BENZENE EXPOSURE FROM AUTOMOBILES FUELLED WITH PETROL

A thesis submitted in accordance with the conditions governing candidates for the degree of

DOCTOR OF PHILOSOPHY

To

The University of Newcastle-upon-Tyne

By

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December 2002

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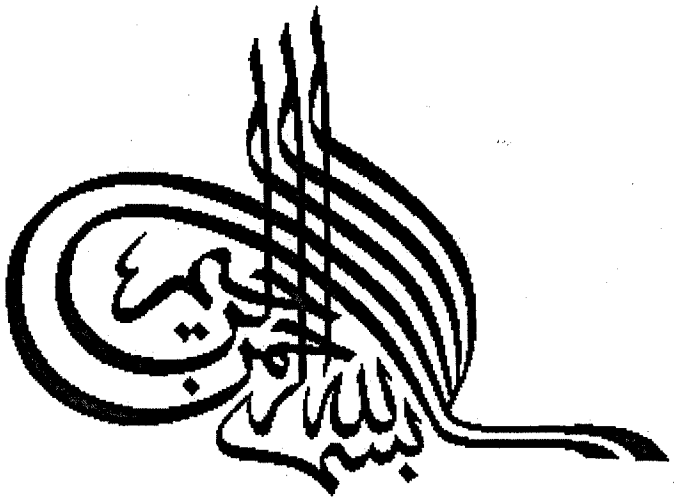

In the name of Allah, Most Gracious,
Most Merciful. Praise be to Allah
The Cherisher and Sustainer
of the Worlds.

(Quran, 1985)

CERTIFICATE

I hereby certify that the work embodied in this thesis is the result of my own investigations, except where reference has been made to published literature.

Candidate:

Directors of Studies: Signed:

Professor P.G. Blain

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DECLARATION

I hereby declare that this work has not been accepted in any substance for any degree, and is not concurrently submitted in candidature for any degree.

Candidate:

To
My Father "Mansour Al-Khulaifi"
My Mother "Nora Al-Obaid"
My Wife "Haya Al-Rukhiess"
My Son "Abdul-Rahman"
My Daughters "Monira & Nora"

ACKNOWLEDGEMENT

First of all, I would like to express my gratitude to the Kuwait Public Authority of Applied Education and Training for the opportunity they gave me to do this research with their financial and ethical support and their understanding of the research difficulties that I've faced during the time course of the study.

The research work was a new approach and consists of a spectrum of technical challenges. I have been privileged to work in the Environmental and Occupational Medicine Department in the University of Newcastle. I am grateful for the supervision of Professor Peter Blain and Dr. Ovnair Sepai. Dr. Faith Williams and Dr. Elaine Mutch have provided encouragement and support for this work. Also, I'm very grateful to Mr. David Sutherland and Mr. David Henderson for their substantial technical help. I wish to acknowledge with thanks the generous contribution from all friends and colleagues to the study. Mr. David Foster, Mr. Gorden Smith and Mr. Andrew Hall (members of Newcastle Occupational Health in Newcastle General Hospital) were very helpful in the fieldwork.

CONTENT

Aim

Develop a new method for extraction of *t,t*-muconic acid in urine and a new analytical method for ambient BTEX levels by applying GC-MS to assess the environmental benzene levels and the effect of benzene on car drivers.

Objectives

1. To validate a new extraction technique for urinary *t,t*-muconic acid based on solvent extraction.

2. To validate a new analytical method using GC/MS to determine benzene, toluene, ethylbenzene, and xylenes (BTEX) levels for environmental application.

3. To measure BTEX inside car cabin while driving and urinary *t,t*-muconic acid for exposed individuals.

4. To determine the relationship between benzene level from automobiles and urinary *t,t*-muconic acid following environmental exposure.

Abstract

Benzene is a leukaemogenic and mutagenic agent, which may pose a risk to the general public even at low levels of exposure. Since petrol fuel contains a high concentration (1-5%) of benzene, there is the potential for exposure to man during car journeys. The main aim of this study was to develop and validate a sensitive method to detect urinary *t,t*-muconic acid (uMA) following low level environmental exposures to benzene. Subjects potentially exposed to benzene were divided into petrol (n= 9) and diesel groups (n= 7). The control group (n=14) consisted of individuals who were not exposed to benzene inside the car. The uMA method developed during this study involved butanol extraction instead of the traditional solid phase extraction followed by UV (259nm) detection. The method was reasonably precise (CV=1.5%) with >80% recovery from urine. Air samples were collected on charcoal tubes and analysed for benzene, toluene, ethylbenzene, and xylenes by GC-MS following extraction with purified carbon disulphide. The benzene concentration of ambient air samples taken from inside the cabins of petrol fuelled cars (7.5 ppb) was about triple that found from diesel-fuelled cars (2.6 ppb)($p=0.01$). The uMA of volunteers exposed to petrol increased ($p<0.01$) post-sample in compared to pre-exposure level (0.66mgMA/gCr and 0.38mgMA/gCr, respectively). There was no increase in uMA for volunteers exposed to diesel. The uMA level of samples collected from individuals 2h-7h after exposure to petrol showed a significant association with the air benzene ($p=0.012$) and toluene ($p=0.042$) concentrations taken inside the car cabins. Half of the 24h-profiles of individuals exposed to petrol had at least one urine with 1 mgMA/gCr or higher, while all of the profiles of controls were below 1 mgMA/gCr. The technique developed in this study for the determination of uMA showed promise as a tool for monitoring levels of benzene arising from low-level environmental exposures to petrol.

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Abbreviations

CODE	DISCRIPTION
%	percent; 1/100 (ratio)
°C	degree centigrade (temperature)
µg	microgram (mass; 1g= 1,000,000 µg)
µg/gCr	microgram per gram creatinine (mass)
µg/kg	microgram per kilogram (mass concentration)
$\mu g/m^3$	microgram per cubic metre of air (mass)
13X	one of the molecular sieve materials
8OHdG	8'droxy-2'deoxyguanosine
ANOVA	analysis of variance (statistics)
BTEX	benzene, toluene, ethylbenzene, and xylenes
CH_4	methane
C_6H_6	benzene
C_6D_6	deutrated benzene
cm/h	centimetre per hour
CNG	compressed natural gas
CO	carbon monoxide
CO_2	carbon dioxide
CS_2	carbon disulphide
CV	coefficient of variation
CYP	Cytochrome P450
DL	detection limit
DNA	deoxyribonucleic acid
EC	electrochemical detector
ELISA	enzyme-linked immunosorbant assay
ETS	environmental tobacco smoking
FID	flame ionisation detector
g	gram (mass) or g-force (rotation velocity)
g/L	gram per litre
g/ml	gram per millilitre (density)
GC	gas chromatography
GC-MS	gas chromatography - mass spectrometer
GSH	glutathione (thiols)
h	hour (IS)
H_2	hydrogen gas
HL-60	human promyelocytic cell
HPLC	high performance liquid chromatography
K	Kelvin (temperature)
kg/cm^2	kilogram per square metre (pressure)
kPa	kilo-Pascal (pressure)
L/tonne	Litre per tonne
LPG	liquefied petroleum gas
m-	meta- isomer
m.w.	molecular weight (mass)
m/z	single ion record (GC-MS)
MA	*t,t*-muconic acid
mb	millibar (pressure)

mg/l	milligram per litre
mg/m^3	milligram per cubic metre
mgMA/gCr	milligram *t,t*-muconic acid per gram creatinine
min	minute (IS)
ml	millilitre
mM	millimolarity
mmHg	millimetre mercury (atmospheric pressure)
mmol/L	millimole per litre
MTBE	methyl tertiary butyl ether
N or n	number of subjects or samples
NADPH	nicotinamide adenine dinucleotide phosphate
NCV	natural gas vehicle
ng/gCr	nanogram per gram creatinine
ng/l	nanogram per litre
NO_x	nitric oxides
o-	ortho- isomer
p-	para- isomer
P450	Cytochrome P450
pH	Logarithmic power of hydrogen ion abundance
PID	photoionisation detector
POST	sample collected after exposure.
POST	sample collected after exposure
ppb	part per billion (volumetric concentration)
ppm	part per million (volumetric concentration)
PRE	sample collected before exposure
r	Pearson correlation
R^2	coefficient of determination
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
SAX	strong anion exchange
SD	standard deviation
sec	second
SIR	single ion record (GC-MS)
SOD	superoxide dimutase
SPE	solid phase extraction column
SPMA	*S*-phenylmercapturic acid
TWA	time-weighted average - 8h threshold limit
UV	ultra-violet
VOC's	volatile organic compounds

SECTION(I):

INTRODUCTION

SECTION (I): INTRODUCTION

The health impact of benzene exposure to man is a world-wide concern. Modern technology has contributed greatly to the elevation of benzene levels in the environment. Major steps have been taken to minimise the health impact and reduce the potential of benzene carcinogenicity.

Determination of *t,t*-muconic acid in urine is a well-known biological marker of occupation of benzene exposure. Urinary *t,t*-muconic acid measurement has been recognised as a good marker of exposure to low concentrations of benzene (Inoue et al., 1989b).

The first aim of this research was to develop and validate a method to determine urinary *t,t*-muconic acid which could be used to monitor the exposure to environmental benzene level. Secondly, a sensitive method to analyse organic in air contaminants was required. Benzene, toluene, ethylbenzene, and (p-, m-, and o-) xylenes (BTEX) analysis by using GC-MS was the second aim after extract it from charcoal with carbon disulphide.

CHAPTER 1

Chapter 1: Benzene

Benzene is a highly volatile organic compound. It consists of six carbon atoms with six-hydrogen atom (C_6H_6) forming a ring-shaped molecule. It is a colourless liquid with an aromatic odour and forms a highly flammable and explosive mixture with air at 1.4% to 8.0% by volume. The ambient odour threshold is 1-12 ppm (3.2-39 mg/m^3). It is very stable in the environment because of its physical properties (Table 1). The molecular weight and density of benzene are 78 m.w. and 0.879 g/ml at 20°C, respectively. Its conversion factor from (ppb) to ($\mu g/m^3$) is to multiply 3.24 by (ppb), when the ambient temperature is 20°C and atmospheric pressure is 1 atmosphere (101.3 kPa)(Murely, 1994).

Benzene has many synonyms (Neumeier, 1993). In alphabetical order, they are: annulene, benzene, benzin, benzine, benzole, bicarburet of hydrogen, carbon oil, coal naphtha, cyclohexatriene, fenzen, mineral naphtha, motor benzol, nitration benzene, phene, phenyl hydride, pyrobenzol, and pyrobenzole.

Table 1: Physical properties for xenobiotics that are associated to benzene exposure (Merian and Zander, 1982).

Name/Synonyms	Empirical Formula	Molecular Weight	Boiling Point (°C)	Vapour @ 25 °C (mm Hg)	Pressure (kPa)	Density @ 20 °C (g/ml)
Benzene	C6H6	78.11	80.1	95.2	12.7	0.879
CAS #71-43-2						
RTECS CY1400000						
Ethylbenzene	C8H10	106.17	136.2	9.6	1.28	0.867
CAS #100-41-4						
RTECS DA0700000						
Toluene	C7H8	92.14	110.6	28.4	3.79	0.867
CAS #108-88-3						
methylbenzene						
RTECS XS5250000						
Xylene	C8H10	106.17				
CAS #1330-20-7	(ortho)		144.4	6.7	0.89	0.880
RTECS ZE2100000	(meta)		139.1	8.4	1.12	0.864
	(para)		138.4	8.8	1.18	0.861
dimethylbenzene(p-xylene)						

1.1.1 Ambient Concentration

The average ambient benzene concentration in 1987 in urban areas taken from 31 cities in California was 2.21 ppb (EPA, 1999). The ambient concentration in commercial, industrial and residential areas were 2.31 ppb (0.90-5.24 ppb). High readings in one of the industrial areas (9.54 ppb) influenced the average. The concentrations of ambient benzene (1998) in Newcastle upon Tyne was 3.16 ppb (personal communication), which not difference from that in California.

In 1993 and 1994, the mean dropped to about 1.14 ppb (4 $\mu g/m^3$) (Wallace, 1996). This fall may be due to one or more factors. Firstly, 50% of the hydrocarbon emission was reduced by enforced modifications to new cars. Secondly, the concentration was affected by Stage II vapour recovery controls. Thirdly, the benzene content in petrol was reduced to 1% by the 1990 Clean Air Act Amendments. The mean air benzene concentration in ten Canadian cities was 1.26 ppb (4.4 $\mu g/m^3$)(Wallace, 1996).

In 1992-93, Austria measured the ambient benzene and found it to range from 1.4 to 5.7 ppb (5 - 20 $\mu g/m^3$) in heavy traffic areas (Hanus-Illnar and Hrabcik, 1996). In Japan (1997), ambient benzene levels were between 0.29 and 2.9 ppb (1-10 $\mu g/m^3$) (PREFECTURE, 1998).

The European Commission has adopted a proposal that set European Union (EU) limit values for benzene in ambient air. The main element of the proposal, which a limit for ambient benzene of 1.5 ppb (5 $\mu g/m^3$), must be met on 1 January 2010 (EC, 1998).

Davoli and co-workers (1996) investigated benzene emissions from cars under different conditions in Italian urban areas. Driving an electrical car in a car park produced half of the air benzene level (9.24 ppb, 30 $\mu g/m^3$) of urban traffic (19.4 ppb, 63 $\mu g/m^3$). Driving a petrol-fuelled car with a catalytic converter halved the emission of benzene (3.9 ppb, 12.7 $\mu g/m^3$) compared to driving without a converter (6.38 ppb, 20.7 $\mu g/m^3$). Smoking two cigarettes while driving doubled the benzene concentration (7.3 ppb, 23.7 $\mu g/m^3$) in the car compared to the level when no one smoked. Toluene was emitted in higher quantities compared to benzene (Table 2). At

low level of benzene (4.7-5.9 ppb, 15.2-19.1 μg/m^3), the benzene/ toluene ratio varied between 0.156 and 0.27. However, with higher level of benzene (53.7-119 ppb, 174.4-386.3 μg /m^3), the benzene/ toluene ratio increased to between 0.311 and 0.337.

Table 2: Benzene and toluene emission from petrol-fuelled car (Davoli et al., 1996).

Benzene	Toluene	Benzene/Toluene
ppb	ppb	ratio
5.0	24.0	0.210
53.7	146.2	0.311
119.0	299.5	0.337

1.1.2 Sources

Benzene sources are either natural or anthropogenic. Benzene is formed naturally in very low concentrations in environment. Ambient benzene concentration in atmosphere ranges between 0.3 and 45 μg /m^3 (0.1 and 14 ppb) throughout the world, even in remote regions. The main source of ambient benzene is believed to be biological rather than industrial (Fishbien and O'Neill, 1988). Biomass burning in African savannas (Zambia and South Africa) is a significant source of carbon monoxide (CO), benzene, ethyne, and propene in the atmosphere. The amount of CO, ethene, ethane, ethyne, propene, propane, and benzene emitted per year from these fires is about 20-95% of the amount released from global industrial activities (Hao et al., 1996).

The main natural source is forest fire disasters. The global scale of the forest fire reflects the serious impact on the atmosphere. In Canada in 1999, 54 fires broke out. The satellite image (Figure 1) showed four fires with grey coloured plumes directed by the southern wind towards the north direction (NCRAN, 1999). This image reflected the actual damage such a disaster could do to the global atmosphere.

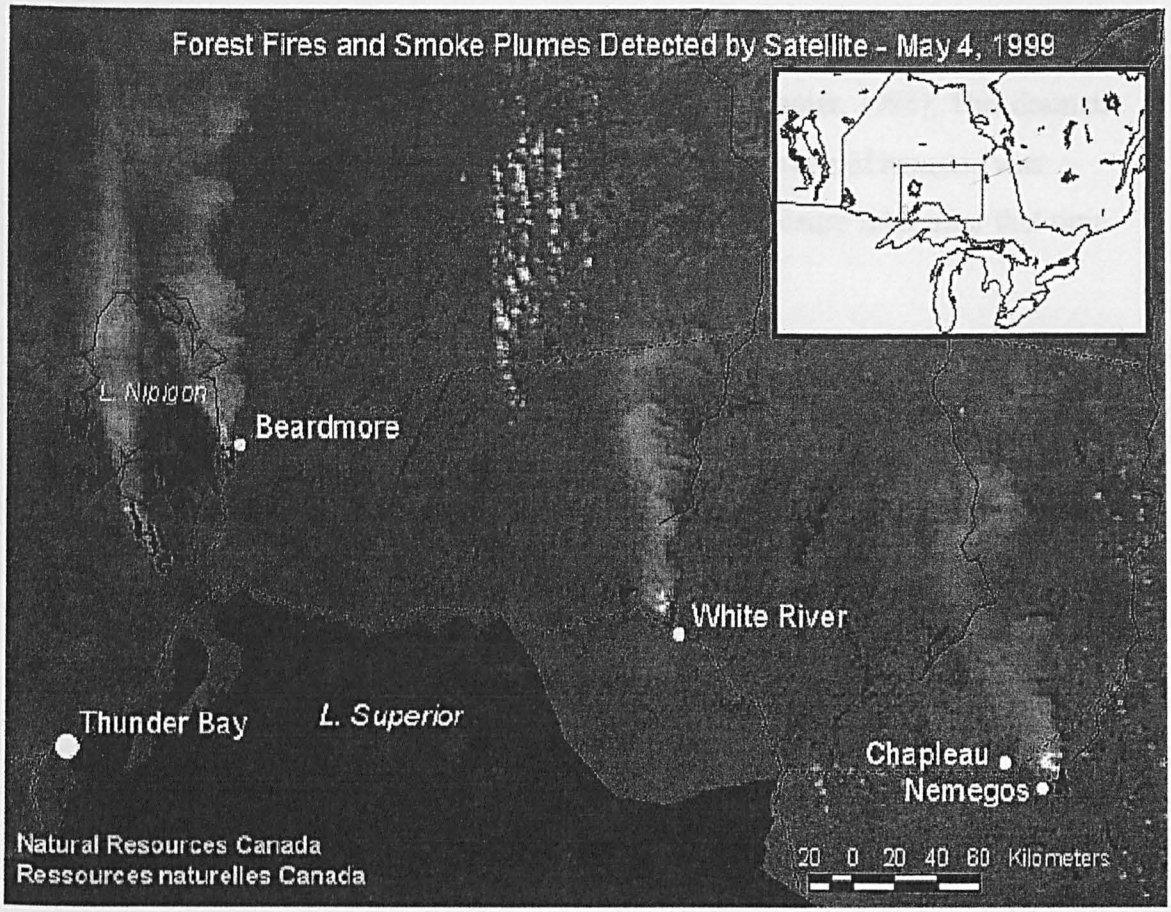


Figure 1: Global impact of fires in the northern forests of Nipigon Lake, Canada (NCRAN, 1999).

Anthropogenic sources that emit benzene into the atmosphere are mainly petrochemical industries, tobacco smoking, and petrol fuel (Wiglusz and Slebioda, 1991). Crude oil might consist of up to 0.4% benzene (Neumeier, 1993). Heating and incomplete combustion of organic material might also add more benzene to the air (Neumeier, 1993). In small industrial wood-fired boilers, benzene was also detected (Hubbard, 1995, Gupta et al., 1998).

The maximum mean of benzene concentration in the workplace reached 1,600,000 $\mu g/m^3$ (500,000 ppb) with peak values of more than 3,200,000 $\mu g/m^3$ (1,000,000 ppb) (Neumeier, 1993). The mean annual averages for occupational benzene concentrations were about 100,000 $\mu g/m^3$ (31,000 ppb) in the late 1960s and less than 50,000 $\mu g/m^3$ (16,000 ppb) in the 1970s. In the 1980s the level of benzene was about 48,000 $\mu g/m^3$ (15,000 ppb) (Moszczynski, 1993). Benzene exposure in more than 100 German enterprises were investigated in the 1990s. Ninety percent of

samples were below 7,870 μg/m^3 (2,460 ppb) in the first half of the decade and 2,944 μg /m^3 (950 ppb) was determined in the second half (Neumeier, 1993). This dramatic drop in benzene concentration can be seen in Figure 2. The use of benzene was abandoned as a solvent in the 1980s, also reducing the exposure level after that time.

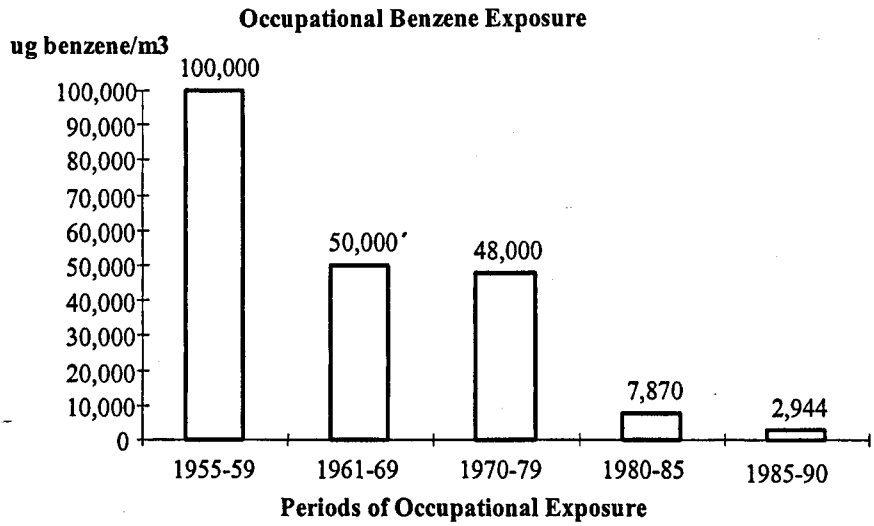


Figure 2: Occupational exposure to benzene from 1955 to 1990 (Neumeier, 1993).

Spillage, leakage, the loading of tanks, shipping, the connection-disconnection of pipes and the cleaning of tanks are responsible for most occupational exposures of more than 1000 ppb in the short term. This can be reduced significantly by applying simple precautions. Furthermore, industries where old technical standards were not up-dated mainly contributed to the cases of more than 1,000 ppb in long-term exposure (Neumeier, 1993). The department of the Environment, Transport and the Regions (DETR) in the United Kingdom estimated that workers exposed to 1,000 ppb, were one-fifth of the UK workplace exposure limit, in 1998. This can be represented as being exposed to 10,000 μg of benzene a day compared to 650 μg day in urban areas (DETR, 1998).

Tobacco smoke contains benzene. The daily intake of benzene for non-smokers is 130-550 μg a day. However, individuals who smoke one pack a day take in 700-1,200 μg of benzene daily and those who smoke two packs take in 1200-1800 μg (Wiglusz and Slebioda, 1991). Twenty-six cigarette brands market and hand rolled tobacco on the UK were tested for volatile organic compounds (VOC's). Smoking

cigarettes with an average yield of 50 μg per cigarette was compared with the occupational maximum exposure limit (16,000 $\mu g/m^3$) concentration (Darrall et al., 1998).

Environmental tobacco smoke (ETS) from a selection of six commercial cigarettes was estimated to contribute 37% to 58 % (1.3 to 8.2 $\mu g/m^3$) of the benzene concentration in a room-size of environmental chamber (Hodgson et al., 1996). ETS is estimated to contribute 5% of the total inhalation exposure of all non-smokers (Miller et al., 1998).

1.1.3 Production

Aromatic hydrocarbons primarily consist of benzene, toluene, xylenes, ethylbenzene, styrene, cumene and halogenated benzene. They are among the most important volatile aromatic compounds and have both commercial and environmental consequences (Fishbien and O'Neill, 1988).

Michael Faraday (1825) isolated benzene from liquid condensed by compressing oil gas and A. W. Hoffman (1845) discovered benzene in light oil derived from coal tar. Charles Mansfield (1849) developed the commercial recovery of benzene from coal tar. Benzene was discovered in coal gas in 1876 which initiated the use of coal gas light oil as a source of benzene. Light oil usually contains more than 80% aromatic compounds and less than 5% unsaturated compounds. The yield of light oil from coke ovens producing furnace coke is approximately 13 - 17 L/tonne in carbonised coal (Fishbien and O'Neill, 1988). Benzene exists in crude oil at a concentration of 0.4% (Neumeier, 1993).

In 1925, the United Kingdom was the first European country to produce petroleum benzene. This was followed by France (1958), Germany (1961), and Italy (1962). The main commercial routes to produce benzene from petroleum sources (Fishbien and O'Neill, 1988) are:

1- **Pyrolysis petrol:** Large quantities of benzene are commercially obtained from pyrolysis petrol in olefin plants, as a by-product of the manufacture of ethylene or propylene, by cracking naphtha or gas oil. In Western

Europe and Japan, it is the largest source of benzene production counting for 55% and 50%, respectively.

2- **Refinery Streams (Primarily Catalytic Reformat):** A low-sulphur naphtha-range petroleum fraction is catalytically reformed to produce a high-octane product for use in petrol blending. Principally platinum (Pt) or palladium (Pd) dehydrocyclisation catalysts are applied for the reform process. Catalytic reformatting was the source of approximately 44-55% of benzene produced in USA in the period 1978-1981.

3- **Toluene transalkylation:** This route is more expensive than the others are. The economic attractiveness of toluene dealkylation is sensitive to the relative amounts of benzene and toluene. Many toluene dealkylation units produce benzene for in-house consumption or from captive toluene supplies not needed for petrol. Toluene alkylation provides 25-30% of the benzene supply in USA (1981).

World production of benzene was estimated in 1977 to be over 12 million tonnes. In 1982, it was estimated at 14 million tonnes. The production in Western Europe in 1979 was 4.8 million tonnes. In 1979, USA production of benzene was 5.44 million tonnes. Around 2.17 and 2.882 million tonnes were produced in Japan in 1979 and 1980, respectively (Fishbien and O'Neill, 1988).

1.1.4 Uses

Two main areas for use of benzene in industry are in petrol and chemical manufacturing (Fishbien and O'Neill, 1988).

Petrol (gasoline) is a complex mixture of volatile hydrocarbons with a boiling point range of 50-200°C predominantly in the C4 - C12 range. Automotive petrol may contain 0-7% (typically contains 2-3%) benzene. Benzene in petrol is used as a solvent to increase the octane rating of unleaded petrol.

The other major areas of benzene consumption lie in utilising benzene as an intermediate in the manufacture of other chemicals. The main organic chemicals

produced from benzene are ethylbenzene, styrene, cumene, cyclohexane, nitrobenzene, alkylbenzene, maleic anhydride and chlorobenzene (Table 3).

Table 3: Main applications for chemicals syntheses from benzene (Fishbien and O'Neill, 1988).

Derived Chemical	Application
Ethylbenzene to Styrene	resins, polystyrene, rubber, polyester.
Cumene to Phenol	phenolic resins, nylon other products.
Cyclohexane	nylon fibres, resins
Nitrobenzene	rubber chemicals, polyurethane foams, dyes
Maleic Anhydride	polyester resins, food additive, pesticides.
Alkylbenzene	surfactants, detergents
Other non-fuel uses	DDT, chlorobenzene, biphenyl,fumaric acid, other chemicals

CHAPTER 2

Chapter 2: Benzene Toxicokinetics

This chapter is related to the toxicokinetics of benzene. This includes the absorption benzene into the living organism, distribution throughout the body of the organism, metabolism within the body, and excretion from the organism. For any substance, the toxic effects depend on dosage, which means that any toxic response will be related to the amount of a chemical taken up by an organism. The complex adverse effects of benzene exposure to man will be discussed in this chapter.

1.2.1 Absorption

Inhalation, ingestion, and dermal contact are routes of exposure, by which benzene absorption occurs. Inhalation is the major and most rapid route of absorption for benzene (ATSDR, 1988). Maximal inhalation absorption was observed within the first minutes of exposure, which then fell to a constant rate within 15 minutes to 3 hours during continuous exposure. In a study of smokers, an average of 64% of the inhaled benzene dose was absorbed through the lung barrier, with the percentage absorbed decreasing with continued exposure (Yu and Weisel, 1996a). F344/N rats, Sprague-Dawley rats, and B6C3F1 mice (Sabourin et al., 1987) inhaled ^{14}C- Benzene. The percentage of benzene obtained after a 6 h exposure by inhalation was inversely proportional to the benzene concentration. Three inhaled doses (11, 130 and 870 ppm) demonstrated an inverse relationship with the absorbed proportions (39%, 24% and 20%, respectively) after a 6 h exposure (Henderson et al., 1989). Benzene was also rapidly absorbed from the gastro-intestinal tract (Henderson et al., 1989).

Dermal absorption of benzene vapour can be considered negligible compared to pulmonary uptake (Blank and McAuliffe, 1985). Absorption of liquid benzene through skin depends on factors such as the thickness and hydration of the stratum corneum, and the perfusion rate of the dermis (Nakai et al., 1997). ^{14}C- benzene was applied to stain in an *in vitro* system. A permeability coefficient of 0.10, 0.14, and 0.26 cm/h under standard conditions at 15°C, 26°C, and 50°C was found, respectively. Prior storage of the skin at -20°C did not affect the penetration of benzene. Application of baby oil, moisturiser, or insect repellent to the skin before exposure did not affect the flux of benzene, but a significant increase was observed

when the skin was pre-treated with sunscreen (permeability coefficient 0.24 cm/h) (Nakai et al., 1997).

1.2.2 Distribution

In animal studies (dogs), higher levels of benzene were found in fat and bone marrow compared to other body organs, reflecting their role in benzene distribution. The distribution in fat and bone marrow was also faster than the distribution in blood (Schrak et al., 1941, Thorne et al., 1986). The distribution half life varied between 0.9 and 2.6 h except for the bone marrow where the distribution was much faster. Human studies showed a similar distribution pattern to the animal studies (Wiglusz and Slebioda, 1991). Blood, bone marrow, adipose tissue and liver were the main depots for benzene in the human body (Wiglusz and Slebioda, 1991).

1.2.3 Metabolism

Benzene metabolism occurs mainly in the liver. However, all tissues contribute to its metabolism, including bone marrow. The relationship between benzene metabolites and toxicity was confirmed by Snyder et al. (1993). Benzene metabolism is competitively inhibited by toluene. As a consequence, benzene toxicity is reduced when co-administrated with toluene (Sammett and Lee, 1979). Thus, hepatic metabolism plays an important role in benzene toxicity. Also, bone marrow contributes to the toxicity as secondary metabolism of benzene occurs in this tissue (Subrahmanyam et al., 1991).

Phenol, catechol, hydroquinone, 1,2,4-benzenetriol, *t,t*-muconic acid, and L-phenylmercapturic acid were recovered in urine after oral administration or intraperitoneal injection of ^{14}C- benzene to rabbits (Parke and Williams, 1953, Parke, 1996). Urinary metabolites represented 32.6% of the total recovered dose (Table 4). Unchanged benzene and small amounts of carbon dioxide in expired air represented 44.5% of the recovered dose.

Table 4: Metabolism of ^{14}C-benzene by rabbits (Parke, 1996).

Source	^{14}C-Benzene	Unlabeled-benzene
Expired air		
Benzene	46.5	48.4
$^{14}CO_2$	1.0	-
Total in expired air	47.5%	48.4%
Urine		
Phenol	22.9	
Catechol	2.9	
Quinol	4.8	
Resorcinol	<0.3	
Hydroxyquinol	0.3	
L-Phenylmeracpturic acid	0.4	1.0
t,t-Muconic acid	1.3	1.0
Total in urine	32.9%	38.0%
Faeces	0.5%	
Tissues	5.0%	
Total accounted for	86.0%	86.0%

1.2.3.1 Cytochrome P450 2E1

The cytochromes P450 are a superfamily of enzymes (Gonzalez et al., 1991). The individual enzymes are named with the prefix (CYP) followed by an Arabic numeral for the enzyme family. A letter is given for the subfamily and another Arabic numeral for the gene number. Therefore, CYP2E1 is identified as Cytochrome P450 2E1, in which **CYP** is the superfamily, **2** is for the enzyme family, **E** for the enzyme subfamily, and **1** is the enzyme number. CYP2E1 is constitutively expressed in human liver (Yoo et al., 1988) and is likely to be expressed in extrahepatic tissue following induction such as by ethanol administration, fasting and uncontrolled diabetes (Song et al., 1987).

