due to the presence of active carboxylic and phenolic groups. Procedures including silylation [12, 14], esterification [13, 15] and alkoxycarbonyl derivatization [6, 16] have been employed. Extractive alkylation has proven to be relatively simple and practical for a range of analytes that may exist as anions in aqueous solution. These include fatty acids, phenols, cyanide, diuretics and so forth [17-24]. This method however has not been used for the simultaneous extraction and derivatization of urinary biomarkers for exposure to benzene and alkyl benzenes. Considering the high concentrations (mg/L or part per million) of relevant metabolites [25, 26] in the sample matrix (urine), alongside the chemical similarities between

analytes themselves, it is likely that time-optimized conditions for extractive alkylation can be attained.

5.2. Materials and methods

5.2.1. Reagents

All reagents and solvents used were of analytical grade. Standards for phenol, *o*-, *m*-, *p*-cresol, mandelic acid (MA), hippuric acid (HA), *o*-, *m*-, *p*-methylhippuric acid (*o*-MHA, *m*-MHA, *p*-MHA), and Phenyl-β-D-glucuronidase (from *H. Pomatia)* were purchased from Sigma-Aldrich (Steinheim, Germany). 2, 3, 4, 5, 6-pentafluorobenzyl bromide (PFBBr) was purchased from Aldrich (Milwaukee, WI, USA). Dichloromethane, methanol and tetrabutylammonium hydrogensulphate (TBA), and 1-iodopropane (IP) was purchased from Fluka (Buchs, Switzerland). [$^2H_6$]Phenol, [$^2H_5$]Hippuric acid, [$^2H_4$]*o*-cresol and [$^2H_5$]mandelic acid was purchased from CDN-Isotopes (Pointe-Claire, Quebec, Canada). *S*-phenylmercapturic acid (*S*-PMA) and [$^2H_2$]*S*-PMA was purchased from Toronto Research Chemicals (Toronto, Canada). Sodium dihydrogenphosphate monohydrate (NaH_2PO_4 . H_2O), 32% hydrochloric acid, and anhydrous sodium acetate was purchased from Merck (Darmstadt, Germany). Deionized water was obtained from a Milli-Q (Millipore, Bedford, MA, USA) water system and used throughout the entire method.

5.2.2 Solutions

Stock solutions of the metabolites and isotope substituted internal standards (IS) were dissolved in methanol. A calculated volume of stock solution for each metabolite was spiked in water and further diluted with water and methanol to obtain calibration standards that contained a mixture of metabolites in a water-methanol (70:30) solution. The procedure for the internal standards was identical to that of the calibration standards and an internal standard mixture was also obtained in water-methanol (70:30). Initial work solutions containing phenol, *o*-, *m*-, *p*-cresol, MA, HA, *o*-, *m*-, *p*- MHA and *S*-PMA were appropriately diluted from stock in the range 0.005 mg/L – 500 mg/L to test for selectivity and linearity. After the initial assessments were completed, solutions for

routine calibration of phenol, o-cresol, MA, HA and all isomers of MHA were prepared as per Table 5.1

Analyte	Standard 1 mg/L	Standard 2 mg/L	Standard 3 mg/L	Standard 4 mg/L	Standard 5 mg/L
Phenol	5	10	20	40	80
o-Cresol	0.4	0.8	1.6	3.2	6.4
MA	10	20	40	80	160
HA	10	20	40	80	160
MHA (total)	30	60	120	240	480

Table 5.1 Concentration of calibration standards.

Separate quality control (IQC) working solution was prepared from different lot stock solutions and further diluted with water-methanol (70:30) to yield control solutions that could be spiked in pooled urine (concentrations given below). The internal standards (IS) were diluted from stock to a concentration equal to Standard 3 of the unsubstituted analog.

Buffer solutions of pH 8.5, 10 and 11.5 respectively were obtained by adjusting a 0.8M phosphate buffer (NaH_2PO_4 . H_2O) with different volumes of a saturated NaOH solution. Solutions of TBA in water were made up at concentrations of 0.1M, 0.2M, 0.4M, 0.8M and 1.6M respectively. PFBBr was dissolved in dichloromethane to obtain solutions with the following volume percentage concentrations: 0.05%, 0.5%, 1%, 5% and 10%. Note that PFBBr is a very strong lachrymator and should be handled under a fume hood, especially at higher concentrations. A 50% acetate buffer (1M, pH 4.5):20% Phenyl-β-D-glucuronidase:30% H_2O enzyme solution was prepared fresh daily for deconjugation. All other solutions were stored at 4°C for a maximum of 6 months.

5.2.3. Equipment

A HP6890 GC system fitted with a HP7683 Auto injector and a HP5973 mass selective detector (MSD) (Agilent Technologies, Palo Alto, CA, USA) was used for mass spectrometric analysis. A microbore DB-5 fused silica capillary column (5 m x 100 µm; d_f 0.1 µm) (Agilent Technologies, Palo Alto, CA, USA) was used. Data collection, integration and signal-to-noise ratio (*S/N*) was performed with HP Chem Station software. The MS was tuned monthly with perfluorotributylamine (PFTBA) using the autotune function as per manufacturer's recommendation. Furthermore,

weekly column maintenance was performed by injection of a chromatography standard that contains a variety of underivatized compounds with reactive functional groups. Activity on the column and/or inlet liner was indicated by tailing peaks of 1-octanol and 2,4-dimethylaniline contained in the chromatography standard. The inlet liner was replaced with a clean deactivated splitless liner and approximately 5-10 cm of the front column end was cut, and reconditioned by a sustained increased column temperature (320ºC) for 15-30 min. Analysis of the highest standard in full-scan mode was performed to re-establish retention times.

5.2.4. GC-MS procedure

A 1 µl volume of derivatized sample was injected in split mode (split ratio 1:200). The inlet temperature was set at 250°C and helium carrier gas at a constant flow-rate of 0.6 ml/min. The average velocity was 70 cm/s. A single-ramp temperature program was used. An initial isotherm of 85°C was maintained for 0.3 min and ramped at 65°C/min to a temperature of 310°C maintained for 0.32 min. This amounted to a total chromatographic time of 4.1 min. The MSD transfer line temperature was set at 280°C and that of the quadrupole at 150°C. The temperature of the source was 230°C. A solvent delay time of 1 min was set before the source was turned on. All mass spectra were recorded at 70 eV in the electron ionisation (EI) mode. Chromatograms were first recorded in full-scan mode (50-550 *m/z*) to identify analytes and their respective retention times. Quantitation was performed in the selected ion-monitoring (SIM) mode. The mass-to-charge ratios (*m/z*) of the quantifier ions used were: phenol, 274 *m/z* cresols, 288 *m/z*; mandelic acid, 107 *m/z*; hippuric acid, 134 *m/z*; methylhippuric acids, 119 *m/z*. The common ion 181 *m/z* from the PFBBr derivative was used as qualifier ion for all compounds. The quantifier ions for the isotope labelled internal standards were as follows: [$^2H_6$]phenol, 279 *m/z*; [$^2H_4$]*o*-cresol, 292 *m/z*; [$^2H_5$]hippuric acid, 139 *m/z*; [$^2H_5$]mandelic acid, 112 *m/z*.

5.2.5. Linearity and Selectivity

Blank urine samples, obtained from ten healthy drug-free, non-exposed volunteers were analyzed and compared to a standard (200 mg/L) containing phenol, *o*-,

m-, *p*-cresol, MA, HA, *o*-, *m*-, *p*- MHA and *S*-PMA. Endogenous concentrations for all analytes except *S*-PMA and *o*-cresol were expected due to dietary constituents forming the relevant metabolites. Selectivity could therefore only be assessed through studying chromatographic resolution of known urine samples spiked with a wide variety of drugs and other metabolites. No interferences could be observed after spiking samples with solutions containing common over-the-counter medicaments (acetylsalicylic acid, acetaminophen, ephedrine, etc.) as well as drugs of abuse (cocaine, morphine, amphetamines etc.). Other industrial exposure metabolites such as furoic acid, *p*-nitrophenol, phenylglyoxylic acid, benzoic acid, dimethylhippuric acids, 1-naphtol, 1-hydroxypyrene etc. were also assessed and no interference observed. Urine was pooled for further experiments.

An aliquot (1 mL) from the urine pool, was placed in a reaction vial (7 mL). Calibration standard solution (1 mL) was added to the sample along with the internal standard mixture (200 µL). Five point calibration curves were set up (5 replicates per level) utilizing the relative response (peak area) of the analyte towards the internal standard. An un-weighted, least-squares regression model was utilized and linearity was established ($r^2 \geq 0.99$) over the concentration ranges indicated in Table 5.1. Back calculated values for standards were all within 10% of their theoretical concentrations.

5.2.6. Routine calibration

Aliquots (1 mL) from the urine pool were placed in reaction vials (7 mL) and calibration standard solutions (1 mL), as indicated in Table 5.1, were added to the samples along with the internal standard mixture (200 µL). Five point calibration curves were set up alongside a urine blank to check for changes in established endogenous concentrations of certain metabolites. This was particularly necessary to ensure the hydrolysis of the phenol conjugate was achieved with every batch analysis. The addition of free phenol to calibrators and IQC samples creates the possibility of incomplete deconjugation in authentic specimens going unnoticed as concentration values for controls would still appear correct. Control samples were spiked in pooled urine aliquots (1 mL) from appropriate solutions to provide two IQC levels (LOW, HIGH) indicated in Table 5.2. The aliquots were stored frozen at -20 °C for up to a year.

Analyte	LOW QC mg/L	HIGH QC mg/L
Phenol	10	40
o-Cresol	0.8	3.2
MA	20	80
HA	20	100
MHA (total)	60	240

Table 5.2 Concentration of internal quality control samples.

5.2.7. Extractive alkylation

A large percentage of phenol is excreted as the glucronide conjugate and subsequently needs to be converted to the corresponding aglycone prior to extraction procedures. Freshly prepared (Section 5.2.2.) beta glucuronidase enzyme solution (300 µL) is added to the urine samples and placed on a heating block (3 hours, 65 °C). The optimized extraction procedure after deconjugation was as follows. Phosphate buffer solution (500 µL, 0.8M, pH 10) was added to the hydrolysed samples. Phase transfer catalyst (200 µL, 0.2M TBA) was added, followed by the introduction of dichloromethane (1 mL) containing PFBBr (5% v/v). The reaction vial was capped with a PTFE lined cap, vortexed (5 sec) and placed on a multishaker (30 min). The sample was centrifuged (1 min, 3000 rpm) to achieve adequate phase separation and the organic phase was transferred into another vial containing dilute hydrochloric acid (1 mL, 0.1M). The sample was vortexed (30 sec) and centrifuged (1 min, 3000 rpm). A volume of the organic extract (200 µL) was transferred to a clean GC-MS vial insert and directly analysed by injection into the GC system. Figure 5.3 summarizes the sample preparation protocol.

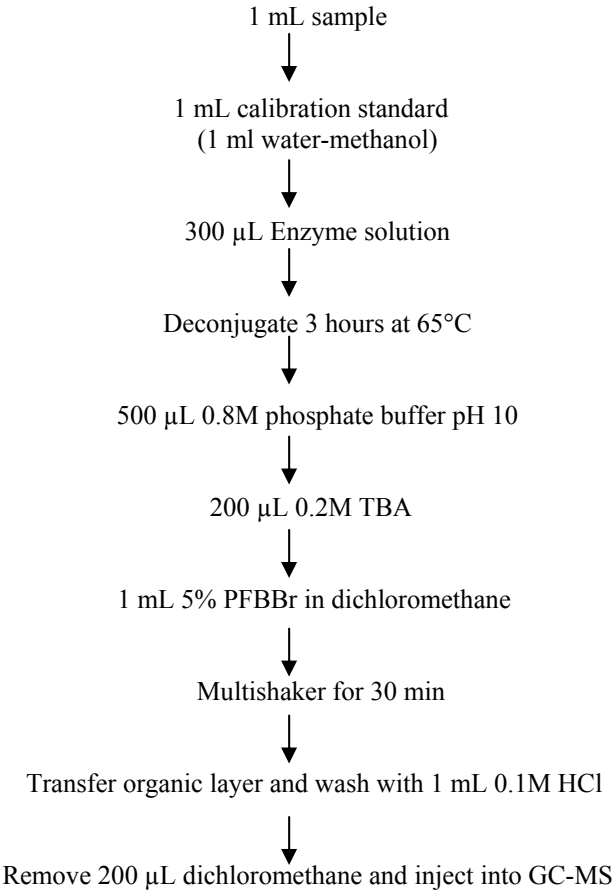

Figure 5.3 Flow diagram of the optimized extractive alkylation procedure.

5.2.8. Optimization

As discussed in Chapter 4, the complex interactions involved in all the various chemical equilibria that exist for a two-phase phase transfer catalysed reaction requires careful consideration of parameters to be optimized. The most important of these are pH of aqueous media, phase transfer catalyst concentration, alkylating agent concentration, organic phase ratio, and temperature.

In the current assay the effect of alkylating agent concentration was studied using increased concentrations of PFBBr in dichloromethane over a period of 120 min. An optimum reaction time of 30 min was established and used for pH and phase transfer catalyst concentration optimization. The optimum phase transfer catalyst concentration

was obtained by subjecting pooled urine samples to five different concentrations of TBA. The effect of pH was studied by the addition of buffers of different pH values.

During the optimization process, reaction conditions were monitored utilizing the absolute response (peak area) as an indication of the extractive alkylation reaction yield. The internal standards could not be used to compensate for sample losses as their response would increase/decrease to the same degree as corresponding unsubstituted analogs. The experiments were repeated twice for each variable.

5.2.9. Validation

The performance of the method was evaluated as described in Chapter 3. Shortly, a daily five-point calibration curve along with three replicates of the two IQC samples (Table 5.2) described in the preceding text were analysed on 8 different days. Accuracy was determined as the percent bias of the mean of the calculated concentrations at the different levels with respect to their nominal concentrations. Precision was assessed by calculating relative standard deviation (% RSD) for within-day (repeatability) and intermediate variation by one-way ANOVA. Criteria for acceptance were equal or less than 15% RSD (20% at LLQ) and within 15% of nominal concentration (20% at LLQ) for precision and accuracy, respectively.

The processed sample stability of the pentafluorobenzyl (PFB) -derivatives was studied over a period of 23 hours by injection of the same sample (MED QC) once every hour. Absolute peak areas of each analyte relative to their initial concentrations were expressed as a percent value versus injection time and plotted. Instability is determined by a negative slope significantly different from zero ($p \leq 0.05$). Freeze/thaw and long term stability at -20 °C were assessed by repeat analysis of external quality control (EQC) specimens received from the Finnish Institute of Occupational Health (FIOH) over a 1 year period alongside stored IQC specimens.

The lower limit of quantification (LLQ) in SIM mode was determined according to the following criteria. Precision was within 20% RSD and accuracy within 20% of nominal concentration at S/N = 10. The limit of detection (LOD) was determined by diluting the lowest calibrator, and criteria for acceptance were set as both qualifier and quantifier ions present at minimum S/N=3.

5.3. Results

5.3.1. GC-MS analysis

A chromatogram, recorded in full-scan mode, of a spiked urine sample containing all metabolites of interest is shown in Figure 5.4. The chromatographic conditions were different than those listed in the Section 5.2.3 and 5.2.4, as a narrowbore DB-5 column (30 m x 250 µm; d_f 0.1 µm) was permanently installed in the available instrumentation at that stage. Chromatographic resolution was more than sufficient for quantitative analysis of all the metabolites, with baseline resolution between positional isomers for both the cresols and methylhippuric acids. The speed at which this separation could be attained was favourable as compared to previous methods [6], however the instrumental cycle time was still more than 15 min per sample. The peaks eluting close to *o*-cresol could easily be resolved in mass difference by selection of a single ion channel specific to the analyte. Figure 5.5 indicates the chromatogram in full-scan mode and SIM mode for an injected calibration standard utilising the previously listed (Section 5.2.4) chromatographic conditions. Please note the large concentration differences between samples analysed in Figure 5.4 and Figure 5.5.

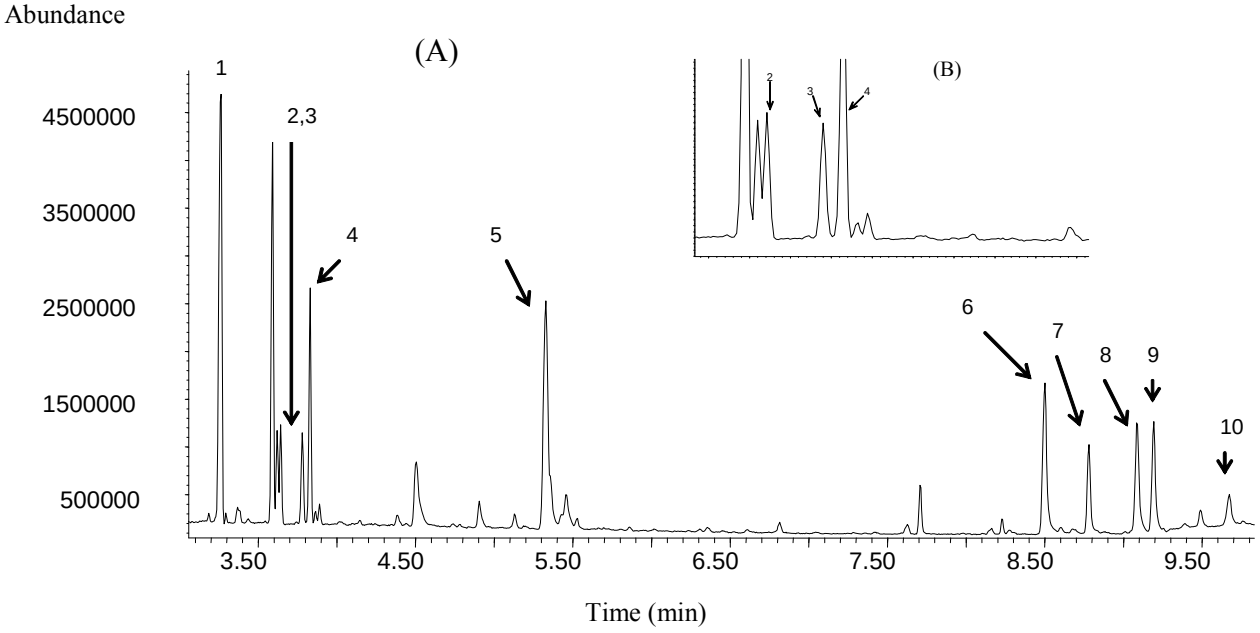

Figure 5.4 (A) Chromatogram of spiked urine (200 mg/L) recorded in scan mode (*m/z* 50-550) on a narrowbore DB-5 column (30m x 250 µm; d_f 0.1 µm). The elution order is as follows: phenol (1) *o*-cresol (2), *m*-cresol (3), *p*-cresol (4), mandelic acid (5), hippuric acid (6), *o*-methylhippuric acid (7), *m*-methylhippuric acid (8), *p*-methylhippuric acid (9), *S*-phenylmercapturic acid (10). (B) Enlargement of the first section indicating the positional isomers of cresol.