Detoxification pathways for benzene are those that produce phenol and water soluble phenyl conjugates (mercapturic acid, glucuronides and sulphates). On the other hand, dihydroxybenzene is a toxic pathway. Dihydroxybenzene is further oxidised to semiquinone and quinone. The ring opening reactions of dihydroxybenzene to the reactive *t,t*-muconaldehyde are considered also to be toxic pathways (Henderson et al., 1989). Hydroquinone glucuronide and *t,t*-muconic acid have been shown to be markers of toxic pathways (Henderson et al., 1989). Both metabolites are generally proportionally greater at lower benzene doses as seen in F344 rats and B6C3F1 mice (Henderson et al., 1989, Sabourin et al., 1989).

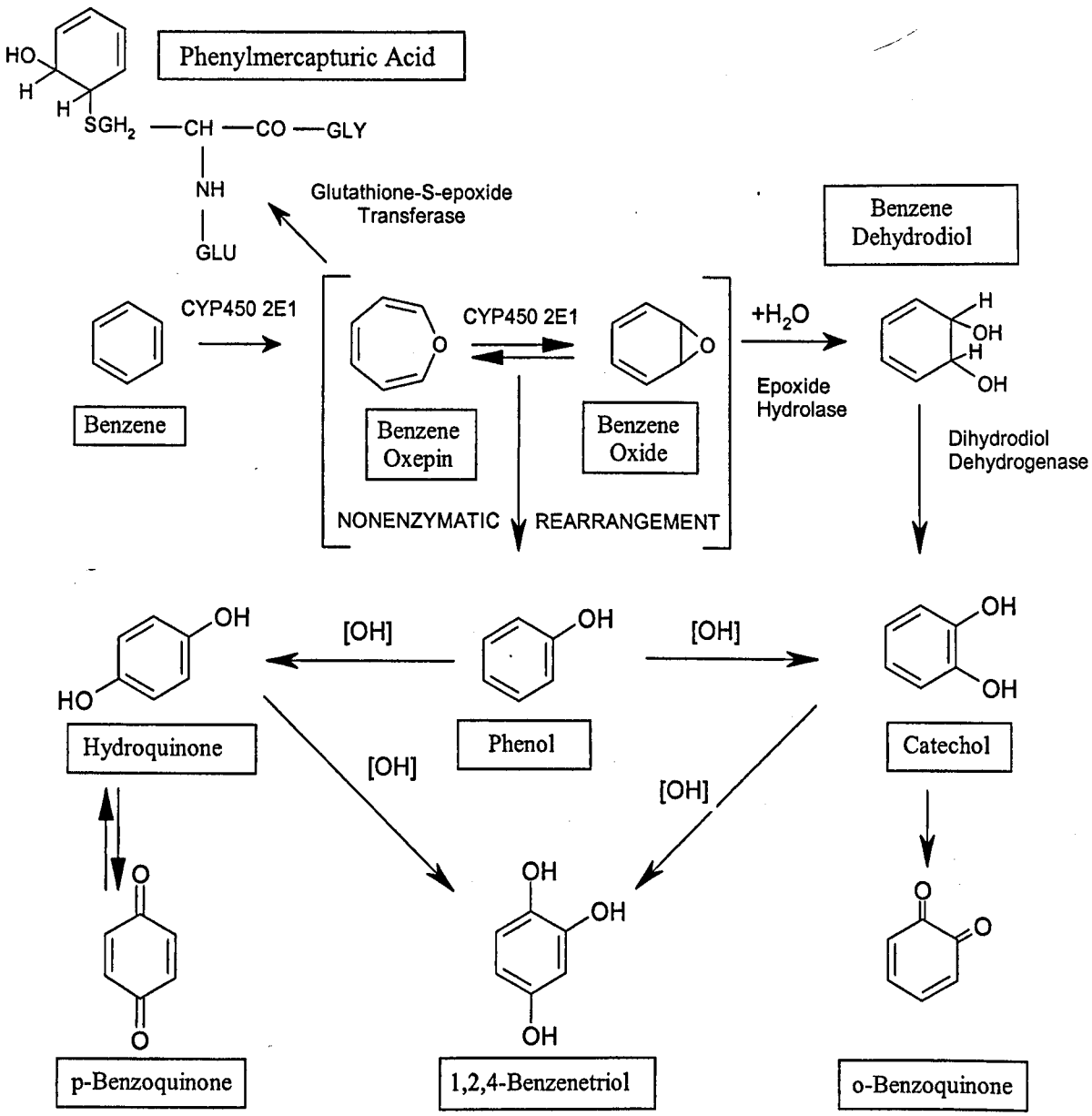


Figure 3: Biotransformation of benzene. The dashed arrow refers to a putative pathway (Klaassen, 1996).

The first step in benzene metabolism is phenol formation (Klaassen, 1996). At very low benzene concentrations, Gorsky and Coon (1985) proposed that a free radical mechanism occurs following induction of P 450 leading to hydrogen peroxide formation. Direct oxidation via the P 450 pathway may occur to form benzene oxide and oxepin, which then may spontaneously rearrange to form phenol. Alternatively, benzene may be hydroxylated non-enzymatically to form phenol regardless of benzene oxide and oxepin formation (Figure 3). Johansson and Ingelmansundberg (1983) concluded that free hydroxyl radicals generated from hydrogen peroxide in rabbit liver microsomes mediated the benzene-phenol reaction. Gorsky and Coon

(1985) suggested that hydroxyl radical-mediated oxidation of benzene microsomes via nicotinamide adenine dinucleotide phosphate (NADPH) reductase may be a significant route of phenol formation in rabbit liver. On the other hand, no free radical oxidation was found following P450-mediated pathways(Gorsky and Coon, 1985).

Rapid destruction of CYP2B1 was caused by inhalation of benzene (4 mg/litre of air) in rat liver. The destruction was reversed by ascorbate and diminished by alpha-tocopherol, suggesting that hydroquinone was not toxic, and that benzoquinone and semiquinone radicals (SQ) caused the effect. Destruction of P 450 *in vitro* caused by hydroquinone (HQ) or benzoquinone (BQ) was not mediated by hydroxyl radical formation or by lipid peroxidation. Furthermore, HQ and BQ inhibited NADPH-mediated lipid peroxidation (Gut et al., 1996a).

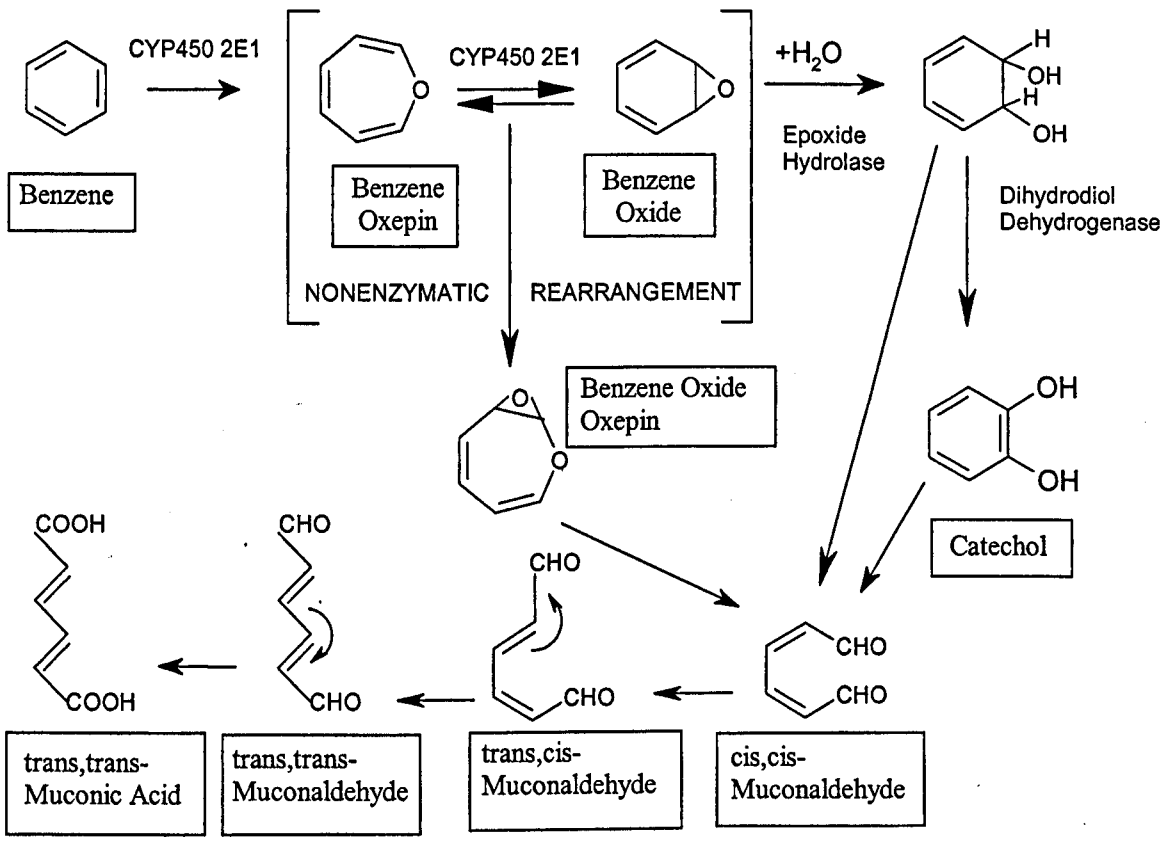


Figure 4: Benzene biotransformation to *t,t*-muconic acid (open the ring). The dashed arrow refer to putative pathways (Neumeier, 1993, Bleasdale et al., 1996).

Apart from phenol formation from benzene, benzene oxide can be hydrolysed via epoxide hydrolase to produce 1,2-benzene dihydrodiol. 1,2-Benzene dihydrodiol can then be oxidised via dihydrodiol dehydrogenase to form catechol. The reaction of benzene oxepin or oxide with glutathione may be catalysed by glutathione *S*-transferase, which leads to the formation of *S*-phenylmercapturic acid. Hydroquinone and catechol formations are the result of further hydroxylation of phenol. Further hydroxylation of hydroquinone may form 1,2,4-benzenetriol (Inoue et al., 1989a). These pathways are presented in Figure 3.

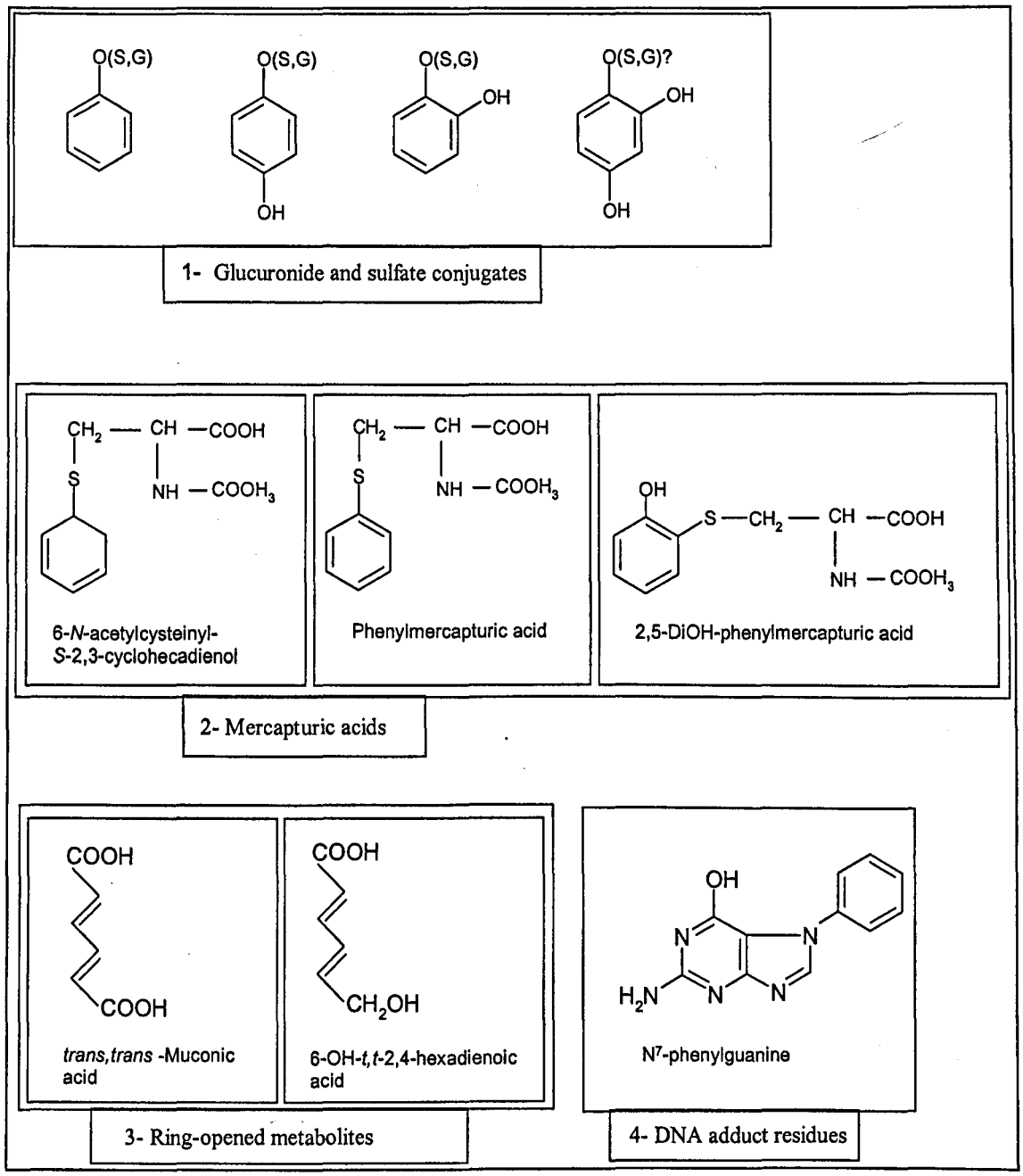


Figure 5: Urinary metabolites of benzene (Snyder and Hedli, 1996).

Benzene oxide or benzene oxepin are precursors to ring opening to give *t,t*-muconic acid (Witz et al., 1996) (Figure 4). In general, four forms of benzene metabolites were found in urine in both animal or human studies(Parke and Williams, 1953, Sabourin et al., 1988a, Sabourin et al., 1988b, Nerland and Pierce, 1990, Kline et al., 1993, Norpoth et al., 1988); (1) sulphate or glucuronide conjugate with any of the phenolic metabolites, (2) mercapturates (including S-phenylmercapturic acid, 6-*N*-acetylcysteinyl-*S*-2,3-cyclohexadienol and 2,5-dihydroxy-phenylmercapturic), (3) ring-opening products (*trans,trans*-muconic acid and 6-hydroxy-*t,t*-2,4-hexadienoic

acid), (4) and N^7-phenylguanine as a residue of DNA adduct formation. The chemical structures of these metabolites are shown in Figure 5.

Covalent binding to cellular macromolecules is an alternative fate of some benzene metabolites. Benzene metabolites bind covalently to proteins in mouse liver, bone marrow, kidney, spleen, blood, and muscle (Snyder et al., 1978, Longacre et al., 1981a, Longacre et al., 1981b). Benzene-resistant C57B1/6 mice had less covalent binding in bone marrow, blood and spleen than the relatively more benzene-sensitive DBA/2 mice (Luke et al., 1988). Genotoxic and cytotoxic damage of DNA may lead to the inhibition of cell replication and so initiate leukaemia and other cancers. DNA extracted from rat liver was shown to contain benzene residues after exposure to labelled benzene vapour (Lutz and Schlatter, 1977). On the basis of ^{32}P-postlabelling, several DNA adducts were determined in the nuclei and mitochondria of rabbit liver following treatment with benzene (Randerath et al., 1981). At very low level exposure of mice to benzene, covalent binding of the metabolites was found (Creek et al., 1994). Covalent binding of hydroquinone to spindle fibre protein could help to explain the inhibition of cell replication by benzene (Irons and Neptun, 1980).

The mitochondria are a crucial organelle for the covalent binding of benzene (Gill and Ahmed, 1981). Inhibition of RNA synthesis in the mitochondria of liver and bone marrow correlated with covalent binding of benzene metabolites to DNA (Kalf et al., 1982). The inhibition of RNA synthesis in mitochondria was related to the inhibition of critical mitochondrial proteins and mitochondrial malfunction. Phenol, hydroquinone, catechol, benzoquinone, and 1,2,4-benzenetriol have the potential to form adducts in bone marrow mitochondria.

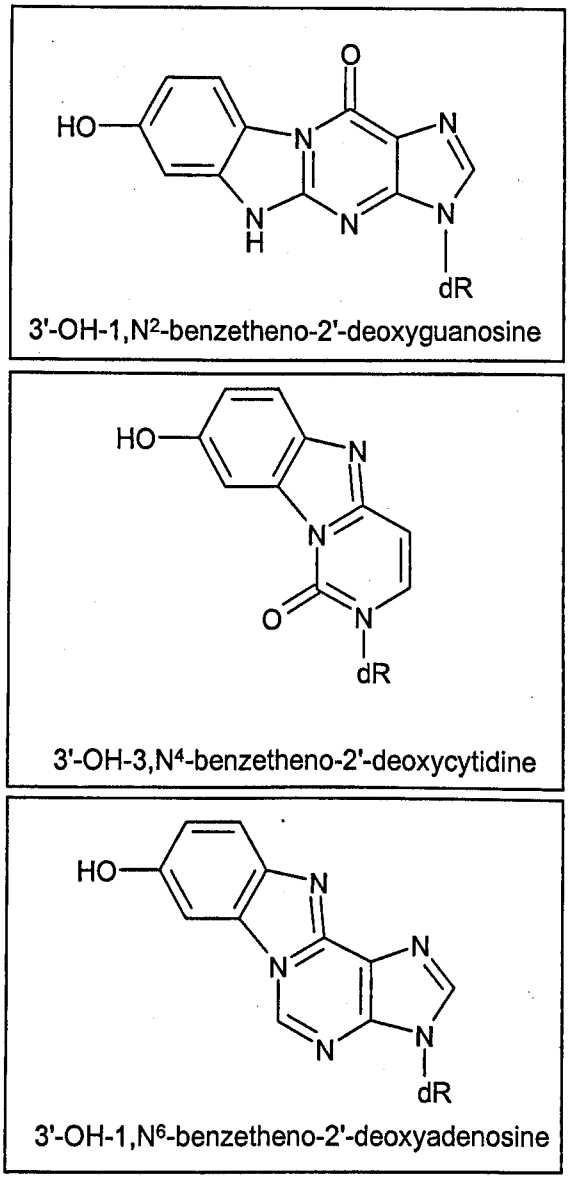


Figure 6: The major deoxyribonucleoside adduct structures of p-benzoquinone (Snyder and Hedli, 1996).

In the presence of an oxidising agent *in vitro*, deoxynucleosides or deoxynucleoside monophosphates reacted with *p*-benzoquinone and hydroquinone to produce 3'-OH-1,N^2- benzethen-2'-deoxyguanosine, a major deoxyguanosine (dG) adduct (Jowa et al., 1990). Three more dG adducts were identified by applying sensitive DNA ^{32}P postlabelling following the *in vitro* reaction of *p*-benzoquinone to calf thymus DNA: (3'-OH)-3,N^4-benzethenodeoxycytidine-3'-phosphate, (3'-OH)-1,N^6-benzetheno-2'-deoxyadenosine-3'-phosphate, and (3'-OH)-1,N^2-benzetheno-2'-deoxyguanosine-3'-phosphate (Pongracz and Bodell, 1991, Levay et al., 1991). These adducts are shown in Figure 6.

Studies *in vitro* have demonstrated covalent binding of benzene metabolites to DNA, however, *in vivo* studies have had more difficulty proving this reaction. Human promyelocytic (HL-60) cells are myeloid cells that have the capacity to differentiate in response to specific chemical stimulants into any of the four classes of hematopoietic cells of the myelomonocytic lineage, i.e., granulocytes, monocytes, eosinophils, or macrophages (Collins, 1987). DNA adduct formation by hydroquinone but not 1,2,4- benzenetriol may play a role in inhibiting cell differentiation in HL-60 cells (Hedli et al., 1996).

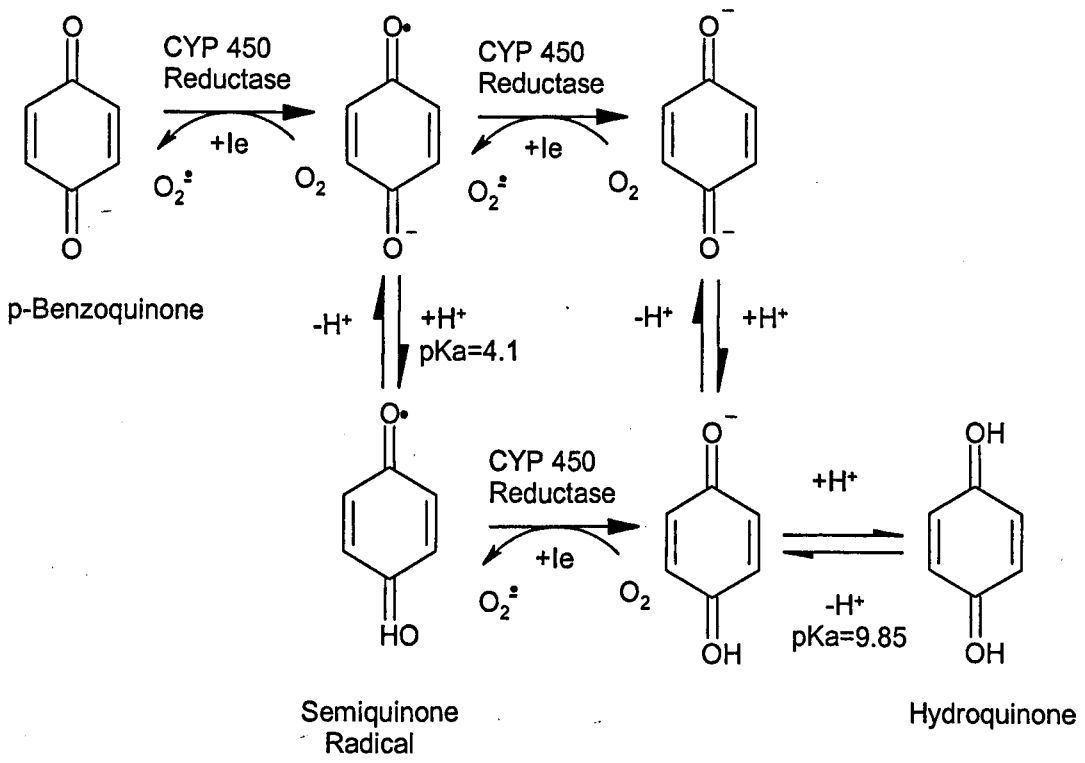


Figure 7: Redox cycling of hydroquinone and *p*-benzoquinone (Snyder and Hedli, 1996).

Some benzene metabolites are capable of producing potential oxidative stress, which may contribute to benzene toxicity. Hydroquinone may be oxidised to *p*-benzoquinone, which is highly reactive and can covalently bind to cellular macromolecules or glutathione. Hydroquinone and *p*-benzoquinone may engage in redox cycling, which involves auto-oxidation of a reduced form of the metabolite to yield an oxidised species plus reactive oxygen. The bone marrow is capable of generating reactive oxygen species as it is a richly oxygenated organ. Four-electron reduction of oxygen (Williams, 1984) may generate superoxide anion radicals, hydrogen peroxide, and hydroxyl radicals. This is shown in Figure 7.

Boersma and his colleagues (1994) showed that the reduction of *p*-benzoquinone may proceed via a reductase such as P450 reductase, in two steps. The semiquinone anion radical, the first step could either be reduced again or more likely may be oxidised before a second reduction. Re-oxidation to *p*-benzoquinone is the next step in redox cycling. This re-oxidation leads to superoxide anion formation, which would inhibit the process. The anionic form would predominate at approximately pH 7.5. The anion dissociation constant (pKa) of the first protonation step (*p*-benzoquinone > semiquinone radical) is pH 4.1. The second protonation step (semiquinone radical > hydroquinone) pKa is pH 9.85. Therefore, it is unlikely that hydroquinone - *p*-benzoquinone undergoes redox cycling at physiological pH.

If *p*-benzoquinone is not reduced, then its metabolic fate may be to react with glutathione to form the premeracpturic acid of hydroquinone (Snyder and Hedli, 1996). It may proceed to form the mercapturic acid, or may undergo slow auto-oxidation leading to production of the reactive oxygen species (Brunmark and Cadenas, 1988). Alternatively, *p*-benzoquinone may be converted to its epoxide either via CYP450 or hydrogen peroxide (H_2O_2), leading to *p*-benzoquinone 2,3-oxide or ultimately to 1,2,4- benzenetriol following either a two-electron reduction by diaphorase or two one-electron reductions by CYP450 reductase. Reaction of *p*-benzoquinone 2,3-oxide with glutathione leads to the formation of glutathionyl 1,2,4-benzenetriol.

The unique feature of glutathionyl 1,2,4-benzenetriol is its ability to spontaneously undergo auto-oxidation and redox cycling. It could be sequentially oxidised to glutathionyl-1,2,4-benzenetriol semiquinone and then to 4-hydroxy-glutathionyl - *p*-benzoquinone (HGB) (Brunmark and Cadenas, 1988). 4-hydroxy-glutathionyl - *p*-benzoquinone is also a product of the reaction between *p*-benzoquinone 2,3-oxide and glutathionyl-1,2,4-benzenetriol semiquinone, which also yields 5-hydroxy-benzosemiquinone. Alternatively, glutathionyl-1,2,4-benzenetriol semiquinone can undergo disproportionation to form glutathionyl- 1,2,4-benzenetriol, which can initiate the redox cycle again. If redox cycling is an important phenomenon in the mechanism of benzene toxicity, it is likely that 1,2,4-benzenetriol plays a significant role in the process.

Table 5: The impact of hydroquinone, p-benzoquinone, and 1,2,4-benzenetriol on oxidative stress and antioxidants: superoxide, hydrogen peroxide, nitric oxide, catalase, superoxide dimutase, vitamin C and thiols in HL-60 cells (Snyder and Hedli, 1996).

Antioxidant	Hydroquinone	*p*-Benzoquinone	1,2,4-Benzenetriol
Superoxide (Reid and Loeb, 1992)	Increase	Increase	Increase
Hydrogen peroxide (Bass et al., 1983)	Increase	Increase	Increase
Nitric oxide (Green et al., 1982)	Increase	Increase	No change
Catalase (Aebi, 1984)	Decrease	Decrease	No change
Superoxide Dimutase (Marklund and Marklund, 1974)	Decrease	No change	Decrease
Thiols (GSH) (Beutler et al., 1963)	Decrease	No change	Decrease
Myeloperoxidase (Himmelhoch et al., 1969)	No change	No change	No change
Vitamin C (Attwood et al., 1974)	No change	No change	No change

Snyder and Heldi (1996) added hydroquinone, *p*-benzoquinone, and 1,2,4-benzenetriol to HL-60 cells to determine their impact on oxidative stress and antioxidant factors (Table 5). Superoxide and hydrogen peroxide levels were increased on addition of the three metabolites. Superoxide dimutase (SOD) and thiol levels decreased after adding hydroquinone and benzenetriol. Nitric oxide was elevated after hydroquinone and benzoquinone were added. On the other hand, catalase activity decreased after adding hydroquinone and benzoquinone. No change was observed in myeloperoxidase activity and vitamin C levels after these metabolites were added. The same was found for nitric oxide and catalase when benzenetriol was added. Change was observed for superoxide dimutase and thiols when benzoquinone was added.

Metabolic pathways appear to be influenced by the level of benzene exposure. Urinary phenol was a suitable biomarker for measuring 8h TWA benzene exposure to 10 ppm (32.5 mg/m^3) or greater (Lauwery, 1979). Urinary phenol was not a specific metabolite when airborne benzene concentration was below 6 ppm 8h TWA. S-

phenyl-mercapturic acid (van Sittert et al., 1993) and *t,t*-muconic acid (Ducos et al., 1992, Ong et al., 1995, Ruppert et al., 1997) were more specific than urinary phenol when benzene exposure was less than 10 ppm 8 h TWA. Sorbic acid in the diet is a significant confounding factor in assessing low level benzene (Ducos et al., 1992, HSE, 1982). It has a similar structure to muconaldehyde, which is used as a food preservative.

1.2.3.2 Competitive Interactions

Animal studies, in which benzene was introduced by the respiratory and oral routes, have shown that toxicity was dose dependant (Sabourin et al., 1989). Urinary metabolites are the best biomarkers to indicate exposure (Sabourin et al., 1987). A possible explanation for the differences seen in urinary metabolic profile for different levels of exposure is the competitive interaction between benzene and phenol. Both are substrates for CYP2E1(Koop et al., 1989, Schlosser et al., 1993) and have similar affinities. Benzene can be an effective inhibitor of phenol oxidation following benzene exposure by the oral route, since the level of phenol in the liver will be much lower than the benzene concentration (Figure 8). Moreover, the formation of hydroquinone conjugates was lower when phenol was administered compared with benzene (Medinsky et al., 1995).

Diabetic rats tend to have an increase in benzene metabolites, which is probably due to increased expression of CYP2E1 observed in diabetes (Costa et al., 1999). Even at high doses of benzene, no detoxification metabolites were observed because of reduced glutathione-S-transferase activity in diabetes. Therefore, if these data were extrapolated to man then occupational exposure to benzene may indicate a higher risk level to diabetic workers.

The dose-response relationship of benzene could not be explained by simple saturable (Michaelis-Menten) kinetics. However, water soluble benzene metabolites could be characterised in human liver microsomes by simple saturable kinetics (Nedelcheva et al., 1999).

Incubating mouse and rat liver microsomes in vitro with water soluble benzene metabolites assessed the metabolism of benzene and phenol (Schlosser et al., 1993).

CYP2E1 was assumed to catalyse all three reaction steps in the model: 1) benzene to phenol, 2) phenol to catechol then trihydroxybenzene, and 3) phenol to hydroquinone then trihydroxybenzene. All are assumed to compete for binding to the same site in CYP2E1 through reversible bonds. Hydroquinone formation demonstrated similar constant rates following benzene and phenol exposures. Phenol, as an intermediate in the oxidation of benzene, competed with the other substrates, which made the production rate of hydroquinone slower than that for phenol exposure. This reflects the competition between benzene and phenol for CYP2E1.

As mentioned previously, phenol has a crucial role in benzene metabolism. It has the ability to be oxidised to form hydroquinone and to be conjugated by the glucuronyl- or sulpho-transferases. Although, benzene carcinogenicity was confirmed at multiple sites in mice following benzene exposure by inhalation or ingestion, no carcinogenicity was observed from phenol (NCI, 1980). Micronuclei were induced in bone marrow cells *in vitro* by benzene but not by phenol (Gadelkarim et al., 1984, Choy et al., 1985, Luke et al., 1988).