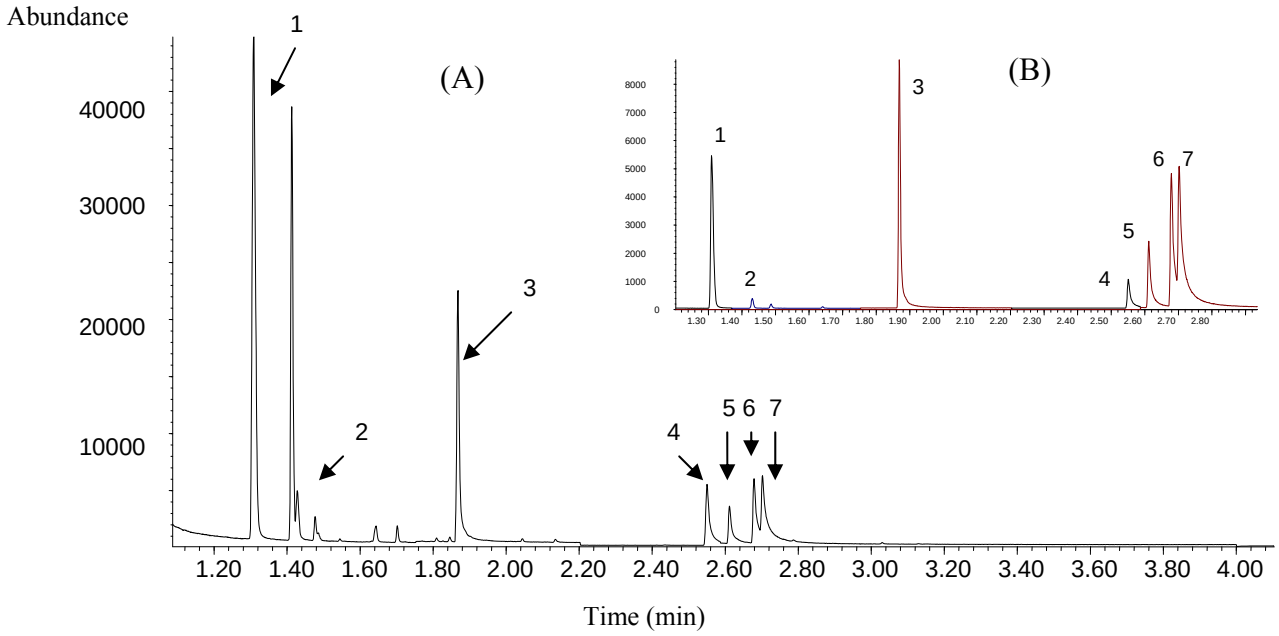

Figure 5.5 (A) Chromatogram of standard 1 (Table 5.1) recorded in scan mode (*m/z* 50-550) on a microbore DB-5 column (5m x 100 µm; d_f 0.1 µm). The elution order is as follows: phenol (1) *o*-cresol (2), mandelic acid (3), hippuric acid (4), *o*-methylhippuric acid (5), *m*-methylhippuric acid (6), *p*-methylhippuric acid (7). (B) The same chromatogram recorded in SIM mode with extracted quantifier ions.

Employing the shorter, smaller diameter column of identical stationary phase, a 3-fold decrease in elution time was observed with minimal loss of resolution (R_s, Chapter 3). The instrument cycle-time was reduced to approximately 6 min per sample, thus 10 samples could be analysed per hour compared to a previous maximum of 4 to 5. Sample throughput, as far as instrument time is concerned, was at the very least doubled.

The full-scan mass spectra of the PFB-derivatives of HA, MA and *o*-cresol are shown in Figure 5.6. Molecular ions could be observed for all the analytes except for MA. The underivatized benzylic hydroxyl group of MA caused a slight tailing as the column deteriorated over time, but never to an extent where accurate quantitation was compromised. The mass spectra of all the derivatized analytes also contained the benzylic ion $(C_7H_2F_5)^+$ (181 *m/z*) that originated from the derivatization agent. This ion formed the base peak in the mass spectra of the cresol and phenol ethers, but had lower abundances in the esters formed with analytes containing carboxylic functional groups.

The mass spectrum for phenol is nearly identical to that for the cresols, with only a difference of 14 *m/z* (phenol lacks a methyl group) between their respective molecular ions. Similarly there is a difference of 14 *m/z* between the molecular (359 *m/z* and 373 *m/z*, respectively) and other diagnostic ions (105 *m/z* and 119 *m/z*, respectively) of HA and MHA.

S-PMA was not included in routine analysis due to insufficient sensitivity. Positional isomers of *o*-cresol are not clinically significant and was only initially included to assess chromatographic resolution.

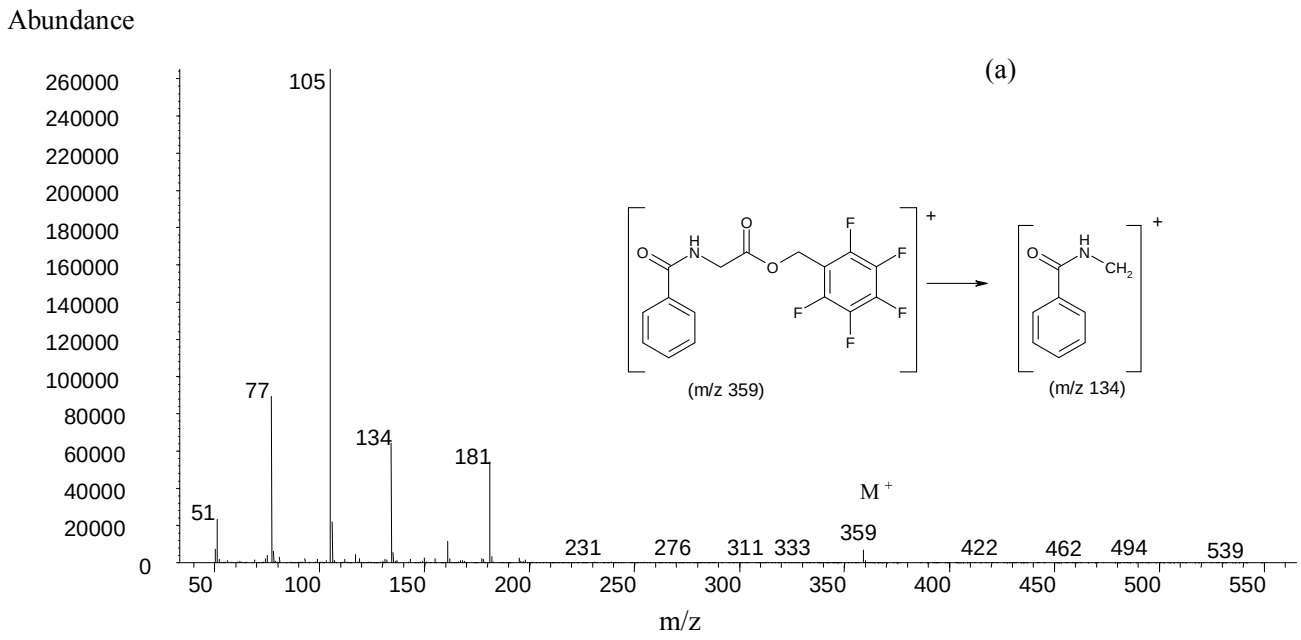

Figure 5.6 Full-scan mass spectra of pentafluorobenzyl-derivatives of hippuric acid (a), mandelic acid (b) and *o*-cresol (c). The mass spectra of all the derivatized analytes contained the benzylic ion $(C_7H_2F_5)^+$ (181 *m/z*) that originates from the PFBBr derivatization agent. M^+ indicates the presence of the molecular ion.

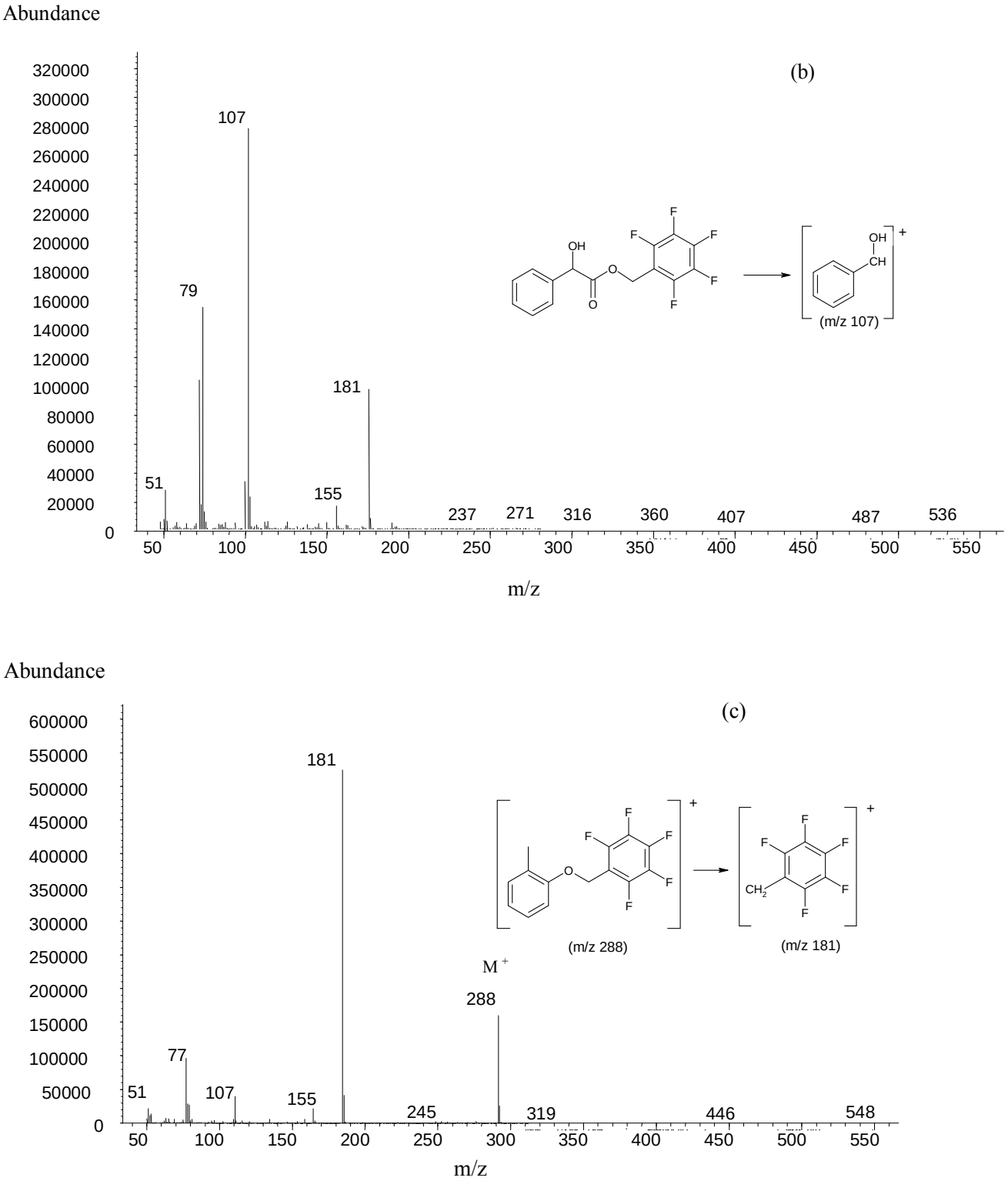

Figure 5.6 Full-scan mass spectra of pentafluorobenzyl-derivatives of hippuric acid (a), mandelic acid (b) and *o*-cresol (c). The mass spectra of all the derivatized analytes contained the benzylic ion $(C_7H_2F_5)^+$ (181 *m/z*) that originates from the PFBBr derivatization agent. M^+ indicates the presence of the molecular ion.

5.3.2. Optimization

In the early method development phases the exact method of Fogelqvist et al [18] was replicated, however unsatisfactory results for HA and MHA were obtained. At increasing concentration values of HA and MHA the calibration curves showed a distinct flattening off effect. This alluded to the possibility of a limiting reagent in the extractive alkylation procedure. In the aforementioned method [18] low concentrations of PFBBr were required since electron capture detection (ECD) was employed. Excess PFBBr creates immense background noise on the ECD, which diminishes the sensitivity and selectivity of the analysis. The use of GC-MS in the current method negated this problem as it introduced the required selectivity by monitoring specific ion channels in SIM mode. The concentration of alkylating agent was the single largest factor influencing the sensitivity of the extractive alkylation. This finding was in accordance to that of Gyllenhaal et al [20] who postulated that alkylation rather than extraction was the rate-determining step in the extractive alkylation procedure.

Figure 5.7 illustrates the effect of PFBBr concentration on the HA response at pH 10 with 0.2M TBA. The unsatisfactory results associated with HA and MHA at the higher concentration end were seemingly due to the low concentration of PFBBr that was used initially (0.05%). The increase of PFBBr to 5% not only improved linearity of the HA and MHA response, but also halved the initial reaction time. No significant increase in response was observed when changing the concentration of PFBBr from 5% to 10%.

Previous studies indicated that a phosphate buffer performed well under extractive alkylation conditions [25]. The effect of different buffer systems was therefore not examined in this study. As expected from the equilibrium equations in Chapter 4, the pH of the aqueous phase strongly influenced the extraction yield of the various analytes. Figure 5.8 indicates that the response for cresol decreased markedly from pH 11.5 to pH 10 with the reverse being true for the rest of the analytes. Although the responses varied significantly between the three pH values, the sensitivity was still sufficient for detection of all the metabolites. All showed linear responses across their respective concentration ranges despite the change in pH. The background noise also increased at higher pH values and pH 10 was selected as the optimum.

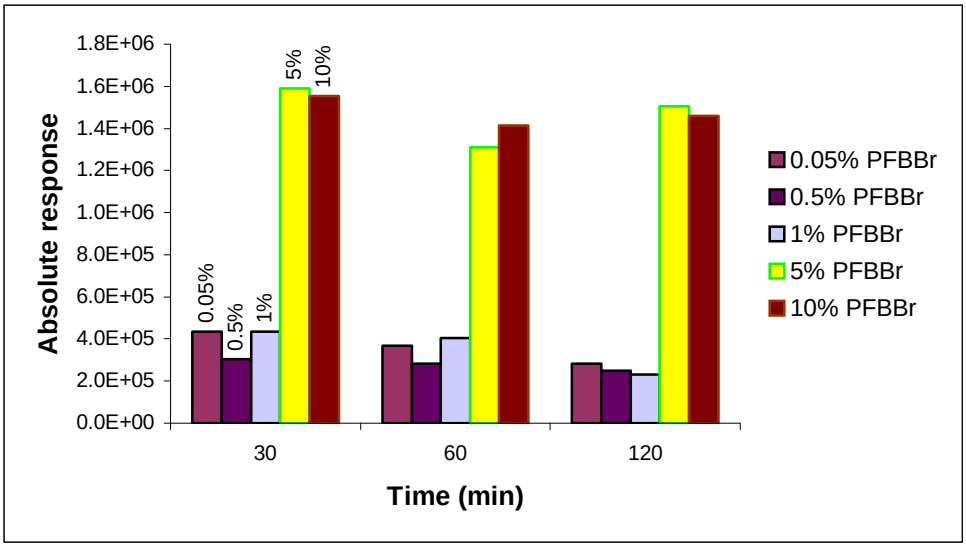

Figure 5.7 Effect of pentafluorobenzyl bromide concentration on the hippuric acid yield in the extractive alkylation procedure at pH 10 using 0.2M TBA.

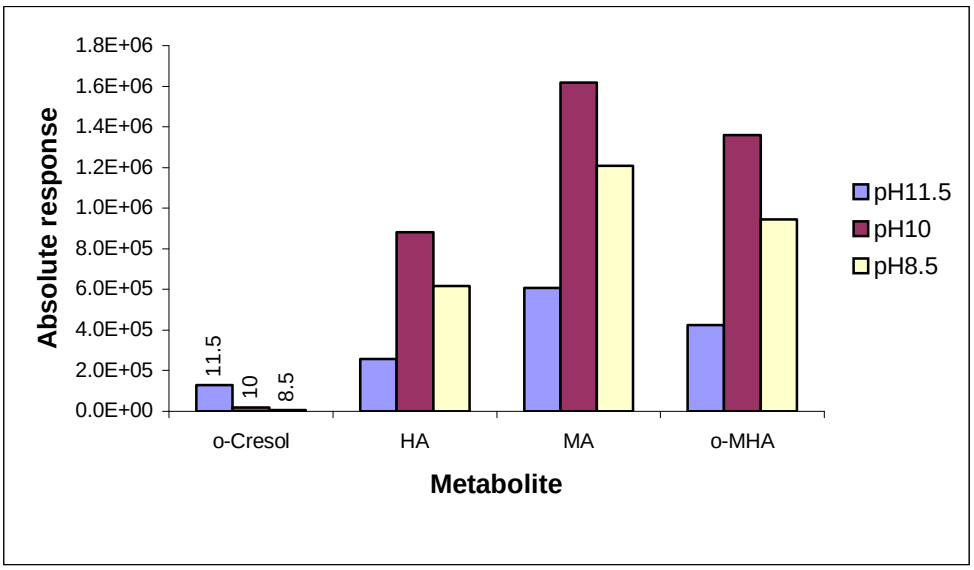

Figure 5.8 Effect of pH on the extractive alkylation of selected analytes using 5% PFBBr and 0.2M TBA. Concentrations of the analytes are that of calibration standard 3 as indicated in Table 5.1.