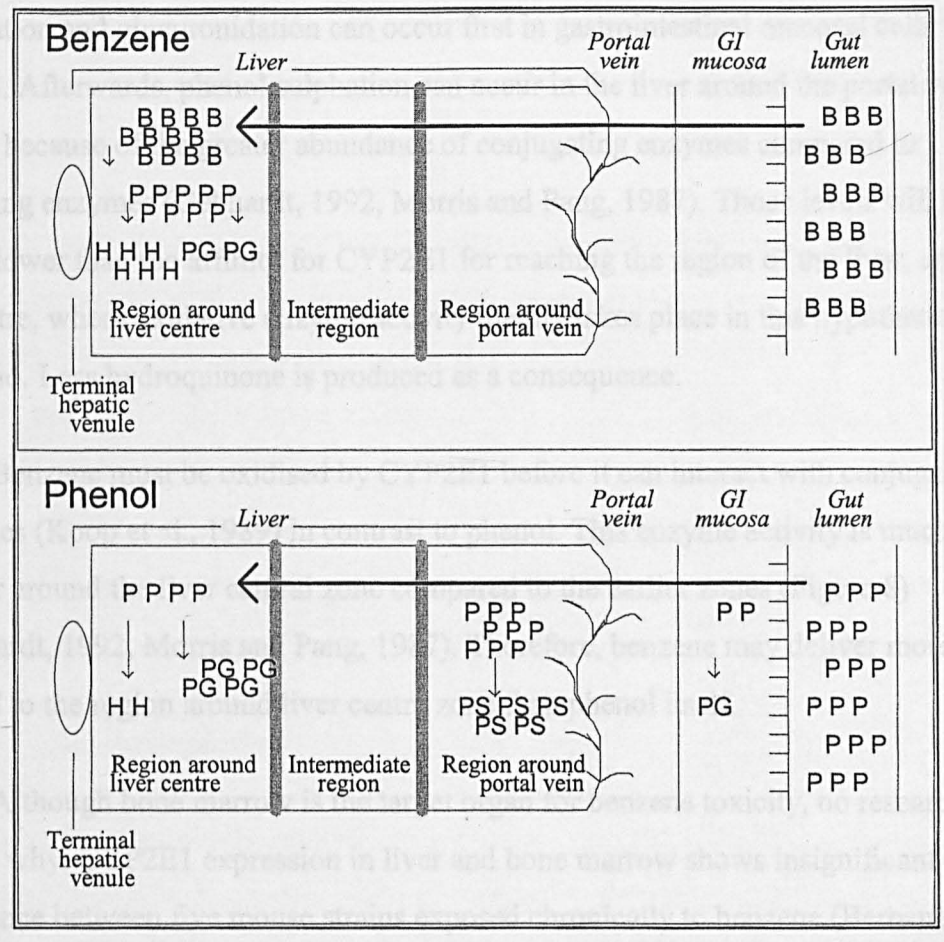


Figure 8: Benzene and phenol absorption models after gavage administration through the major liver zones. (B) benzene, (P) phenol, (PG) phenol glucuronide, (PS) phenol sulphate, (H) hydroquinone (Medinsky et al., 1995).

Hydroquinone induced sister chromatid exchanges *in vitro* in human lymphocytes (Erexson et al., 1985) and *in vivo* in mouse bone marrow (Erexson et al., 1985, Ciranni et al., 1988b, Ciranni et al., 1988a). The carcinogenicity of benzene related to the level of urinary metabolites after oral uptake of benzene (Kenyon et al., 1995). On the other hand, the lack of phenol carcinogenicity parallels the lack of other benzene metabolites. For example, hydroquinone and muconaldehyde production after benzene administration was greater than that following phenol administration.

Medinsky and his colleagues (Medinsky et al., 1995) discussed a probable explanation for the differences between the observed benzene and phenol metabolic profiles. This research discussed the differences in urinary metabolites of benzene compared with phenol after oral administrations. The quantitative differences in enzyme zonal distribution within liver are shown in Figure 8. Absorbed phenol from the gut lumen has comparatively more chance of conjugation than oxidation.

Sulphation and glucuronidation can occur first in gastrointestinal mucosal cells for phenol. Afterwards, phenol sulphation can occur in the liver around the portal vein region because of the greater abundance of conjugating enzymes compared to oxidising enzymes (Gebhardt, 1992, Morris and Pang, 1987). Those levels will be much lower than the affinity for CYP2E1 for reaching the region of the liver, around its centre, where oxidative enzyme activity mainly takes place in this hypothetical scenario. Less hydroquinone is produced as a consequence.

Benzene must be oxidised by CYP2E1 before it can interact with conjugating enzymes (Koop et al., 1989) in contrast to phenol. This enzyme activity is much greater around the liver central zone compared to the earlier zones (Figure 8) (Gebhardt, 1992, Morris and Pang, 1987). Therefore, benzene may deliver more free phenol to the region around liver centre zone than phenol itself.

Although bone marrow is the target organ for benzene toxicity, no research has shown why. CYP2E1 expression in liver and bone marrow shows insignificant difference between five mouse strains exposed chronically to benzene (Bernauer et al., 1999). In man, the P 450 superfamily enzymes in bone marrow (1.14-3.97 ng/mg cellular protein) are about 1000-fold less than in liver (1.2-41.2 μg/mg protein) (Bernauer et al., 2000, Carriere et al., 1996).

Conversely, hydroquinone formation following phenol oxidation by CYP2E1 was confirmed after rats were exposed to benzene by inhalation (Gut et al., 1996b). Furthermore, the oxidation of phenol was found to mediate its further spontaneous oxidation to benzoquinone (Gut et al., 1996b). Differences among human subjects in CYP2E1 expression could contribute to differences in susceptibility to cancer and other toxicities (Nedelcheva and Gut, 1994). The expression and activity of CYP2E1 in human liver biopsy samples from alcoholics was two- (Perrot et al., 1989) or three-fold (Ekstrom et al., 1989) higher than in controls. However, no correlation was found between alcohol consumption and CYP2E1 expression and activity (Lucas et al., 1993). Moreover, CYP2E1 activity in liver samples from transplant donors varied more than seven-fold (Peter et al., 1990, Ekstrom et al., 1989). Using chlorzoxazone oxidation as a marker reaction (Girre et al., 1994), alcoholics have twice the CYP2E1 activity of controls. However, in the same study the activity of some of the alcoholic subjects was similar to control. It is not clear whether alcohol-induced CYP2E1

expression would influence the toxic effect to benzene in man in spite of the fact that alcohol intake increased benzene metabolism and toxicity in rats (Nakajima et al., 1985).

The level of the benzene metabolites in urine, *t,t*-muconic acid and S-phenylmercapturic acid, were investigated in 59 non-smoking bus drivers (Rossi et al., 1999). The individuals were genotyped for CYP2E1, CYP 2D6, GSTT1, GSTP1, GSTM1 and NAD(P)H:quinone oxidoreductase (NQOR) by polymerase chain reaction-based methods. Urinary *t,t*-muconic acid excretion was highly correlated with the GSTT1 null genotype. Also, S-phenylmercapturic acid was lower in drivers lacking quinone oxide NQOR activity and in CYP 2D6 extensive-metabolisers. These genotypes may influence the toxic outcomes of benzene.

1.2.3.3 Polymorphism

Many xenobiotic-metabolising enzymes are polymorphically distributed. These polymorphisms have been identified as impaired or altered metabolism *in vivo*. Cloning many genes, which encode xenobiotic-metabolising enzymes (Nedelcheva et al., 1999), has identified aditional polymorphisms. Also, new techniques for the identification of allelic variants at the DNA level have contributed to determining even more polymorphisms (Daly et al., 1993).

Defects originally detected at the metabolic level are now well understood at the DNA level. In general, metabolic polymorphisms may have several consequences. Adverse reactions to a drug may occur in patients who lack a particular enzyme when that enzyme is involved in the metabolism of that drug. Also, deficiency in the expression of an enzyme, may be either an advantage or a disadvantage in determining susceptibility to disease, depending on whether the enzyme is involved – the activation or detoxification of the drug.

CYP2E1 is now known to metabolise a range of low molecular weight organic compounds including benzene and ethanol (Guengerich et al., 1991). Several polymorphisms have been detected by restriction fragment length polymorphism (RFLP) analysis leading to interindividual differences in expression of the protein. A group of lung cancer patients in Japan showed a significantly decreased incidence of a

rare genotype compared with controls (Uematsu et al., 1991). A polymorphism of the human CYP2E1 gene was detected using the restriction endonuclease DraI. The population was divided into three genotypes: heterozygotes (C/D) and two forms of homozygotes (C/C and D/D). Two linked polymorphism detectable with Rsa I and Pst I in the 5'-flanking region of the gene have been identified, both have been shown to affect transcription levels (Hayashi et al., 1991).

NAD(P)H: quinone oxidoreductase 1 (NQO1) is a two-electron reductase that detoxifies quinones derived from the oxidation of phenolic metabolites of benzene (Moran et al., 1999). A polymorphism in NQO1 has been identified, and individuals homozygous (T/T) have no detectable NQO1. Exposed workers with a T/T genotype have an increased risk of benzene hematotoxicity. This finding suggests NQO1 is protective against benzene toxicity, which is difficult to reconcile with the lack of detectable NQO1 in human bone marrow. The human promyeloblastic cell line, KG-1a, was used to investigate the ability of hydroquinone to induce NQO1. A concentration-dependent induction of NQO1 protein and activity was observed in these cells cultured with hydroquinone. Multiple detoxification systems, including NQO1 and glutathione protect against benzene metabolite-induced toxicity. Indeed, exposure to a noncytotoxic concentration of hydroquinone induced both NQO1 and soluble thiols and protected against hydroquinone-induced apoptosis. NQO1 protein and activity was higher in wild-type (C/C) human bone marrow, whereas no NQO1 was induced by hydroquinone in bone marrow cells with the T/T genotype. Intermediate induction of NQO1 by hydroquinone was observed in heterozygous bone marrow cells (C/T). NQO1 also was induced by hydroquinone in wildtype (C/C) human bone marrow CD34(+) progenitor cells. Moran and co-workers reported that failure to induce functional NQO1 might contribute to the increased risk of benzene poisoning in individuals homozygous for the NQO1 mutation (T/T)(Moran et al., 1999).

1.2.4 Excretion

The total excretion of ^{14}C- benzene administered to rabbits by the oral route varied between 84 and 89% (Parke and Williams, 1953). The excreted dose was

32.6% in urine, 44.5% in expired air as unchanged benzene and small amounts of carbon dioxide, and 5% to 10% in the faeces and other tissues.

A study on environmental tobacco smoking (passive smoking) investigated the excretion after exposure to airborne benzene in man (Yu and Weisel, 1996b). Benzene was exhaled as unchanged benzene and carbon dioxide. Benzene in breath was confirmed as a short-term biomarker of environmental benzene exposure at lower than ppm levels. Less than 10% of the inhaled benzene was expired within three hours following two-hour inhalation exposures. Shorter exposure is followed with greater percentage expiration. Benzene half-lives of 7.6 and 68 minutes were calculated based on the exponential benzene decay curve after correcting for background breath levels.

The half-time for urinary excretion of benzene metabolites has been estimated to be about 4.5 h (McMahon and Birnbaum, 1991). The main metabolites excreted in urine are phenol-conjugates such as glucuronides, sulphates or as phenylmercapturic acid, and in small quantities conjugated quinol, catechol, *t,t*-muconic acid and 1,2,4 trihydroxybenzene. Animal studies (Inoue et al., 1988) have shown that aged male mice had lower levels of benzene in urine and higher amounts of benzene in the faeces.

For optimal monitoring technique, urine samples were recommended to be collected in the morning before the beginning of a work shift and after the end of the shift.

1.2.5 Benzene Toxicity

There have been extensive investigations of benzene toxicity at high dose. Benzene genotoxicity is strongly supported by animal studies. Significant results were found in the bone marrow and lymphocyte of mice and rats. Chromosomal aberrations, sister chromatid exchange, and micronuclei were analysed in these studies. A positive elevation was found in chromosomal aberration in bone marrow cells after acute exposure (100-7500 ppm) (Anderson and Richardson, 1981, Styles and Richardson, 1984).

Although, DBA/2 mice showed a significant elevation is sister charomatoid exchange in bone marrow cells after an acute exposure (3100 ppm), no significant elevation was found in chromosomal aberrations (Tice et al., 1980, Tice et al., 1982). DBA/2 and C57BL/6 mice showed a significant linear increase in lymphocyte sister chromatid exchange after the exposure to range of benzene concentrations (28-5000 ppm) (Tice et al., 1982, Erexson et al., 1985). Also the same relation was found significant in Sprage-Dawley rats.

The increase of micronuclei in peripheral blood polychromatic erythrocytes (PCE) allows an assessment of bone marrow acute damage. The life span of the PCEs is about one day (~24 h). On the other hand, peripheral blood normochromatic erythrocytes (NCE) allow the assessment of bone marrow accumulation damage. The life span of NCEs is about 30 days. Therefore, it has been considered as a good indicator of chronic damage was found (Hibbs et al., 1997). Luke and his colleagues (Luke et al., 1988b) have studied the relationship between the benzene exposure duration via inhalation and PCEs and NCEs in peripheral blood in DBA/2 mice. The mice were exposed to 300 ppm benzene fro 6 h/day either for 5 day/ week or 3 day/week for 1-13 weeks. A significant dose-response increase was found between micronuclei frequency in male DBA/2 bone marrow PCEs and benzene acute exposure (6 h expose to 1-1000 ppm) (Erexson et al., 1985). Also, male NMRI mice showed the same pattern after continuous and periodic acute (Toft et al., 1982). Sprage-Dawley rats have demonstrated the same pattern of dose-response with even lower range of benzene exposure (3-30 ppm) (Erexson et al., 1985).

Exposure duration has affected NCEs after exposed to 5 day/ week only. However, the inhaled benzene has affected PCEs without any significant relationship with the exposure duration. Male DBA/2 mice were more sensitive to benzene exposure than females (Luke et al., 1988b). Three strains of male mice (DBA/2, C57BL/6, and B6C3F1) were compared in similar study (Luke et al., 1988a). As mentioned previously, the effects of benzene exposure on the PCEs were related significantly, and no relationship was found with the exposure duration. Micronulei increases in PCEs were found in DBA/2 strain more than the other strains. On the other hand, micronuclei elevation in NCEs was reported to be influenced by strain and exposure duration. Unlike PCEs, micronuclei increases in NDEs were higher in

C57BL/6 and B6C3F1 strains than in DBA/2 strain. Also, the increases were significant in 5 day/ week compare to 3 day/ week. Luke and his colleagues concluded that the genotoxicity of inhaled benzene can be dependent upon exposure duration and animal sex, strain.

Cell mutation was reported by Ward and co-workers (Ward et al., 1992) from benzene exposure. CD-1 mice were exposed to purified air and different benzene concentrations (0.04, 0.1, and 1.0 ppm) for 22 h/ day (7 day/ week) for 6 weeks. The frequency of mutant spleen lymphocytes increased significantely at 0.04 and 0.1 ppm, but not 1.0 ppm. Another study was conducted on male C57BL/6 mice to be exposed to 300 ppm benzene 6 h/ day (5 day/ week) for 12 weeks (Mullin et al., 1995). DNA was purified from the lungs, spleen, and liver tissues. Mutation increased in lacI transgene in lung and spleen, but not in liver. Plappert and his colleagues (Plappert et al., 1994a) exposed female BDFI mice to 0, 100, 300, and 900 ppm benzene for 6 h/ day, 5 d/ week for up to 8 weeks. After exposure to 300 or 900 ppm for 3 days up to 4 weeks, DNA damage increased in the collected peripheral blood. After 5 day of benzene exposure to 100 and 300 ppm, DNA damage was noticed in bone marrow. The DNA damage was not accumulative after 4 weeks after 5 days of the exposure to 300 ppm, changes were found in liver, but not in blood. The DNA repair was measured after 1 and 2 days of exposure. The repair was almost complete in blood within 2 days. However, the repairs for damages in liver after exposure to 100 and 300 ppm were complete after 5 days. This finding changed after applying chronic exposure. After 4 weeks of exposure to 300 ppm, the repair was not complete in the blood. In liver after 4 weeks of exposure to 100 and 300 ppm, the repair was not complete.

Another study was conducted by Plappert et al. (Plappert et al., 1994b) to include the co-exposure to toluene effects. Again, female BDFI mice were exposed to benzene (300 or 900 ppm) and / or toluene (250 or 500 ppm) for 6 h/ day, 5 day/ week, upto 8 weeks. A significant increase in DNA damage was found in blood, liver, and bone marrow cells after exposed to benzene only. The co-exposure of benzene and toluene did not show any significant increase in DNA damage.

Inhibition of DNA synthesis in certain cell types is one of benzene adverse effects, which probably could be considered genotoxic. Acute inhalation exposure (4

h) of female ICR mice to 2000 and 3000 ppm benzene inhibited DNA synthesis in bone marrow cells 15% and 33%, respectively (Lee et al., 1988).

In human studies, occupational benzene toxicity can be either reversible or irreversible. Hematotoxicity is reversible when the exposure is short and occurs at low doses. However, bone marrow damage is irreversible with prolonged exposure to high doses (Snyder and Hedli, 1996). A range of hematotoxic effects including anaemia, leukopenia, and thrombocytopenia, were observed in workers who used benzene as a solvent (Snyder and Hedli, 1996). More than one cell type was decreased in some cases. Irreversible bone marrow aplasia is characterised by a reduction in the levels of erythrocytes, leukocytes, and blood platelets in the circulation. Aplastic anaemia is mostly fatal. The marrow appears to grow abnormally in those who survive aplasia. As a preleukemia, a developmental abnormality syndrome of the spinal cord is probably an early stage of acute myeloid leukaemia (Snyder and Hedli, 1996).

Two percent benzene concentration (20,000 ppm) is fatal to human when exposed for 5- 10 min via inhalation (Flury, 1928). The death cause attributed to asphyxiation, respiratory arrest, central nervous system depression, or suspected cardiac collapse (Avis and Hutton, 1993, Hamilton, 1922, Winek and Collom, 1971, Winek et al., 1967).

The haematological effects of benzene exposure were noticed when the concentration increased more than 60 ppm (Midzenski et al., 1992). Leukopoenia (white blood cells reduction), anaemia (red blood cells reduction), and thrombocytopoenia (platelets reduction) are all indicate pancytopoenia. All major blood cells are produced in the red bone marrow of the verterbrae, sternum, ribs, and pelvis. Immature cell in bone marrow (multipotent cyeloid stem cell) differentiate into the various mature blood cells. Reduction of ability of bone marrow to produce mature blood cells causes pancytopoenia (Aksoy and Erdem, 1978). Kipen and his colleages (Kipen et al., 1989) showed a strong positive correlation between the white blood cells count fluctuations and exposure profile. Also, more severe effects of benzene can be found in aplastic anemia when bone marrow stops function and stem cells stops developing mature cells. At low airborne benzene (280 ppb), a case of aplastic anemia was reported by (Baak et al., 1999).

Occupational benzene toxicity can be either reversible or irreversible. Hematotoxicity is reversible when the exposure is short and occurs at low doses. However, bone marrow damage is irreversible with prolonged exposure to high doses (Snyder and Hedli, 1996). A range of hematotoxic effects including anaemia, leukopenia, and thrombocytopenia, were observed in workers who used benzene as a solvent (Snyder and Hedli, 1996). More than one cell type was decreased in some cases. Irreversible bone marrow aplasia is characterised by a reduction in the levels of erythrocytes, leukocytes, and blood platelets in the circulation. Aplastic anaemia is mostly fatal. The marrow appears to grow abnormally in those who survive aplasia. As a preleukemia, a developmental abnormality syndrome of the spinal cord is probably an early stage of acute myeloid leukaemia (Snyder and Hedli, 1996).

The risk of leukaemia in a cohort of workers exposed to 500 ppb of benzene over a working lifetime increasing would be very small in any study (EPAQS, 1994). The 500 ppb level was divided by 10 in order to take account of the difference between the working lifetime (~77000h) and chronological-life (~660000h). Also, for the most vulnerable population such as young children and immune deficient patients, a further safety factor was applied by expert panel in air quality standards in the department of the environment in UK. A further factor of 10 was recommended by the panel for the lack of any scientific data on interindividual differences in metabolic capacity (EPAQS, 1994). Exposure to benzene should be kept minimal as it is a genotoxic carcinogen. The panel recommended a target standard for benzene in ambient air to be 1 ppb instead of 5 ppb (EPAQS, 1994).

CHAPTER 3

1.3.1 Urinary Creatinine

Creatinine is an endogenous substance of myocyte origin. The amounts excreted reflect the volume of fluid filtered at the glomerulus since it is virtually uninfluenced by tubular secretion or resorption (Greenberg and Levine, 1989). Its excretion rate is dependent upon body weight, body size and muscular mass, confounded by diet and is assumed to be stable despite the variability of urinary flow rates (Greenberg and Levine, 1989). It is worth noting that the confounding variables are usually ignored in studies utilising xenobiotics' urinary concentrations for biological monitoring as a tool for risk assessment. Its uses are similar to specific gravity; however, there is no enough data to conclude whether adjustment of biological indices in spot urine samples is equally valid for both tests in biological monitoring programmes. In other words, a question is raised as to whether these two parameters can be used interchangeably for the adjustment of biomarkers' urinary concentration. The literature is inconclusive. Berlin and his colleague concluded that for adjusting the urinary cadmium concentrations there was no advantage offered by the use of either creatinine excretion or specific gravity (Berlin et al., 1985). In Alessio's study, creatinine correlated with specific gravity was tested in 207 individuals and it was found to be relatively weak (correlation 0.49). This was suggested that these two parameters could not be invariably used for adjusting spot urine samples and that they partly express the same phenomenon (Alessio et al., 1985). It should be remembered that the actual usefulness of both parameters should be tested and validated for each biological indicator. Additionally different analytes have different toxicokinetic profiles, which should be carefully considered when evaluating the use of concentration correction parameters.

The most widely employed methods for creatinine determination are Jaffe's reaction, the enzymatic method, and an HPLC method. Max Jaffé (1841-1911), a German biochemist, was a professor of pharmacology known for his studies in physiological chemistry. His achievements included his reaction for the determination of creatinine (1886). The reaction between creatinine and picric acid in alkaline solution results in the formation of a red compound and is used to measure the amount

of creatinine (as in creatinuria) colorimetrically (Marriam-Webster, 1986). The enzymatic method employs creatinine immunohydrolase with NADPH as the cofactor (DIPRO, 1997). High performance liquid chromatography (HPLC) is the most advanced of creatinine methods. The description of the HPLC method is in the methods section of this thesis.

These techniques may suffer from glucose interference. A good example of that is what occurs with dialysate. Dialysate is the material that passes through membrane in dialysis. A comparison of creatinine values obtained by Jaffe's reaction, the enzymatic method and HPLC is in Ferry's study (Ferry et al., 1996). Plasma, urine and dialysate of dialysis patients were tested with various concentrations of glucose. Interference between creatinine and glucose was observed with Jaffe's reaction and the enzymatic method in dialysis solutions. However, lower creatinine values were obtained by HPLC in plasma, urine and dialysate. Therefore, HPLC is the method of reference.

Creatinine concentration is expressed either in mmol/L or g/L. The average normal value of creatinine concentration excreted in a general population is 10 mmol/L (milli-molarity) of 1 g/L. This means that data is expressed either as an amount per millimole creatinine by dividing by 10 (Pryde and Competz, 1994) or by 11.3 (based on creatinine density, which is 11.3 g/l).

The specific gravity of urine is the ratio of urine mass to the mass of an equal volume of water at 4°C, which is related to urinary total solute concentration. The purpose of it is to eliminate the influence of dilution of urine samples on urinary concentrations of substances. The correction is done by referring the results of urinary concentrations to the specific gravity of the urine (Alessio et al., 1985).

1.3.2 Benzene Biological Markers

Five metabolites were recognised to be biomarkers for benzene exposure other than excreted benzene. They are: phenol, S-phenylmercapturic acid, *t,t*-muconic acid, hydroquinone, and 8'hydroxy-2'deoxyguanosine.

1.3.2.1 Blood Benzene

Few studies have investigated benzene in blood to evaluate the environmental and occupational exposures. It is a specific marker because the measurement reflects the pollutant itself. The half-life of benzene in the blood stream has been cited differently in two studies (Brugnone et al., 1992, Popp et al., 1994). Brugnone and co-workers estimated the half-life at 8 h (Brugnone et al., 1992), while, Popp and co-workers estimated it to be between 30 and 60 min (Popp et al., 1994).

Three methods were developed to determine blood benzene levels. Gas chromatography equipped with either a mass spectrometric detector (Brugnone et al., 1992), a photoionisation detector (Ong et al., 1996) or a flame ionisation detector (Popp et al., 1994). New developments have suggested that this marker is becoming increasingly applicable. The sample is injected into the injection chamber then the benzene is volatilised by increasing the chamber temperature. This method of extraction is called "head-space technique", which precedes the GC. This technique eliminates any interference that might result from any chemical or organic matrix. The sample quantity required for the assay was 20 ml, which was reduced to the range between 1 and 3 ml (Brugnone et al., 1992, Popp et al., 1994). The detection limit for the mentioned assays range between 15 and 50 ng/l. This is sufficient to determine satisfactorily the blood benzene concentrations in a non occupationally exposed population.

Caution must be taken during sampling to avoid any contamination of the sample. Benzene is a volatile and miscible compound, so ambient benzene must be prevented from entering the sample solution after sampling. Also, sample loss by volatilisation in air must be avoided. Therefore, the sample must be sealed after collection and handled in a benzene free environment as much as possible (Ghittori et al., 1995).

Benzene in blood was investigated for subjects who were not occupationally exposed to benzene. Non smokers had an average of 205 ng/l with 381 ng/l for smokers (Brugnone et al., 1992). 883 non-exposed individuals were involved in a study to determine the mean of blood benzene (Wallace, 1996). The mean concentration of blood benzene in the study was 131 ng/l. High occupational

exposure, such as in automobile mechanics, chemical and petrochemical workers, results in levels that can reach as high as 2000 to 3300 ng/l (Ong et al., 1996, Popp et al., 1994). Interindividual variations in blood benzene were on the order of 10- to 100-fold for the same level of exposure (Ong et al., 1996).

A significant correlation ($r=0.66$, $n=145$, $p< 0.0001$) between ambient benzene and blood benzene levels reported by Ghittori and co-workers for benzene in air that was between 0.01and 31.6 ppm (Ghittori et al., 1995). Another study (Ong et al., 1996) reported a significant correlation ($r=0.64$, $n=61$, $p<0.05$) between ambient benzene and blood benzene levels, which ranged between 0.011 and 34.6 ppm. Ong et al. reported non-significant correlation after separating the ambient benzene samples into less and more than 0.25 ppm.

Smoking has considerable influence on blood benzene levels. The correlation between the number of cigarettes smoked and blood benzene concentration was found to be significant ($r = 0.20$; $p< 0.00001$) (Brugnone et al., 1992). The contribution of one cigarette has been estimated at 12 ng/l of benzene in blood. However, this study did not specify the time difference between the time of smoking and blood sample collection. One hour after smoking is enough to interfere with measurements (Brugnone et al., 1992, Popp et al., 1994). Also, blood benzene levels were lower in rural (200 ng/l) than in urban (296 ng/l) workers (Brugnone et al., 1992).

1.3.2.2 Urinary Benzene

About one part per thousand of benzene absorbed into the human body is eliminated unchanged in urine. As for blood benzene, the measurement of urinary benzene is specific because the determination reflects the pollutant itself.

Analytical methods for benzene in urine are based on gas chromatography (GC) either by using photoionisation (PID) (Ong et al., 1995, Ong et al., 1996) or flame ionisation (FID) (Ghittori et al., 1995) detectors. The detection limits were 40 ng/l for PID and 100 ng/l for FID. The "head-space technique" precedes the GC as discussed in the blood benzene section. This technique eliminates any interference in the chromatogram from any chemical or organic matrix.

Urinary benzene sampling and analysis must be treated carefully because of its volatility. The samples should be prevented from contamination with ambient benzene or from sufficient losses by volatilisation (Ghittori et al., 1995). Also, A large urine quantity is required (50ml) to obtain reliable measurements.

The urinary benzene concentration of a non-exposed population ranged between 93 and 139 ng/l. For occupationally exposed populations, the range was between 718 and 9936 ng/l. Interindividual variations in urinary benzene were about 10- to 20-fold for the same exposure level (Ong et al., 1995, Ghittori, 1995).

The ambient benzene level correlated significantly with urinary benzene concentrations even when the amount of benzene in the air was low. The urinary benzene measurements correlated with air concentrations in the range 32 to 800 $\mu g/m^3$ (Ong et al., 1995, Ghittori, 1995 #52). As an exposure marker, urinary benzene is the most sensitive.

1.3.2.3 Phenol

Urinary phenol has limited use as a biomarker of benzene exposure at air levels exceeding 3 mg/m^3 up to 620 mg/m^3 (Inoue et al., 1986, Drummond et al., 1988). Sources of phenol other than from benzene exposure were found such as in the diet and ingestion of medicine (Wartiz, 1985).

1.3.2.4 t,t-Muconic Acid

Ducos and co-workers reported that the relationship between ambient benzene and urinary *t,t*-muconic acid was significant (Ducos et al., 1992). Whether *t,t*-muconic acid was corrected for creatinine concentration or not the association remained significant. Therefore they recommended not correcting for creatinine when measuring *t,t*-muconic acid level. A contradiction to Docus' conclusion regarding correcting for creatinine was brought up by van Sitter and Boogaard (van Sittert et al., 1993).

The benzene dose relationship to *t,t*-muconic acid in urine has been reviewed (Table 6). The mean half life of *t,t*-muconic acid was 5.0 h ± 2.3 h (Boogaard and van Sittert, 1995). At relatively high benzene dose (10-1000 µg/kg), 2% of ambient

benzene was biotransformed to urinary *t,t*-muconic acid (Inoue et al., 1989b). On the other hand, with a lower benzene dose (0.02-0.3 μg/kg), a higher proportion of ambient benzene (21%) was biotransformed to urinary *t,t*-muconic acid (Lindstrom et al., 1994, Bucklay et al., 1992). Therefore, extrapolating potential health risks from high to low dose must be treated carefully. Furthermore, Rothman and co-workers (Rothman et al., 1998) found an inversely proportional relationship between urinary *t,t*-muconic acid and total urinary benzene metabolite concentrations (phenol, catechol, hydroquinone, and *t,t*-muconic acid). They concluded that "the risk for adverse health outcomes due to benzene may show a superlinear relationship with external dose, and that linear extrapolation of the toxic effects of benzene from highly exposed workers to less exposed populations may be inappropriate, to the extent that compounds related to hydroquinone and muconic acid play critical parts in benzene toxicity, and could lead to an underestimation of risk" (Rothman et al., 1998). A synergistic toxicity was found when *t,t*-muconic acid and hydroquinone co-exist, which inhibit the proliferation of red blood cells from bone marrow stem cells(Lee et al., 1981).

Most of the studies in Table 6 found a significant correlation between urinary *t,t*-muconic acid and ambient benzene, except in the Ruppert study (Ruppert et al., 1997). In the study, spot urine samples were collected at the end of the study when the air samples were taken. The study design for urine sample recruitment was not clearly justified, which might explain the insignificant correlation between *t,t*-muconic acid and ambient benzene. Different designs were used to determine a relationship between urinary *t,t*-muconic acid and ambient benzene. Four studies considered spot samples after exposure (POST) (Popp et al., 1994, Ghittori et al., 1995, Ong et al., 1996, Javelaud et al., 1998). Two other studies tested the difference between urine samples that were collected before (PRE) and after (POST) exposure (Lee et al., 1993a, Ong et al., 1996). The linear regression (R^2) in these two studies varied between 0.80 and 0.88. One study tested urine samples after 8 h exposure and 12 h exposure. The linear regression for the 8 h samples (R^2= 0.959, n= 58) was higher than the 12 h samples (R^2= 0.862, n= 28).

Table 6: The relationship between benzene exposure by inhalation and urinary *t,t*-muconic acid.

No	Study	N*	R^2	p-value	Equation MA (y) =benzene (x)	Urine Samples
1	(Ducos et al., 1992)	105	0.91	<0.0001	-	-
2	(Lee et al., 1993a)	19	0.88	close association	0.02+0.8502x	POST -PRE
3	(Popp et al., 1994)	20	0.54	0.016	-	POST
4	(Ghittori et al., 1995)	145	0.58	<0.0001	log (y)= -0.304 +0.429(x)	POST
5	(Ong et al., 1995)	64	0.80	<0.001	Log (y)= 0.2 +1.05Log (x)	POST -PRE
6	(Boogaard and van Sittert, 1995)	58	0.959	<0.0001	y= -0.9+2.38x	POST 8h
7	(Boogaard and van Sittert, 1995)	28	0.862	<0.0001	y= 0.39+1.98x	POST 12h
8	(Ong et al., 1996)	131	0.53	<0.01	Log (y)= 0.09+ 0.69Log(x)	POST
9	(Ruppert et al., 1997)	69	0.164	0.179	-	at end of study
10	(Javelaud et al., 1998)	30	0.46	0.009	Log (y)= 2.22 +0.482Log(x)	POST
11	(Carriere et al., 1996)	22	0.64	-	-	POST vs. exhaled benzene

* Number of subjects.
(-) Not available data.

1.3.2.5 S-Phenylmercapturic Acid

Urinary S-phenylmercapturic acid is a product of the ring hydroxylation pathway of benzene. Conjugation of benzene with glutathione forms S-phenylmercapturic acid, which is excreted in the urine. It is considered to be a very specific biomarker for benzene exposure (Ghittori et al., 1995). S-Phenylmercapturic acid metabolic pathways demonstrate a very minor biotransformation and excretion (0.11%) of inhaled benzene (van Sittert et al., 1993). The interindividual variation coefficient was 50 % and the intraindividual variation coefficient was 37%. S-Phenylmercapturic acid half life was 9 h therefore this measure tends to accumulate in the body when exposure exceeds 0.5 ppm (Boogaard and van Sittert, 1995).

High performance liquid chromatography (HPLC) (Jongeneelen et al., 1987, Ghittori et al., 1995) and gas chromatography- mass spectrometry (GC-MS) (Ruppert

et al., 1995) methods have been used to analyse S-phenylmercapturic acid. The detection limit has been shown to be reduced to 1 μg/l for GC-MS compared to 6 μg/l for HPLC (Boogaard and van Sittert, 1995, van Sittert et al., 1993, Einig and Dehnen, 1995).

A competitive enzyme-linked immunosorbant assay (ELISA) has been developed as a rapid, easy, and cheap method to determine urinary *S*-phenylmercapturic acid (Aster et al., 2002). At low levels of urinary *S*-phenylmercapturic acid, the assay showed some problems.

Urinary S-phenylmercapturic acid concentration was found to be between 1.5 and 12.5 μg/g creatinine (μg/gCr) in control populations (without any occupational benzene exposure) (Ghittori et al., 1995, Boogaard and van Sittert, 1995, Popp et al., 1994). The median exposure for a highly exposed population (2.6 mg benzene /m^3) was 33 μg/gCr (Popp et al., 1994). Vast interindividual variations in S-phenylmercapturic acid concentrations for the same exposure level have been observed, varying between 2 and 20 μg/gCr (Boogaard and van Sittert, 1995, Bartczak et al., 1994). Differences in metabolic capacity between subjects may partly explain this variation. The variation increased when samples were obtained at the end of a workday (Popp et al., 1994). Therefore, the sampling time must be considered in the design of any studies, which use S-phenylmercapturic acid as a biomarker for benzene exposure.

Significant correlation ($p<0.0001$) between ambient benzene concentration and urinary S-phenylmercapturic acid levels in exposed subjects were reported in three studies(Ghittori et al., 1995, Boogaard and van Sittert, 1995, Popp et al., 1994). The correlation coefficient in the three studies were 0.74(n=145), 0.81 (n= 26) and 0.968 (n= 52), respectively.

Smoking was found to be a confounder for benzene exposure (Ghittori et al., 1995, Boogaard and van Sittert, 1995). However, there was no relationship between the number of smoked cigarettes and S-phenylmercapturic acid excretion (Boogaard and van Sittert, 1995, van Sittert et al., 1993). Another confounder of urinary S-phenylmercapturic acid excretion, is that carbamazepine (an anti-epileptic drug) has been cited as an interfering agent (Boogaard and van Sittert, 1995).

1.3.2.6 Hydroquinone

Urinary hydroquinone has been investigated less frequently than *t,t*-muconic acid or S-phenylmercapturic acid as a biomarker of benzene exposure (Lee et al., 1993b, Ong et al., 1995, Rothman et al., 1998). Two analytical techniques have been reported: HPLC with variable-wavelength fluorimetric detection (Lee et al., 1993b), and GC-MS has also been used to analyse hydroquinone (Rothman et al., 1998).

The median of hydroquinone level in non-exposed subjects was 1.6 ng/gCr and levels ranged between 0.9-6.7 ng/gCr. Subjects exposed to 1-25 ppm benzene had 16.4 ng hydroquinone/gCr (3.8-82.2 ng/gCr). Subjects who were exposed to more than 25 ppm benzene had 65.3 ng hydroquinone/gCr (8.3-196.6 ng/gCr). The correlation between ambient benzene concentration and urinary hydroquinone level in these subjects was significant ($r=0.86$, $n= 38$, $p <0.0001$).

As discussed earlier for *t,t*-muconic acid, an inversely proportional relationship exists between urinary hydroquinone and total urinary benzene metabolite concentrations (phenol, catechol, hydroquinone, and *t,t*-muconic acid) (Rothman et al., 1998). A higher urinary background level was found for hydroquinone compared to *t,t*-muconic acid and S-phenylmercapturic acid (Qu et al., 2000).

1.3.2.7 Urinary 8-Hydroxy-2'-Deoxyguanosine

Oxidative DNA damage is caused by exposure to a number of organic and inorganic compounds, such as benzene, potassium bromate, 2-nitropropane, paraquat, and hydroquinone (Toraason, 1999). During oxidative metabolism of benzene, the phenolic- benzene metabolites produce active oxygen species that are capable of causing oxidative damage to DNA.

One of the most abundant oxidative DNA adducts is 8-hydroxy-2'-deoxyguanosine (8OHdG). Formation of 8OhdG is shown in Figure 9. 8OHdG formation has been estimated that about to be 5% of all oxidative adducts. 8OHdG is highly mutagenic and results in predominately G-T transversions, which are frequently found in relevant tumour genes (Toraason, 1999). 8OHdG results from a

wide array of treatments that cause oxidative damage and has been implicated in carcinogenises. It can be measured in hydrolysed DNA from lymphocytes where it represents the steady state between damage and repair. It may also be measured in the urine of individuals exposed to benzene.

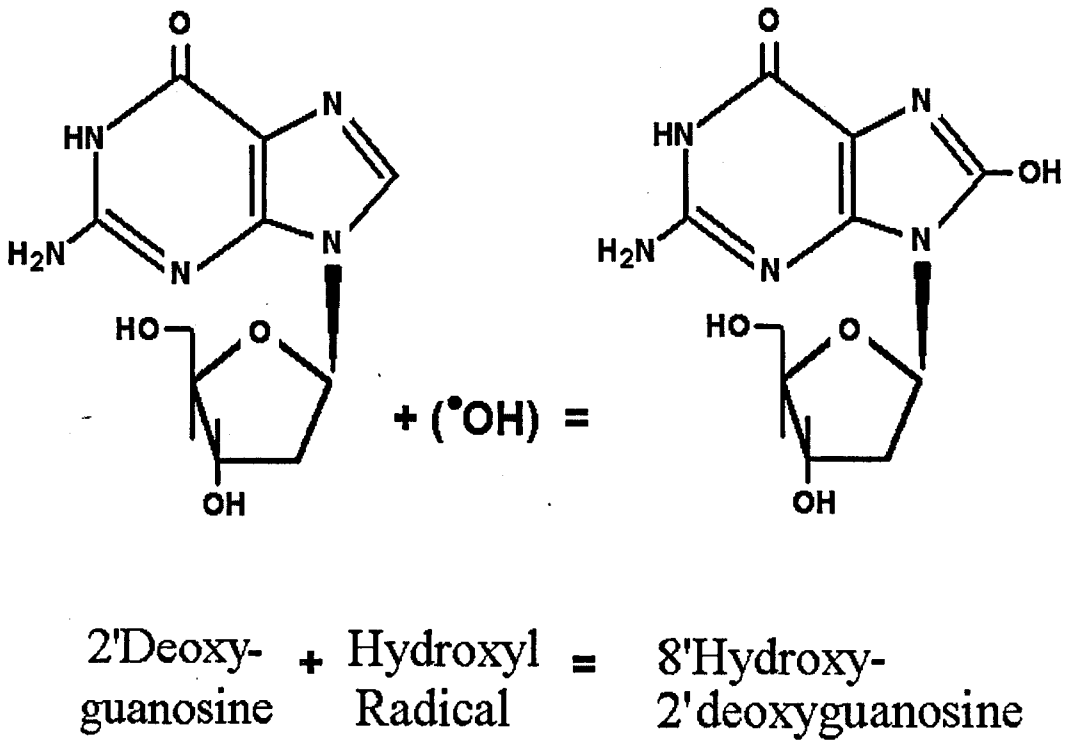


Figure 9: Oxidative damage for deoxyguanosine and 8OHdG formation (Kasai and Nishimura, 1984).

Several methods are available for measuring 8OHdG in human biological samples: High Performances Liquid Chromatography equipped with an electrochemical detector (HPLC-EC), gas chromatograph equipped with a mass-spectrometer (GC-MS), and enzyme-linked immunosorbant assay (ELISA). Some of these methods are discussed in the remainder for this chapter.

High performance liquid chromatography coupled with an electrochemical detector (HPLC-EC)is the most commonly used method (Shigenaga et al., 1989). The level of detection for HPLC-EC is frequently expressed as the ratio of 8OHdG/10^5 deoxyguanosine in serum and microgram 8OHdG per gram creatinine. For several reasons this method has become the most widely used technique for assessing oxidative DNA damage (Halliwell, 1999). (1) 8OHdG is a sensitive assay, (2)

formation of 8OHdG in DNA via range of species (i.e. •OH and $^1O_2$) also possible to be measured, (3) the mutagenicity in inducing transversions is known *in vivo*, and (4) multiple repair mechanism or prevention of its incorporation into DNA are known.

The enzyme-linked immunosorbant assay (ELISA) is based on a non-competitive antibody monoclonal assay. It is based on screening class-specific antibodies and is applicable particularly to the detection of a specific immunoglobulin (Degan et al., 1991, Yin et al., 1995).

In spite of the apparent agreement that oxidative damage is an adverse biological response (Ames, 1989, Kasai, 1997), it is unclear and, certainly debatable, as to how adverse or how critical this is to the disease process.

Several factors contribute to this uncertainty (Toraason, 1999):

1- Free radical biology is extremely complex and many aspects of it are not well understood. Therefore, linking measurable endpoints with disease often requires considerable conjecture.

2- Humans maintain, as oxygen breathing creatures, a balance between the continual production of high reactive oxygen radicals and antioxidant defences. The important elements of an adequate defence have not been defined. As a result, cross-sectional intervention studies, including energy restriction and antioxidant supplements, have produced mixed results in terms of influencing oxidative DNA damage (Loft et al., 1992).

3- Proof of a causal relationship between cancer and a chemical exposure that causes oxidative DNA damage is still lacking, although the evidence from experimental studies supports the theory of oxidative DNA damage as an important mutagenic and carcinogenic factor.

4- Severe oxidative stress may be acutely toxic, however, it is considered to contribute to chronic degenerative diseases.

Therefore, testing the role of a specific workplace exposure, which is usually far removed in time from an adverse outcome, from all the other possible factors that can contribute to degenerative disease, can be nearly impossible. There is a need to

demonstrate that the rate of oxidative DNA damage is an independent risk factor for cancer.

In control subjects, 8OHdG levels in urine varied between 0.32 and 23.9 μg/gCr (Bogdanov et al., 1999, Drury et al., 1998, Witherell et al., 1998). Three other studies showed that the levels ranged between 78 and 527 pmol/(kg*day) (Degan et al., 1991, Loft et al., 1992, Shigenaga et al., 1989). Conversely, Tsuboi and his colleagues claimed that the mean was 0.46 +/- 0.1 pmol/(kg*day)(Tsuboi et al., 1998). The mean concentration of 8OHdG in blood was 1.52 +/- 0.45 8OHdG/ 10^5 dG (Marczynski et al., 1997).

1.3.3 Confounding Factors

There are several potential confounding factors, which interfere with benzene metabolism. Alcohol consumption, active and passive smoking, co-exposure with other chemicals, the area benzene level (whether individual level in an urban or rural area), seasonal variation, day and night, indoor and outdoor, dietary benzene, and other factors are discussed in this section.

1.3.3.1 Sorbic Acid

Sorbic acid intake and the smoking status of the individual are potential confounders of urinary *t,t*-muconic acid determinations. Sorbic acid is a food additive and, less frequently, a preservative in certain cosmetics and medicines (Ducos et al., 1990, Gadelkarim et al., 1985). It was reported that sorbic acid varied between 500 and 800 mg/kg as an additive in cheese, yoghurt and smoked fish (Ruppert et al., 1997). Also, drinks such as in soft drinks and wine have between 150 and 300 mg/l.

Ingestion of sorbic acid caused minor interference with *t,t*-muconic acid excretion in two volunteers (Ducos et al., 1990). Also, Ruppert (1997) investigated the effect of the dietary supplements on *t,t*-muconic acid excretion. About 1.2 part per thousand of sorbic acid is oxidised to *t,t*-muconic acid (Ruppert et al., 1997). Prior to intake of 200 mg of sorbic acid, the urinary *t,t*-muconic acid concentration was below the limit of detection (0.04 mg/l) (Ducos et al., 1990). After three hours, the *t,t*-

muconic acid concentration was more than 0.6 mg/l and after seven hours, the level was about 1.7 mg/l. The level then returned to back ground after 24h. Ruppert and co-workers (1997) also investigated this effect on eight non-smokers (Table 7). During a four days experiment, the subjects consumed on days 2 and 3 a dietary supplementation containing 500mg sorbic acid. An elevation of about 0.5 mgMA/gCr was reported. Then on the forth day, the *t,t*-muconic acid concentration returned back to the background levels (detection limit was 0.04 mgMA/gCr). An average daily dietary intake of 6-30mg sorbic acid gave rise to 0.005-0.03 mgMA/gCr (Ruppert et al., 1997). Therefore, the dietary intake of sorbic acid must be considered when studying urinary *t,t*-muconic acid levels following low-level benzene exposures (Pezzagno et al., 1999, Renner et al., 1999, Ruppert et al., 1997, Scherer et al., 1998).

Table 7: *t,t*-Muconic acid excretion in eight non-smokers following ingestion of 500 mg of sorbic acid on day 2 and 3.

Day	mgMA/24h mean (range)	mgMA/gCr mean (range)
1	0.08 (0.04-0.12)	0.05 (0.02-0.07)
2 & 3*	0.88 (0.57-1.48)	0.55 (0.31-0.84)
4	back to the background	

(Ruppert et al., 1997)
** ingestion of 500mg sorbic acid.*

1.3.3.2 Alcohol Consumption

Alcohol consumption was found to be a major confounder for low level benzene exposure. Javelaud et. al (1998) found a significant (p=0.006) increase in urinary *t,t*-muconic acid among a group of individuals who abused alcohol. In the alcohol abuse group, spot urine samples of seven individuals showed a mean of 0.826 +/- 0.641 mg *t,t*-muconic acid /gCr. In the same study, 27 individuals who didn't consume alcohol showed a mean of 0.176 +/-0.197 mg/gCr.

1.3.3.3 Smoking

Smoking of tobacco is an important confounder since it interferes with urinary *t,t*-muconic acid excretion. One cigarette emits an average of 600 µg of benzene (Davoli et al., 1996). Two studies have indicated the range of exhaled benzene to be

between 2 and 7.8 μg/m^3 (0.57 and 2.23 ppb) in non-smokers' breath compared to 14-21 μg/m^3 (4 and 6 ppb) in smokers (Wester et al., 1986, Wallace, 1989). Thus, ninety percent of the benzene level in smoker's breath was derived from active smoking of tobacco (Wallace, 1996).

Table 8: The influence of smoking on urinary *t,t*-muconic acid excretion.

	Smokers (mg *t,t-muconic acid*/gCr)				Non-Smokers (mgt,t-muconic acid/gCr)			
Source	N	Mean	SD	Range	N	Mean	SD	Range
(Lee et al., 1993a)	35	0.190	0.090	0.06 - 0.43	23	0.140	0.07	0.01-0.29
(Boogaard and van Sittert, 1995)	14	0.058	0.013	-	38	0.037	0.016	-
(Ruppert et al., 1995)	10	0.090	0.035	0.041-0.144	10	0.054	0.017	0.032-0.081
(Ruppert et al., 1997)	32	0.130*	-	0.19 - 0.61	82	0.065*	-	0.05-0.21
(Javelaud et al., 1998)	7	0.089	0.052	0.025-0.175	20	0.035	0.035	0.011-0.155
(Ghittori et al., 1995)	20	0.228	0.139	-	20	0.062	0.042	-

* Median.
(-) Not available data.

Table 8 shows six different studies, which investigated smoking and non-smoking groups, and showed a significant increase in urinary *t,t*-muconic acid with cigarette smoke. The average background urinary *t,t*-muconic acid level in non-smoking, non-occupationally exposed populations, varied in several studies and showed levels of between 0.035 and 0.140 mgMA/gCr (Lee et al., 1993a, Boogaard and van Sittert, 1995, Ghittori et al., 1995, Hotz et al., 1998, Javelaud et al., 1998, Ruppert et al., 1995). On the other hand, urinary *t,t*-muconic acid levels in smokers that were not-occupationally exposed to benzene varied between 0.058 and 0.228 mgMA/gCr. The influence of smoking on excreted *t,t*-muconic acid is shown in Table 8. There were significant increases in *t,t*-muconic acid excretion in smokers compare to non-smokers. Although it is known that benzene from tobacco smoke will influence the level of urinary *t,t*-muconic acid excretion, enzyme competitors in the smoke could also inhibit the biotransformation of benzene.

No significant correlation was found between the number of cigarettes smoked and *t,t*-muconic acid excretion (Boogaard and van Sittert, 1995). Among the non-

smoking group, *t,t*-muconic acid concentrations were higher in the subjects who had been subjected to ambient benzene (p=0.05) (Hotz et al., 1998).

1.3.3.4 Passive Smoking

Passive smoking was found to explain less than 15% of the variation in benzene exposure (Scherer et al., 1995). Benzene levels during the evening for smoking and non-smoking homes were 2.97 and 2.3 ppb, respectively. However, benzene concentrations in non-smoking homes located in an urban area were significantly higher ($p < 0.05$) than in suburban non-smoking households (Scherer et al., 1995). In another study (Heavner et al., 1996) the median benzene level values in homes with smokers was 1.14 ppb compared to 0.69 ppb in homes with no smokers.

1.3.3.5 Co-exposure

Handling petrol during re-fuelling is another confounder for benzene exposure when driving cars (Javelaud et al., 1998). Javelaud (1998) has tested 34 subjects who handled petrol on the day of sampling and demonstrated a higher level of *t,t*-muconic acid compared to 22 whom did not handle it (0.184 +/- 0.172 mgMA/gCr compared to 0.105 +/- 0.068 mgMA/gCr).

Benzene toxicity in individuals exposed to benzene along with other chemicals may be increased or decreased depending upon the properties of the other chemicals. Toxicity was expected to decrease when toluene exposure was also high because they both compete for the same binding site on CYP2E1. However, when male mice were pre-treated with acetone in water to induce CYP2E1 and then exposed to 600 ppm benzene, a significant increase in urinary hydroquinone glucuronide and *t,t*-muconic acid was seen. This increase was paralleled by three- to four-fold higher steady-state concentrations of phenol and hydroquinone in blood and bone marrow in the acetone-pre-treated mice compared with controls (Kenyon et al., 1998).

1.3.3.6 City and Suburbs

Petrol exhaust emission in urban areas is higher than in suburban areas (Wallace, 1996). The Benzene concentration in urban areas in Canada ranged between

5.1 and 7.6 $\mu g/m^3$ (1.5 and 2.17 ppb), but in the rural areas, the concentration ranged between 0.6 and 1.2 $\mu g/m^3$ (0.17 and 0.34 ppb).

When comparing homes in rural and urban areas, the urinary *t,t*-muconic acid level of those living in the city was shown to be significantly ($p<0.05$) higher than those in the suburbs (Ruppert et al., 1997).

1.3.3.7 Seasonal Variation

In the northern hemisphere, the maximum seasonal ambient benzene variation in California was demonstrated to be between November and January and the minimum between May and August (Wallace, 1996). The gradual change in levels between months may be explained by atmospheric inversion and /or changes in the blend of petrol leading to greater volatility in summer (Figure 10).

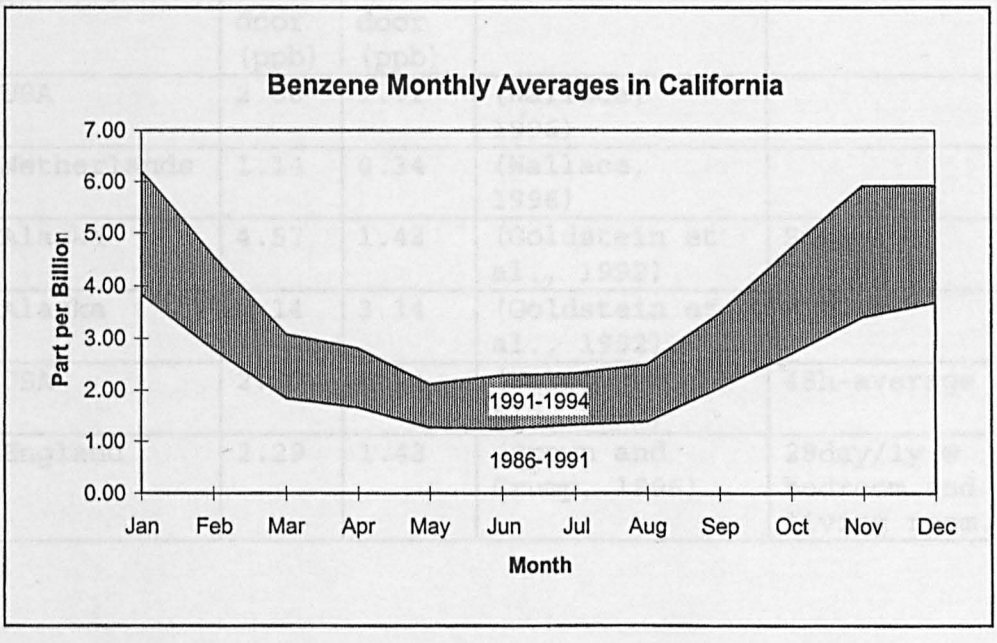


Figure 10: The monthly average of benzene concentration in California (Wallace, 1996).

This was supported by a Canadian study (Fellin and Otson, 1993), which found the mean indoor benzene concentration in 754 homes to be 1.83, 1.6, 0.78, and 1.99 ppb for winter, spring, summer and autumn, respectively. However, another study demonstrated higher indoor benzene concentrations in summer (1 ppb) compared to the concentration in winter (0.77 ppb) (Bell et al., 1994).

1.3.3.8 Day and Night

No systematic pattern in benzene concentration was observed between day and night for 11 studies (Wallace, 1996). The day /night ratio varied in these studies and ranged from 48% to 179%. This variation may be due to factors other than variations between day and night.

1.3.3.9 Indoor and Outdoor

Benzene levels have been shown to be higher indoor than outdoor environment (Wallace, 1996, Goldstein et al., 1992, Wilson et al., 1993, Brown and Crump, 1996). Many studies conducted around the world have confirmed this finding (Table 9).

Table 9: Benzene concentrations in indoor and outdoor environments.

Country	In-door (ppb)	Out-door (ppb)	Reference	Notes
USA	2.86	1.71	(Wallace, 1996)	
Netherlands	1.14	0.34	(Wallace, 1996)	
Alaska	4.57	1.43	(Goldstein et al., 1992)	Summer
Alaska	7.14	3.14	(Goldstein et al., 1992)	Winter
USA	2.37	1.74	(Wilson et al., 1993)	48h-average
England	2.29	1.43	(Brown and Crump, 1996)	28day/1y @ bedroom and living room.

Indoor micro-environments vary according to the nature of their application. The indoor benzene concentration in air at home and work was varied between 1 and 1.3 ppb, respectively (Wallace, 1996). Higher concentrations were found while transportation by cars (4.49 ppb) (Bell et al., 1994). Another study investigated indoor micro-environments in more detail (Wallace, 1996). Benzene concentration in the home (0.63 ppb) was half that in a hotel room (1.3 ppb). Concentrations during commuting tripled (1.71 ppb) the concentrations found in the home. Bingo halls (5.97

ppb) demonstrated almost ten times the level and pubs about fifteen times (9.9 ppb) the level at home.

1.3.3.10 Dietary Benzene

An individual exposure to benzene is mainly due to airborne levels. No benzene was found in significant quantities in water, food or beverages. Benzene was found in eggs at ppm levels (MacLeod and Cave, 1975), but no increase was detected in breath samples due to eating eggs (Wallace, 1996). The following conclusion was suggested: "It is possible that minor levels of benzene in foodstuffs could still have been present and not detected in breath due to efficient metabolisation by the liver, which receives materials from the gut directly before they enter the blood stream. However, it was thought that major concentrations in food would be detectable in breath; since they were not, it was concluded that food and beverages were an unimportant pathway for benzene exposure" (Wallace, 1996).

Two studies have confirmed that the amount of benzene in nearly all foods that were measured is negligible. The Food and Drug Administration (FDA) in the USA conducted the first study when more than 50 foods were analysed for benzene (McNeal et al., 1993). In most cases, the benzene concentration was equal to or less than 2 ng/g, this included egg when no preservatives were included. Slightly higher levels were present in some foods and beverages containing both ascorbic acid and sodium benzoate. Some exceptions were found, for instance strawberry preserves (38 ng/g), taco sauce (9 and 22 ng/g), duck sauce (7 ng/g), and barbecue sauce (5 ng/g) had a small a mount of benzene. The second study was conducted by the American Petroleum Institute (API, 1992). Among the 57 foods tested, shelled peanuts and fried eggs showed the only significant result (30 ng/g). These two studies suggested that the benzene concentration in food was far below the ppm levels previously reported (Wallace, 1996).

1.3.3.11 Other Confounders

Well-designed experiments to assess benzene emission considered that traffic conditions and refuelling (Wixtrom and Brown, 1992), and air-conditioning and

window opening (Davoli et al., 1996) were considered to be micro-environmental factors. Sex may contribute to the extent of benzene metabolism, as mouse studies have demonstrated (Kenyon et al., 1998, Corti and Snyder, 1998, Rothman et al., 1998). Age might also be a confounding factor, as indicated in a study of mice (Corti and Snyder, 1998, Rothman et al., 1998). Polymorphism has a significant influence on benzene metabolism, which suggested a marker of susceptibility (Moran et al., 1999).

1.3.4 Biological Regulations

Benzene exposure has been regulated in the workplace to reduce the exposure impact of benzene on workers. The British occupational maximum exposure level (MEL) for benzene was amended in 1997 to implement 1ppm (8 hours – total weighted average (TWA)). This level should be achieved in a two-step phased reduction by June 2003. In June 2000, the first step was achieved in a reduction from 5ppm to 3ppm. The American Conference of Governmental Industrial Hygienists (ACGIH) has set threshold limit values (TLV) and biological exposure indices (BEI) for chemical substances (ACGIH, 1996). The TLV is the threshold limit value expressed as a time-weighted average, it is the concentration of a substance to which most workers can be exposed without adverse effect. There are two threshold limits; time weighted average (TWA) and short-term exposure limit (STEL). The time weighted average (TLV-TWA) gives the level at which nearly all workers may be repeatedly exposed, day after day, without adverse effect for a conventional 8 hours workday and a 40 hours' workweek. The short-term exposure limit (STEL) is the concentration that should not be exceeded at any time in 15 minutes' time weighted average exposure, even if the TLV-TWA is not exceeded. The TLV-TWA and TLV-STEL for benzene are 0.5 ppm (1.6 mg/m^3) and 2.5 ppm (8 mg/ m^3), respectively.

The biological exposure index (BEI) is a reference value intended as a guideline for the evaluation of potential health hazards in industrial practice. The BEI for benzene at work was determined (ACGIH, 1996) as shown in Table 10. ACGIH listed benzene as a confirmed human carcinogen.

Table 10 The BEI for benzene (ACGIH, 1996)

Biological Exposure Determinant	Sampling Time	Biological Exposure Index (BEI)
Total phenol in urine	end of shift	50 mg/ g creatinine
Benzene in exhaled air: mixed exhaled end-exhaled	prior to next shift	 0.08 ppm 0.12 ppm
S-Phenylmercapturic acid in urine	end of shift	25 μg/ g creatinine

SECTION (II):

MATERIALS AND METHODS

SECTION (II): MATERIALS & METHODS

This section describes the analytical methods for determining of creatinine, *t,t*-muconic acid, 8-hydroxy-2'-deoxyguanosine, and BTEX. It also gives details of this study.

CHAPTER 4

Chapter 4: Creatinine Analysis

To determine *t,t*-muconic acid as a biomarker of benzene, creatinine was determined to adjust the urine samples for the dilution factor. Routine collection of 24 hourly samples was not practical (Eikmann et al., 1992). As a result, "spot" specimens are usually taken for biological monitoring purposes. Either specific gravity or urinary creatinine methods are generally applied to correct for urine dilution factor (Araki, 1980, Araki et al., 1986). Other methods may also be applied such as osmolarity and urinary volume. Because they are commonly used in occupational toxicology studies involving biological monitoring, the following chapters will focus mainly on the methods for measurement of specific gravity and urinary creatinine.

2.4.1 Determination of Creatinine in Urine

Based on what was discussed in the introduction, it was decided to use creatinine to standardise for urine dilution. Besides to the fact that it is cheap and easy to conduct, the required equipment for creatinine measurements was readily available at the department.

2.4.2 Creatinine Urine Samples

The materials that were used in the creatinine assay are:

1- Creatinine, SIGMA, 60-27-5
2- Acetonitrile (HPLC grade)
3- Ammonium dihydrogen orthophosphate ($NH_4H_2PO_4$)
4- Hydrochloric acid (HCl), BDH (ARISTAR), Prod 450027X
5- HPLC column: Partisil 10 SCX, 30 cm x 4.6 mm i.d.
6- Guard column: TG -- P10SAX guard cartridge
7- HPLC-UV (Kontron plc.)

The mobile phase was prepared by adding acetonitrile to 20 mM $NH_4H_2PO_4$ in 1:9 ratio. 20 mM $NH_4H_2PO_4$ Buffer was prepared by adding 2.3 g of $NH_4H_2PO_4$ to

one litre of deionised water. Mobile phase flow rate was 2 ml/min (isocratic). UV-detector absorbance wavelength was 254 nm. After the end of the run, the column washed up with 50% acetonitrile and 50% deionised water for 20 min.

Standards were prepared in mobile phase. Creatinine stock solution was prepared by adding 113 mg of creatinine on 10 millilitre of mobile phase to produce 100 mM creatinine. One or two drops of HCl were added in order to facilitate complete dissolute of creatinine. Creatinine stock solution diluted 100 times as well as urine samples. Six standard concentrations of creatinine were prepared by adding 0.1, 0.2, 0.5,1.0,2.0, and 5.0 ml of the 100 mM creatinine stock solution on 10 ml of mobile phase to produce 1, 2, 5, 10, 20, and 50 mM creatinine (Muirhead et al., 1986).

Another method (James, 1997) claimed to be faster and safer than the one used. This method was found after the work was accomplished. It is exactly the same as the used method except using different mobile phase, UV-wave length and flow rate. The mobile phase is 50 mM sodium acetate (pH=6.25) with 20% methanol. The UV-wave length was 230 nm, which was close to the 256 nm that was used in our assay. The flow rate was 1.25 ml/min with 3 min acquisition time. This assay is capable to analyse 15 samples per hour compare to less than 9 samples per hour in our assay. When 100 samples analysed, the methanol method requires 6 h and 40 min to accomplish the analysis compare to 11 h and 40 min for the acetonitrile method. Furthermore, the waste from the methanol method for the proposed analysis is 0.5 litre compare to 1.4 litre for the acetonitrile method. Also, the toxicity of acetonitrile (140ml) compared to methanol (100ml) must be considered.