The concentration of the phase transfer catalyst influences the alkylating reaction rate by directly affecting the rate of extraction [17, 24]. The results obtained for *o*-cresol and HA, with increasing concentrations of TBA at pH 10 and 5% PFB-Br, are shown in Figure 5.9. A ten-fold increase in response could be observed for *o*-cresol as TBA concentration increased. The increase was more pronounced than the effect of increased

concentration of PFBBr (five-fold). This was not the case for HA and the rest of the analytes, which only had a two-fold increase associated with TBA concentration versus a five-fold increase associated with PFBBr concentration. An increased background signal resulted at concentrations above 0.4M TBA and the buffer capacity was also exceeded beyond this concentration. The increase from 0.1M to 0.2M was associated with the largest increase in response for HA (1.7 fold) and 0.2M TBA was chosen as the optimum concentration. An increase in the phase transfer catalyst concentration did not have an influence on the reaction time under the selected conditions, opposed to the effect of increased alkylating agent concentration. This was in agreement with Gyllenhaal et al [20] and as a rule of thumb an excess of alkylating reagent may be used, keeping in mind the possibility of catalyst poisoning by liberated anions from the organic phase.

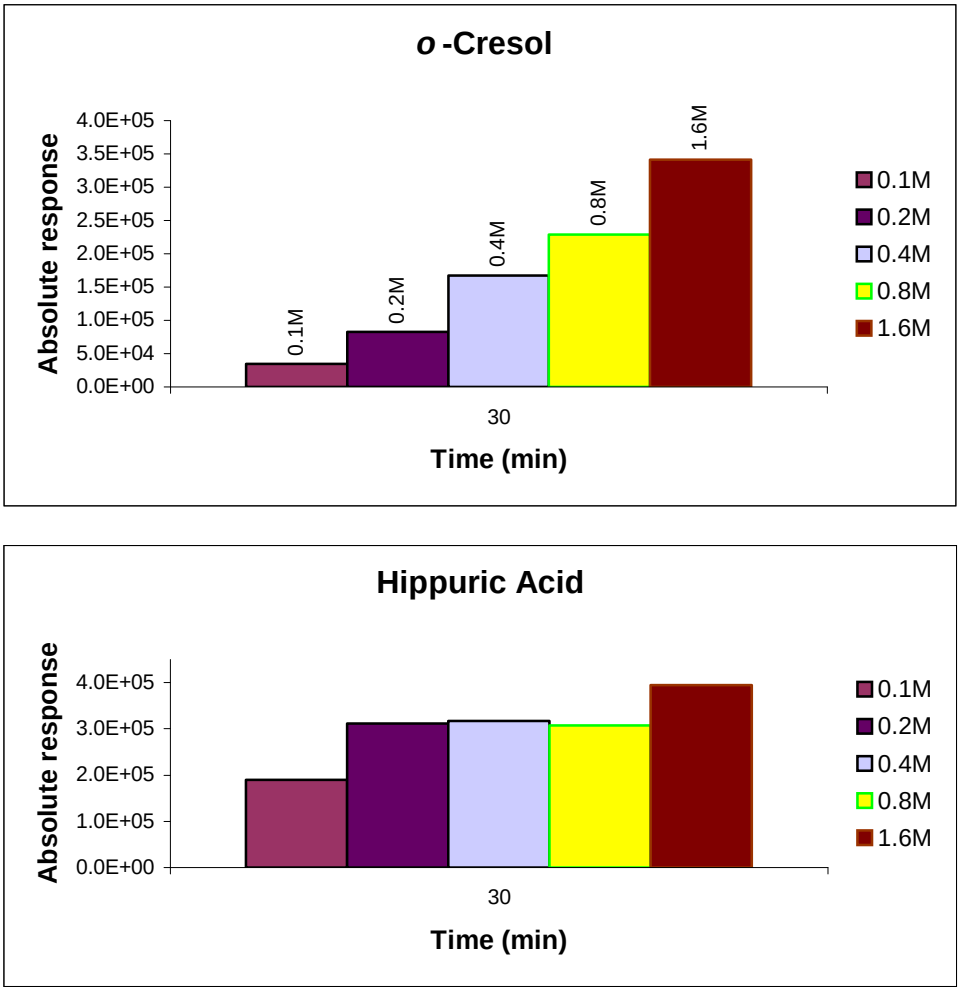

Figure 5.9 Effect of tetrabutylammonium hydrogensulphate concentration on extraction yield at pH10 and 5% PFB-Br.

5.3.3. Validation

Validation data is shown in Table 5.3. The calibration curves showed linear responses for each analyte with correlation coefficients in the range of 0.91 – 0.99. The superior behaviour of isotope labelled internal standards assisted in improved accuracy and repeatability of the assay by accurately simulating the chemistry of the respective analytes. The isotope substituted HA performed sufficiently well to be used as internal standard for the MHA isomers as well.

Analyte (IS)	Control level (mg/L)	Accuracy (% Bias)	Repeatability (% RSD)	Intermediate precision (% RSD)	LOD (mg/L)
Phenol ([$^2H_6$]Phenol)	LOW (10) HIGH (40)	-3.5 -1.8	4.7 3.4	5.9 5.1	0.01
o-Cresol ([$^2H_4$]*o*-Cresol)	LOW (0.8) HIGH (3.2)	- 3.4 - 1.1	5.2 3.5	4.8 4.9	0.02
MA ([$^2H_5$]MA)	LOW (20) HIGH (80)	1.1 3.2	4.8 5.2	7.9 7.2	0.05
HA ([$^2H_5$]HA)	LOW (20) HIGH (100)	-4.7 -6.5	8.3 7.1	10.3 8.9	0.1
Total MHA ([$^2H_5$]HA)	LOW (60) HIGH (240)	- 8.1 - 5.7	9.7 6.4	12.0 9.7	0.1

Table 5.3 Extractive alkylation assay validation data.

The accuracy, precision and limits of detection of the method compares well to those in literature [6, 13, 15] and are within the acceptable criteria for bio-analytical methods.

The stability of the PFB-derivatives is an important factor, as the application of the method would largely be geared towards routine batch analysis. Unstable derivatives lead to sample deterioration on the autosampler tray and subsequently provide unreliable results. Figure 5.10 shows the stability of phenol over 23 hours. All the derivatives had positive slopes when plotted as percentage initial abundance against time. This indicates

sufficient stability for overnight batch analysis. Freeze/thaw stability was confirmed for all the metabolites routinely included by analyzing 3 replicates of each control level (LOW/HIGH) after 3 freeze/thaw cycles. The mean of the replicate specimens maintained a 91% - 97% accuracy relative to the reference values of the IQC specimens. Stability under long term storage at -20 °C was confirmed by repeat analysis of EQC samples (phenol, MA, and MHA) received from the FIOH. Accuracy values remained within 15% of the reference concentrations.

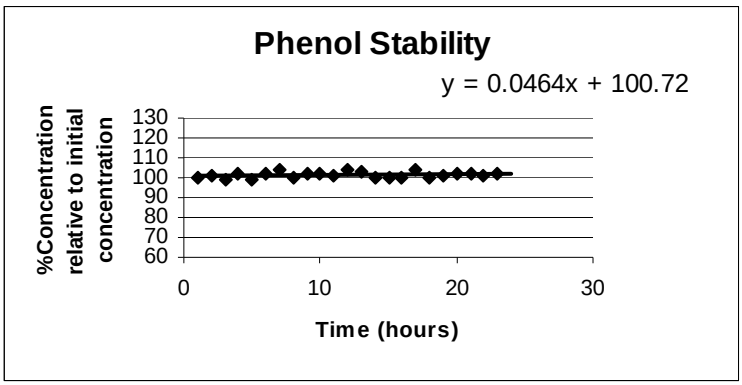

Figure 5.10 Stability results obtained for phenol-PFB by injecting the same sample consecutively, once every hour, over a period of 23 hours. The *y*-axis shows the concentration as a percentage of the initial concentration.

5.3.4. Routine screening

The maximum permissible amounts of the metabolites are indicated in Table 5.4 as the Biological Exposure Index (BEI) [25, 26] along with the LLQ (S/N=10) in urine after adjustment with average creatinine excretion values [27]. The limit of quantification after creatinine adjustment for phenol, *o*-cresol, HA, MA and MHA were well below the maximum permissible amounts, thereby allowing the current methodology to be applied as sensitive screening method for biomonitoring of exposure to benzene and alkyl benzenes.

Table 5.4 Biological Exposure Index (BEI) values compared to quantification limits of assay in urine.

Metabolite	BEI (mg/g creatinine)	BEI (mmol/mol creatinine)	LLQ (mmol/mol creatinine)[a]
Phenol	50	69.65	0.035
o-Cresol	1	1.212	0.051
MA	1500	1293	0.642
HA	2500	1829	0.748
MHA (total)	1500	1018	0.512

[a] A value of 4.18 mmol creatinine/L was used based on the assumption that 1.7 L urine is excreted per day. The normal reference range for creatinine is 7.1-17.7 mmol creatinine/day [27].

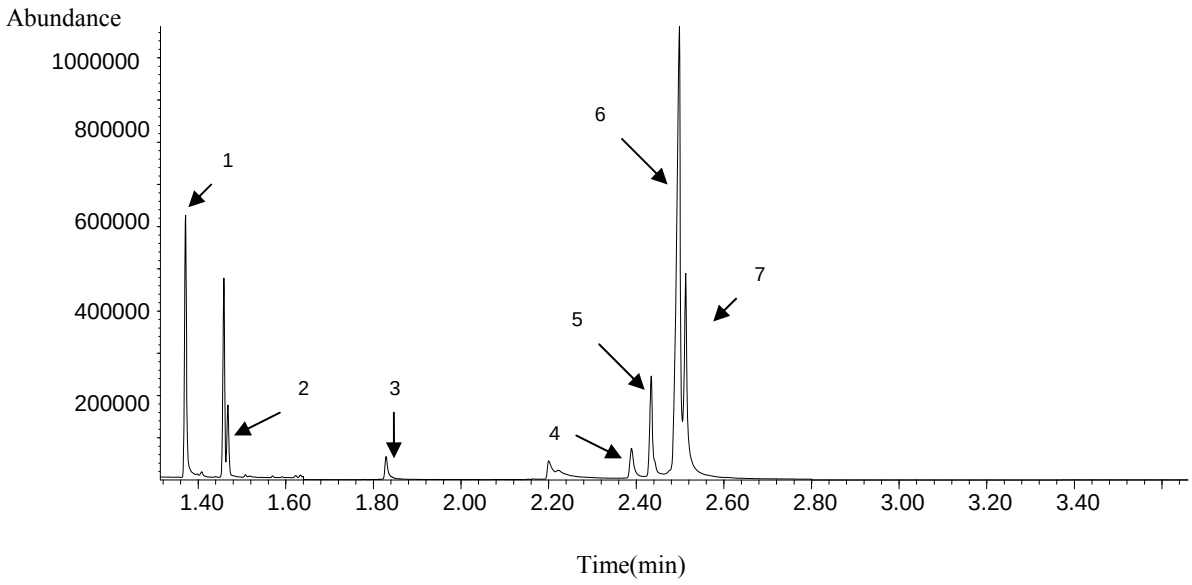

Figure 5.11 Total ion chromatogram of urine extract recorded in SIM mode for a worker exposed to high levels of xylene. Elution order:[$^2H_6$]Phenol (1),[$^2H_4$]*o*-cresol (2), [$^2H_5$]mandelic acid (3), hippuric acid (4), *o*-methylhippuric acid (5), *m*-methylhippuric acid (6), *p*-methylhippuric acid (7).

A chromatogram of a urine extract for a person exposed to high quantities of xylene is shown in Figure 5.11. No other metabolites aside from MHA and HA was detected, so exposure to pure solvent rather than a mixture is likely. The presence of HA is not necessarily indication of toluene exposure, as dietary intake of benzoate preservatives present in numerous foodstuffs leads to the formation of HA. The calculated concentration for total MHA was 1201 mg/L, which translates to approximately 1488 mmol/mol creatinine based on a conservative estimate of urinary

excretion of 4.18 mmol creatinine/L. This is clearly above BEI values and a short leave of absence or rotation to another work area is recommended.

5.4. Discussion and Conclusion

5.4.1. Extractive alkylation

In assessing the complex reaction schemes and multiple variables that are part of a phase transfer catalytic reaction, it is perhaps prudent to turn attention to the simplest and most consistent reaction required for the whole process to work – the dissociation of an analyte to form an anion in aqueous media. Similar to the need for neutral species in successful extractions by simple LLE procedures or SPE techniques utilising C18 solid phases, the essential requirement in phase transfer catalysis is the shift in equilibrium to form the required anionic species. This can very easily be performed by adjusting the pH to 2 units above the pK_a value of a specific compound. Table 5.5 indicates the pK_a values for some of the current metabolites [28].

Analyte	T_1°C	pK_a
Phenol	20	9.89
o-Cresol	25	10.20
m-Cresol	25	10.01
p-Cresol	25	10.17
MA	25	3.85
HA	25	3.62
o-MHA	25	3.91
m-MHA	25	4.27
p-MHA	25	4.36

Table 5.5 Dissociation constants of selected urinary metabolites of benzene and alkyl benzenes.

It is clear to see that the metabolites with carboxylic acid functional groups (MA, HA, MHA) are almost completely dissociated even at neutral pH. Concentrated base solutions are required as aqueous media however for dissociation of the cresols (pH >

12), which is likely to degrade most analytes. Figure 5.12 shows a simplified illustration of the most important equilibria in phase transfer catalysis [29].

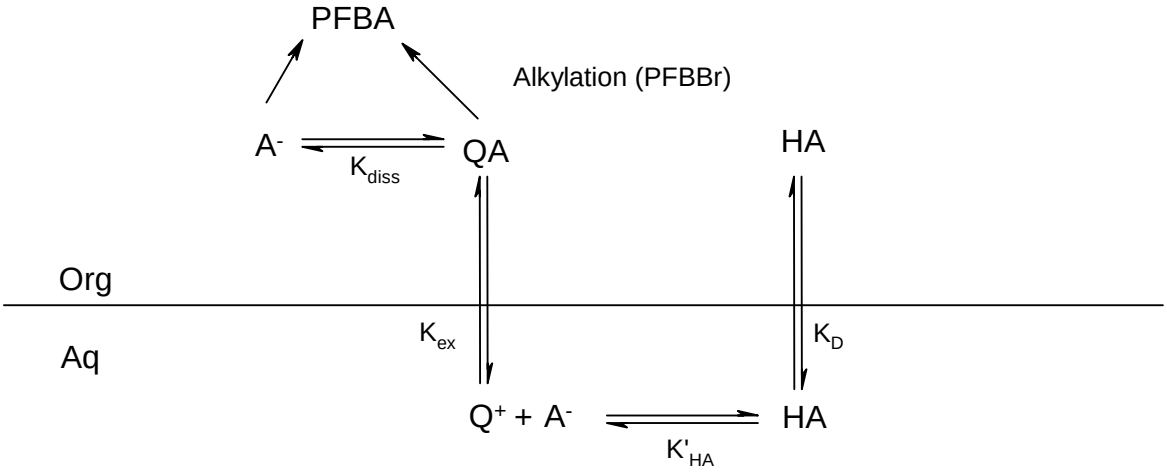

Figure 5.12 Simplified compilation of the essential equilibria in a two-phase system utilizing phase transfer catalysts. Adapted from [29]. HA = Acid/Base pair; Q^+ = Quaternary ammonium ion; A^- = Analyte anion; QA = Ion pair; PFBA = Alkylated derivative; K'_{HA} = Acid dissociation constant of HA; K_D = Distribution constant of HA; K_{ex} = Extraction constant of QA; K_{diss} = Dissociation constant of the ion pair QA

Consider the results for the optimization experiments in the current assay. The rate limiting factor for anions formed by optimum pH values appeared to be the initial low concentration of alkylating reagent. Hippuric acid (HA) largely appears as the free anion in aqueous media of pH 5 and up, and would likely form ion pairs with available cations such as the quaternary ammonium salts. The ion pair may move freely into the organic phase where it can undergo dissociation to form the free anion of HA again. Re-association with any available cation due to poor solvation in the organic phase is likely to occur if no other reaction such as alkylation is possible. If the concentration of the alkylating reagent is low the anion may thus be shuttled back and forth into the organic phase numerous times before it is in close enough proximity of the reagent for the alkylation reaction to take place. This would prolong the reaction rate excessively and, in addition, if the alkylating reagent concentration is so low that it is exhausted, the reaction cannot go to completion and the extractive-derivatization procedure won't be quantitative.

These are precisely the effects that were apparent during optimization of alkylating reagent. Improved linearity and the five-fold increase in yield were due to increased reaction rates that allowed derivatization to follow to completion in a certain time-course (quantitative) for HA. The quantitative extractive-derivatization further occurs in a shorter time period (half of initial) because it approaches the maximum reaction rate for the current reaction scheme. No further increase in derivative yield for higher concentrations of alkylating agent was observed, which indicated that it was at a maximum sensitivity for HA. A possible further reduction in reaction time rather than increased derivative yield was unfortunately not investigated for increasing concentrations of PFBBr.