CHAPTER 5

Chapter 5: Analytical Method for *t,t*-Muconic Acid in Urine

Among the urinary benzene biomarkers, *t,t*-muconic acid was chosen as biomarker in this study. *t,t*-Muconic acid was chosen for the following reasons: First: *t,t*-muconic acid and *S*-phenylmercapturic acid are the most sensitive markers for low level benzene exposure (Hotz et al., 1997). The other markers (i.e. phenol) were less sensitive because of their high background in urine (Qu et al., 2000). Second: *t,t*-muconic acid was selected as a marker of effect for one of the benzene toxic pathways. Unlike *t,t*-muconic acid, *S*-phenylmercapturic acid is a marker of exposure, which is produced by one of the detoxifying pathways. Third: the *S*-phenylmercapturic acid has been appreciated by several researchers (Ghittori et al., 1995, Popp et al., 1994, Ong et al., 1995). Recently, and the end of the lab work in this study, a new fast and easy method for measurement of urinary *S*-phenylmercapturic acid has been developed(Aston et. al., 2002). This method was based on a competitive enzyme-linked immunosorbant assay (ELISA).

Therefore, *t,t*-muconic acid and not *S*-phenylmercapturic acid was used in this research as a biomarker for risk assessment following low level benzene exposures (Lee et al., 1993a). Urinary *t,t*-muconic acid was determined by Docus and his colleagues (Ducos et al., 1992). Before percolate urine sample through strong anion exchange (SAX) column, elevating the pH of urine up to 10 improved *t,t*-muconic acid recovery (Lee et al., 1993a, Boogaard and van Sittert, 1995). Centrifuge urine at least for 1 min at 10000 g improved the chromatogram (Lee et al., 1993a). With the mobile phase (10% methanol in 1% acetic acid aqueous), the elution time of *t,t*-muconic acid drifted dramatically after each injection of samples. An improvement in the consistency of elution time was achieved by adding sodium acetate to the mobile phase. The addition of sodium acetate to the mobile phase enhanced also the stability of the chromatographic conditions. Boogaard and van Sittert (1995) achieved a lower detection limit of 0.01 mg/l than what was reported in another study (0.025 mg/l) (Lee et al., 1993a). Even measurement of *t,t*-muconic acid by GC-MS could not achieve a limit of detection of less than 0.01 mg/l (Ruppert et al., 1995).

The Docus method could not be reproduced in this study. The results suggested slower elution of *t,t*-muconic acid and vanillic acid from the SAX column. In these experiments, SAX material was conditioned with methanol and deionised water. Under these conditions, no peaks were obtained from the eluant. However, when methanol was ignored during SAX conditioning, *t,t*-muconic acid recovery was improved dramatically. Solvent extraction has been considered as an alternative to the SAX columns. However, no data were obtained regards the validation of the applied solevents(Rothman et al., 1998, Bucklay et al., 1992). One of this study objectives is to use solvent extraction instead of SAX.

2.5.1 HPLC Method

Chemicals and materials have been used in this study are listed as:

1- Methanol, FISHER, code#M/4056/17
2- Acetic acid, FISHER, code#A/0400/PB17
3- trans, trans-Muconic acid, ALDRICH, cat#M9000-3
4- Vanillic acid, SIGMA, 121-34-6
5- Butan-1-ol, FISON, code B/4850/17
6- SAX column, ALLTECH, 500 mg
7- Sodium hydroxide (NaOH), BDH (AnalaR), Prod 104384F
8- Hydrochloric acid (HCl), BDH (ARISTAR), Prod 450027X
9- HPLC C18 column: phenomenex, LUNA 5μ C18 (2), size 150x4.6 mm
10- HPLC-UV (Kontron)
11- Precolumn: Guard-Pak Insert (μ Bondapack C18), Waters Corp., part no. WAT088070.
12- Nitrogen gas

Two mobile phases were prepared. MP-A consist of acetic acid/methanol/5mM sodium acetate (aqueous), 1/10/90 by volume, pH 3.25 (Lee et al., 1993a) and MP-B (50% methanol in 1% acetic acid aqueous.). However, MP-B consist of 1/50/50 by volume as well, respectively, pH 4.25. A great improving was achieved in retention time stability with adding sodium acetate (buffer) to the two mobile phases. A gradient elution programme was applied to enhance the chromatogram and reduced

the assay time period. From the beginning of the assay until 15min pass, MP-A was applied to allow for the best separation for *t,t*-muconic acid. The gradient transformation from MP-A to MP-B starts after 15min and finish after 20min. MP-B wash the system for 5min (20-25min). Then the gradient transformation reverses back from MP-B to MP-A in 5min (25-30min). Then MP-A conditions the system for 5min (30-35min) after, which the next run can be executed (see Table 11). HPLC pump flow rate was 1 ml/min, and UV-detector set-up was wavelength 259 nm, range 0.005, response time 2 sec, and channel 1.

Table 11: The gradient programme during HPLC analysis of *t,t*-muconic acid assay.

Time range (min)	MP-A	MP-B
0-15	100%	0%
15-20	100% → 0%	0% → 100%
20-25	0%	100%
25-30	0% → 100%	100% → 0%
30-35	100%	0%

2.5.1.1 Solid phase extraction

The strong anion exchange (SAX) column was conditioned with 3 ml methanol then 3 ml deionised water. To make sure that the sample pH fell between pH 7 and 10, a 35% HCl solution was applied on an aliquot of urine (5 ml) if needed. One ml of the urine was percolated through the SAX column then the column was washed with 3 ml of 1% aqueous acetic acid. Then 4 ml of 10% aqueous acetic acid eluted *t,t*-muconic acid from the column. An aliquot (2ul) of the eluant was injected into the HPLC.

The mobile phase was 10% methanol in 1% aqueous acetic acid. The UV wavelength was 259 nm. The flow rate was 1 ml/min and the analysis was carried out isocraticly. Stock solutions for *t,t*-muconic acid and vanillic acid were prepared by dissolving 10 mg in 8 ml of methanol then deionised water added up to 10 ml. Then from those stock solutions, standards were prepared. Different concentrations of *t,t*-muconic acid had been prepared by spiking a urine sample with *t,t*-muconic acid to

achieve 0,0.2,1, 5, 10 and 20 mg/l. The internal standard was vanillic acid (20 mg/l) (Lee et al., 1993a).

2.5.1.2 Solvent Extraction

Ethyl acetate (Rothman et al., 1998) and ethyl ether (Bucklay et al., 1992) were used in other studies. Insufficient details of these methods were available in the literature. Six solvents were tested for the solubility of *t,t*-muconic acid and vanillic acid (internal standard). This is shown in Table 12. Saturated butanol was the best choice among the other solvents. Saturated butanol was prepared by mixing deionised water and butanol in 1:9 ratio. Two sets of tubes were prepared; the first set contained 5 mg of *t,t*-muconic acid, and the second had 5 mg of vanillic acid. Adding 10 ml of diethyl ether, ethyl acetate, dichloromethane, *n*-hexane, toluene, or saturated butanol were applied to test the solubility. *t,t*-Muconic acid and vanillic acid were completely soluble in butanol only.

Table 12: *t,t*-Muconic acid and vanillic acid solubility in six solvents.

Solvent	*t,t*-Muconic acid	Vanillic acid
Diethyl Ether	very poor (rapid precipitation)	very good (clear)
Ethyl Acetate	poor (cloudy)	very good (clear)
Dichloromethane	poor (cloudy)	good (small quantity precipitated)
n-Hexane	very poor (rapid precipitation)	
Toluene	poor (cloudy)	
sat. Butan-1-ol	very good (clear)	very good (clear)

Different internal standards to urine samples were applied based on the different methods that used. Vanillic acid was used mainly in the HPLC-UV method (Lee et al., 1993a). However, 2-bromo-hexanoic acid (Ruppert et al., 1995, Ruppert et al., 1997) and benzene $^{13}C_6$ (Rothman et al., 1998) were used in the GC-MS methods. In this study, vanillic acid was the internal standard. As internal standard, 0.1 ml of 0.25g vanillic acid /l added to 1ml urine sample to obtain 25mg/l concentration.

0.4 ml 1M HCl was applied to 1ml of urine to reduce the pH to between 1.5 and 2 acidified. It was then spiked with vanillic acid as an internal standard to achieve 25 mg/l (0.1 ml 0.25 g/ml). Three millilitres of saturated butanol was mixed with 1 ml aliquot of the urine by sample rotator. Then mixture was centrifuged for 20 min at 2000g. The centrifuge produce two layers separated. The upper layer (butanol) was transferred to a glass tube by using pasture pipettes. A nitrogen stream was gently (flow rate: 5ml/min) applied to evaporate the butanol under temperature 40°C until dryness. The residue was dissolved with 1 ml of 10% methanol in 1% acetic acid. Then, 20 µl of the sample was injected into the HPLC.

Stock solutions for *t,t*-muconic acid and vanillic acid were prepared by dissolving 10 mg in 8 ml of methanol then add deionised water up to 10 ml, and each chemical was treated separately. From these stock solutions, standards were prepared. The saturated butanol was prepared by adding 10%-deionised water to 90% butanol. Water density is higher than butanol density. Therefore butanol float over water and urine.

The effect of centrifugation before the beginning of the extraction was also tested. A blank urine was obtained to determine the centrifuge effect on *t,t*-muconic acid extraction. The sample centrifuged up to 2000 x g for 20 min.

CHAPTER 6

Chapter 6: Urinary 8-Hydroxy-2'- Deoxyguanosine Analytical Method

8-Hydroxy-2'-deoxyguanosine (8OhdG) was chosen along with *t,t*-muconic acid to determine the genetic impact following benzene exposure. An amperometric electrochemical detector is available. An assay was investigated for the urinary 8OHdG analysis.

2.6.1 Materials

1. 8-Hydroxy-2'-deoxyguanosine: mw=353, Sigma (St. Luis, MO)
2. 5-Hydroxymethyl-2'-deoxyuridine
3. *n*-Hexane: Fisher Scientific (Leicestershire, UK)
4. Methanol: (Leicestershire, UK)
5. Acetic Acid: (Leicestershire, UK)
6. Potassium dihydrogen orthophosphate (KH_2PO_4): Fisher Scientific (Loughborough, UK)
7. C18-OH (SPE) 3ml columns
8. Ultra Violet-detector: Phillips Pye Unicam Pu 4021 Multichannal detector.
9. Electrochemical detector: Waters 464 pulsed electro-chemical detector.
10. Working cell: single glassy carbon electrode (Water).
11. HPLC pump: Waters 510.
12. In-line filter: 0.2 um Nylon membrane filter, Gelman Sciences -Ann Arbor, Michigan.
13. Guard column-C18: Waters.
14. Autosampler.
15. IBM compatible personal computer.
16. Data logger:Konton Instruments 450-MT2.
17. Two C18 columns length is 25cm, and one them is C18-OH.
18. Temperature control oven.

2.6.2 Extraction Procedure

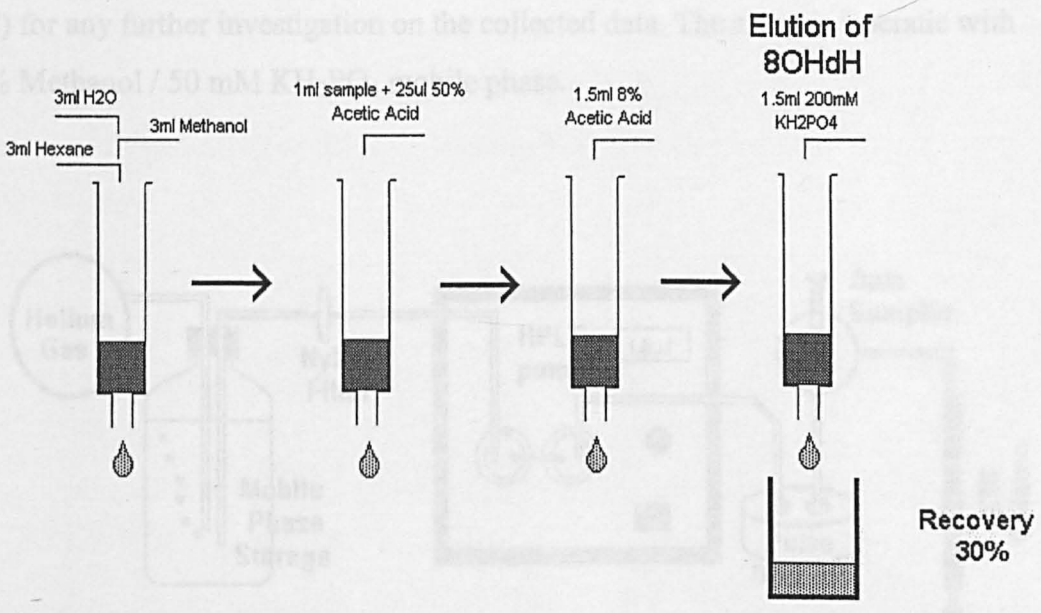


Figure 11: Extraction procedure for urinary 8OHdG.

The extraction procedure in this assay is based on solid phase extraction (SPE). C18-OH (SPE) 3ml columns were used to clean the urine samples. Conditioning solid phase extraction cartridges by applying 3ml hexane, 3ml methanol, then 3ml deionised water, successively. Next, apply the acidified samples or the standards into the SPE. After that, washing the sample in the SPE with 1.5ml 8% acetic acid (correct the response with multiply it with 1.5). The sample is eluted from the SPE by adding 1ml 200mM KH_2PO_4. This procedure is presented in Figure 11.

2.6.3 Instrumentation

The high performance liquid chromatography (HPLC) in the 8OHdG assay equipped with two detectors: Ultra Violet-detector and electrochemical detector as in Figure 12. The tubing line between mobile phase storage and HPLC pump has in-line to remove gases from mobile phase. After the pump, pulse damper installed to reduce the noise in the base line. Autosampler was placed after that Guard column-C18 proceeds the two HPLC columns, which placed in temperature control oven (25 ° C). The two C18 columns length is 25cm, and one them is C18-OH. Afterward, electrochemical detector then UV detector are connected to the system. The data

acquisition system consists of an IBM compatible personal computer (PC) with data logger. The signals monitored instantly on the PC. The analyses were saved on the (PC) for any further investigation on the collected data. The assay is isocratic with 10% Methanol / 50 mM KH_2PO_4 mobile phase.

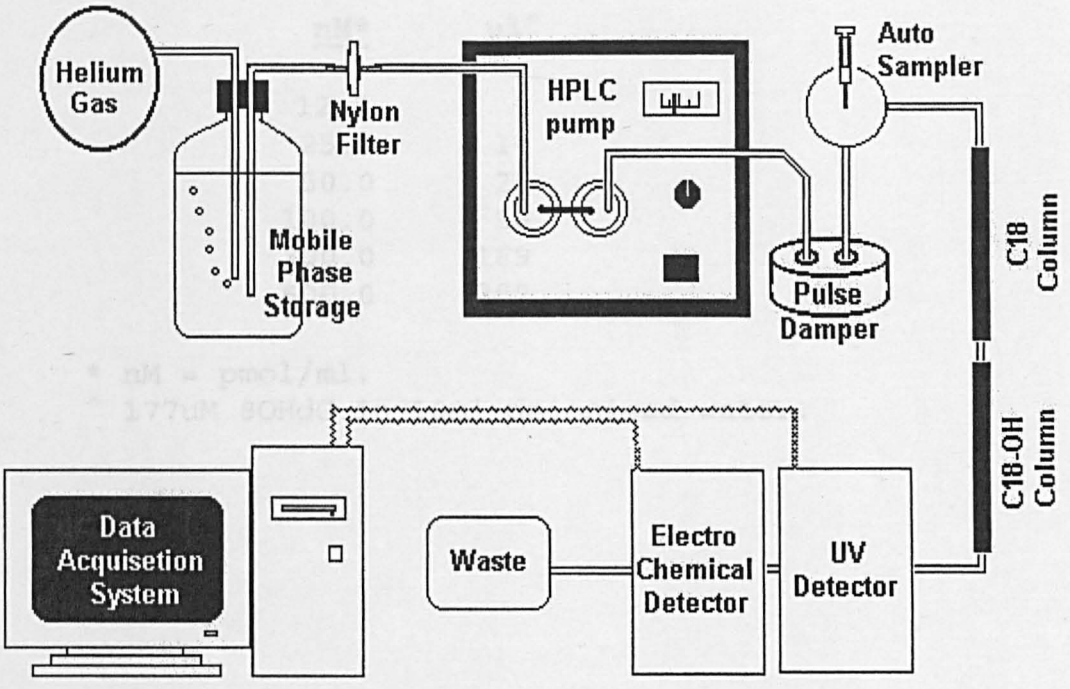


Figure 12: Schematic diagram for the HPLC-electrochemical detector system used to determine urinary 8OHdG.

The HPLC pump flow rate was 0.8 ml/min. The sample was injected (100µl) by the autosampler. The UV detector wavelength was 260nm with 0.16 absolute range. The electrochemical detector potential (E) is 600mV with 100nA I range. The two detectors were in serial, and tested with either electrochemical to UV or UV to electrochemical sequence. No difference was found between the two settings. The retention time for 8OHdG was 45 min.

2.6.4 Procedure

5 mg 8OHdG (molecular weight=353) was dissolved in 10% methanol in 50 mM KH_2PO_4, which was equivalent to 1.77 mM. A second stock standard was

prepared by diluting 1 ml of the 1.77 mM standard into 10 ml of deionised water (177 μM). These standards were stored at -20°C. Seven standards were prepared (Table 13).

Table 13: Preparation of 8OHdG standards in deionised water.

nM*	ul^
12.5	6
25.0	14
50.0	28
100.0	56
300.0	169
500.0	282

* nM = pmol/ml.
^ 177uM 8OHdG in 10ml deionised water.

CHAPTER 7

Chapter 7: BTEX Analytical Method

The BTEX analytical method in this study was based on using gas chromatograph-mass spectrometer (GC-MS). It consists of two main parts. First: the gas chromatograph, which separates gases and vapours. Second: the mass-spectrometer detector, which detects the output of the gas chromatograph by measuring the molecular weight of the separated chemicals. Only the study of Crebelli et al. (2001) used charcoal to collect air samples followed by analysis by GC-MS. The mass spectrometer detector allows the analyst to specify the mass of the targeted chemical to avoid any the interference with chemicals that have different masses. Air samples collected by charcoal were extracted using carbon disulphide (CS_2). Because benzene is a CS_2 impurity, alternative solvents and CS_2 purification were investigated in this study.

2.7.1 Instrumentation

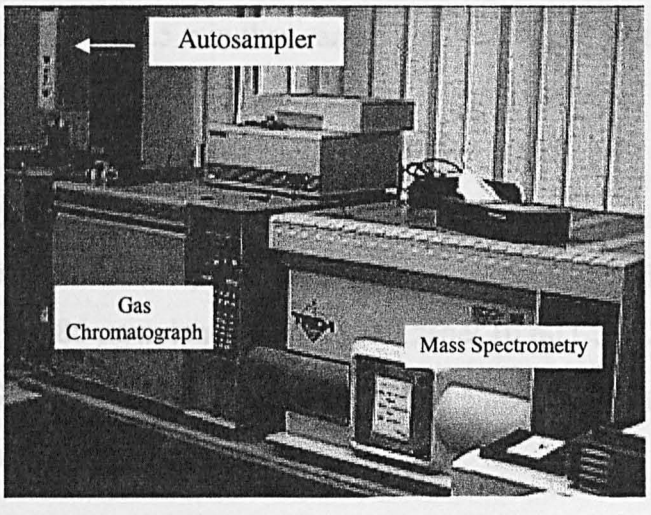


Figure 13: GC-MS combination

The GC-MS consist of a VG TRIO-1 quadrupole mass spectrometry, a gas chromatograph (HP 5890 -- series II), an autosampler (HP 7973), and LAB-BASE™ data system (see Figure 13). The gas chromatograph equipped with HP Ultra 1 capillary column (crosslinked Methyl Silicone Gum "dimethyl polysiloxane" -- 25m x 0.32 mm x 0.52 μm film thickness). The carrier gas was helium. Total flow gas was 60 ml/min, and the column pressure adjusted to 10 kPa. The temperature was set to

200°C for injector. The injected amount was 1μl. The temperature programme was 3 min initial time at 40°C, 70°C /min ramp rate, and the final temperature was 200°C.

LAB-BASE™ data system was based on IBM compatible personal computer. LAB-BASE™ provides full control of the system from tuning of the mass spectrometer (in either manual or automatic modes), through data acquisition (full scan or selected ion recording mode) to an extremely powerful suite of data processing software. The gas chromatograph temperature programme and autosampler are fully controlled by LAB-BASE™. During the study progress, the software was upgrade with MSS Maspec II32 Data system for windows 2.0 (TRIO version).

Electron impact ionisation was used, monitoring the positive ion fragments. Mass fragments have been determined for benzene (ALDRICH, cat# 15,462-8), deutrated benzene -d6 (SIGMA, 1076-43-3), toluene, and xylene isomers (p-, m-, and o-xylene) and ethylbenzene (ALDRICH, cat# 21,473-6). These chemicals were analysed by GC-MS by using scan mode (m/z 30-130). The temperature programme was initiated with 40°C for 1.5 min then ramped in 20°C /min rate then held at 100°C for 3 min. The benzene standard was prepared as diluting 2.2 μg of benzene and 2.175 μg of toluene in 10 ml of redistilled CS_2 (SIGMA-ALDRICH, cat# 42,464-1). After the previous standard had been analysed, 4.75 μg of benzene -d6 was added to the standard. Toluene standard was prepared as 2.175 μg of toluene added on 10 ml of redistilled CS_2. Also, 86 μg xylenes and ethylbenzene/ 10 ml CS2 (ALDRICH, cat# 15,470-9, Lot 02937AN), which consist of ethylbenzene, para-, meta-, and ortho-xylenes, was added.

2.7.2 Back and Front Portions of Charcoal Tubes

The back and front portions of tubes were transferred to a separate vial on the day of analysis. The vials were then capped with Teflon liners to allow solvent addition. Two millilitres of solvent was added to the front portion. To the back portion, one millilitre of solvent was added. Both were analysed by GC-MS or GC-

FID. Breakthrough of BTEX was determined by extracting the back portion of air samples. No breakthrough was detected for any of the contaminants.

2.7.3 Ideal Solvent

An investigation was carried on to determine an ideal solvent to desorb BTEX from charcoal. The ideal solvent supposes to achieve three criteria: (a) recovery factor more than 75%, (b) minimum detection by analytical instrument, (c) minimum impurities interferes with the targeted chemicals. Carbon disulphide (CS_2), dichloromethane (DCM), cyclohexane (C_6H_{12}), ethyl acetate, and diethyl ether (DEE) were tested. The properties of each were demonstrated in Table 14. Gas chromatograph-flame ionisation detector was applied to determine impurities in these solvents.

Table 14: Validation of five solvents for used by GC-FID.

Solvent	Comment
Carbon disulphide (CS2)	500 ppbV of benzene in the solvent.
Dichloromethane (DCM)	recovery factor was less than 60%
Cyclohexane (C6H12)	had broad peak and more than 30 peak detected benzene eluted either very close to or in cyclohexane shoulder
Ethyl acetate	had broad peak and more than 12 peak detected, one of them is toluene. Benzene eluted near one of the impurities.
Diethyl ether (DEE)	Although very few impurities exist, benzene was eluted closely before these peaks.

Mixtures of some solvents were tested. The relatively high benzene content in CS_2 was diluted with dichloromethane (DCM) in 1/4, 1/9, and 1/19 was tested for the mentioned criteria. Also, mixtures of diethyl ether (DEE) and dichloromethane (DCM) were tested in 3/7, 1/9, and 1/19 ratios. The mixture's recovery factors failed to achieve 75%. But the CS_2 that diluted in dichloromethane (DCM) in 1/4 ratio demonstrated the best among the mixtures.

Also, carbon disulphide (CS_2) and a mixture of carbon disulphide and dichloromethane (DCM) in ratio of 1/4 were tested by GC-MS to determine the ideal solvent for the study. SIR in the GC-MS was m/z 78 for benzene and m/z 92 for toluene.

The criteria for the ideal solvent demonstrated the advantage of using carbon disulphide over the mixture in extracting air sample. The recovery of benzene by the mixture was 61% compare to 94% by carbon disulphide. Also, the recovery of toluene by the mixture was 66% compare to 96% by carbon disulphide. The baseline noise and the tail of the mixture are much higher than carbon disulphide. Although less benzene in the mixture compare to carbon disulphide, toluene is one of the impurities that found in the mixture when none detected in carbon disulphide. Therefore, carbon disulphide would be the solvent of choice. Solvent purification was the next step to minimise benzene abundance in the solvent.

2.7.4 Carbon Disulphide Purification

Carbon disulphide was not ideal to determine benzene at low levels in ambient air. Because of the amount of benzene in carbon disulphide as an associated impurity. Benzene concentration in high by purified carbon disulphide varied between 1 and 50 μg/ml.

Therefore different solutions have been suggested by different studies. These solutions were based either on avoiding carbon disulphide (portable GC-MS) (Davoli et al., 1996), or by purifying carbon disulphide (chemical and physical benzene removal). Chemical removal based on extracting hydrogenated hydrocarbon, then extracting nitro-compounds for aromatic after nitration (Levdie and MacAskill, 1976).

Physical removals based on using molecular sieve 13X (60/80) (Michalko and Phillips, 1989). When 5 ml of carbon disulphide (5mg benzene /ml) was passed through 1g of 13X, about 90% of benzene was removed. No benzene was detected up to the 4ml/g elutes. Also, it is possible to regenerate molecular sieve 13X, to use it again (Aldrich, technical information bulletin, number AL-143). In this work, regeneration of molecular sieve 13X and its reuse were investigated.

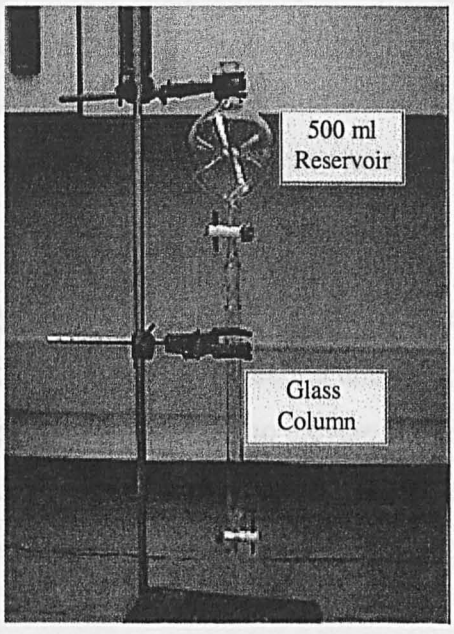


Figure 14: The purification system consisting of a carbon disulphide reservoir (upper part) and glass column (lower part)

A clean up procedure was suggested using 13X. It is simply a chromatographic method. Benzene removal efficiency varied with respect to the carbon disulphide flow rate, column diameter, molecular sieve, mesh size, and benzene concentration in the purified carbon disulphide (Michalko and Phillips, 1989). Molecular sieve 13X (60/80 mesh size) was purchased from Alltech Associates Inc. (Deerfield,IL, USA). Carbon disulphide (spectrophotometric grade) was bought from Aldrich Chemical Co. (Milwaukee, WI, USA). It is worth mentioned that benzene concentration varied in different bottle even with the same catalogue number, and because lot number 02937AN has less benzene variation between bottles from the same lot (420μg /l ≡ 480 ppbV). Also, redistilled carbon disulphide was purchased from Sigma-Aldrich (Sigma Chemical, St. Louis, MO, USA, and Aldrich Chemical, Milwaukee, WI, USA). Glass column for the removal process was designed with 250mm length, 20mm diameter, and with valve at the end of the column. An independent reservoir (500-ml) was joined with the column (see Figure 14). Glass fibres were placed in the bottom of the column to retain the molecular sieve.

Benzene concentration was determined by VG TRIO-1 quadrupole mass spectrometry accompanied with HP5890--series II gas chromatograph. This system

equipped with HP7973 autosampler and LAB-BASE data system. The GC column was HP Ultra 1 capillary column (crosslinked methyl silicone gum "dimethyl polysiloxane"--25m x 0.32mm x 0.52 um film thickness). The pressure of helium as carrier gas was 10kPa and the total flow was 60 ml/min. The temperatures for injector and over were 200 and 40°C, respectively. Single ion chromatography (SIR) was applied at m/z 78.

The regeneration is a typical recycling system, which based on the removal of the adsorbate from the molecular sieve bed by heating and purging with a carrier gas (AlDRICH, 1993). Adequate heat must be used to elevate the temperature of the adsorbate. The adsorbent and the vessel to vaporise the liquid and offset the heat of wetting the molecular sieve surface. The bed temperature is important in regeneration.

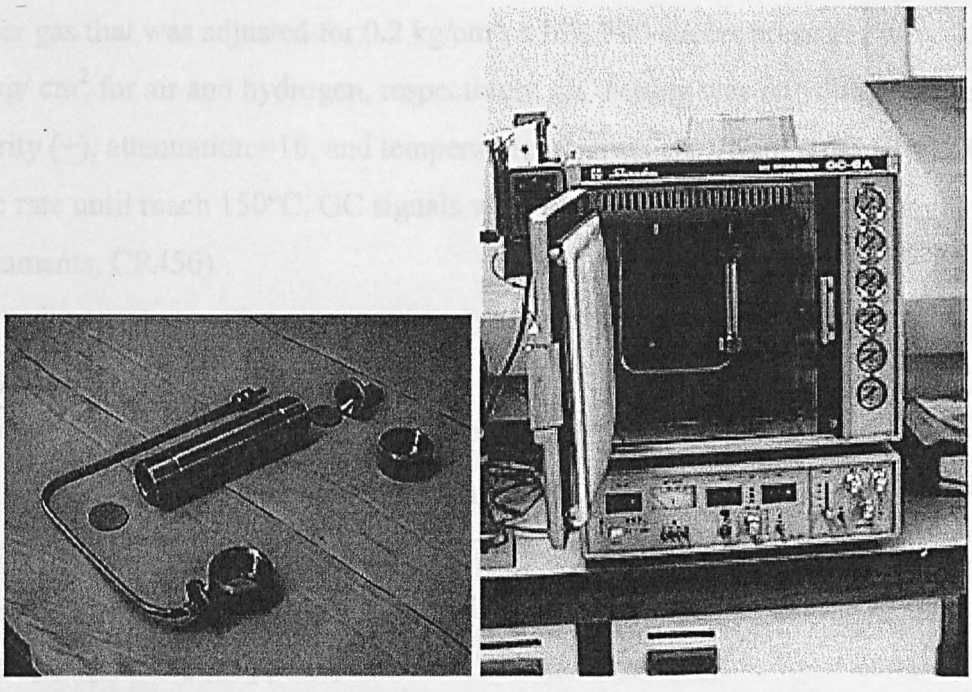


Figure 15: The purging kit was used to regenerate 13X following CS_2 purification. The purging kit after disassembly (Left). Shimadzu GC-8A after the kit was installed (Right).

The lower regeneration temperature minimises polymerisation of olefins on the molecular sieve surface when it is present in the carrier gas. Slow heating is recommended since most olefinic materials will be removed at 200°C. 13X bed temperature range is in between 200 and 315°C. After regeneration, a cooling period is crucial to reduce the molecular sieve temperature within 15°C of the temperature of the stream to be processed. This is most conveniently done by using the same gas

stream as for heating, but with no heat input for optimum regeneration. Alternatively, small quantities of molecular sieve may by dried in the absence of a purge gas by oven heating followed by slow cooling in a closed system such as a desiccator.

Purging container was designed for this purpose. The container was made from stainless steel metal as in Figure 15. It has a cylindrical shape. The dimensions are 127-mm length, 30-mm outer diameter, and 22-mm inner diameter. The capacity of the container is 30 mg of 13X. A conical metal and a porous disc were mounted in both ends of the container. The conical metals were welded with metal tubing to be connected with GC.