It is not surprising that phosphate buffers are considered as ideal aqueous media in phase transfer catalysis. The PO_4^{3-} anion is very hydrophilic (Chapter 4) and will not interfere in any reactions. Additionally, competing for solvation with other less hydrophilic anions in the aqueous phase may be advantageous for increasing extraction constants. This is akin to salting out effects in classical LLE extraction. Addition of potassium carbonate to a water-dichloromethane two-phase system, employing terabutylammonium bromide as transfer catalyst, increased extraction constants (Figure 5.12 – K_{ex}) up to a thousand-fold [30].

The importance of pH effects on extraction rates and derivatization yields has been made abundantly clear and may be considered the key starting point for phase transfer catalytic reactions. The optimization experiments involving *o*-cresol illustrate the relation between extraction rates and derivatization yield for given time-periods quite well. The optimization of the alkylation reagent was performed at pH 10 in the presence of 0.2M TBA as catalyst. At this pH value nearly 50% of *o*-cresol may be in the anionic form, however nearly 100% of all the acid metabolites are expected to be anionic. One can imagine that there is a rush or flood of anions at any one moment in the vicinity of the available active sites presented by the phase transfer cations. It thus becomes an issue of anion concentration or density near the active site of the catalysts. Complete ionization to the anionic species as for HA, MA and MHA, translates to total availability, all at once, near the active sites and therefore a better chance for extraction.

Increased rate of alkylation brings about increased rate of dissociation and availability of the phase transfer catalyst as well. For a set time period, anions with complete availability to form ion pairs and subsequently react in the organic phase, there exists a good likelihood of maximum derivatization yield. For the same time period, if

only a few anions are available to form ion pairs, through what may be deemed as fortunate proximity, and subsequently react in the organic phase, there exists a very low likelihood of significant derivatization yield. Increased alkylation agent concentration (0.05%-5%) increased *o*-cresol derivatization yield five-fold at pH 10 in 30 min, whereas increased phase transfer catalyst (0.1M-1.6M TBA) at the same pH and time parameters indicated a ten-fold increase. Examining Figure 5.9 it appears almost a linear relationship between increased TBA concentrations and derivatization yield for *o*-cresol exists. Because the neutral and anionic species exist in equilibrium, removal of the anion from the aqueous phase shifts the equilibrium toward formation of a new anion at the same rate as the rate of removal (extraction rate) at sub-optimal pH values. The extraction rate was therefore the limiting factor in derivatization yield for *o*-cresol.

Increasing concentrations of TBA had a minimal effect on derivative yield for HA (Figure 5.9) which indicated extraction rates where close to maximum for the current system (pH optimum) and derivative yield was therefore directly linked to alkylation rate.

To summarize, the various equilibria shown in Figure 5.12 may be assessed. Optimum pH negates the influence of two terms, K'_{HA} and K_D. Eliminating K_D means no associative/dissociative effects in the organic phase other than the ion pair can take place, and only the required reactions will occur. Removal of K'_{HA} means that the rate of extraction is determined by the only terms left, K_{ex} and K_{diss}. These two equilibria are largely a function of the concentration and type (lipophilicity) of the phase transfer catalyst and the rate of reaction in the organic phase.

Using HA as example, K'_{HA} was negligible because of optimum pH, K_{ex} was at a maximum because of catalyst concentration, and the reaction rate and derivatization yield was therefore largely a function of K_{diss} (alkylation agent concentration). With *o*-cresol, K'_{HA} and K_D were still having an effect, which was reflected in K_{ex} and the increased reaction yield with increasing catalyst concentrations. Increased alkylation reagent on its own maximized K_{diss}, which increased reaction rates and yield only slightly for *o*-cresol, largely because of increased availability of free catalyst after quick dissociation with other analyte anions such as HA.

Other parameters that have to be taken into account in the extractive alkylation process are phase ratio and temperature. These parameters are by no doubt secondary to those discussed above, but may have enough influence to make a difference in trace compound analysis. Close proximity of the anion target molecules to the cation transfer

catalysts are required for ion pair formation. Depending on the specific lipophilicity of a selected phase transfer catalyst it is postulated that the phase boundary between aqueous and organic media is where the quaternary ammonium ions find themselves.

One can compare it to a buoy anchored in the ocean with the surface of the water as the phase boundary between the ocean and atmosphere. The buoy represents the catalyst, the ocean the organic phase, and the atmosphere the aqueous phase. At any point in time the buoy is in contact with both phases but more strongly associated (anchored) to the ocean. As the tides change it is exposed to the atmosphere to a greater or lesser degree. Similarly, the phase transfer catalyst disperses itself between the aqueous and organic phase to various degrees around the phase boundary. Target anions must be close to, or be able to move toward, the phase boundary for extraction to occur.

If the concentrations are high the phase ratio has very little effect, however if concentrations are low a larger volume of aqueous phase matrix is normally used to increase the absolute amount of analyte available for extraction. The volume of organic phase cannot be too small as interaction between the catalyst and the target anions becomes unlikely. Sufficient agitation of both phases allows greater surface volume exposure of the catalysts to take place, as the organic phase is dispersed in small droplets into the aqueous phase.

Temperature may play a role by affecting extraction rate or derivatization reactivity. Higher temperature imparts greater energy to molecules thereby increasing movement in both phases and increasing extraction rates. Derivatization may also be increased due to greater reactivity of reagents at higher temperatures. Exothermic reactions may however be inhibited at elevated temperatures and thereby decrease the derivatization rate and yield.

The use of stable isotope substituted analogs as internal standards in the extractive alkylation procedure are recommended and largely contributes to its success, as the variable extraction parameters may affect diverse compounds to highly different degrees. This variation must be equally reflected by the IS and the analyte to ensure correct quantitation. If substituted analogs are not available, compounds with similar functional groups and pK_A values must be selected.

5.4.2. Method performance

Different derivatization reagents were assessed initially. Propyl derivatives from derivatization with 1-iodopropane showed superior chromatographic performance on the DB-5 (5% phenyl-, 95% dimethylpolisiloxane) stationary phase. The use of this reagent was however prohibited by the appearance of mixed derivatives (methyl, ethyl, butyl), most likely originating from impure reagent. PFBBr was shown to give abundant singular derivative yields and adequate chromatographic resolution under the selected chromatographic conditions (Figure 5.4 and 5.5). As indicated by the figures, resolution decreased with increased separation speed upon switching to a smaller internal diameter column. The resolution term R_s (Chapter 3) for two adjacent bands or peaks may also be defined as follow:

$$R_s = 2(t_2 - t_1)/(w_1 + w_2) \qquad \text{(Eq. 5.1)}$$

$(t_2 - t_1)$ = Retention time difference for adjacent peaks

$(w_1 + w_2)$ = Baseline width in time units for each peak

For routine quantitation purposes it is ideally preferred that $0.8 \geq R_s \leq 1.5$. This allows sufficient resolution in minimum elution time. The isomers of cresol were still separated with baseline resolution ($R_s > 1.25$) allowing accurate quantitation of *o*-cresol, the only significant isomer as biomarker for toluene exposure. The quantitation of total MHA concentration, as a reflection of total xylene exposure, was a function of the sum of all MHA isomers. Baseline resolution was thus not required, although an $Rs = 1$ was maintained between *m*-MHA and *p*-MHA (peak size ratio = 1/1).

Slight tailing is observed in Figure 5.5 for the acidic metabolites (MA, HA and MHA) and performance on different stationary phases were assessed. DB-1 (100% dimethylpolysiloxane) and DB-17 (50% phenyl-, 50% dimethylpolisiloxane) capillary columns of similar dimensions (5m x 100 µm; d_f 0.1 µm) were employed. The higher polarity of the DB-17 stationary phase yielded more symmetrical peak shapes for the analytes in question, however the high workload of other routine procedures in the laboratory utilising a DB-5 column, coupled to the additional time required to alternate between columns, precluded its use.

S-PMA was initially included in the assay as sensitive biomarker for low-level exposure to benzene (Figure 5.4). The optimized extraction conditions alongside the selected instrument parameters were a compromise between the required sensitivities of

the various analytes and their respective physicochemical properties. In simultaneous analysis of the various metabolites the required detection limits for *S*-PMA could not be reached (< 0.001 mg/L). The high split ratio (200:1) and the extraction conditions were not optimized for this analyte in the current assay. The extractive alkylation procedure was therefore altered and splitless injection employed to reach the required sensitivities.

The high concentration of HA in authentic specimens and its co-extraction with *S*-PMA caused significant sample overload on the GC column in the splitless mode, and accurate quantitiation could not be performed. Subsequently, S-PMA could not be successfully quantified under the current extractive alkylation system. Additional sample preparation steps to increase selectivity for the analyte is required [31]. The simultaneous analysis of *S*-PMA alongside the other metabolites could not be performed.

The elevated pH of the urine specimens caused non-selective extraction of basic metabolites from the matrix. These compounds remained underivatized during sample preparation and caused deterioration of the chromatographic column with repeat analytical cycles. Non-linear chromatography due to additional active sites in the chromatographic system was observed. The addition of a solvent wash with 0.1M HCl (1 mL) removed these co-extracted compounds and also decreased the concentration of TBA that remained in the organic phase after extractive alkylation.

Pyrolytic alkylation is known to occur in the presence of quaternary ammonium salts at elevated temperatures (Chapter 4, Section 4.5.2) encountered in the injection port of a GC-MS system. It has been shown that high concentrations of co-injected phase transfer catalyst may interfere in the analysis of certain compounds or cause degradation of the stationary phase over a prolonged period [32]. Thermal degradation of TBA was evident in the elution of its corresponding tertiary amine (tributylamine) in the solvent front, but no non-selective alkylation of any of the relevant compounds was identified.

A high split ratio was originally selected simply to avoid overloading the capacity of the microbore, thin-film capillary column (100 µm; d_f 0.1 µm), however it presented additional advantages. Utilizing a high split ratio in the analysis further served in reduction of co-injected reagents (alongside the solvent wash procedure) and no additional column deterioration could be attributed to the phase transfer catalyst when compared to other routine methods. Retention times for the various analytes were always within 10% of the relative retention time of corresponding standards irrespective

of injection sequence. Approximately 1000 to 2000 analyses were performed on a single column before peak shape for the acidic metabolites (MA, HA, MHA) were judged too distorted by visual inspection. Columns could be successfully recycled with solvent rinsing procedures (1-propanol/dichloromethane) allowing approximately 500-1000 more injections per column upon re-installation.

The sensitivity and selectivity of the assay is illustrated in Figure 5.5. The extracted ion chromatogram of the quantifier ions show an absence of peaks other than those of the measured analytes. Furthermore, the abundance of the selected ions produces strong signals for quantitation, even at the high split ratio employed and utilising only a fraction of the organic phase (200 µL) without further sample enrichment procedures. The total ion chromatogram elution pattern is consistent and easily recognisable when comparing an authentic specimen (Figure 5.11) and calibrator (Figure 5.5). The precision, accuracy and stability data from validation experiments further supports the value of the current assay for routine application.

Participation in the external quality assessment scheme of the Finnish Institute of Occupational Health (FIOH) over a 1 year period yielded excellent results. Three batches of 2 unknown concentration levels each for phenol, MA and MHA in urine were received during the year as part of the scheme. Scoring criteria were as follow: a score of 1 is assigned if reported values are within 20% of assigned reference values (accuracy 80%-120%); a score of 2 is assigned if reported values are within 10% of assigned reference values (accuracy 90%-110%). Scores are added up to a total out of 12 (2 for each analysis of each level) for each analyte at the end of the assessment period. Final scores were 10 out of 12 (phenol and MHA) and 12 out of 12 (MA) for the year 2006, thereby further validating the applicability of the current assay as EQC specimens are the ultimate indicators of method performance.

A small volume of urine sample (1 mL) was used due to the high concentrations of the metabolites in the matrix and the high sensitivity of the assay. Subsequently a small volume of solvent (1 mL) was employed in the extraction procedure. In conventional LLE procedures the same solvent volume may have sufficed, but increased solvent handling (e.g. sample-concentration), less selective extraction (e.g. matrix background), and lengthier derivatization reactions (e.g. silylation) would be unfavourable compared to the current assay. Both extractive alkylation and LLE are cheaper options compared to SPE. The reduced solvent volumes carry reduced costs for waste disposal, whereas SPE would typically include two wash steps and an elution step

with different organic solvents. The costs of SPE cartridges far outweigh that of the phase transfer catalyst, which was used in fairly low concentration (0.2M). In terms of speed, GC-MS necessitates derivatization irrespective of extraction methodology, therefore combining extraction and derivatization in one step creates the fastest possible option for sample preparation.

The deconjugation of phenol was considered the limiting factor in sample turn-around time for the current assay. Enzymatic hydrolysis was selected above acid hydrolysis despite increased reagent cost and reaction time. Conditions for complete deconjugation utilising acid hydrolysis was associated with increased background noise and degradation of MA, amongst others. Active handling of specimens and reagents (pipetting, sample transfer, aliquot) by laboratory personnel during sample preparation may be termed occupied time. Sample incubation time, centrifuge time and time spent on the analytical instrument are all examples of unoccupied time for personnel as they may perform other duties during these events.

The turn-around time is the total time taken from receiving to the reporting of a result for a specific sample and is a reflection of analysis speed. Sample throughput is the amount of samples that may be analyzed in a given time-period. Regarding labour by analysts, this consists of both occupied and unoccupied time. Optimal turn-around times may be achieved by condensing unoccupied time into occupied time by what may be termed sequential-batch analysis. The deconjugation time is a set factor, irrespective of batch size for analysis of phenol. A batch of 100 urine specimens may be prepared and placed on a heating block for 3 hours. During this time the analyst may label and ready the required vials and reagents, create the instrument analysis sequence, perform maintenance or some other duties relating to the analysis.

The extractive alkylation procedure for 30 samples takes approximately 1 hour (30 min extraction; 30 min sample transfer etc.) and for 100 samples approximately 2 hours. The instrument analysis time is set at approximately 10 hours (10 samples per hour). Data review and integration is approximately 3 min per sample. Sequential-batch analysis prioritises the activities by placing smaller batches through the various stages at different time intervals. Extractive alkylation of 30 samples will have an instrumental analysis time of 3 hours. The batch of 30 samples is placed on the instrument and the analysis is started. A second batch of 30 is prepared by extractive alkylation in the hour while the first batch is analysed, and also placed on the instrument. The remaining instrument analysis time for the 50 samples is 5 hours.

Data interpretation and integration for the first 10 samples already analysed in the initial run may now be performed (0.5 hours). The final batch of samples is prepared and placed on the instrument to be analyzed. Using the sequential-batch analysis, the fastest total turn-around time for the first 10 samples is the sum of 3 hours (deconjugation), 1 hour (sample preparation – 30 samples), 1 hour (instrument analysis) and 0.5 hours (data handling) – a total of 5.5 hours. In a strict batch analysis, the fastest total turn-around time for the first 10 samples is the sum of 3 hours (deconjugation), 2 hours (sample preparation – 100 samples), 1 hour (instrument analysis) and 0.5 hours (data handling) – a total of 6.5 hours. The sample throughput in 12 hours is 100 samples for both approaches.

In specimens where quantitation of phenol is not required, the turn-around time for sequential-batch and batch analysis is 2.5 and 3.5 hours respectively, with a sample throughput of 100 samples in 9 hours. With respect to an 8 hour workday for laboratory personnel, the results for a batch of 100 samples can therefore be reported within 24 hours of reception for routine specimens. Results for possible emergency cases can be reported within 2.5 - 5.5 hours depending on the inclusion of phenol.

5.4.3. Concluding remarks

The extractive alkylation assay is sensitive and selective for biomonitoring of the variety of metabolites in urine as required by authorities for the exposure to benzene and alkyl benzenes. The method is simple, rapid and cost-effective. The speed at which the analysis can take place makes the assay especially applicable for routine quantitation of exposure to these vapours. A metabolite profile of occupationally exposed persons can easily be attained using a single method. This may replace a combination of analytical methods that are currently used for biomonitoring of exposure to aromatic vapours. The possibility exists for a range of different metabolites to be included alongside the current ones by altering the extraction parameters.

5.5. References

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Chapter 6

EXTRACTIVE ACYLATION OF AMPHETAMINE TYPE STIMULANTS IN URINE

6.1. Introduction

"There is but one right, and the possibilities of wrong are infinite." (Thomas Huxley, 1825-1895)

Stimulant abuse has increased worldwide over the past decade with the classical phenethylamine derivatives amphetamine (AMP, "Speed") and methamphetamine (MET,"Ice") at the forefront. In South Africa, there has been a high incidence of methamphetamine abuse in the Western Cape region since 2003, where it has currently become the most prevalent drug of abuse, superseding the use of historically more commonly abused substances such as cocaine, heroin and methaqualone [1]. In other regions, the synthetic designer drug methcathinone (MCA, "Cat") has risen in popularity, especially amongst youth involved in the so-called club culture. Likely reasons for this trend are due to both the low cost and ease of synthesis of these compounds from standard over-the-counter preparatives, namely ephedrine (EPH) or pseudoephedrine (PEP). Ring substituted derivatives of amphetamine and methamphetamine such as the methylenedioxy designer drugs methylenedioxymethamphetamine (MDMA, "Ecstacy"), methylenedioxyamphetamine (MDA, "Adam"), methylenedioxyethylamphetamine (MDEA, "Eve") and N-methyl-1-(3,4 methylenedioxyphenyl)-2-butanamine (MBDB) represents a lower, yet still significant percentage of abused psychostimulants, especially under recreational drug users. Figure 6.1 indicates the structures of the common amphetamine type stimulants.

An extension of occupational monitoring is so-called workplace drug testing. The same risks to individual health, co-worker safety, as well as increased employer expenditure due to employee absenteeism, traditionally associated with occupational exposure to harmful substances, is also linked to illicit substance abuse [2]. Workplace drug testing in Europe and the United States have resulted in positive incidence rates of 8-35% [3]. It was estimated that 10-23% of workers used illicit substances while on duty. The economic impact of such substance abuse was estimated to range from 46-85

billion dollars per annum. Claims were made that an employee involved in recreational drug use was more than twice as likely to request time-off, three times as likely to be late for work, and three to five times as likely to be involved in workplace accidents and file worker's compensation claims.

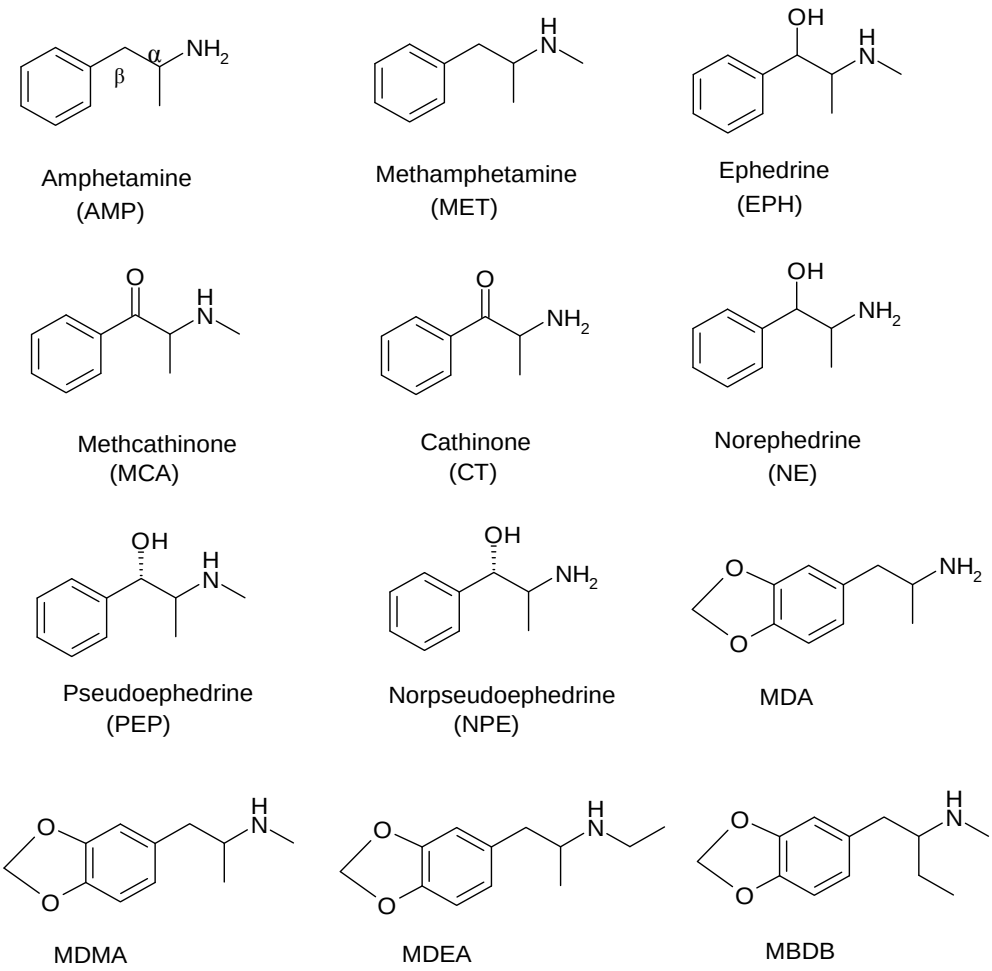

Figure 6.1 Chemical structures of relevant compounds: amphetamine (AMP), methamphetamine (MET), ephedrine (EPH), methcathinone (MCA), cathinone (CT) norephedrine (NE), pseudoephedrine (PEP), norpseudoephedrine (NPE), methylenedioxyamphetamine (MDA), methylenedioxymethamphetamine (MDMA), methylenedioxyethylamphetamine (MDEA), N-methyl-1-(3,4 methylenedioxyphenyl)-2-butanamine (MBDB).

6.1.1. Effects and metabolism

AMP and MET are potent central nervous system stimulants that produce increased alertness and euphoria [4, 5]. MCA has been shown to have similar central

stimulant effects as MET [6, 7] and is more potent than cathinone (CT) [8], however there is a reported greater degree of impaired judgment and inability to concentrate. Ring substitution on the classical phenethylamine backbone has largely produced compounds that potentiate hallucinogenic effects. The methylenedioxy derivatives of AMP, however, show none to very mild hallucinogenic properties, but induce strong feelings of euphoria, increased sociability and empathy [9, 10]. These properties have earned them the classification of entactogens [11, 12]. The metabolism of AMP and most derivatives have been elucidated [13, 14, 15] but the complete metabolism and expected half-life of MCA was not documented in any published work at the time of this study. Published urinary excretion profiles for CT and MCA have however appeared [16, 17]. Metabolism of amphetamine and derivatives occurs mainly by cytochrome P450 isoenzymes CYP2D6, CYP1A2 and CYP3A2/4. The principle phase 1 metabolic pathways for amphetamine and N-substituted derivatives are N-dealkylation, ring hydroxylation with subsequent methylation of the hydroxyl group, and oxidative deamination [14]. The methylenedioxy derivatives undergo demethylenation to catechols with subsequent methylation of one of the hydroxyl groups, and N-dealkylation [15]. Figure 6.2 illustrates the major metabolism pathways.

6.1.2. Screening and confirmation

Detection of illicit substances in humans is a very serious matter due to the legal implications and personal repercussions of the possible results. Two major issues need to be taken into consideration in workplace drug testing. The first is the protection of the individual, employer and co-workers against the harmful effects of substance abuse. The second is the protection of the individual liberties and human rights of people subjected to testing. The choice of sample matrix, sampling procedure and analytical testing strategy all occur with these two issues in mind.

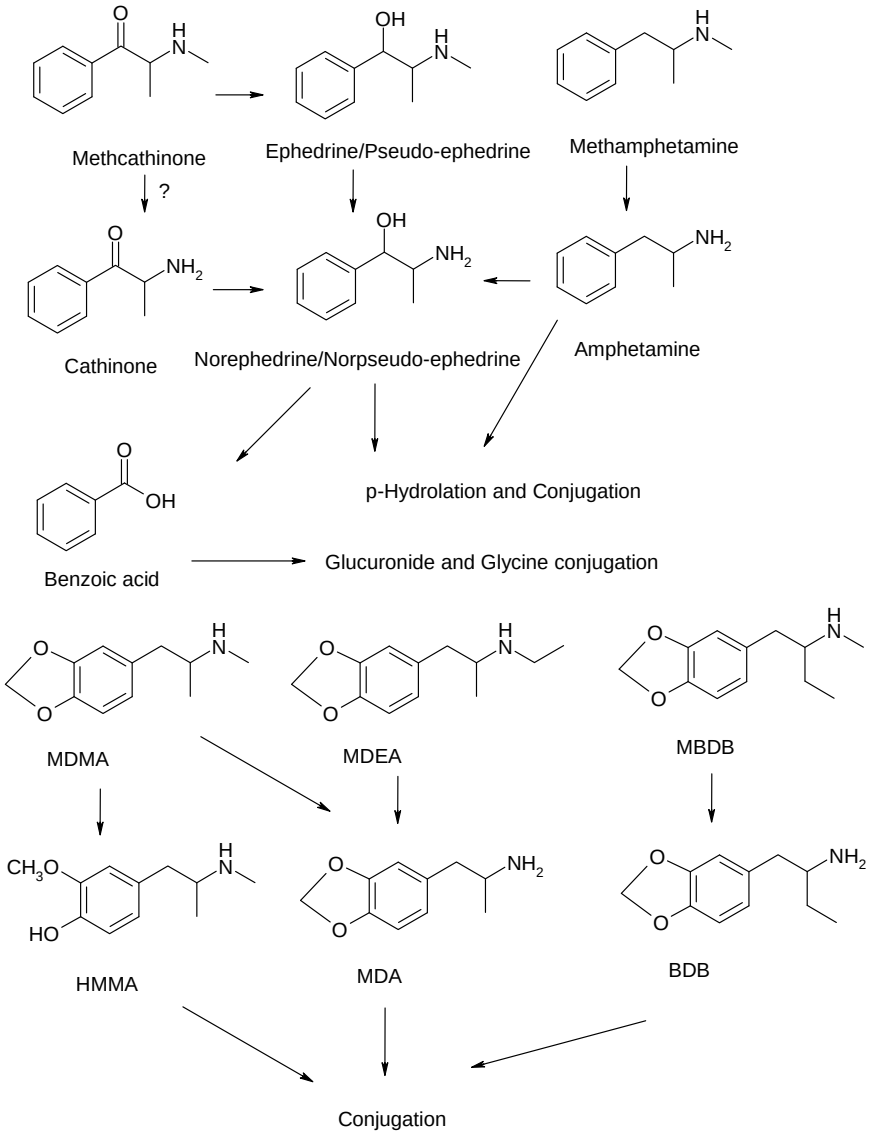

Figure 6.2 The major metabolism pathways of amphetamine and related analogs [13, 14, 15].

Various matrices such as plasma, urine, saliva, hair, nails etc. may be utilized for detection of illicit compounds. The physicochemical properties of the compound and its metabolism largely determines which matrix to be utilised (Chapter 1), however the invasiveness of the collection procedure and the information required from the analysis also play a significant role. Saliva, hair and nails are the least invasive, but offer very different information with regards to substance abuse. The latter only reflect long-term use (weeks to months) and are subject to environmental contamination. Saliva indicates very recent use (hours) but is subject to oral pH and buccal contamination from smoking. Plasma samples are the most invasive as they require a trained phlebotomist to draw blood from a venupuncture. It reflects recent use and may be used to judge current

intoxication status in situations where compounds have well-described pharmacokinetics (e.g. alcohol). Urine has traditionally been the matrix of choice for detection of illicit compounds. Depending on the metabolism and half-life of the specific compound, it reflects short to moderate long-term use (hours to weeks). It may be collected by untrained persons and is considered relatively non-invasive. Testing for structure specific metabolites or metabolic profiles circumvents external contamination.

Urine testing is however subject to adulteration, both *in-vitro* and *in-vivo*. Ingestion of water or substances to hasten or inhibit excretion, or substitution of a urine specimen with a drug free sample is *in-vivo* adulteration. Addition of a foreign substance to alter the structure of the compounds themselves (e.g. oxidation) or to affect the principles of the analytical methodology is *in-vitro* adulteration. A proper sampling protocol largely prohibits the occurrence of adulteration.

Sampling protocols are established to ensure the entire drug testing process is capable of legal scrutiny. It provides safeguards to protect the integrity of specimen donors and guarantees accurate and reliable information about their drug use. It is beyond the scope of the current work to detail all the steps in a sampling protocol [18, 19], but the key points are that the specimen is freshly voided, not subjected to external contamination, protected against tampering and adulteration, and traceable back to the donor. Generally the donation is monitored within a secured area to prevent adulteration, split into two separately sealed samples, and sent for analysis.

The analytical strategy in testing for illicit substances in urine usually follows a two-prong approach. Screening tests employing immunoassays, either as point-of-care devices (immuno-chromatographic) or automated laboratory instrumentation, are most widely available. These analyses are based on the interaction between an antibody raised against a specific antigen (drug). It is usually a competitive binding assay where the drug competes with a labelled analogue of some kind. The antibody-antigen binding is only class specific due to the binding sites available in the raised antibody, and significant cross reactivity between compounds with similar structures occur. The tests are only semi-quantitive due to the variability of cross reactivity to each compound in a class. For instance, a benzodiazepine immunoassay may have 100% reactivity toward oxazepam but only 50% toward flunitrazepam. Any assigned numerical value is therefore an approximation of the total cross reactivity occurring, as both analytes may be present but no differentiation is possible. It is precisely the uncertainty, due to variable cross reactivities in immunoassay screening, that only allow them to distinguish

between negative and presumptive positive tests. A secondary, structure specific analysis is therefore utilised to confirm the results of positive screening test. This is usually performed by GC-MS.

6.1.3. Analytical approach

The majority of samples received by our laboratory are direct confirmation requests by clinicians. Presumptive positive urine samples that result from laboratory screening and workplace point-of-care devices based on immunoassay technology are also received on a regular basis. These are known to be subject to a variety of cross reactivities [20], especially with reference to amphetamine type stimulants [21, 22]. CT and MCA are not listed as cross reactants in such amphetamine immunoassay systems, and the potential metabolic products ephedrine (EPH), pseudoephedrine (PEP), norephedrine (NE) and norpseudoephedrine (NPE) are commonly listed at less than 1% cross reactivity. A high probability therefore exists of MCA abuse going undetected in these screening methods, which calls into question the traditional approach of exclusive confirmation of screen-positive specimens only. Multi-analyte confirmation for amphetamine type stimulants is therefore a requirement to ensure that a clear picture of possible abuse is realised.

Quantitative analyses of amphetamine and associated analogues have been reviewed [15]. Analytical strategies including gas chromatography (GC) [23, 24], high performance liquid chromatography (HPLC) [25, 26], gas chromatography-mass spectrometry (GC-MS) [16, 23, 27-32] and liquid chromatography-tandem mass spectrometry (LC-MS/MS) [33, 34] have been employed. Recent work that has focused on multi-analyte procedures has shown their applicability in a routine laboratory setting [32-34]. LC-MS/MS procedures [33, 34] have the advantage of decreased work-up time due to the ability to directly analyse underivatized compounds. These techniques have also shown the ability to adequately separate structural isomers such as NPE and NE [33] with relatively short analysis times, having a reported instrument cycle-time of 8 minutes per sample [34].

Currently, GC-MS is still the preferred method for forensic confirmation in urine [35] and has the advantage of lower equipment costs. Utilizing a single step extractive-derivatization technique with heptafluorobutyric anhydride (HFBA), it has been shown

that derivatization need not be a timely step in GC-MS sample preparation procedures for amphetamines [32]. Aside from direct injection methodologies that may suffer from their own pitfalls such as ion suppression/enhancement, similar sample preparation times for GC-MS and LC-MS/MS may exist.

Rapid chromatographic separation with microbore capillary columns [36] and quick temperature gradients [27, 37] in fast GC-MS analysis have indicated the possibility of accurate quantitation in relatively short analysis times with adequate resolution between structurally close-related components (Chapter 4). Care must however be taken in addressing the number of data points that define a chromatographic peak for forensic confirmations in such methodologies [38, 39, 40]. Combining extractive-derivatization techniques with fast GC-MS creates an attractive analytical approach for routine confirmations, only if analytes of interest can be sufficiently separated chromatographically and accurately quantified.

6.2. Materials and methods

6.2.1. Reagents

All reagents and solvents used were of analytical grade. The following methanolic standards were purchased from Cerilliant (Round Rock, Texas, USA), each at 1mg/mL: AMP, MET, MCA, CT, NPE, NE, EPH, PEP, MDA, MDMA, MDEA, phentermine (PHM), fenfluramine (FFA,) and 4-hydroxy-3-methoxymethamphetamine (HMMA). Deuterium substituted internal standards were also obtained from the same manufacturer, each at 100 µg/mL in methanol: [$^2H_{11}$]AMP, [$^2H_9$]MET, [$^2H_3$]NE, [$^2H_3$]EPH, [$^2H_5$]MDA, [$^2H_5$]MDMA and [$^2H_6$]MDEA. Dichloromethane (CH_2Cl_2), methanol (CH_3OH) and 2, 2, 3, 3, 3-pentafluoropropionic anhydride (PFPA) were purchased from Fluka (Buchs, Switzerland). Sodium hydrogen carbonate ($NaHCO_3$) was purchased from Merck (Darmstadt, Germany). Deionized water was obtained from a Milli-Q (Millipore, Bedford, MA, USA) water system. External quality control (EQC) samples (Liquichek™) were obtained from Bio-Rad (Irvine, California, USA).

6.2.2 Solutions and biosamples

Stock solutions of the compounds of interest and internal standards were dissolved in methanol. Initial work solutions containing AMP, MET, MCA, NE, EPH, MDA, MDMA, MDEA and MBDB (solution A) and CT, NPE, PEP, PHM, FFA, and HMMA (solution B) were appropriately diluted with methanol from stock in the range 125 ng/mL to 100 000 ng/mL for studying the linearity and selectivity of the assay. After the initial assessments were completed, solutions for calibration and quality control were prepared as follow: A calculated volume of stock solution for each analyte was further diluted in methanol to obtain a calibration working solution of 8000 ng/mL containing a mixture of AMP, MET, MCA, NE, EPH, MDA, MDMA, MDEA and MBDB. Further serial dilutions were made to obtain final calibration standards of 4000, 2000, 1000, 500, 250 and 125 ng/mL.

A separate quality control (QC) working solution was prepared in a similar manner, but from different lot stock solutions to avoid bias, and further diluted with methanol to yield control solutions that could be spiked in pooled urine (concentrations given below).

The internal standards (IS) were diluted from stock to a concentration of 2000 ng/mL in methanol. Solutions were stored at 4°C in the dark for a maximum of 3 months. Authentic urine specimens were stored at 4°C in the dark and analysed within 2 days of reception. Thereafter, aliquots were made and stored frozen at -20°C.

6.2.3 Equipment

A Hewlett-Packard 6890 GC system fitted with a Hewlett-Packard 5973 mass selective detector (MSD) (Agilent Technologies, Palo Alto, CA, USA) and a GERSTEL MPS2 injector (Gerstel GmbH & Co.KG, Müllheim an der Ruhr, Germany) was used for mass spectrometric analysis. The injector was run in the standard liquid sampling mode utilising a 10 µL syringe. A 220V oven shroud was installed on the GC. A microbore DB-5 fused-silica capillary column (5 m x 100 µm; d_f 0.1 µm) (Agilent Technologies, Palo Alto, CA, USA) was used to effect fast separation. The MS was tuned monthly with perfluorotributylamine (PFTBA) using the autotune function as per manufacturer's recommendation. Data collection, integration and signal-to-noise ratio

(*S/N*) was performed with HP Chem Station software. System maintenance was performed as described previously (Chapter 5, Section 5.2.3).