Gas chromatograph-flame ionisation detector (Shimadzu, GC-8A) has been used to monitor the regeneration progress as in Figure 15. Nitrogen gas was the carrier gas that was adjusted for 0.2 kg/cm^2, while FID gasses pressures were 0.2 and 1.7 kg/ cm^2 for air and hydrogen, respectively. GC setting was on voltage range 5, polarity (+), attenuation =16, and temperature program start from 50°C with 5°C/min ramp rate until reach 150°C. GC signals were plotted by a recorder (J.J. Lloyd Instruments, CR450).

2.7.5 Standard Preparation

Deuterated benzene (C_6D_6) as an internal standard was added to purified carbon disulphide as the best analytical solvent. The solvent was prepared by adding 3.75 ml C_6D_6 to 250 ml of purified carbon disulphide to achieve 1.3 mg carbon disulphide - d6/ ml purified CS_2 (15 ppmV).

Table 15: Preparation of BTEX standards preparation.

ppmV	Benzene (mg/ml)	Toluene (mg/ml)	Ethylbenzene + p-, m-, o-xylene (mg/ml)	µl / 10ml CS_2 - d6
1	0.088	0.087	0.348	100 (Stock B)
3	0.264	0.234	1.044	300 (Stock B)
5	0.440	0.435	1.740	500 (Stock B)
10	0.880	0.870	3.480	100 (Stock A)
30	2.640	2.340	10.440	300 (Stock A)
50	4.400	4.350	17.400	500 (Stock A)

100	8.800	8.700	34.800	1000 (Stock A)

Two dilutions were prepared to achieve the expected range of ambient concentrations. Dilution (A) was prepared by adding 10 µl benzene, 10 µl toluene, and 40 µl mixture of ethylbenzene and xylene on 10 ml purified carbon disulphide. Dilution (B) was prepared by adding 1 ml (A) to 10 ml carbon disulphide. The working standards were presented in Table 15.

2.75.1 Air Concentration Conversion

The measurements obtained from GC-MS or GC-FID represented the concentration of contaminants desorbed from the charcoal. The ambient concentration of that chemical can not be achieved unless several factors are considered: recovery factor, added solvent, injected amount, measured mass, the sampling flow rate and the sampling duration.

The linear relationship between prepared standards' masses and instrumental responses were adjusted with benzene -d6 (internal standard). From the relationship between mass (nanogram) and responses, the desorbed pollutants' masses could be obtained. A recovery factor was determined for the chemicals based on their CS_2 extraction. It varied with each batch of charcoal. The recovery varied between 81% and 100% among BTEX compounds.

The added solvent refers to the quantity of solvent added to charcoal to desorb the contaminant. As mentioned previously, 2 ml of solvent was added to the front portion and 1 ml to the back portion. The proportion of added solvent over the injected quantity was calculated to restore the desorbed pollutants' masses. If the added solvent was 2ml and the injected amount was 2ul, then the proportion will be 1000 (as 2000µl/2µl). The multiplication of that proportion with the measured mass (ng) obtained from the GC divided by the recovery factor determined the actual mass of pollutants desorbed from charcoal. The following equation shows how the actual was mass calculated.

$$\text{Actual mass } (\mu g) = \frac{1}{\text{Recovery factor}} \times \frac{\text{Added solvent } (\mu l)}{\text{Injected amount } (\mu l)} \times \text{measured mass (ng)}$$

2.7.5.2 *Microgram per Cubic Meter*

Air pollutant's concentrations are expressed in microgram per cubic metre ($\mu g/m^3$). To interpret the ambient concentrations from the obtained masses, the air pump flow rate and the sampling duration is required to get the drown volume of air through the charcoal tube. The following equation is to determine mass per volume concentration ($\mu g/m^3$)

$$\text{Ambient Concentration } (\mu g/m^3) = \frac{\text{Actual mass } (\mu g)}{\text{Flow Rate (L/min)} \times \text{Sampling Duration (min)} \times 10^{-3} (m^3/L)}$$

2.7.5.3 *Part per Billion (ppb)*

Part per billion is a unit that mainly calculated to make the mass/volume ($\mu g/m^3$) concentrations comparable world wide. The conversion formula is to convert from mass per volume to volume per volume concentration (ppb)

2.7.5.4 *$\mu g/m^3$ to ppb Conversion Formula*

$$\text{ppm} = 22.4 \times \frac{\mu g / m^3}{\text{mol.wt}} \times \frac{760 \text{mmHg}}{\text{A mbar} \times 0.750062 \text{mmHg / mbar}} \times \frac{\text{B°C} + 273.15\text{K}}{273.15\text{K}}$$

When:
(A) Atmospheric pressure in mbar
(B) Ambient temperature in Celsius

Table 16: The conversion factors for BTEX analysis.

Chemical	M.W.	µg/m3 to ppb multiply by	ppb to µg/m3 multiply by
Benzene	78	0.308	3.245
Toluene	92	0.261	3.826
Ethylbenzene	106	0.227	4.408
para-Xylene	106	0.227	4.408
meta-Xylene	106	0.227	4.408

ortho-Xylene	106	0.227	4.408

The conversion factor for benzene becomes an international standard from $\mu g/m^3$ to ppb (Murely, 1994). Two factors were assigned to ease the equation; ambient temperature (20°C) and atmospheric pressure (1013 mbar or 759.8 mmHg). Based on that, conversion factors for the other chemicals were calculated (Table 16).

CHAPTER 8

Chapter 8: Monitoring Study Design

Subjects who were exposed to emissions from inside petrol or diesel vehicles were recruited for this study. Questionnaire data, urine samples and air samples were collected from some subjects (see Figure 16). The questionnaire was included in the study to define the participants life style (i.e. age, sex, health status, diet and drinks), study parameters (sampling time, fuel type, vehicle information ... etc.) and potential confounders (active and passive smoking, drinking alcohol, sorbic acid, ... etc.). After collection, urine samples were stored at -20°C. Creatinine and *t,t*-muconic acid levels were determined. Urine samples were also stored for 8OHdG. Air samples were collected for BTEX analysis.

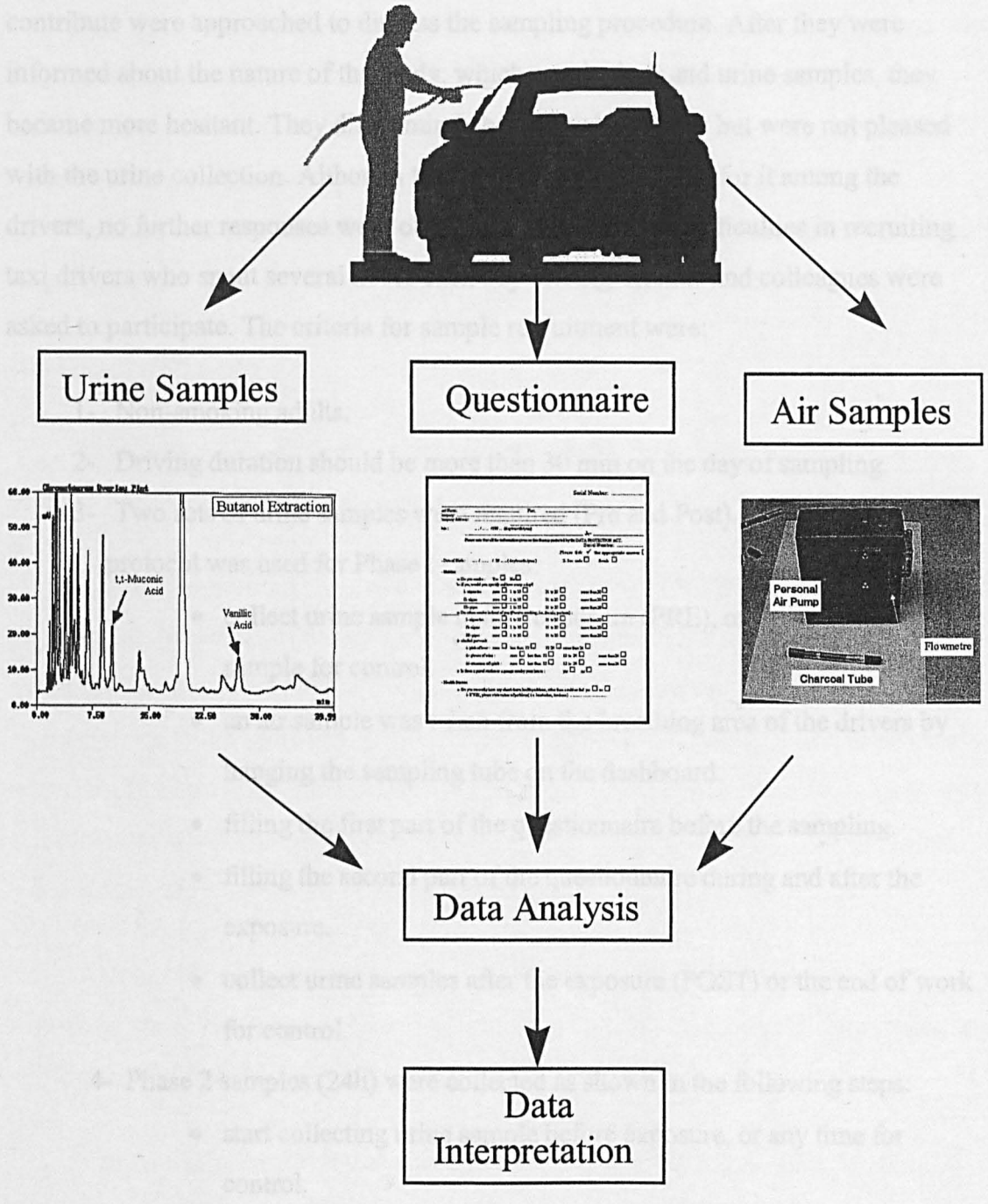


Figure 16: Schematic diagram for the study.

2.8.1 Recruitment of Volunteers

The initial design was to study taxi drivers. However after contacting several companies, only few a positive responses were obtained. Those who agreed to contribute were approached to discuss the sampling procedure. After they were informed about the nature of the study, which required air and urine samples, they became more hesitant. They didn't mind about the air samples but were not pleased with the urine collection. Although they promised to advertise for it among the drivers, no further responses were obtained. Because of the difficulties in recruiting taxi drivers who spent several hours each day driving, friends and colleagues were asked to participate. The criteria for sample recruitment were:

1- Non-smoking adults.
2- Driving duration should be more than 30 min on the day of sampling.
3- Two sets of urine samples were required (Pre and Post). The following protocol was used for Phase I samples:
    - collect urine sample before exposure (PRE), or the early morning sample for control.
    - an air sample was taken from the breathing area of the drivers by hanging the sampling tube on the dashboard.
    - filling the first part of the questionnaire before the sampling.
    - filling the second part of the questionnaire during and after the exposure.
    - collect urine samples after the exposure (POST) or the end of work for control.
4- Phase 2 samples (24h) were collected as shown in the following steps:
    - start collecting urine sample before exposure, or any time for control.
    - Every single sample within the next 24h collected after measuring it's volume.
    - no air sample taken.
    - filling the first part of the questionnaire before the sampling.

- filling the second part of the questionnaire during and after the exposure.
- specifying the exposure periods in the day of sampling.

2.8.2 Questionnaire

The questionnaire was divided into two main parts (Appendix III). The first one consisted of personal, habits, health, vehicle, and career data. At the end a consent agreement was attached. The second part included sampling data. Air sampling record, driving data, smoking data, and diet and drink status obtained. This questionnaire was discussed with a statistician and some modifications were made as a consequence.

2.8.2.1 First Part

Volunteer name, company name and address, date of filling the first part, date of birth, and sex were required in the personal data.

Habitual smoking and alcohol consumption were asked for. Three types of tobacco products were to determine cigarettes, cigar, and pipe. Four choices are available for each type. NONE choice was for not smoking that type. If the smoking was from one to ten units then 1 to 10 choices suppose to be chosen. The third choice was from 11 to 20 units. Finally, the fourth choice was the more than 20 units. Passive smoking was when another smoked in the presence of the volunteer. The questions were exactly the same as the smoking questions.

Alcohol consumption was measured in units per week. Four choices are available; none, less than 12, 12 to 24, and more than 24 units a week. Beer, wine, and spirit were expressed in pint, glass, and measure, respectively.

The volunteer health status was asked for any short or long health problem. If there was a problem, then the volunteer was asked to specify the name of medication and the duration of using it.

Vehicle data that required include the car make, year of production, fuel type, and partition existence between the driver and passengers. Fuel types were petrol, diesel, and electricity. No electric vehicles involved in the study, which make the electricity choice useless. Further details was added to specify whether the petrol was unleaded or premium (4star). Although the fuel supplier company was asked for, sample size and the diversity of the local suppliers made this question useless as well. Sixteen choices were available for this question. ASDA, BP, ESSO, FINA, IMPERIAL, JET, MOBILE, Q8, SAVE, SHELL, SPOT, TEXACO, TESCO, TOTAL, UK petroleum, and other were the addressed choices. Being in the neighbourhood of a petrol station or garage was considered in the questionnaire.

The volunteer career was also included in the questionnaire. This question and the car partition were prepared before approaching taxi companies. But it were kept in the questionnaire even after changing the population to general drivers because part of actual samples collected already at that point. At the end of the first part, a consent form was included to publish the produced results anonymously, unless further consent agreed upon.

2.8.2.2 Second Part

On the day of sampling (part 2), further questions were addressed. An air sampling table was designed to determine the time and flow rate of the sampling session. Air sample flow rates were measured in litre per minute. The sampling sessions were operated during the actual driving periods. If more than one session was obtained then the summation for these sessions was considered to be the driving duration. The driving duration was applied to the calculation of the chemicals' ambient concentration (Air Concentration Conversion subsection in Part II).

Smoking status was asked before, during, and after driving. Every one had the same choices. Passive smoking status was also acquired. Alcohol consumption was addressed as units consumed in the sampling day. The choices were none, 1, 2, and more than 2 units. The driving section consisted of questions about the driven distance (mile), driving period, refuelling the vehicle, window status, air conditioning, and traffic status. In the diet and drinks section, volunteers were asked if they had

consumed preservatives. Also an instruction sheet was attached to the questionnaire especially in the first contribution for the volunteer. The sheet demonstrated in the following bordered text.

Dear Sir,
It is great to have you in the study, and hopefully this sheet will be helpful to you.

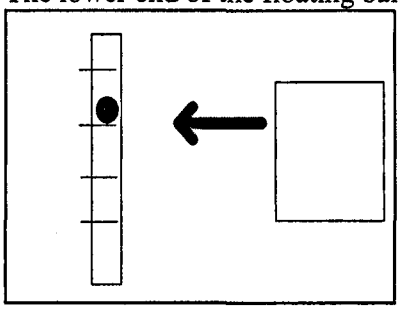
Questionnaire:
It consists of two parts. The first part contains questions regards the volunteer in general, that can affect the level of exposure. Therefore it is very helpful to answer this part before the day of sampling.
However, the second part regards different factors that can be determined in the day of sampling.

Urine Samples:
Two samples required from the volunteer. One, before the volunteer expose to fumes inside the car cabin, which is preferable to be obtained after wake up in the morning. The other is after the end of the air sampling (0 - 4h).
Note:

Urination during the air sampling does not affect the study.
Mid-stream urine sample recommended.
The collected sample temperature must be kept as low as possible.

Air Sample:
Air sampling system consists of air pump, charcoal tube, and hose (to connect the pump with charcoal). Charcoal tube has two open ends covered with red caps. An arrow can be observing on the tube shows the proper flow direction of air sample (SKC <---). Assembling the system can be done as in the following diagram. Two long wires are also supplied; to hold the pump and to hold charcoal tube.

Notes:
Air samples ideal site is in the exposed person breathing area.
The lower end of the floating ball considers airflow readings.



I apologise for the inconvenient and thank you for your great help.

YOURS,

Nabeel Al-Khulaifi

2.8.3 Air Samples

An air sample was collected into the charcoal tube placed on the car dashboard to collect a sample from the breathing zone within the car. The time of starting and

completion of air sampling were recorded. The air sample was only collected during a period of driving. Volunteers recruited for this research were drivers of petrol and diesel cars in Newcastle upon Tyne and Sunderland.

The kit for collecting air samples consists of a personal air pump and a charcoal tube. An icebox was supplied with the kit to reduce the pump noise, which was inconvenient to the drivers. An instruction note accompanied the questionnaire to instruct the volunteer clearly how to install and handle the sampling kit. Also, most of the expected questions were answered. SKC AIRCHEK samplers were used in the study (cat # 224-43XR).

The air sample was collected by drawing air through a sorbent material (Charcoal tube) for a certain period of time. ANASORB CSC charcoal tubes (400/200) had been used (cat. no. 226-09). The front and back portions of the tube consist of 400 and 200 mg of charcoal, respectively (see Figure 17). A sampling container was supplied with the kit to reduce the pump noise (see Figure 18). Two holes were made to allow the sucked air to the pump, and the other to balance the pressure inside with outside the box. Benzene, toluene, ethylbenzene, and xylenes (BTEX) were measured.

The sampler flow rate was very high (up to 4 Litre /min) in the study, which make it difficult to measure it with the ordinary flowmeter. Therefore, a dry gasometer was applied to measure the volume of the collected air after the charcoal tube. This volume was divided on the time that required obtaining this volume to determine the flow rate. This method was used to check the samplers before each sampling event. Charcoal broken ends used to be capped with red caps to avoid contamination before the sampling. Each subject had a simple training for the sampling kit usage. One of the subjects forgot to remove the second end cap in some of his samples. This loses of air samples, fortunately, did not occur with the other subjects. These samples were capped and stored at room temperature. This was discussed as in the air samples storage subtitle (2.9.3.1). Therefore, air and urine samples were analysed in batches.

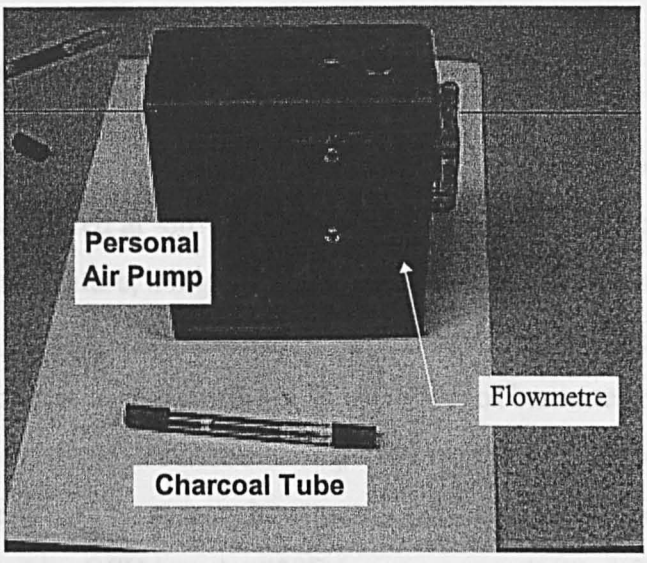


Figure 17: Personal air pump and charcoal tube capped with two red plastic caps.

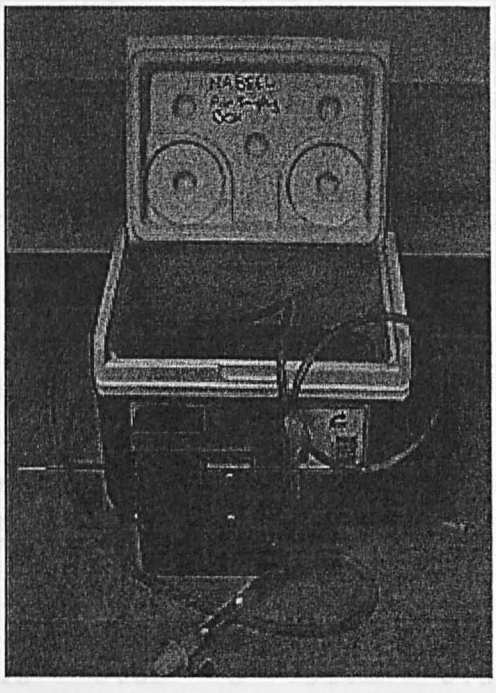


Figure 18: Sampling kit includes an icebox to reduce the pump noise.

2.8.3.1 *Storage of Air Samples*

Urine samples were stored in a freezer at -20°C after the sampling. Some charcoal tubes were stored at -80°C. However, when these were removed from the freezer to room temperature, a high internal pressure built up in these tubes and end up having shooting the plastic caps, which block the both ends of the tube. Through four months, a triple set of two standards (5 and 50µl) of benzene spiked into fresh front charcoal tubes was stored at room temperature. Due to malfunctioning in GC-MS, the results were lost. However, the storage efficiency for charcoals spiked with standards was tested by OSHA under 23°C (room temperature) and -25°C temperatures (OSHA, 1985). They generated 1.03ppm samples at 80% relative humidity, 22°C and 643 mmHg (85.71kPa). These samples were taken at flow rate 0.2 L/min for 50min. Six samples were immediately analysed. Also, fifteen samples were analysed for the refrigerated and others for ambient storage over a period of fifteen days. No significant difference was found between the samples that stored in refrigerator and that stored at room temperature (two-sample t-test, n=21, p=0.93, self-compiled).

Table 17: Storage tests. Values are the percentage of recovered benzene.

Days of	Refrigerated			Ambient		
Storage	N	Mean	S.D.	N	Mean	S.D.
0	6	98.9	1.44	6	98.9	1.44
2	3	95.9	0.41	3	96.3	0.65
5	3	93.3	0.66	3	93.6	0.85
9	3	94.6	0.78	3	95.8	0.59
13	3	94.4	0.66	3	93.5	1.80
15	3	95.6	1.06	3	95.0	1.52

A significant difference was found between the immediately analysed samples and the rest of the samples (one-way ANOVA, $p<0.001$). However, no significant differences were found between 2, 5, 9, 13 and 15 storage days samples (Tukey's pairwise comparisons). Also, no significant difference was found between the mentioned storage conditions the OSHA applied to this test.

Air samples were collected from inside vehicles. Not all of the subjects who exposed collected air samples. In some occasions, more than one volunteer were in the same journey. Some air samples failed because of technical problems. The 24h samples model was design without considering air samples, instead exposure period was acquired to determine the driving sessions influence on *t,t*-muconic acid excretion. No air samples were collected for control subjects. Ten vehicles were involved in the study. Six petrol cars (9 volunteers) and four diesel cars (8 volunteers) were obtained. Some volunteers participated with a number of occasions per one volunteer. The number of samples per volunteer varied between 1 and 10 except for whom 18 samples were collected. The mean of samples for the same subject was considered in testing the differences between petrol and diesel car exposures.

2.8.4 Urine Samples

Unlike occupational exposure, environmental exposure is not stricted to a certain period of time. For example, driving the cars may have several ways of exposure such as driving the car continuously for a long time or driving for more than one short-time period. The driving duration in the study varied from 50min to 10h. Urine sample analysis assessed the exposure to the hydrocarbons emitted from the driven car or other cars driven on the road at that time. The time-difference between the samples that were collected before and after exposure varied widely. The half life for urinary *t,t*-muconic acid excretion after exposure was 5.0h ± 2.3h (Boogaard and van Sittert, 1995). Therefore, special care was taken regarding the timing of post-samples. The post-samples were divided into two groups based of time of collection. POST1 samples were collected from 3 to 7 h from the start of driving. POST2 samples were collected 7 and 21 h after the start of driving. These samples were called "phase one". Because of the nature of the study, the average of the POST samples for each event was also considered. POST samples were averaged when more than one sample collected after the exposure. Control subjects were defined as those who were not exposed to petrol or diesel emissions from inside a vehicle. Pedestrians were included in the control group.

Phase II samples were collected at 24h to determine *t,t*-muconic acid variability for subjects who were exposed or not exposed to vehicle emissions.

Standards were applied in mobile phase and urine. Urine for standards was obtained from early morning specimen. To avoid the temperature influence for the elution time for *t,t*-muconic acid and vanillic acid, standards were injected alternatively with samples.

SECTION (III):

RESULTS

SECTION (III): RESULTS

The results section includes four chapters: method development for *t,t*-muconic acid in urine, 8OHdG measurements in urine, BTEX analysis in air, and the monitoring data. Firstly, results for *t,t*-muconic acid in urine were addressed including the solvent extraction validation and optimisation. Secondly, urinary 8OHdG assay methodology was validated. Some obstacles were found in the 8OHdG assay. Thirdly, the analytical method to determine BTEX concentration in atmosphere was assessed. Fourthly, the volunteer monitor study results were evaluated based on questionnaire responses, and data from the air and urine samples. The potential relationships between some factors and the air and urine analyses were investigated in this chapter.

CHAPTER 9

Chapter 9: Method Development for *t,t*-Muconic Acid in Urine

This chapter starts with the creatinine and *t,t*-muconic acid assay validation. Butanol was validated as a solvent to extract *t,t*-muconic acid from urine, which is the main contribution to this study. A comparison was conducted between butanol extraction and solid phase extraction. Finally, the extracted *t,t*-muconic acid statistical distribution was tested in this chapter.

3.9.1 Creatinine Level in Urine

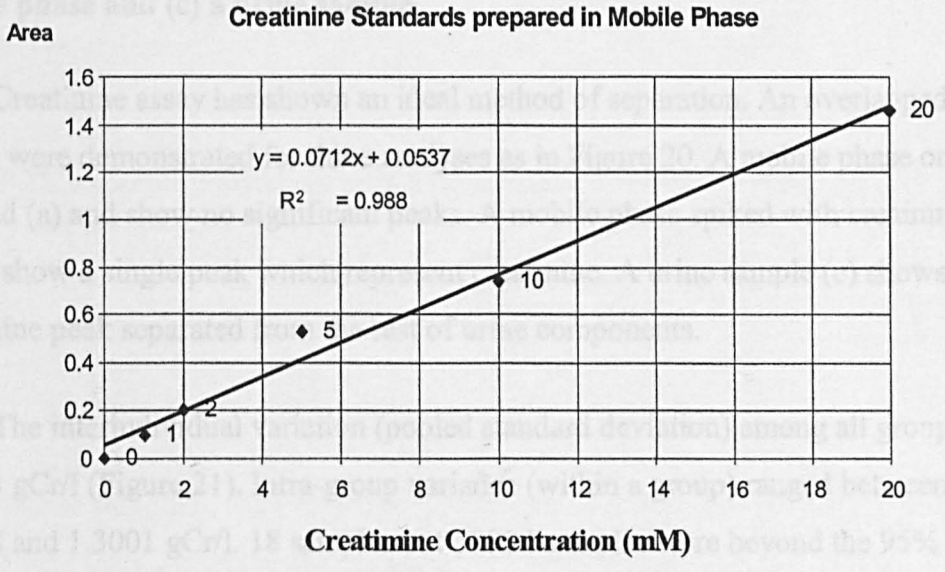


Figure 19: Calibration line for creatinine concentration mMCr (mmol/L) versus UV detector response at 254 nm wavelength.

The creatinine calibration line showed high precision (R^2=0.988) as seen in Figure 19. The average creatinine concentration for all urine samples (N=399) in the study was 1.62 gram creatinine per litre urine (gCr/l), which is equivalent to 143 mM. The values varied between 0.05 and 6.66 gCr/l (4.4 - 589.4 mMCr). The standard deviation was ± 0.9409 gCr/l (83.3 mMCr).

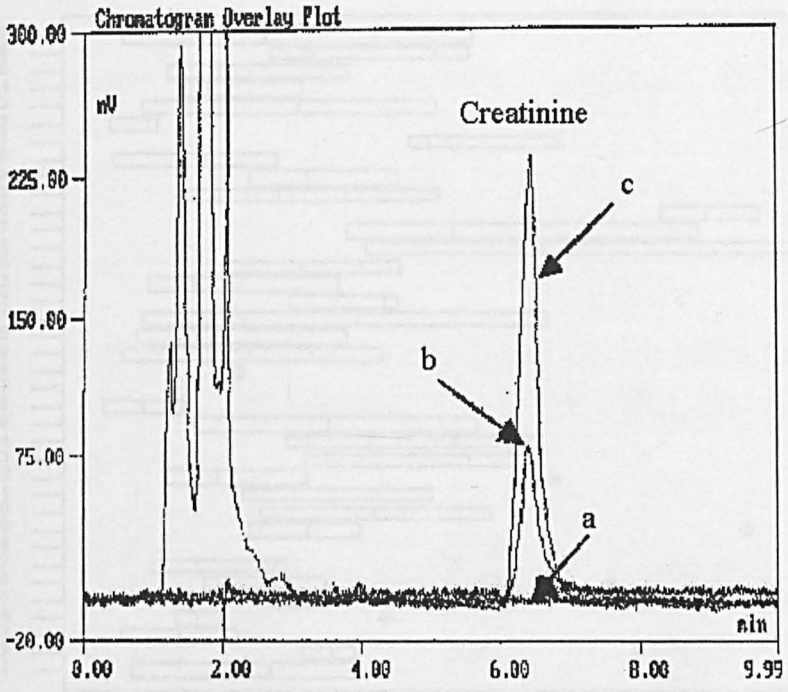


Figure 20: Analysis of creatinine in urine (a) mobile phase alone (b) creatinine in mobile phase and (c) a urine sample.

Creatinine assay has shown an ideal method of separation. An overlapped curves were demonstrated for three analyses as in Figure 20. A mobile phase only was injected (a) and show no significant peaks. A mobile phase spiked with creainine (b) which show a single peak which represent creatinine. A urine sample (c) shows the creatinine peak separated from the rest of urine components.

The interindividual variation (pooled standard deviation) among all groups was 0.8293 gCr/l (Figure 21). Intra-group variance (within a group) ranged between 0.0568 and 1.3001 gCr/l. 18 samples out of 322 samples were beyond the 95% confidence intervals (about 5% of total samples). The outlier samples were included in the study as the total number of samples was small.

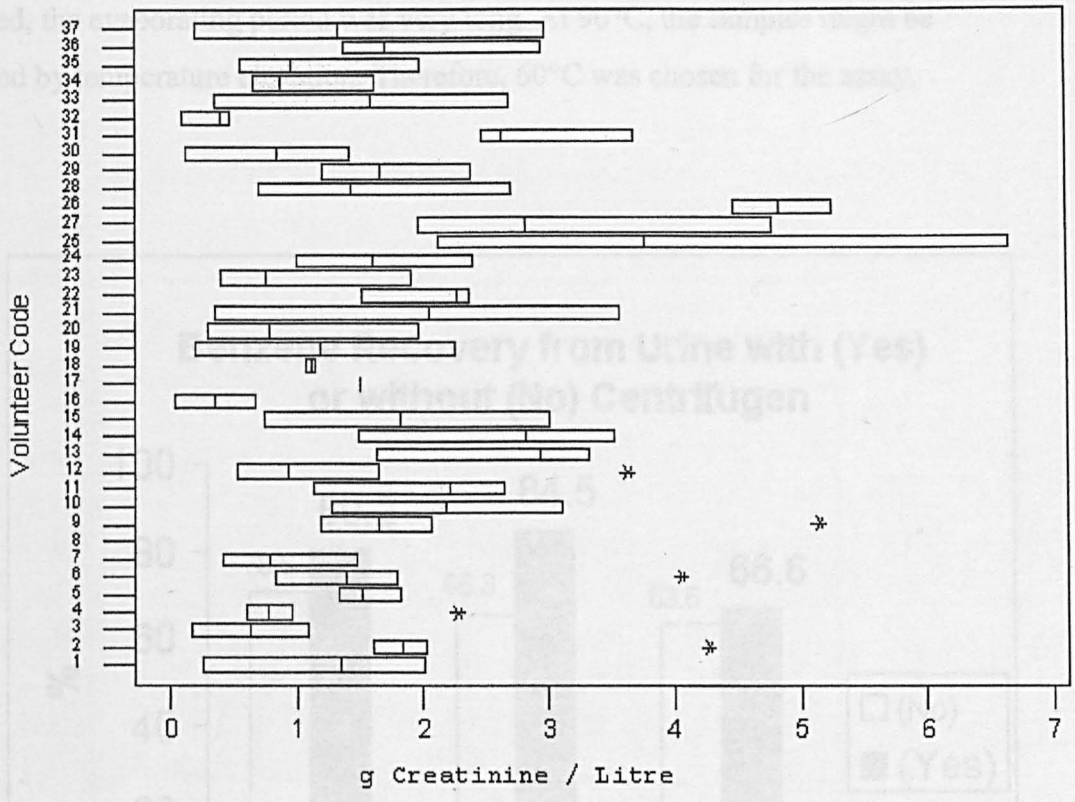


Figure 21: Interindividual variation for urinary creatinine levels. Outlier points plotted as (*) sign.