6.2.4 GC-MS procedure

A 1 µl volume of derivatized sample was injected in splitless mode with a purge time of 0.1 min and purge flow of 20 mL/min. The inlet temperature was set at 280°C and helium carrier gas at a constant flow-rate of 0.6 ml/min. The average velocity was 70 cm/s. A solvent delay time of 0.9 min and two-ramp temperature gradient was used. An initial isotherm of 85°C was maintained for 0.35 min and ramped at 62°C/min to a temperature of 115°C, which was kept for 0.8 min. The second ramp rate was 65°C/min to an isotherm of 300°C, which was maintained for 0.52 min. This amounted to a total chromatographic time of 5 min. The MSD transfer line temperature was set at 280°C and that of the quadrupole and source at 150°C and 230°C respectively. A solvent delay time of 0.9 min was set before the source was turned on. All mass spectra were recorded at 70 eV (electron impact, positive mode) with an EM offset of +200 V.

Chromatograms were first recorded in full-scan mode (50-550 *m/z*) to identify analytes and their respective retention times. Routine confirmation and quantitation was performed in the selected-ion monitoring (SIM) mode with respect to three significant ions for each compound and two for each IS. Positive confirmation utilizing three significant ions was in accordance with published recommendations [41]. The mass-to-charge ratios (*m/z*) of the analyte ions used for quantitation in SIM mode are indicated in Table 6.1. Eight SIM time-windows were set to monitor the aforementioned analytes, with a maximum of eight ions (10 ms dwell-time per ion) monitored per window for collection of sufficient number of data-points that adequately defined chromatographic peaks.

Analyte	Qualifier ion 1 *(Relative abundance %)*	Qualifier ion 2 *(Relative abundance %)*	Quantifier ion *(Relative abundance %)*
Amphetamine	91 *(42)*	118 *(79)*	190 *(100)*
Methamphetamine	118 *(24)*	160 *(28)*	204 *(100)*
Norephedrine	253 *(6)*	280 *(14)*	190 *(100)*
Ephedrine	160 *(20)*	294 *(4)*	204 *(100)*
Methcathinone	105 *(95)*	160 *(31)*	204 *(100)*
MDA	135 *(100)*	325 *(16)*	162 *(45)*
MDMA	160 *(32)*	339 *(17)*	204 *(100)*
MDEA	162 *(44)*	353 *(12)*	218 *(100)*
MBDB	218 *(100)*	353 *(12)*	176 *(59)*
[$^2H_{11}$]AMP	128 *(74)*		194 *(100)*
[$^2H_9$]MET	163 *(28)*		211 *(100)*
[$^2H_3$]NE	283 *(15)*		193 *(100)*
[$^2H_3$]EPH	297 *(5)*		207 *(100)*
[$^2H_5$]MDA	330 *(16)*		167 *(43)*
[$^2H_5$]MDMA	344 *(14)*		208 *(100)*
[$^2H_6$]MDEA	359 *(11)*		224 *(100)*

Table 6.1 The mass-to-charge ratios (*m/z*) of the analyte ions used for quantitation in SIM mode.

6.2.5 Sample preparation

The method of Beck and Faull [42] was adapted and modified as follow. Solid $NaHCO_3$ (150 mg) was added 'volumetrically' to urine aliquots (950 µl) containing IS (50 µl) and vortexed (10 sec). Dichloromethane (1500 µl) was added, followed by addition of PFPA (50 µl). The acylation reaction started immediately after addition of PFPA, and samples were shaken (2 min) on a mechanical multi-shaker. Thereafter, samples were centrifuged (2 min, 3500 rpm) to achieve adequate phase separation and the organic phase (±1000 µl) transferred into a clean GC vial. Care was taken not to aspirate and transfer any of the excess solid $NaHCO_3$ along with the organic phase. The extract was gently dried under nitrogen (± 5 min) and additional PFPA added (50 µl) for derivatization (10 min, 80°C) of β-hydroxy groups of ephedrine analogs. Excess PFPA was removed at room temperature under a stream of dry nitrogen (± 5 min) and reconstituted with CH_2Cl_2 (60 µl) prior to GC-MS analysis. Figure 6.3 summarizes the process.

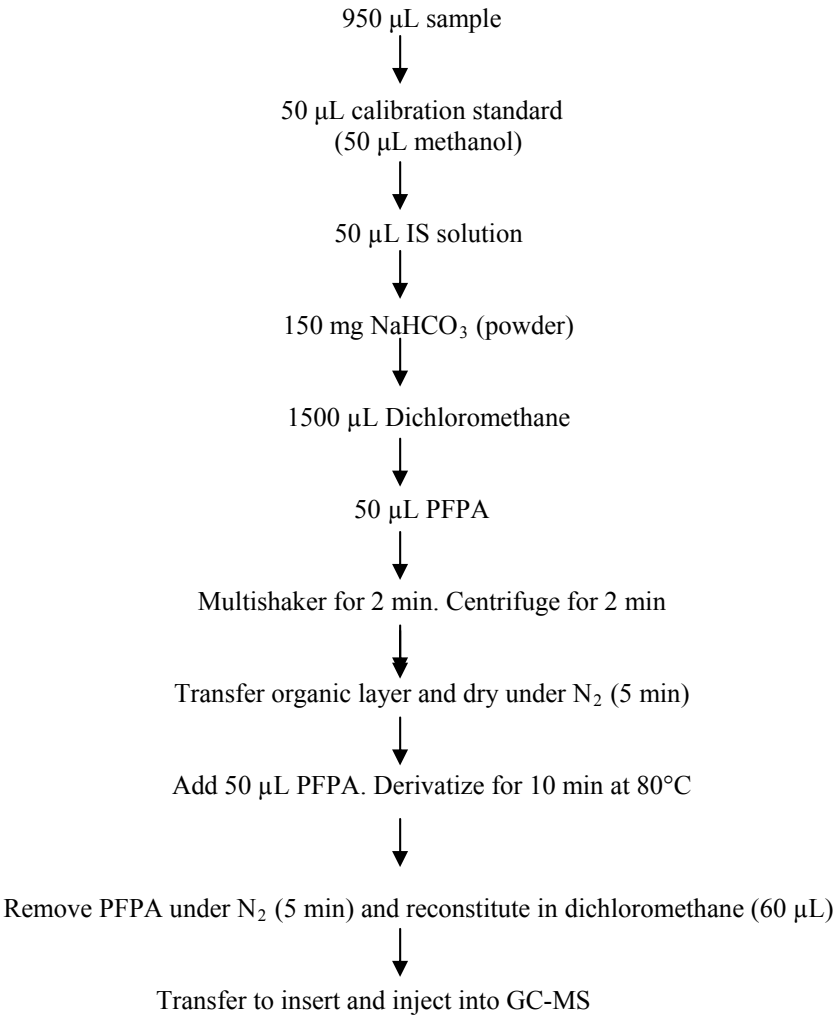

Figure 6.3 Flow diagram of the extractive acylation procedure.

6.2.6 Linearity and selectivity

Blank urine samples, obtained from ten healthy drug-free volunteers, were analyzed and compared to a standard (200 ng/mL) containing AMP, MET, MCA, CT, NE, NPE, EPH, PEP, PHM, FFA, MDA, MDMA, MDEA, MBDB and HMMA. The comparison was based on the presence of three characteristic ions (one quantifier ion, two qualifier ions) that eluted at the same retention time as the standards. CT, NPE, PEP, PHM, FFA and HMMA were included to assess the assay selectivity as far as adequate chromatographic resolution between closely related compounds and suspected

interferences from authentic samples were concerned. In the absence of interfering peaks from the ten blank samples, the urine was pooled for further experiments.

Aliquots (900 µL) from the urine pool were spiked with corresponding standard solutions (50 µL of each calibration standard obtained from solution A and B respectively) along with the internal standard mixture (50 µL) to obtain an eight point calibration of 5, 25, 50, 100, 200, 500, 1000 and 5000 ng/mL with five replicates at each level. This was a wide-range linearity assessment, and from these results a routine calibration range could be determined. Criteria for acceptability of wide-ranging linearity were correlation coefficients (r^2) should exceed or equal 0.99 and back-calculated values of standards should fall within 10% of respective theoretical values (20% at lower limit of quantification, LLQ). An un-weighted, least-squares regression model was utilized to describe the peak area ratios of analyte versus the internal standards in the final linear range 5 – 500 ng/mL.

6.2.7 Calibration curves and control samples

An aliquot (950 µL) from a urine pool was placed in a reaction vial (7 mL). Calibration standard solution (50 µL) containing AMP, MET, MCA, NE, EPH, MDA, MDMA, MDEA and MBDB was added to the sample along with the internal standard mixture (50 µL) to yield final calibration standards of 6.25, 12.5, 25, 50, 100 and 200 ng/mL in urine. Each calibration contained a reagent-blank (no urine or IS), a matrix-blank (urine with no IS), and a zero-blank (urine with IS) to check for any possible background contributions to measured signals. Before analysis of an authentic specimen, the zero-blank was re-analysed to monitor for possible carry-over from previous injected samples. Control samples were spiked in pooled urine (1 mL) from appropriate solutions to provide four QC levels: 6.25 ng/mL (LLQC), 50 ng/mL (LOW), 100 ng/mL (MED), and 200 ng/mL (HIGH).

6.2.8 Accuracy and Precision

The performance of the method was evaluated according to international recommendations [43, 44] as described in Chapter 3. A daily six-point calibration curve along with three replicates of the four QC samples (LLQC, LOW, MED, and HIGH)

described in the preceding text were analysed on 8 different days. Accuracy was determined as the percent bias of the mean of the calculated concentrations at the different levels with respect to their nominal concentrations. Precision was assessed by calculating relative standard deviation (% RSD) for within-day (repeatability) and intermediate variation by one-way ANOVA. Criteria for acceptance were equal or less than 15% RSD (20% at LLQ) and within 15% of nominal concentration (20% at LLQ) for precision and accuracy, respectively. Additional EQC (Liquichek™ Level C1) samples at the concentration values of MED control were also analysed over a period of six months. Precision and accuracy data for analytes that occur in both QC and EQC samples were compared.

6.2.9 Stability

The processed sample stability of the pentafluoroproprionyl (PFP) -derivatives was studied over a period of 24 hours by injection of the same sample (MED QC) once every 2 hours. Absolute peak areas of each analyte versus injection time was plotted and instability indicated by a negative slope significantly different from zero ($p \leq 0.05$) [44].

Stability studies in the present work were reduced to only freeze-thaw and long-term stability of MCA. Replicates (n=3 each level) of spiked urine (50 ng/mL and 200 ng/mL) before and after 3 freeze-thaw cycles were analyzed and the accuracy compared. The appearance of any of the ephedra components in the thawed samples (NE, NPE, EPH, PEP) were closely monitored. Long-term storage at -20°C for MCA was assessed through repeat analysis of an authentic urine sample that was stored for 3 months in standard polypropylene specimen collection containers.

6.2.10 Limits of quantification and detection

The lower limit of quantification in SIM mode was determined to be the lowest calibrator (6.25 ng/mL) according to the following criteria. All three significant ions in the sample spectrum had to be within 20% of the mean ion ratios for calibrators at minimum S/N=10, while precision was within 20% RSD and accuracy within 20% of nominal concentration. The limit of detection (LOD) was determined by diluting the lowest calibrator, and criteria for acceptance were set as three significant ions present at

minimum *S/N*=3. Peak height above baseline noise, as determined in the aforementioned analysis of 10 blank urine specimens, was used to calculate *S/N*.

6.3. Results

6.3.1. GC-MS analysis

The PFP-derivatives of the studied compounds produced mass spectra with structure-specific ions in relative high abundances. The fragmentation patterns of the analytes of interest were similar, as could be expected from the structural similarities shared between amphetamine and related analogs. A typical fragmentation, common to all studied analytes, occurred on the side chain between the α- and β-carbon (see Figure 6.1) to yield two main positively charged fragments used for identification and quantitation. Abundant molecular ions were only attained for the methylenedioxy derivatives MDA, MDMA, MDEA and MBDB. The CT mass spectrum indicated a significant ion at *m/z* 105 from the $[C_7H_5O]^+$ fragment, and a much smaller signal at *m/z* 190 $[C_5H_5NOF_5]^+$. This is in accordance with findings on the heptafluorobutyric- (HFB) derivative of CT by Paul et al [10]. MCA mass spectrum also proved to have *m/z* 105 as base peak, but with *m/z* 204 $[C_6H_7NOF_5]^+$ in a near one-to-one ratio. Quantitation was supported through the use of the more structure-specific ion. The use of common ions (Table 6.1) imposed even more stringent requirements on chromatographic resolution as separation in mass was impossible. Figure 6.4 illustrates the mass spectra obtained for AMP, MCA and MDMA which typically represents the fragmentation patterns of the PFP derivatives.

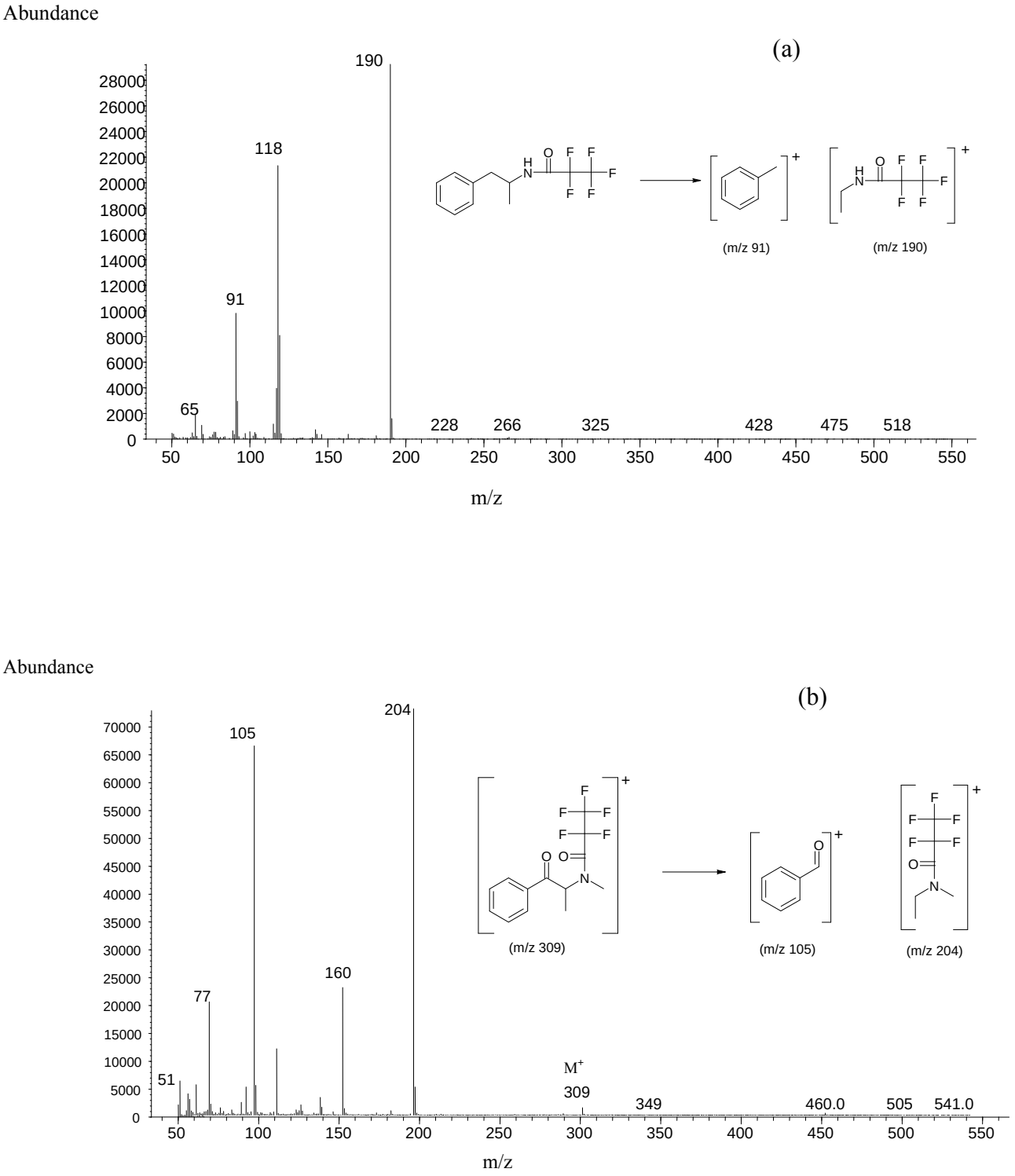

Figure 6.4 Full-scan mass spectra of pentafluoroproprionyl-derivatives of amphetamine (a), methcathinone (b) and MDMA (c).

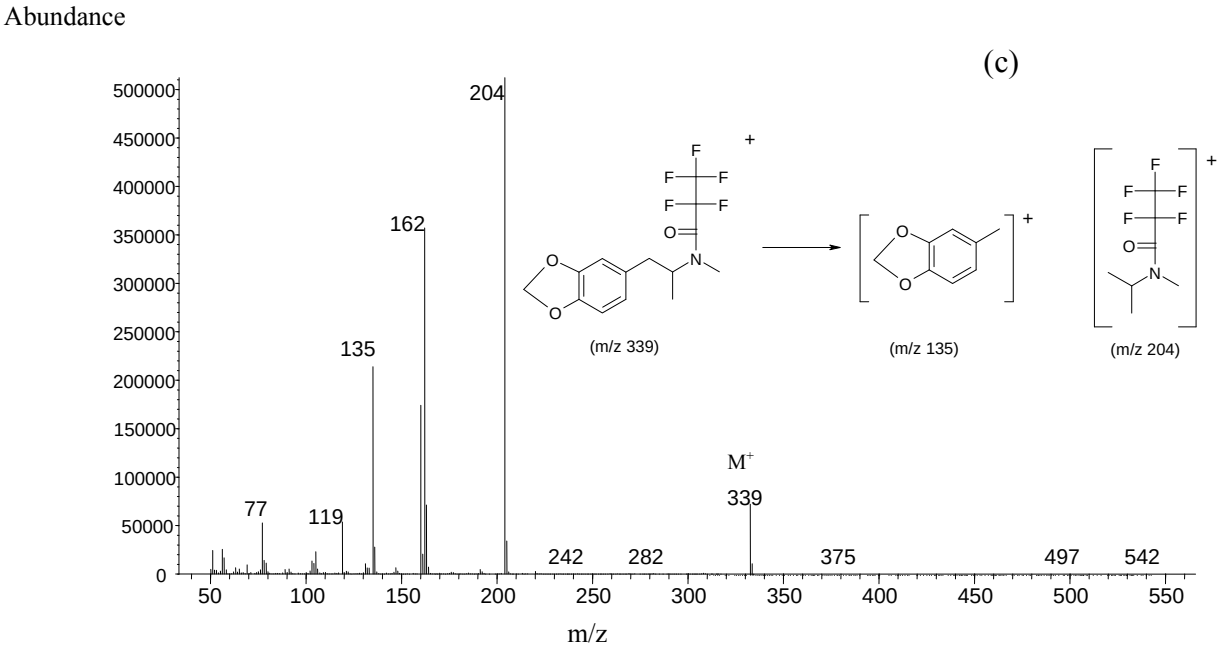

Figure 6.4 Full-scan mass spectra of pentafluoroproprionyl-derivatives of amphetamine (a), methcathinone (b) and MDMA (c).

An extract of urine spiked with relevant analytes and potentially interfering compounds (200 ng/mL) recorded in SIM mode is shown in Figure 6.5. Only common ions between analytes of interest and interferences, as determined in full scan mode, were included in SIM mode. None of the suspected interferences co-eluted with the analytes of interest at concentrations of 200 ng/mL and 2000 ng/mL. Fenfluramine is not indicated on the chromatogram due to the change of monitored SIM window at its retention time, but elutes with baseline separation between CT (peak 7) and MCA (peak 8).

Analysis of the zero-blank samples indicated no contribution from deuterium substituted internal standards to selected quantitation ions for relevant compounds. The total absence of chromatographic peaks containing the aforementioned quantitation ions indicated that no carry-over from samples containing high concentrations of analytes to the following samples in a sequence occurred. No contribution was observed during validation experiments when LLQ samples were continually injected after HIGH QC specimens.

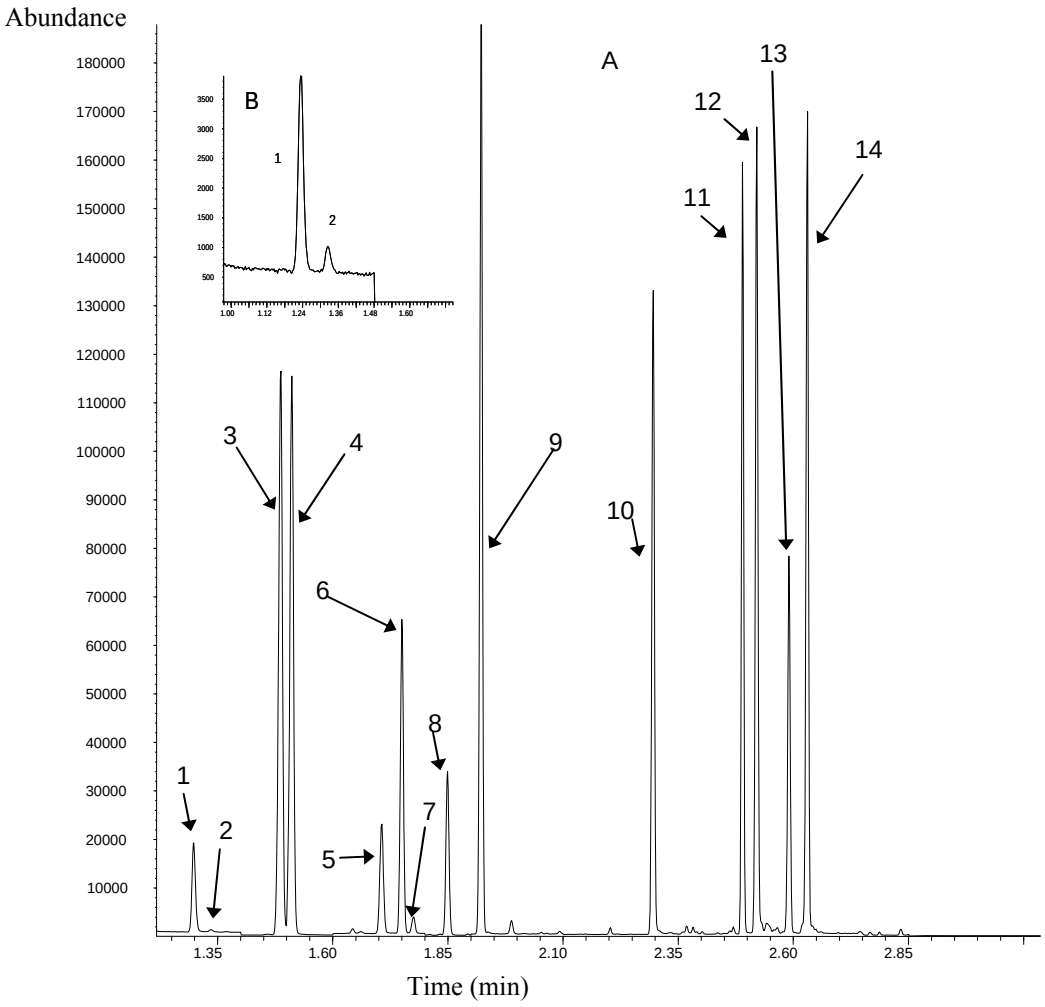

Figure 6.5 (A) Total ion chromatogram in SIM mode of spiked urine containing analytes of interest, alongside possible interferences, at 200 ng/mL. The elution order is as follow: (1) Amphetamine, (2) Phentermine, (3) Norpseudoephedrine, (4) Norephedrine, (5) Methamphetamine, (6) Ephedrine, (7) Cathinone, (8) Methcathinone, (9) Pseudoephedrine, (10) MDA, (11) HMMA, (12) MDMA, (13) MDEA, (14) MBDB. (B) Enlargement of section indicating adequate resolution between amphetamine and phentermine for the shared ion 91 *m/z*.

Resolution between diastereomers such as NE and NPE, EPH and PEP, as well as structurally close related compounds such as MDEA and MBDB were achieved in less than 3 min using the selected chromatographic conditions. A total instrumental cycle-time of 6 min per sample was obtained, which compares favourably with current rapid chromatographic methodologies for both GC-MS and LC-MS/MS [27, 32]. The total sample preparation time for a batch of 20 specimens, performed by a single analyst, was approximately 45 minutes. Considering the instrument cycle-time of 6 min

per sample, the total analysis time was therefore less than 3 hours for such a batch. A single analyst could thus comfortably start the instrumental analysis and continue serially preparing new specimen batches, allowing up to 100 specimens to be analysed in approximately 10 hours.

Specific ions for CT, NPE and PEP were monitored in the final GC-MS SIM method and their appearance in authentic urine specimens qualitatively assessed.

6.3.2 Validation

A minimum of 5 data points were collected over the peak area. This has been shown to be sufficient for accurate quantitation with a conventional quadrupole mass spectrometer in fast GC-MS analysis [38, 40]. Variation that results from integration of inadequately defined signals would be reflected as decreased precision (increased % RSD), especially at LLQ concentrations.Variation arising from sample preparation steps was however likely to play a more significant role.

The established criteria were satisfied for all the relevant compounds across the calibration range. Table 6.2 indicates validation data for precision, accuracy and LOD for the current GC-MS assay. Precision and accuracy of internal quality control specimens were compared with external quality control specimens (n=12) that have been analyzed over a period of 6 months. Not all analytes of interest were available in the EQC specimens. Results are shown in Table 6.3.

The limits of detection for AMP, NE and EPH were equal to the lower limit of quantification as only two significant ions were present when diluted below a concentration of 6.25 ng/mL. The LOD for the different analytes compared well to those published elsewhere [15].The correlation coefficients for the calibration curves were in the range 0.993 -0.999 for all the analytes. Precision and accuracy were within to the aforementioned criteria.

Analyte (IS)	Control level (ng/mL)	Accuracy (% Bias)	Repeatability (% RSD)	Intermediate precision (% RSD)	LOD (ng/mL)
Amphetamine ([$^2H_{11}$]AMP)	LLQC (6.25)	- 8.1	6.2	8.0	6.25
	LOW (50)	3.5	5.7	5.9	
	MED (100)	1.8	4.1	7.3	
	HIGH (200)	3.1	5.3	7.1	
Methamphetamine ([$^2H_9$]MET)	LLQC (6.25)	- 4.1	5.5	9.6	3.1
	LOW (50)	3.4	3.0	4.3	
	MED (100)	0.9	3.5	4.8	
	HIGH (200)	1.1	2.5	8.2	
Norephedrine ([$^2H_3$]NE)	LLQC (6.25)	- 9.5	10.7	12.3	6.25
	LOW (50)	5.1	4.8	5.4	
	MED (100)	2.9	3.2	5.0	
	HIGH (200)	1.2	5.2	5.8	
Ephedrine ([$^2H_3$]EPH)	LLQC (6.25)	7.3	9.3	10.5	6.25
	LOW (50)	4.1	4.1	5.3	
	MED (100)	2.3	2.3	5.2	
	HIGH (200)	2.5	3.5	7.9	
Methcathinone ([$^2H_3$]EPH)	LLQC (6.25)	- 1.3	11.7	12.2	3.1
	LOW (50)	- 6.0	9.7	12.0	
	MED (100)	- 1.7	6.4	13.8	
	HIGH (200)	- 4.6	6.7	12.6	
MDA ([$^2H_5$]MDA)	LLQC (6.25)	- 7.9	6.5	13.8	1.5
	LOW (50)	4.6	4.6	6.8	
	MED (100)	2.5	2.6	8.0	
	HIGH (200)	0.4	3.0	6.7	
MDMA ([$^2H_5$]MDMA)	LLQC (6.25)	0.8	5.9	6.3	1.5
	LOW (50)	3.2	2.7	6.0	
	MED (100)	1.4	2.4	4.8	
	HIGH (200)	- 1.5	1.8	6.4	
MDEA ([$^2H_6$]MDEA)	LLQC (6.25)	- 8.7	6.1	10.8	1.5
	LOW (50)	3.1	4.3	4.6	
	MED (100)	1.3	2.8	8.5	
	HIGH (200)	- 2.2	2.9	9.3	
MBDB ([$^2H_5$]MDMA)	LLQC (6.25)	- 1.7	6.6	7.0	1.5
	LOW (50)	- 2.3	4.4	7.3	
	MED (100)	- 0.2	3.3	8.1	
	HIGH (200)	- 1.6	6.5	8.9	

Table 6.2 Validation data for extractive acylation GC-MS assay.

Analyte	Internal quality control (MED, 100 ng/mL)		External quality control (Liquicheck C1, 100 ng/mL)	
	Accuracy (%)	RSD (%)	Accuracy (%)	RSD (%)
Amphetamine	99	7.2	89	5.8
Methamphetamine	101	6.8	90	5.7
MDA	98	5.9	86	3.9
MDMA	101	6.2	91	3.2
MDEA	102	7.1	92	4.1

Table 6.3 Accuracy and precision comparison of internal quality control (QC) and external quality control (EQC) samples.

The PFP-derivatives were sufficiently stable over a period of 24 hours with no evidence of degradation observed as indicated by Figure 6.6. Comprehensive stability studies by Jiménez et al [23] as well as published work by other authors [33, 45, 46] have indicated the viable stability of amphetamine and related derivatives under various freeze-thaw and storage conditions. Freeze/thaw stability for MCA was also positively confirmed in agreement with reports in literature [33]. Re-analysis of an authentic urine specimen positive for MCA indicated that only concentrations for NE and EPH were within acceptable limits [44], whereas MCA was almost completely missing. Positive MCA urine specimens stored at 4°C are reported as unstable [16]. Long-term stability data from this work suggests that storage at -20°C for longer than two months is also not advised.

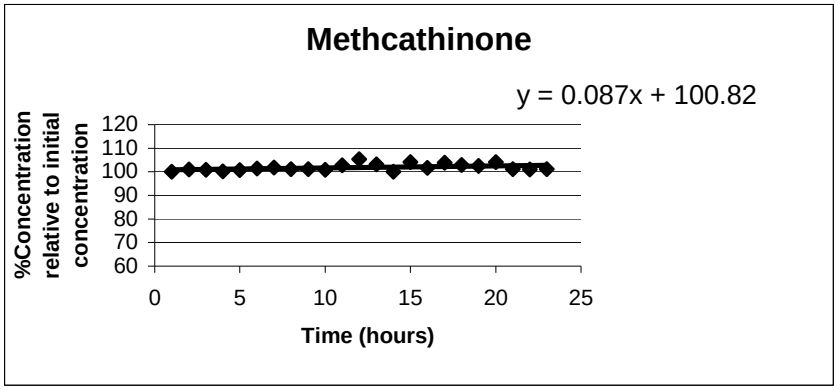

Figure 6.6 Stability of methcathinone-PFP by injecting the same sample consecutively, once every hour, over a period of 24 hours. The *y*-axis shows the concentration as a percentage of the initial concentration. Results are typical for all PFP derivatives in the assay.

6.3.3 Routine confirmation

A typical chromatogram for a MCA positive urine specimen is presented in Figure 6.7. High concentrations of EPH were observed with lower concentrations of NE and PEP present. NPE was excreted at much lower concentrations. These observations were in agreement with published findings [16, 17], however, no inferences with regards to metabolism can be made as no information about the precise composition of the ingested substance nor the timeframe could be obtained. The quantified concentrations in this sample were as follow: 157 463 ng/mL EPH, 6752 ng/mL MCA, and 783 ng/mL NE.

A somewhat atypical chromatogram is presented in Figure 6.8, as the concentration of PEP is higher than EPH. The most likely explanation for this is ingestion of a PEP contaminated preparation. This sample was also positive for MDMA and MDA, which had been indicated in a presumptive positive immunoassay screen. The quantified concentrations were as follow: 36 056 ng/mL EPH, 22 582 ng/mL MCA, 2080 ng/mL NE, 250 ng/mL MDMA, and 48 ng/mL MDA.

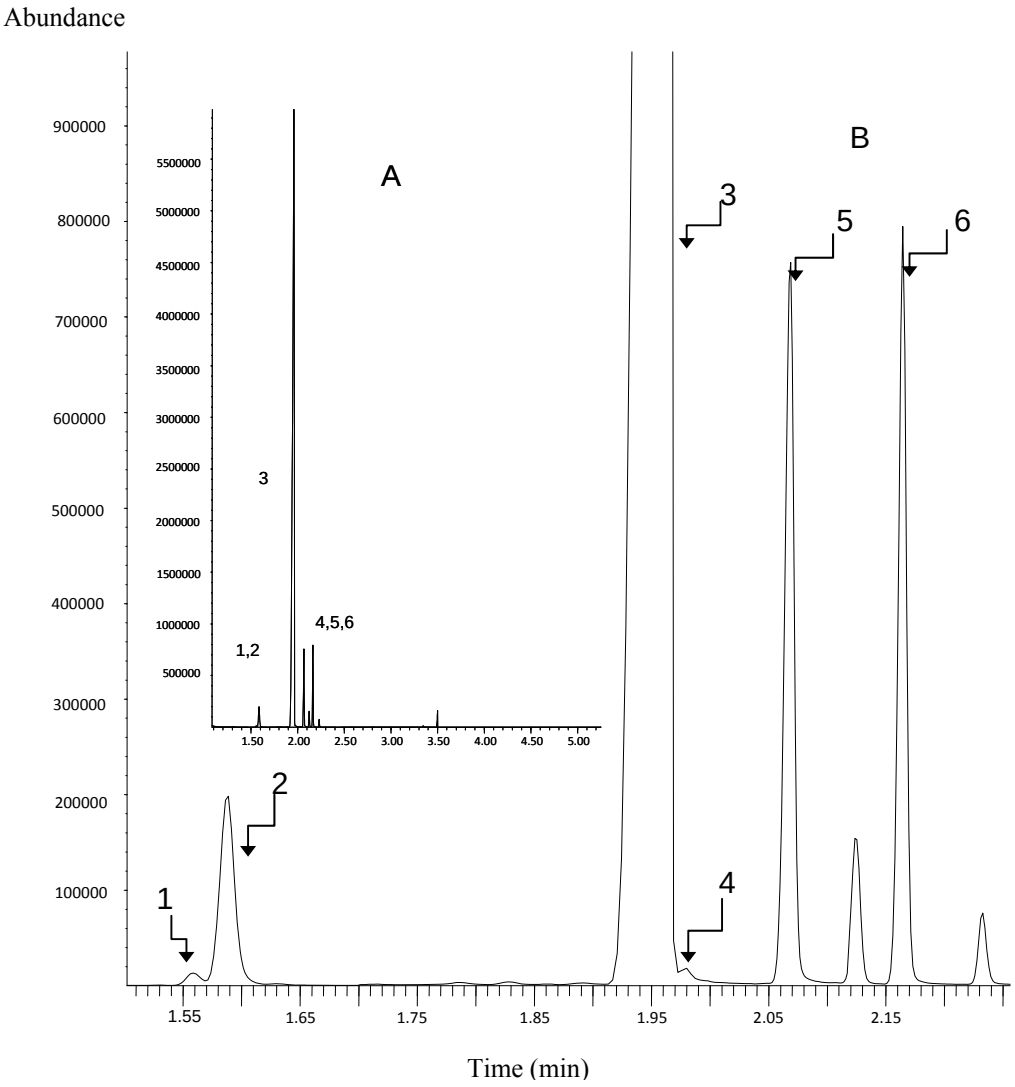

Figure 6.7 (A) Total ion chromatogram of authentic urine specimen in SIM mode before dilution to fall within calibration range for quantification. (B) Enlargement of section indicating positive findings for the following compounds: (1) Norpseudoephedrine, (2) Norephedrine, (3) Ephedrine, (4) Cathinone, (5) Methcathinone, (6) Pseudoephedrine.