3.9.2 *t,t*-Muconic Acid in Urine

Three standards were prepared in water and control urine; 0, 20, 50, and 80 mg/l. These standards were also prepared in mobile phase-A (MP-A)(Figure 22). Centrifugation of the urine samples improved the subsequent extraction of *t,t*-muconic acid with butanol (Table 18).

Butanol evaporation was compared in 4-ml and 10-ml conical tubes. At 60°C, the evaporation period ranged between 1 h: 15 min and 1 h: 50 min (N= 30) for 4-ml tube compare to 3 h: 18 min - 5 h: 20 min (N= 72) in 10-ml tube. The evaporation time improved 64%, which saved time and nitrogen gas.

With the 10-ml tubes , a test was prepared to compare samples evaporated at 40°C, 60°C and 90°C 8:10, 4:19 and 3:30, respectively. Although the time was

reduced, the evaporating period was very long. At 90°C, the samples might be affected by temperature elevation. Therefore, 60°C was chosen for the assay.

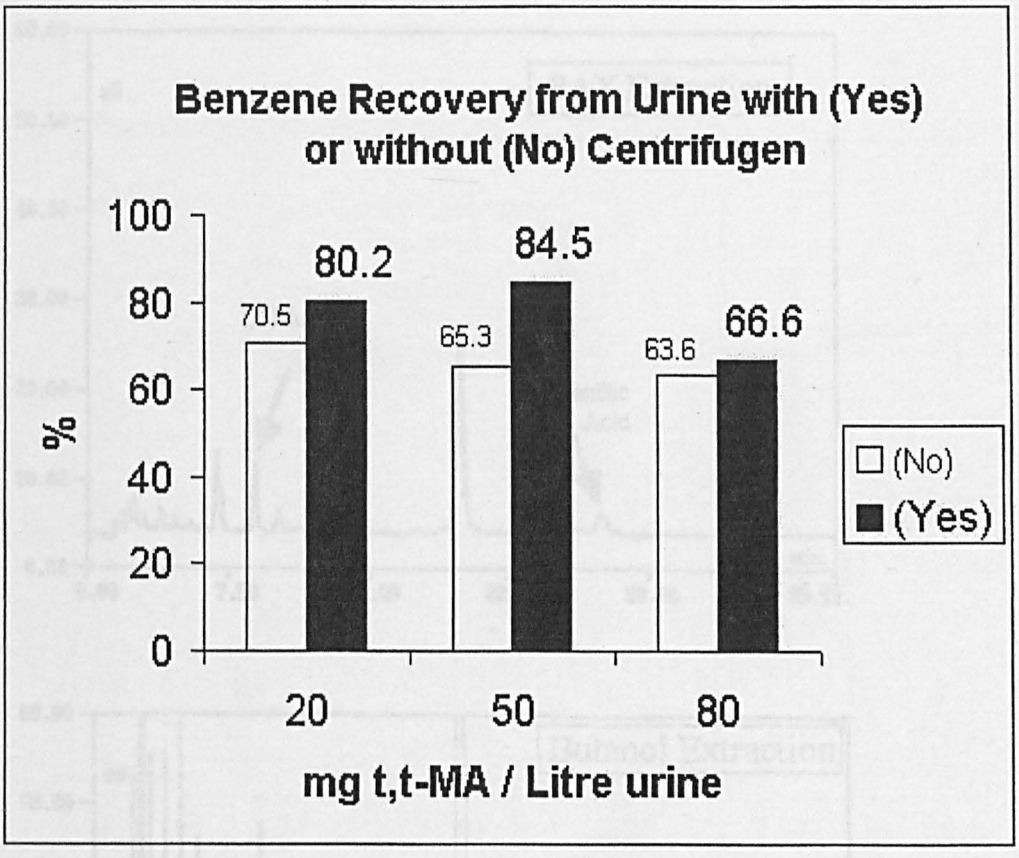


Figure 22: The recovery of *t,t*-muconic acid from blank urine spiked with 20, 50, and 80mg *t,t*-muconic acid /l. Centrifuged urine (Yes) tends to have a higher extraction efficiency with butanol compared to non-centrifuged (NO).

Table 18: Comparison between peak areas for standards not centrifuged before extraction and centrifuged. The not extracted standards were prepared in mobile phase (MP). The extracted *t,t*-muconic acid samples was either centrifuged (Yes) or not centrifuged (No).The recovery of *t,t*-muconic acid was compared the efficiency for the not centrifuged with the centrifuged extracts.

		Centrifuged			
mg/l	MP	No	(%)*	Yes	(%)*
20	1.00	0.71	70.5%	0.80	80.2%
50	2.52	1.64	65.3%	2.13	84.5%
80	4.10	2.61	63.6%	2.73	66.6%
	Mean		**66.5%**		**77.1%**
	S.D.		**3.6%**		**9.4%**

* Recovery % = 100 x ((No or Yes)/MP)

3.9.2.1 *Solvent Extraction versus Solid Phase Extraction*

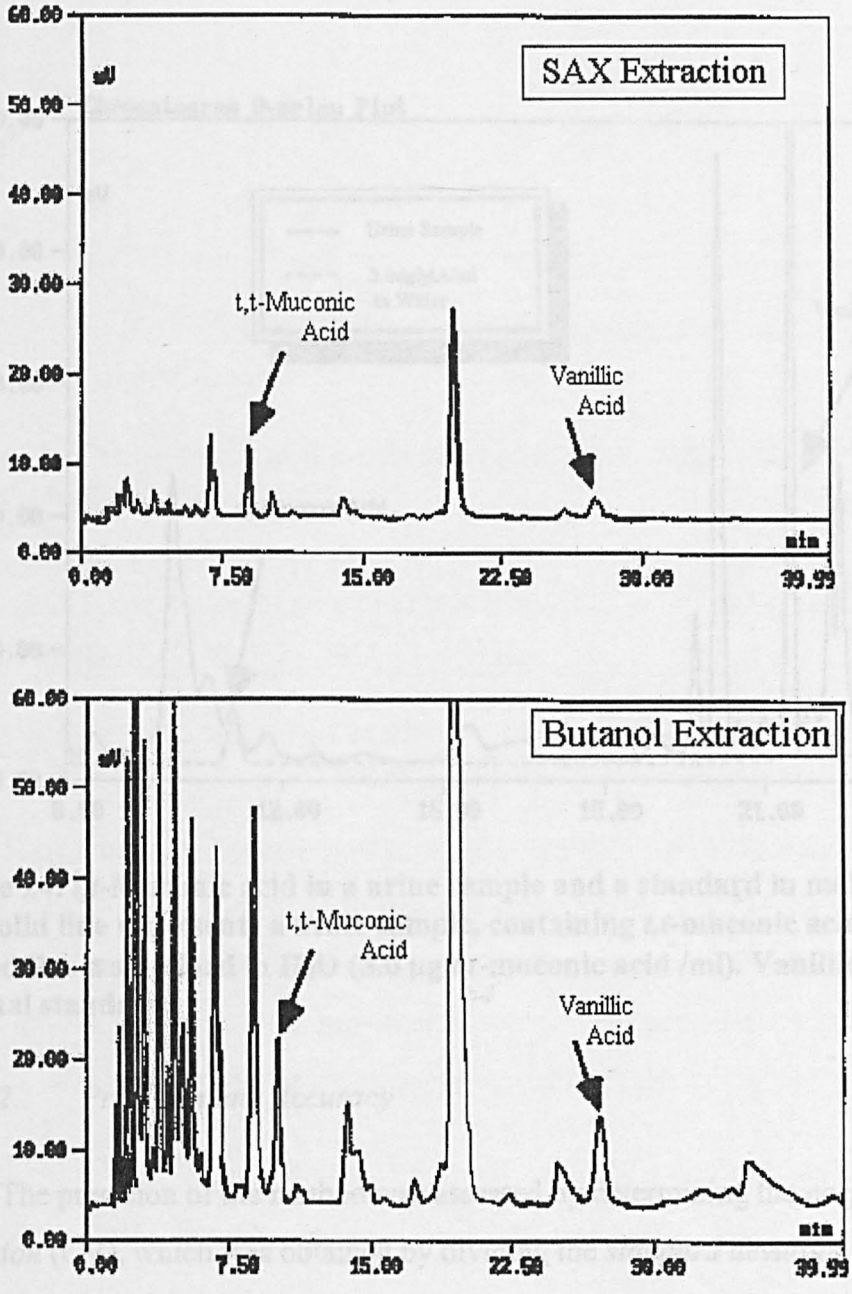


Figure 23: A comparison between *t,t*-muconic acid (1 mgMA/gCr) extracted with SAX (upper) and Butanol (lower).

The analyses indicated that the concentration of *t,t*-muconic acid was four-times greater than after SAX. A comparison of the sensitivity of the two methods is shown in Figure 23. The area of SAX extracted *t,t*-muconic acid was 44% of that following

butanol extraction. Only 7% of vanillic acid that extracted by butanol was extracted by SAX.

Figure 24 shows *t,t*-muconic acid in a urine sample compared with a 3.0 μgMA/ml standard in deionised water.

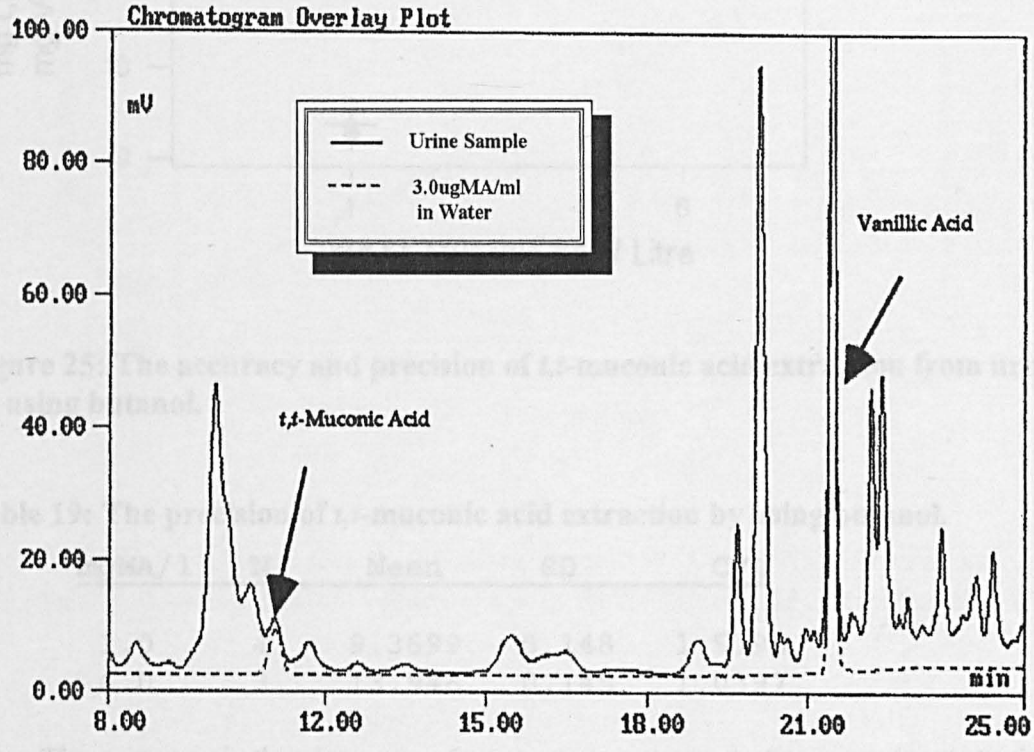


Figure 24: *t,t*-Muconic acid in a urine sample and a standard in mobile phase. The solid line represents a urine sample, containing *t,t*-muconic acid and the dashed line a standard in H_2O (3.0 μg *t,t*-muconic acid /ml). Vanillic acid was the internal standard.

3.9.2.2 Precision and Accuracy

The precision of the method was assessed by determining the *coefficient of variation* (CV), which was obtained by dividing the *standard deviation* by the *mean* of the data, which was expressed as a percentage. A blank urine sample (below the detection limit) was spiked with *t,t*-muconic acid. Two standards were prepared; 1 mg/l and 6 mg/l. Four aliquots of 1 mg/l and three aliquots of 6 mg/l were extracted (Table 19). The CVs for the two standards were 1.58% and 1.04%, respectively (Figure 25). *t,t*-Muconic acid responses were divided by the area of vanillic acid (internal standard).

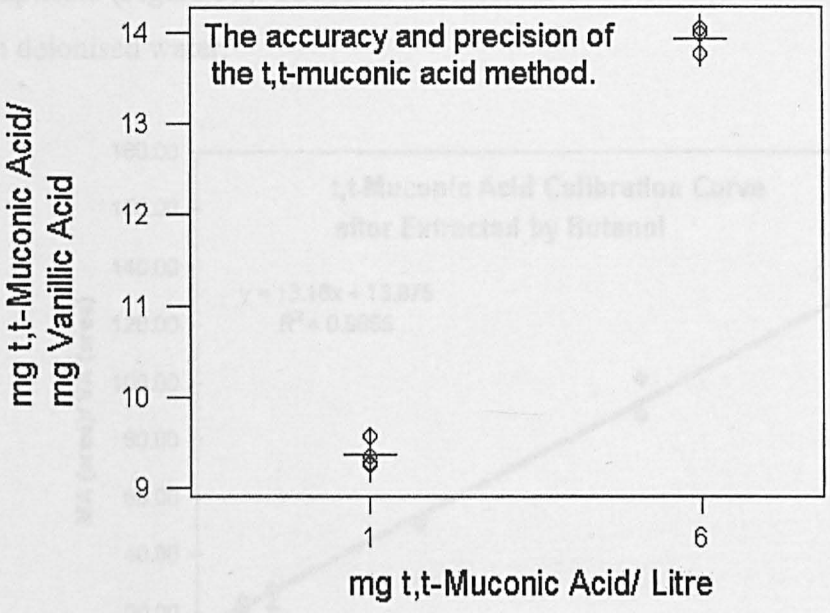


Figure 25: The accuracy and precision of *t,t*-muconic acid extraction from urine by using butanol.

Table 19: The precision of *t,t*-muconic acid extraction by using butanol.

mgMA/l	N	Mean	SD	CV%
1.0	4	9.3699	0.148	1.5795
6.0	3	13.946	0.145	1.0397

The accuracy is the closeness of a measurement or set of measurements to the accepted value and is expressed in terms of error (Skoog et al., 1988). The prediction was determined for six standards prepared in urine and water: 0.2, 0.5, 1.0, 3.0, 6.0, 10.0 mg/l. All standards were extracted five times. The presician (R^2) was 0.975 ($p < 0.001$), which indicated a strong association between the extracted standards and responses.

3.9.2.3 Calibration Curve

t,t-Muconic acid concentration was linear with to the HPLC-UV response. The response was calculated by a ratio for *t,t*-muconic acid peak area divided by vanillic acid peak areas. Very good linearity was obtained for urine samples spiked with several concentrations of *t,t*-muconic acid. Extracted urine standards were analysed. The extracted standards (0.2, 0.5, 1.0, 3.0, 6.0, 10.0 mg/l) were carried out in

duplicate (Figure 26). The limit of detection was 0.01 mgMA/l in standards prepared in deionised water.

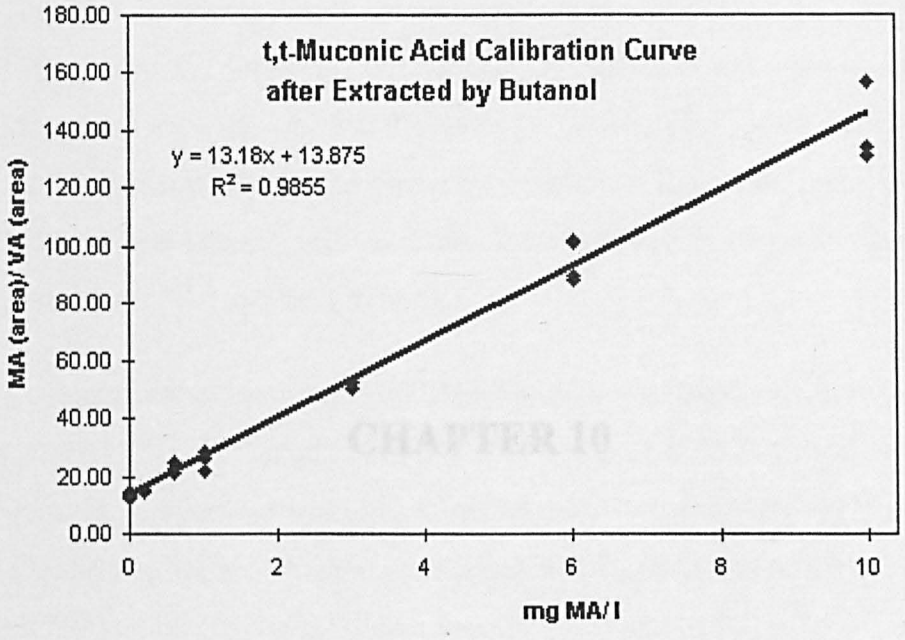


Figure 26: A calibration line for *t,t*-muconic acid standards prepared in urine and extracted with butanol. Values are area *t,t*-muconic acid / area vanillic acid (IS).

CHAPTER 10

Chapter 10: Urinary 8-Hydroxy-2'- Deoxyguanosine

The electrochemical detector showed four- fold lower detection limit for 8OHdG than UV. The detection limit, reported in the literature for electrochemical detection, varied between 0.07 and 0.2 ng/ml (Bogdanov et al., 1999, Loft et al., 1992). However, in ELISA-based method, the detection limit was 1ng/ml (Witherell et al., 1998). The electrochemical detection limit in this study was 0.44 ng/ml, and 1.77ng/ml using UV-detection (260nm).

A typical calibration curve for 8OHdG prepared in deionised water is shown in Figure 27 for UV-detection and Figure 28 for electrochemical detection. Six standards were prepared in deionised water; 12.5, 25, 50, 100, 300, and 500 nM (35, 70, 141, 282, 847, and 1412ng/ml). A good association was found between 8OHdG standards and both UV and electrochemical detectors (R^2=0.96 and 0.997).

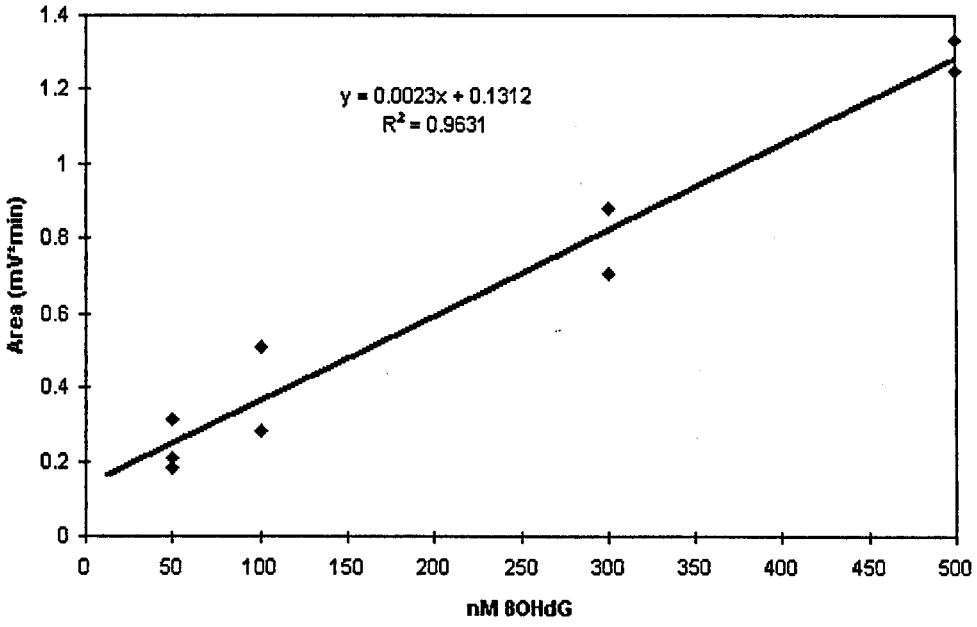


Figure 27: A calibration line for 8OHdG standards (injected quantity=100ul, UV wavelength= 260nm).

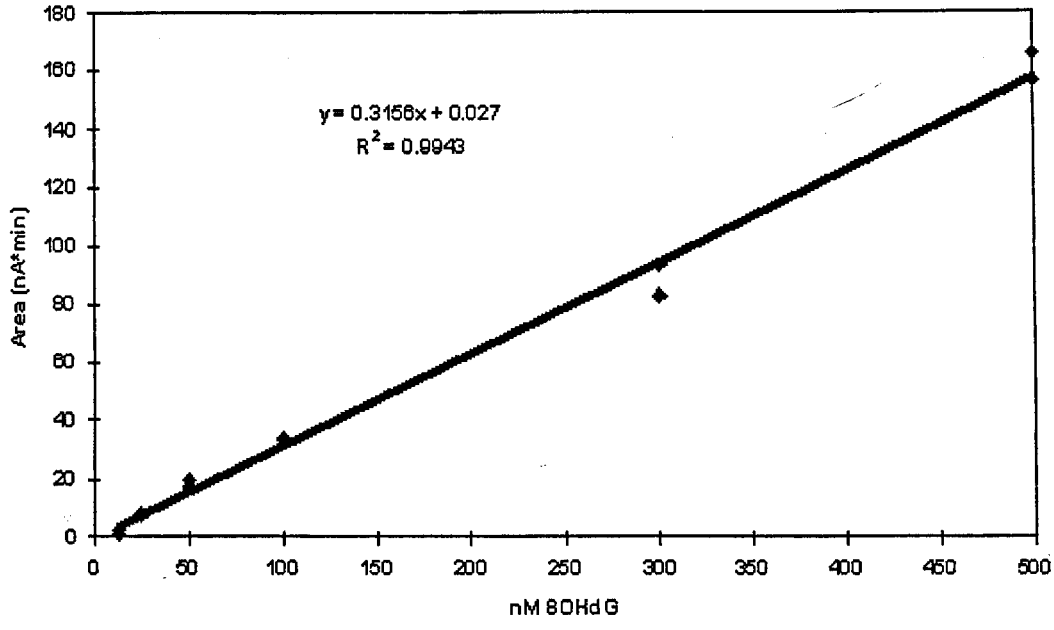


Figure 28: A calibration line of 8OHdG standards in electrochemical detection (injected quantity= 100ul, E=600mV, I range= 100nA).

3.10.1 Internal Standard

Radiolabelled 1',2'-^{3}H OHdG has been shown to be a good internal standard for measurement of 8OHdG (Shigenaga et al., 1990). Unfortunately, it is not produced commercially and it's synthesis and analysis required very sophisticated instrumentation that was not available in our laboratories. To determine a suitable alternative internal standard, the following chemicals were assessed:

(1)	4-Chloroaniline	(2)	1-Naphthol
(3)	Pseudouridine	(4)	Cytidine
(5)	3-Methylcytidine	(6)	Uridine
(7)	1-Methyladinosine	(8)	Thiocytidine
(9)	Methylquanosine	(10)	2'-O-Methylcytidine
(11)	Inosine	(12)	Ribothymidine
(13)	5-Hydroxy methyl-2'-deoxyuridine	(14)	3-Deazauridine

None of the tested chemicals were suitable as an internal standard. None of them was detectable by electrochemical detection except for 4-chloroaniline and 1-naphthol. 4-Chloroaniline took about 3h to be eluted under the assay set-up while

8OHdG was eluted at about 45min. 1-Naphthol couldn't be eluted until the column was washed with 50% methanol.

3.10.2 8-Hydroxy-2'-Deoxyguanosine Recovery

A standard (50 nM 8OHdG) was prepared in phosphate buffer (0.1 M Na_2HPO_4) and its recovery determined. The recovery was 38%. The extraction was carried out as mentioned previously. 8OHdG standards (10, 20, 30, and 50 nM) give a regression coefficient of 0.996 (Figure 29).

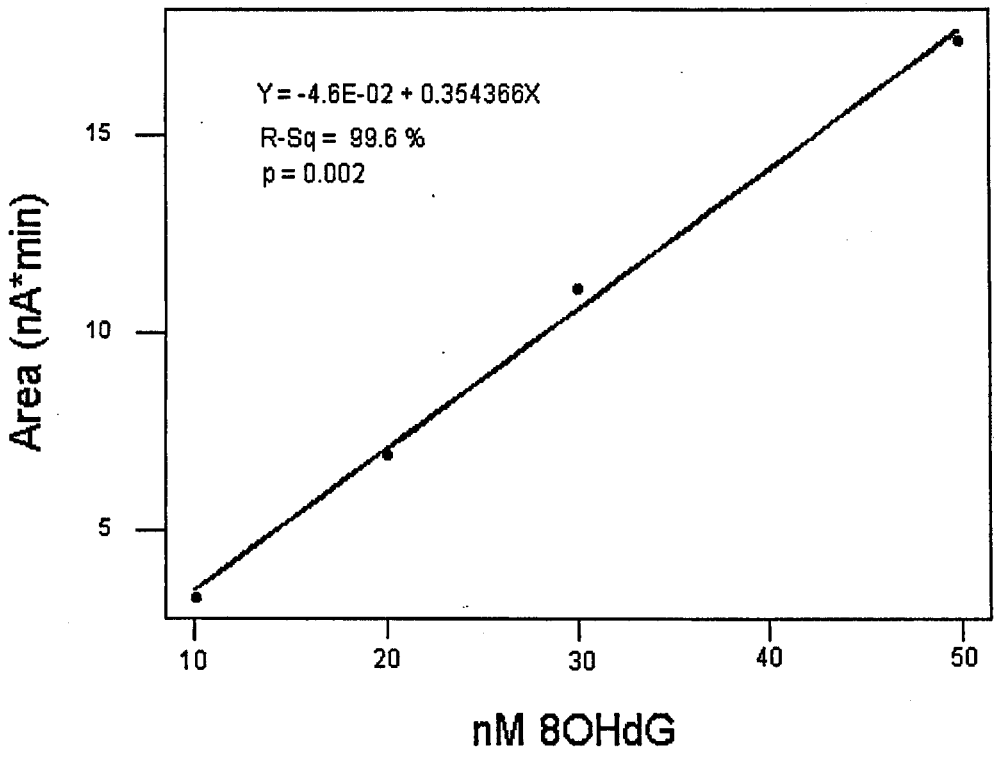


Figure 29: A calibration line for standards of 8OHdG extracted from water standards by SPE and detected by-electrochemical detector.

Two 8OHdG standards were also extracted from urine. Two 0.5-ml aliquots of the urine were spiked with high concentrations of 8OHdG, 88 and 880 μg/ml. Although the standards extracted from water showed a huge peak by electrochemical and UV detection, no peak was observed in the standards extracted from urine.

Several difficulties were faced with 8OHdG assay. Urinary 8OHdG extraction was not reproducible even after modification of the sample pH. 8OhdG in urine could not be recovered from the SPE tube, unlike the standard prepared in deionised water. Also, no suitable internal standard was found. Moreover, the length of the assay (60 min) was long compared to the ELISA assay. The glass cell in the electrochemical detector deteriorated rapidly (2-4 days).

CHAPTER 11

Chapter 11: BTEX in air

This chapter discusses the validation of the analytical method used for BTEX determination. Carbon disulphide (CS_2) was purified using 13X. Various BTEX mass fragments were assessed to improve the chromatograph and to minimise interfering peaks during GC-MS. A calibration line was prepared and the relationship between BTEX concentrations and GC-MS responses determined.

3.11.1 CS_2 Purification and 13X Regeneration

The method used to purify CS_2.reduced its benzene concentration. Benzene eluted more slowly from the 13X column than carbon disulphide. Therefore the faster the carbon disulphide passed through the column, the lower the benzene concentration was obtained. Some contaminants were found in the purified carbon disulphide by GC-FID even after the purification. But because these contaminants eluted later than the xylenes, they did not interfere with the analytical method.

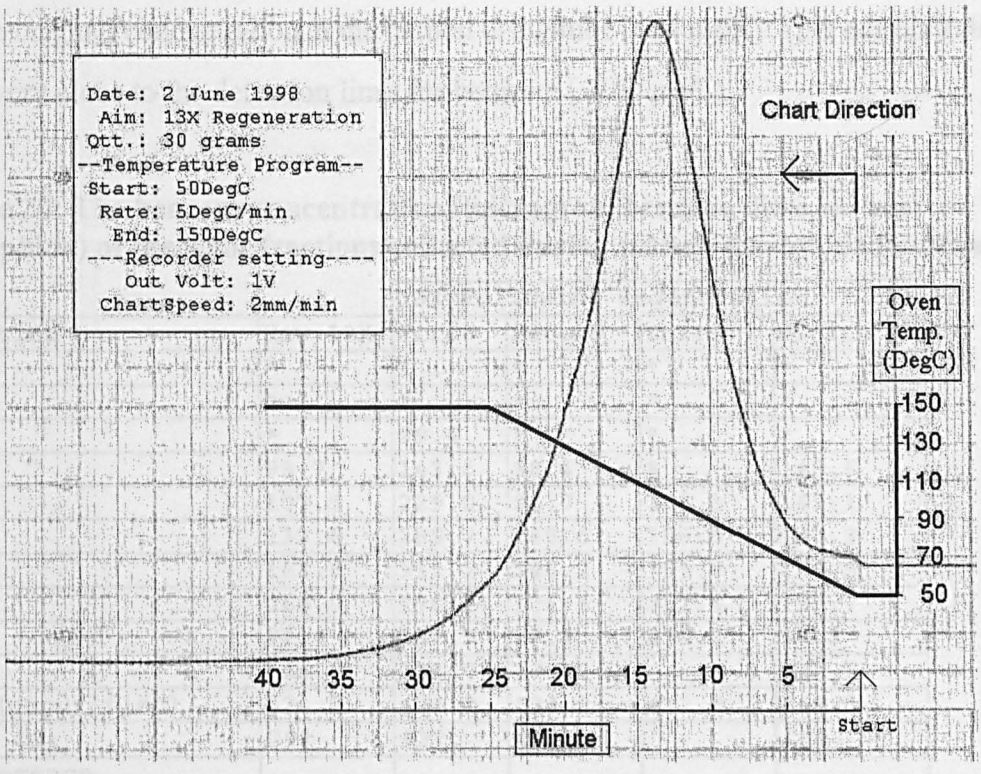


Figure 30: Regeneration of 13X and the analysis carried on by GC-FID

The 13X material was regenerated once it had been used to purify CS_2.four times. The accumulated retained hydrocarbons were removed by heating up the used 13X until it reached 150°C while passing nitrogen gas through it. One hour was enough to clear 13X of retained hydrocarbons (Figure 30). The 13X was regenerated four times.

However regeneration of 13X demonstrated a reduction in the benzene removal efficiency. The more the 13X was regenerated, the lower the benzene removed efficiency. Different breakthrough conditions were obtained from the process. The 13X bed might be disturbed if the wetting process for the bed wasn't prepared properly. The fast flow of carbon disulphide might produce an air bubble, which would reduce the filtration efficiency. The column affluent must not be blocked during the wetting process, which might end up with the same problem. In fresh 13X, the first three collected fractions (1-3 ml carbon disulphide /g13X) all had no detectable benzene. However after the first regeneration, only the first two fractions has no benzene. It was recommended that the first eight fractions (1-8ml carbon disulphide /g13X) of purified carbon disulphide should be collected in order to achieves low benzene concentration (Michalko and Phillips, 1989). The benzene concentration in the pooled eight carbon disulphide fractions (124 μg/L) was one third

of the original concentration in the carbon disulphide (424 μg/L). This concentration was very close to the detection limit for benzene (~100 μg/L).