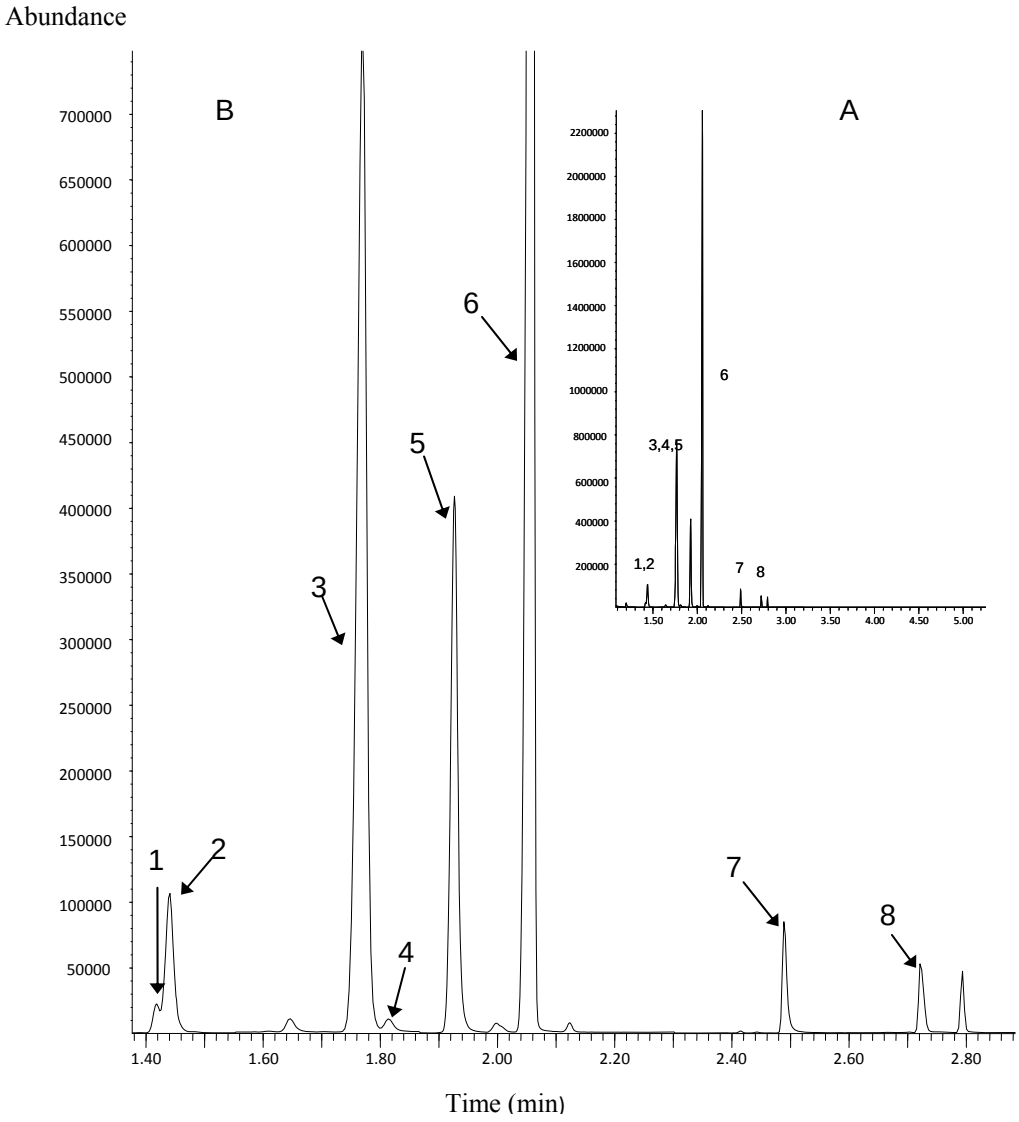

Figure 6.8 (A) Total ion chromatogram of authentic urine specimen in SIM mode before dilution to fall within calibration range for quantification. (B) Enlargement of section indicating positive findings for the following compounds: (1) Norpseudoephedrine, (2) Norephedrine, (3) Ephedrine, (4) Cathinone, (5) Methcathinone, (6) Pseudoephedrine, (7) MDA, (8) MDMA.

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6.4. Discussion and Conclusion

6.4.1. Extractive acylation

During the early development phase of the assay different acylation reagents such as acetic anhydride (AA), trifluoroacetic anhydride (TFA), pentafluoropropionic anhydride (PFPA), heptafluorobutyric anhydride (HFBA) and pentafluorobenzoyl

chloride (PFB-Cl) were all tested. The most suitable reagent was PFPA, as it allowed sufficient volatility for successful GC separation without adding too much mass to the relevant analytes and increasing boiling points, which in turn would prolong elution time. Using the described sample preparation technique it also gave the largest yield of derivatized product. Solid $NaHCO_3$ was weighed off initially (150 mg) and a volumetric device that would repeatedly contain the correct gravimetric amount (150±5 mg) to be added to each sample was improvised. The volumetric addition eliminated the time to weigh out the correct amount for each sample, thereby reducing sample preparation time. The addition of $NaHCO_3$ was a crucial step in the reaction as low derivatization efficiency was obtained when not present in excess, in accordance with Schotten-Baumann reaction conditions (Chapter 3, Section 6.1).

Acylation of primary and secondary amines as well as phenolic hydroxyl groups was achieved during the initial extractive-acylation, in agreement with Beck and Faull [32]. Derivatization *in-situ* was advantageous as the PFP-derivatives were less subject to associated free-base amphetamine volatility and subsequent analyte loss in sample concentration procedures with nitrogen gas. Prolonged drying steps (>25 min) resulted in decreased signal strength for AM, MET and MCA. Alcoholic functional groups such as the β-hydroxy groups of NE, NPE, EPH and PEP remained underivatized after the initial acylation reaction. No mixed derivatives of mono- (mono-*N*-PFP) and diacylated (bis-*N*,*O*-PFP) compounds in appreciable yield were found as reported in the extractive-derivatization technique of Kankaanpää et al [32]. Formation of the diacylated derivatives of NE, NPE, EPH and PEP was preferred, as better chromatographic resolution and separation speed was achieved. Quantitation of EPH and NE as potential metabolites of MCA was required, and the additional derivatization step with PFPA was introduced.

Thin-film microbore capillary columns have reduced sample loading capacity and are subject to loss of performance in the event of less volatile components being introduced into the chromatographic system that lead to active sites. The addition of $NaHCO_3$ to urine samples raises the pH between 8 and 9, and compounds that contain basic groups will likely be co-extracted but not necessarily acetylated in the initial extractive-acylation. The secondary derivatization step would derivatize most functional groups subject to acylation, and hence form more volatile compounds amenable to gas chromatographic analysis. It hence found that less column maintenance along with

prolonged column-lifetime was gained when an extra derivatization step was introduced.

It was found that the best approach for good chromatographic performance using microbore capillary columns in this analysis was to push column efficiency to its maximum through relatively high carrier-gas flow rates and steep temperature gradients, assuming that acceptable stationary phase selectivity (constant resolution) was present. The absolute increase in chromatographic efficiency results in relatively narrow Gaussian-type peaks, therefore baseline resolution is easily attained as analytes move through the column in narrow bands with little opportunity for longitudinal diffusion. Adequate separation for quantititation ($0.8 \geq R_s \leq 1.5$) with good resolution between diastereomers such as NE/NPE ($R_s = 1$) and EPH/PEP ($R_s > 1.5$), and structurally close related compounds such as MDEA/MBDB ($R_s > 1.5$), were achieved in less than 3 min using the selected chromatographic system.

6.4.2. Confirmatory urinalysis

A routine calibration range with low concentrations compared to international recommendations for confirmatory cut-offs [47] of amphetamine and related derivatives was used in the present method. The decision for this was based largely on the reported concentrations for MCA [16] along with the lack of information regarding the detection time window for MCA in positive urine specimens. Analysis of urine specimens in certain cases resulted in certain analyte concentrations to be within the calibration range while others were present at much higher concentrations. In such events, the analytes that were within the calibration range were immediately quantified and the urine specimens were appropriately diluted with deionized water, re-analysed and the previous higher-concentration analytes quantified.

Synthesis of MCA can be performed by relatively simple means through oxidation of EPH or PEP with a strong oxidizing agent such as potassium permanganate [48]. Illicit substances with MCA as the proposed major active ingredient can therefore be expected to contain EPH and/or PEP in appreciable amounts due to incomplete synthesis or as diluents in the so-called ‘cutting’ process. The presence of EPH and PEP in the urine of MCA abusers can also be expected as a result of metabolism [49]. Detection of CT and MCA by analysis of 4-carbethoxyhexafluorobutyryl chloride (4-

CB) derivatives with GC-MS has been performed [50]. The presence of high concentrations of EPH and PEP, as may be expected for MCA users due to ingestion of precursor/contaminant EPH and PEP alongside their metabolic formation, presents a particular difficulty with this method. Detection of MET as an artifact of EPH and PEP resulting from derivatization with a.o. 4-CB, PFPA and HFBA have been reported [50]. Degradation of EPH and PEP by addition of sodium periodate prior to extraction has been recommended [51] and is an acceptable option for exclusive forensic confirmation of AMP and MET. Simultaneous detection of MCA alongside AMP and MET is however excluded, as the proposed reaction also destroys MCA [16] and is therefore not applicable in a multi-analyte confirmation procedure that includes MCA. Two separate sample preparations must be performed in the case of a positive screen for both MCA and MET, one with periodate treatment (AMP, MET) and one without (CT, MCA). Urine spiked with 2000 ng/mL EPH and PEP did not indicate the presence of MET following the current prescribed procedure. Authentic urine specimens with quantified concentrations of up to 100 000 ng/mL of EPH and 30 000 ng/mL of PEP also did not indicate the presence MET, before and after appropriate dilutions down to calibration range. It is our conclusion that the current GC-MS assay does not suffer from MET artifact formation in the presence of high concentrations of EPH and PEP. Simultaneous quantitation of MCA, MET, AMP, EPH and NE is therefore possible while qualitative data for CT, PEP and NPE is retained.

A previously unreported qualitative observation in certain MCA positive authentic urine samples is the appearance of CT (Figure 6.7 and 6.8). The major metabolic pathway for MCA metabolism has been indicated to occur through stereoselective reduction of the ketone group to form EPH and PEP, which is further metabolised to NE and NPE respectively by *N*-dealkylation [49]. The reported concentrations for MCA in this study are much higher than those previously indicated [16]. Formation of CT may thus be a very minor pathway where MCA follows a *N*-demethylation route after intake of large dosages, and may possibly be detected in the very early phase of excretion. The possibility of ingestion of a CT contaminated preparative can unfortunately not be excluded, and the appearance of the analyte may be artifactual in this sense. The appearance of higher concentrations of MCA and EPH in the specimens excludes the possibility of pure CT ingestion however.

Amphetamine-class confirmatory analysis on 119 urine specimens, forwarded by referring clinicians who requested direct amphetamine type stimulant confirmations

irrespective of a positive or negative immunoassay screen, resulted in a total of 21 positive specimens (18%) of which 17 of the positive specimens (81%) confirmed for MCA use. Only 2 of these positive MCA samples were presumptive positives in on-site immunoassay screening kits, likely due to the synergistic cross-reactive effect of extremely high concentrations of EPH, PEP, NE and NPE. The need for accurate screening techniques for MCA is however indicated.

Many specimens only tested positive for a combination of EPH, PEP, NE and NPE. This may well be due to sampling in the late excretion phase after MCA usage, but the availability of these compounds as common over-the-counter medicaments denotes that such detection cannot be considered as characteristic for MCA abuse. However, with no valid explanation for their appearance in a urine specimen, further investigation using alternative matrices such as hair may be prudent to establish possible historic abuse. Care in reporting true positive MCA specimens is also required, as alkylated homologs such as the anorexant dimethylpropion (*N*,*N*-dimethylcathinone) is known to have MCA as metabolite [49].

6.4.3. Concluding remarks

In a clinical laboratory setting various analysts usually perform expedient sample preparation and data interpretation, but reporting of results may be delayed due to slow data acquisition from lengthy instrumental analysis. Instrument availability in such events is the most limiting time factor [39]. Literature reports, with similar fast sample preparation times for GC-MS multi-analyte analysis of amphetamines [32] as the current assay, seem to be hindered by lengthy analytical runs (up to 15 min). The most rapid chromatographic techniques with similar instrument cycle-times are LC-MS/MS methodologies [33, 34], which in this instance have lengthier and more costly sample preparation steps than the current assay. The extractive acylation assay is sensitive and selective for routine confirmation of amphetamine type stimulants in urine. Similar to extractive alkylation, the method is simple, rapid and cost-effective The complementary approach of fast GC-MS alongside relatively rapid sample preparation fulfilled the short turn-around time requirements of a routine toxicology laboratory that deal in forensic urine confirmations for drugs of abuse.

6.5. References

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Chapter 7

CONCLUSION: CONTRIBUTION OF EXTRACTIVE-DERIVATIZATION SAMPLE PREPARATION TO THE ROUTINE CLINICAL TOXICOLOGY LABORATORY

7.1. Introduction

"When I have clarified and exhausted a subject, then I turn away from it, in order to go into darkness again." (Carl Friedrich Gauss, 1777-1855)

In the work thus far, the complexity of analytes occurring in biological matrices (Chapter 2), the methods to visualize and quantify them (Chapter 3), the techniques to isolate and enhance their detectability (Chapter 4), and the practical implementation of all of the above principles in a routine laboratory setting (Chapter 5 and Chapter 6) have been discussed in detail. This chapter will highlight the analytical considerations for routine clinical toxicology laboratories, in the context of how the developed extractive-derivatization methods from the current research act to fulfil those requirements.

7.2. Extractive-derivatization applications

The analytical chemist burdened with method development in routine settings understands the needs for any given analysis, sets goals to meet those needs, envisions the theoretical path toward the goals, and finally develops and tests the approach in practise. All decisions in the analytical approach must therefore address the purpose of the results. Criteria for quantitation must be met (selectivity, accuracy, precision) while taking into account several constraints that may exist (analysis time, costs, available technology, sample matrix, sample size, other analyses). These constraints must be prioritized and balanced to attain the desired quality of result in the most efficient overall process. The desired attributes for an ideal analytical method may be summarized as follow:

- very sensitive (low quantification limits)

- highly selective (analyte discrimination)
- rugged and repeatable
- fast
- inexpensive
- easy to perform
- minimal waste production
- widely applicable

Regarding instrumentation, GC-MS and LC-MS (or LC-MS/MS) possess most of the abovementioned features; however, GC-MS is much more widely available and at much lower instrument cost. Optimising the GC-MS analysis for speed and efficiency (fast GC-MS) by utilizing short microbore columns also translates to reduced analytical costs as short sections (5m) from a purchased column (20m) are used. Each installed column length doesn't put the entire purchased column length stationary phase through the various temperature cycles as would be the case in conventional GC-MS, prolonging purchased column lifetime.

Sample preparation methods for GC-MS largely entail the additional step of derivatization after extraction to allow sufficient volatility for analysis of analytes from biological matrices. Combining the extraction and derivatization procedures into a single step allows for sample enrichment, reduced solvent/reagent costs, reduced analysis time, and introduction of an additional mechanism for enhanced selectivity during extraction, to further approach the ideal analytical method described above. Furthermore, the application of extractive-derivatization is complementary to fast GC-MS regarding sample throughput and sample-turn around time. Total laboratory output is further increased as the minimum amount of time possible is spent on sample preparation and instrumental analysis for each sample, which allows more time for other analytical methods to also be completed during a 24 hour cycle. The extractive-derivatization assays discussed in this research are not novel in themselves, however their application in analyzing the analytes from Chapter 5/Chapter 6 and combination with fast GC-MS is a novel approach. As the validation data in the aforementioned chapters indicate, the assays are rugged and repeatable.

The assays have successfully been adjusted and applied to other analytes for routine quantitation. In-house, validated methods for the extractive alkylation of a.o. *p*-nitrophenol, 1-naphtol, 1-hydroxypyrene, furoic acid, and thiocyanate have been

developed. Extractive acylation has been successfully applied to routine quantitative analysis of a variety of aromatic- and biogenic amines.

Extractive-derivatization sample preparation alongside fast GC-MS is an efficient analytical process for routine clinical toxicology laboratories, and meets all the analytical requirements while successfully approaching the analytical ideal.

7.3. Future developments

The analytes in this research were specifically selected to exemplify the application of extractive-derivatization techniques combined with fast GC-MS for

1. physicochemically different molecules (i.e. acidic vs. basic)
2. Analytically different scenarios (i.e. screening of urinary metabolites for occupational monitoring vs. forensic confirmatory urinalysis)

From the results obtained in the practical application of the methods, it is clear to see that the assays are able to successfully cover a wide spectrum of applications in the same type of biological matrix. Future research may be directed toward applications in alternative matrices such as hair, or more complex matrices such as whole blood.

The analytical step in the current work is optimal (i.e. sample preparation and instrumental analysis), however it is only one component in the overall process of generating a result for a given sample. Other processes (sample collection, sample transport, sample storage, data processing, quality assurance etc.) are equally important to increase total laboratory output, but are harder to optimize as they are dependent on a complete laboratory support system. All factors must be addressed to truly improve productivity and efficiency. It may be prudent to aim future research in assessing the impact of decreased analysis time in the context of the additional factors mentioned.