Table 20: The benzene concentration (microgram benzene /litre carbon disulphide) of the eight fractions collected during carbon disulphide purification.

		13X Following Regeneration			
Fraction	New 13X	First	Second	Third	Fourth
1	*	*	*	*	*
2	*	*	*	*	*
3	*	99.4	*	*	*
4	98.7	143.6	98.4	*	145.6
5	107.5	229.0	96.7	364.3	149.1
6	133.6	289.7	145.6	356.5	152.9
7	188.7	311.2	146.2	359.7	156.5
8	250.9	270.1	124.3	352.4	157.7
Mean	**97.4**	**167.9**	**76.4**	**179.1**	**95.2**
1-8	123.7	224.7	178.9		148.5
CS_2 (unpurified)	424.2	424.2	424.2	412.9	248.1
1-8/ CS_2	*29%*	*53%*	*42%*		*60%*
Difference (1-8)- Mean	9.9	39.3	38.8		20.2
md6/ CS_2	*14%*	*33%*	*17%*		*34%*

* below the limit of detection.

The concentration of benzene in the combined six fractions of CS_2.was determined. In the first, second, and fourth regenerations of 13X, benzene concentration fell between 17% and 34%. The maximum benzene concentration after purification was about one third of original concentration. *1-8/* CS_2 unpurified was calculated by dividing *1-8* by CS_2 then multiplying by 100. The expected benzene concentration of the six combined fractions is presented in percentage in *md6/* CS_2 raw, calculated by adding the total benzene in the first six fractions plus *Difference (1-8) -Mean.*

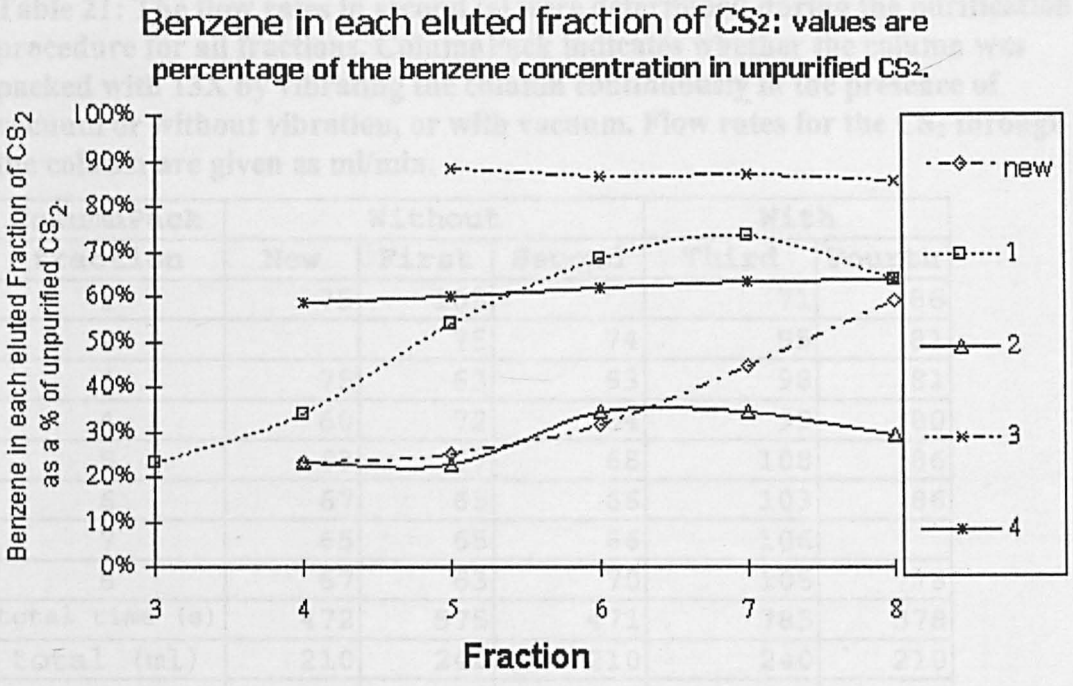


Figure 31: Benzene concentration in each eluted fraction of CS_2.

Table 21: The flow rates in second (s) were determined during the purification procedure for all fractions. ColumnPack indicates whether the column was packed with 13X by vibrating the column continuously in the presence of vacuum or without vibration, or with vacuum. Flow rates for the CS_2 through the column are given as ml/min.

ColumnPack	Without			With	
fraction	New	First	Second	Third	Fourth
1	75	105		71	86
2		75	74	95	81
3	75	63	63	98	81
4	60	72	64	99	80
5	63	67	68	108	86
6	67	65	66	103	86
7	65	65	66	106	
8	67	63	70	105	78
total time (s)	472	575	471	785	578
total (ml)	210	240	210	240	210
g 13X	27	30	27	30	27
ml/min	26.69	25.04	26.75	18.34	21.80

There was a gradual increase of benzene concentration in new13X (Standard Deviation 6 - 20%)(Figure 31). The third and fourth regenerated 13X demonstrated almost flat profile for benzene concentration in CS_2 and contained 34% of the benzene concentration of unpurified CS_2. This flat pattern of concentration might be due to compaction of the packing, which reduced the flow rate. Therefore, the regenerating process can be carried on up to four times and still produce CS_2, which is one third of the benzene concentration of unpurified carbon disulphide.

Purified carbon disulphide was therefore collected from the first eight fractions when new 13X was used. When regenerate 13X were collected used only the first six fractions were collected.

3.11.2 Mass Fragmentation

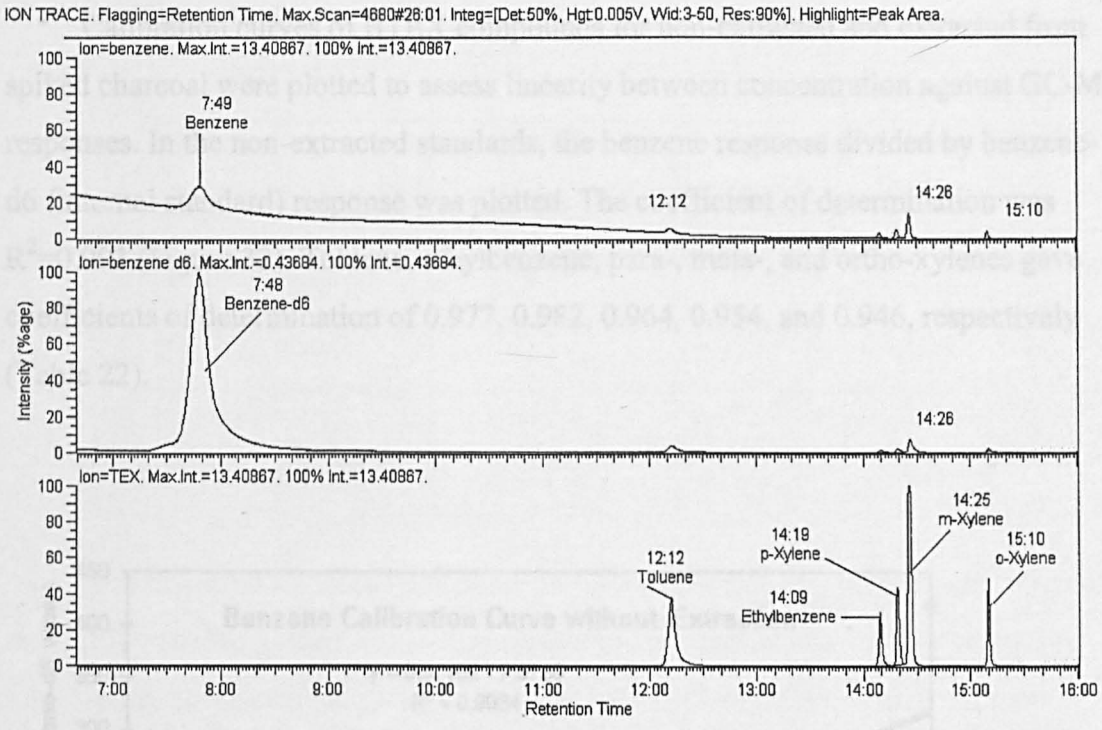


Figure 32: GC-MS in SIM mode was used to optimise single ion fragments in BTEX compounds. Benzene (r.t.= 7:49), toluene (r.t. = 12:12), ethylbenzene (r.t. = 14:09), and (p-,m-,and o-) xylenes (r.t. = 14:19, 14:25, and 15:10, respectively) were monitored. Benzene ion monitored at m/z 78 (top). Deutrated benzene ion (r.t. = 7:48) was monitored at m/z 84 (middle). Toluene, ethylbenzene, and (p-,m-,o-) xylenes major ion fragments were monitored at m/z 91 (bottom). r.t.: retention time.

Mass fragments for BTEX compounds were determined. The chemical mass spectrum was acquired by using SCAN mode. Benzene (m.w.78) was diluted in redistilled CS_2 and the dominant mass was at m/z 78 (100%). In toluene (m.w.92) mass spectrum, m/z 91 (100%) was the most dominant mass, while m/z 92 was 60%. Deutrated benzene (m.w.84) was the internal standard, and the dominant detected mass was m/z 84. Xylene isomers and ethylbenzene (m.w.106) dominant mass was for the fragment mass m/z 91. Neither m/z 106 nor m/z 105 mass had major detection in the xylene mass spectrum.

The detection limit for benzene and toluene were determined in single ion recording (SIR) mode. The benzene detection limit was 0.22 ng at m/z 78 and the detection limit for toluene was 0.21 ng at m/z 91.

3.11.3 BTEX Measurement in Air

Calibration curves of BTEX compounds for non-extracted and extracted from spiked charcoal were plotted to assess linearity between concentration against GC-MS responses. In the non-extracted standards, the benzene response divided by benzene-d6 (internal standard) response was plotted. The coefficient of determination was R^2=0.903 (Figure 33). Toluene, ethylbenzene, para-, meta-, and ortho-xylenes gave coefficients of determination of 0.977, 0.982, 0.964, 0.954, and 0.946, respectively (Table 22).

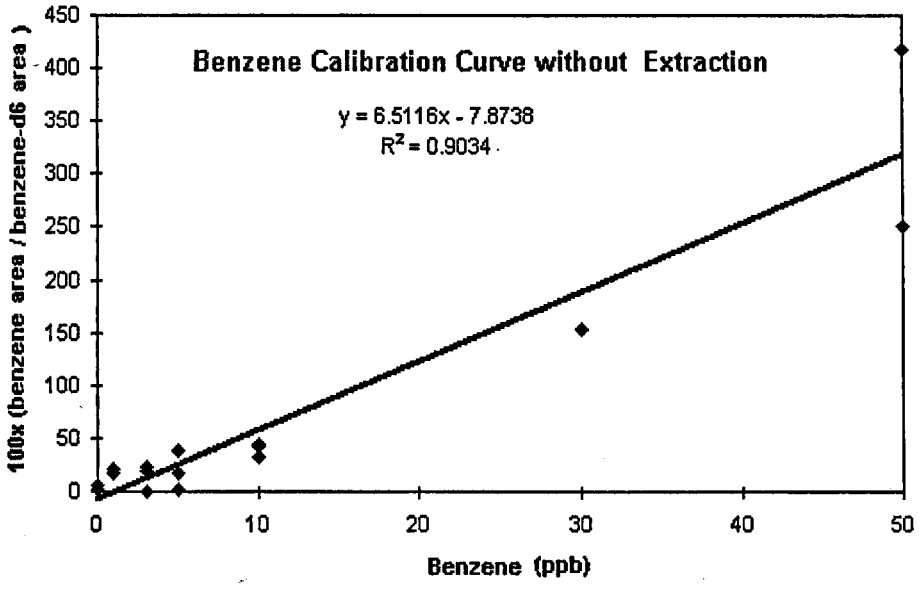


Figure 33: A benzene calibration line for non-extracted standards using benzene -d6 as internal standard..

Table 22: Calibration curves for BTEX components adjusted using benzene-d6 as internal standard.

Chemical	Intercept	Slope	R^2
Benzene	- 0.079	+ 0.065	0.903
Toluene	- 0.132	+ 0.092	0.977
Ethylbenzene	- 0.095	+ 0.035	0.982
p- Xylene	- 0.125	+ 0.042	0.964
m- Xylene	- 0.372	+ 0.127	0.954
o- Xylene	- 0.116	+ 0.037	0.946

For extracted standards, the benzene response divided by benzene-d6 (internal standard) was also plotted. The coefficient of determination was 0.976 (Figure 34). For toluene, ethylbenzene, para-, meta-, and ortho-xylenes, the coefficients were 0.970, 0.917, 0.934, 0.941, and 0.907, respectively.

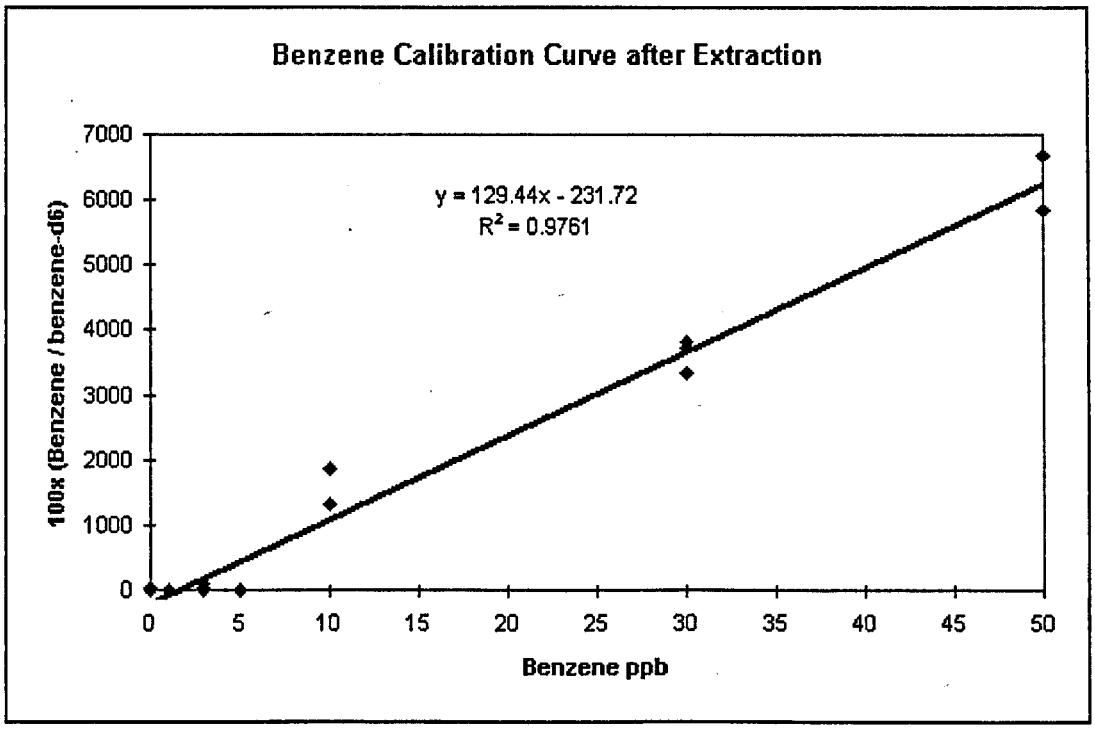


Figure 34: Benzene calibration line for extracted standards using benzene -d6 as internal standard.

Table 23: The linearity of extracted BTEX standards using benzene -d6 as internal standard.

Chemical	Intercept	Slope	R2
Benzene	- 2.32	+ 1.29	0.976
Toluene	- 3.86	+ 2.07	0.970
Ethylbenzene	- 1.70	+ 0.80	0.917
p- Xylene	- 1.54	+ 0.76	0.934
m- Xylene	- 5.19	+ 2.29	0.941
o- Xylene	- 1.08	+ 0.520	0.907

CHAPTER 12

Chapter 12: Monitoring of Samples from Car Drivers

The recruitment of volunteers started from 28/1/1998 until 12/12/2000. Air and urine sample collections for benzene exposure measurements began in 29/1/1998 until 4/6/1999. The age of the volunteers varied between 16 to 68 years on the day of sample collection.

The volunteer study was designed to study the relationship between benzene exposure and *t,t*-muconic acid excretion in urine for car drivers and to consider the influence of potential confounders on this relationship. A further extension of the study was to determine twenty-four hour profiles for urinary *t,t*-muconic acid excretion.

The normal distribution (Mendenhall et al., 1999) is characterised with a symmetric sampled population. The samples that have detectable *t,t*-muconic acid levels (n=224) was tested for normality. The direct calculation for the original data fails to behave in normal distribution pattern.

After transforming the data to be logarithmic to the base 10, the distribution show two curves. One of them is for the detectable samples and the other for the undetectable samples. Because the undetectable samples were assigned 0.01 mgMA/gCr (half the limit of detection), therefore, sharper curve can be observed in the left side of Figure 36. This pseudo-normal distribution was due to the variation of creatinine levels, not based on the level of *t,t*-muconinc acid. Two undetectable *t,t*-muconinc acid with 0.05 and 4.277gCr are calculated as 0.2 and 0.00233mgMA/gCr.

This difference was totally due to creatinine level. Therefore excluding the undetectable samples would avoid facing this bias. Also after excluding the undetectable samples, the the normal distribution was found (Anderson-Darling Normality Test, p= 0.472) as in Figure 37 for Pre-samples, and Figure 38 for Post-samples. Other studies confirmed this finding (Ghittori et al., 1995, Ong et al., 1995, Ong et al., 1996, Javelaud et al., 1998). Even After subdividing the data to three groups (control, petrol exposed, diesel exposed). Therefore, the parametric statistical tools were used in this study. Different tools were selected carefully based on the study design. Minitab (release 12) statistical package was used to do the statistical

analysis (Minitab, 1997). The statistics were either to compare one variable in two or more groups of samples or testing the association between two variables.

If two independent groups of samples were tested for the same variable, then two-sample t-test was the choice. Two-sample t-test is used to perform a hypothesis test and compute a confidence interval of the difference between two population means when the population standard deviations are unknown (Mendenhall et al., 1999). In the following example, benzene concentration (ppb) in air was tested after obtained from inside cars cabin that fuelled with petrol and diesel.

If two samples were taken from the same group under different circumstances (before and after exposure), then paired t-test is the choice. When observations are paired, the paired t -test is used to compute a confidence interval and perform a hypothesis test of the difference between population means (Mendenhall et al., 1999). A paired t-procedure matches responses that are dependent or related in a pairwise manner. This matching allows you to account for variability between the pairs usually resulting in a smaller error term, thus increasing the sensitivity of the hypothesis test or confidence interval. Typical examples of paired data include measurements on twins or before-and-after measurements.

If more than two groups of samples were tested, then one-way analysis of variance is chosen. Analysis of variance extends the two-sample t-test for testing the equality of two population means to a more general null hypothesis of comparing the equality of more than two means, versus them not all being equal (Mendenhall et al., 1999). One-way analysis of variance tests the equality of population means when classification is by one variable. The classification variable, or factor, usually has three or more levels (one-way ANOVA with two levels is equivalent to a t-test), where the level represents the treatment applied.

The one-way procedure to examine differences among means using multiple comparisons. The multiple comparison methods compare different means and use different error rates. Tukey-Kramer method was chosen to examine all pairwise comparisons of means. The Tukey method result is calculated in confidence interval form to assess the practical significance of differences among means, in addition to statistical significance. As usual, the presumed hypothesis of no difference between

means is rejected if and only if zero is not contained in the confidence interval. The example for one-way ANOVA is when differences between three categories of urinary t,t-muconic acid before exposure were determined. Control (n), diesel (d), and petrol (p) groups were tested in the result section.

Testing the two variables (bivariate data) association determined by correlation (r), linear regression (R^2), and chi-square (χ^2). The correlation between two continuous variables could be determined by correlation coefficient (r), which measure the strength of linear relationship. Pearson correlation coefficient (r) is used to measure the degree of linear relationship between two variables (Mendenhall et al., 1999). The (r) value is ranged between +1 to –1. If one variable tends to increase as the other decrease, the (r) is negative. Vice versa, if the two variables tend to increase together, the (r) is positive.

However, the best-fitting line (least squares) can be determined by the coefficient of determination (R^2). Regression analysis is used to investigate and model the relationship between a response variable and one or more predictors (Mendenhall et al., 1999). Least squares procedures are used when response variable is continuous. Least-squares method estimates parameters in the model so that the fit of the model is optimised. Least squares minimise the sum of squared errors to obtain parameter estimates.

If the two variables are categorical data, then chi-square (χ^2) will be the choice. The chi-square tests the association in a two-way classification (Mendenhall et al., 1999). It can be used when the probabilities of items or subjects being classified for one variable depend upon the classification of another variable. The χ^2 statistics investigate a test of hypothesis of the independence of two methods of classification. This hypothesis is based on H_0 (the two methods of classifications are independent) and H_1 (the two methods of classifications are dependent).

3.12.1 *t,t*-Muconic Acid in Urine Samples

Urinary *t,t*-muconic acid levels for all the individuals investigated are shown in appendix I. The recruited numbers of subjects in the control, petrol and diesel groups were 14, 9, and 8, respectively. Before transforming the data, the data behaved non-

parametrically. The general distribution for *t,t*-muconic acid levels has skewed positively. The *t,t*-muconic acid level of very extreme sample is 68.36 mgMA/gCr. This sample was unique because the volunteer 15 had smoked more than 11 cigarettes and drank more than 2 pints of beer prior to sampling. This sample was excluded from the study. The samples' median after excluding the extreme sample become 0.180 mgMA/gCr and the levels varied between 0.0004-7.71 mgMA/gCr.

3.12.1.1 Urinary t,t-Muconic Acid Levels in Petrol and Diesel Car Drivers

Distribution for urinary *t,t*-muconic acid levels were subdivided into three groups; control, petrol and diesel exposures (Figure 35). The distributions were heavily skewed because of the samples that were below the detection limit. The data was transformed to logarithmic values (log MA/gCr) and two different peaks were obtained (Figure 36).

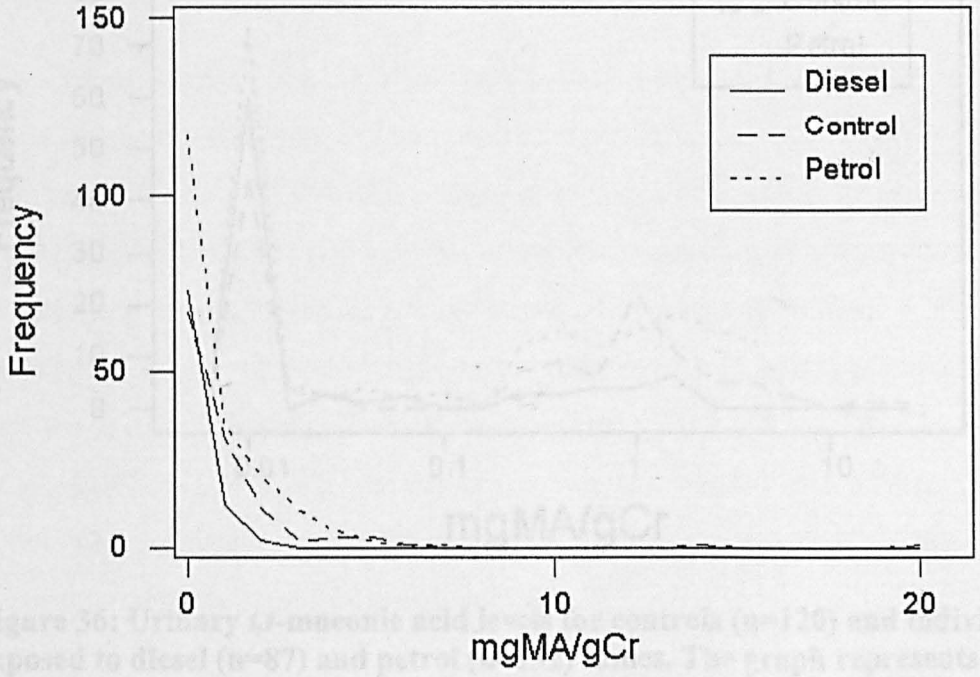


Figure 35: Urinary *t,t*-muconic acid levels for controls (n=120) and individuals exposed to diesel (n=87) and petrol (n=192) fumes.

Kruskal-Wallis test was used to determine the difference between the three groups in the study for non-parametric distributed data. The median of all samples of diesel (n=87), control (n=120), and petrol (n= 192) groups are 0.074, 0.22, and 0.26

mgMA/gCr, respectively. After excluding the undetectable samples, the medians become 0.38 (n=32), 0.36 (n=72), and 0.58 mgMA/gCr (n=120), respectively. Both tests demonstrate highly significant difference among these groups. Unfortunately, one limitation of the non-parametric distributed data is the lack of pairwise tests between the tested groups. Therefore, the data transformation was aimed to test that if the data become normally distributed.

The data transformation was carried on by calculating the logarithm of base 10 for the sample mgMA/gCr level. After executing the statistical tests, the parameters were reversed back to by reversing the log 10 transformations (Figure 36). The data in the rest of this chapter has been transferred unless the un-transformation mentioned.

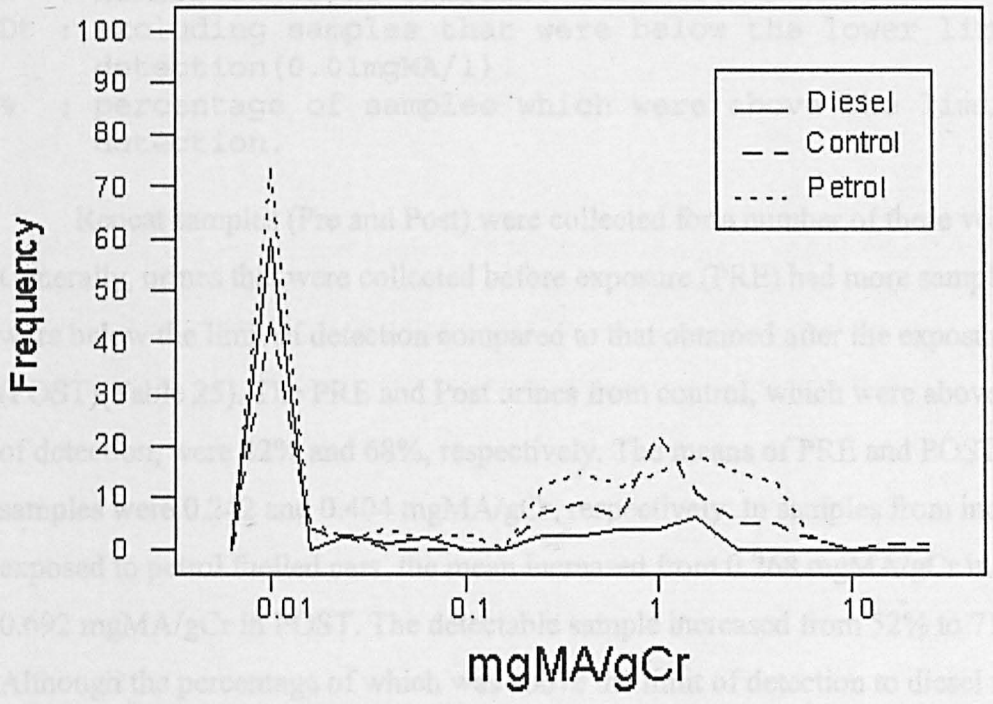


Figure 36: Urinary *t,t*-muconic acid levels for controls (n=120) and individuals exposed to diesel (n=87) and petrol (n=192) fumes. The graph represents logarithm mgMA/ gCr.

After transforming the data, fifty three percent of the samples (Table 24) were less than the limit of detection for *t,t*-muconic acid in urine (0.01 mgMA/l). The *t,t*-muconic acid concentration mean was 0.079mgMA/gCr for all samples (n=399). The mean, after excluding the samples that below the limit of detection, was 0.376mgMA/gCr (n=224). Sixty percent of urine samples that obtained from exposure

to petrol fuelled cars and controls had *t,t*-muconic acid levels which were above the limit of detection . However, only thirty six percent of urine samples that obtained from individuals who exposed to diesel fuelled cars were above the limit of detection.

Table 24: Urinary *t,t*-muconic acid levels including and excluding samples which were below the limit of detection (0.01mgMA/l) values are expressed as mgMA/ g Creatinine.

	All		All Dt		
Group	(N)	Mean	(N)	Mean	%
Control	(120)	0.096	(72)	0.361	60%
Petrol	(192)	0.121	(120)	0.468	63%
Diesel	(87)	0.024	(32)	0.181	36%
Total	(399)	0.079	(224)	0.376	56%

N : number of urine samples.
Dt : excluding samples that were below the lower limit of detection(0.01mgMA/l).
% : percentage of samples which were above the limit of detection.

Repeat samples (Pre and Post) were collected for a number of these volunteers. Generally, urines that were collected before exposure (PRE) had more samples that were below the limit of detection compared to that obtained after the exposure (POST)(Table 25). The PRE and Post urines from control, which were above the limit of detection, were 42% and 68%, respectively. The means of PRE and POST control samples were 0.242 and 0.404 mgMA/gCr, respectively. In samples from individuals exposed to petrol fuelled cars, the mean increased from 0.268 mgMA/gCr in PRE to 0.692 mgMA/gCr in POST. The detectable sample increased from 52% to 71%. Although the percentage of which was above the limit of detection to diesel fumes exposure, the mean *t,t*-muconic acid decreased.

Table 25: The mean and percentage of PRE and POST urines which were above the limit of detection for *t,t*-muconic acid. The percentage represents the detectable samples over the total samples in these groups.

	PRE (mgMA/gCr)			POST (mgMA/gCr)		
Group	(N)	Mean	%	(N)	Mean	%
Diesel	(12)	0.268	28%	(20)	0.143	46%
Control	(16)	0.242	42%	(56)	0.404	68%
Petrol	(43)	0.268	52%	(77)	0.692	71%
Total	(78)	0.254		(157)	0.462	

N : number of urine samples
% : percentage of urine samples which were above the limit for *t,t*-muconic acid.

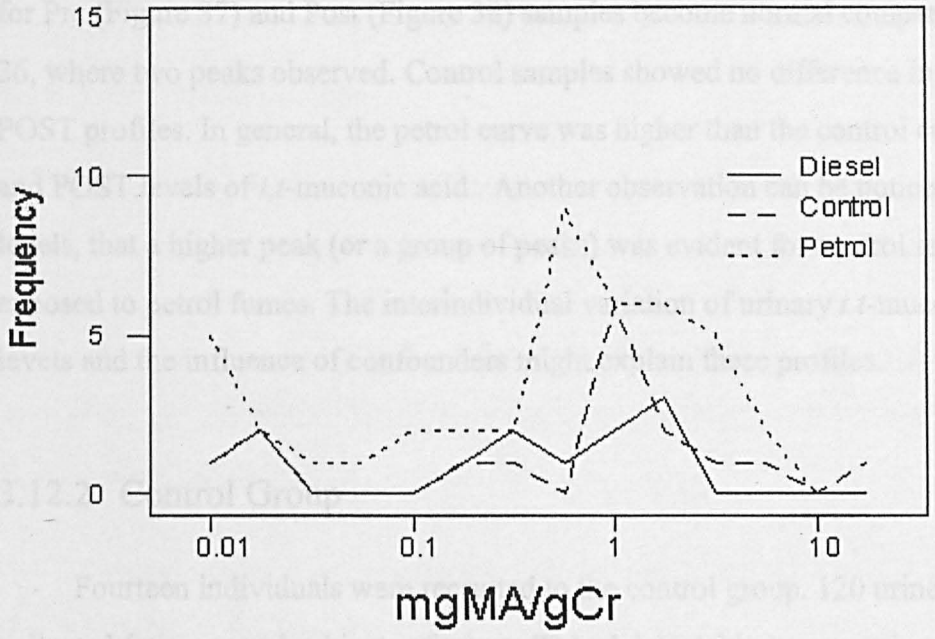


Figure 37 : Urinary *t,t*-muconic acid levels for controls (n=16) and individuals before exposure (PRE) to diesel (n=12) and petrol (43) fumes. Show only samples that above limit of detection. The x-axis is log mgMA/gCr.

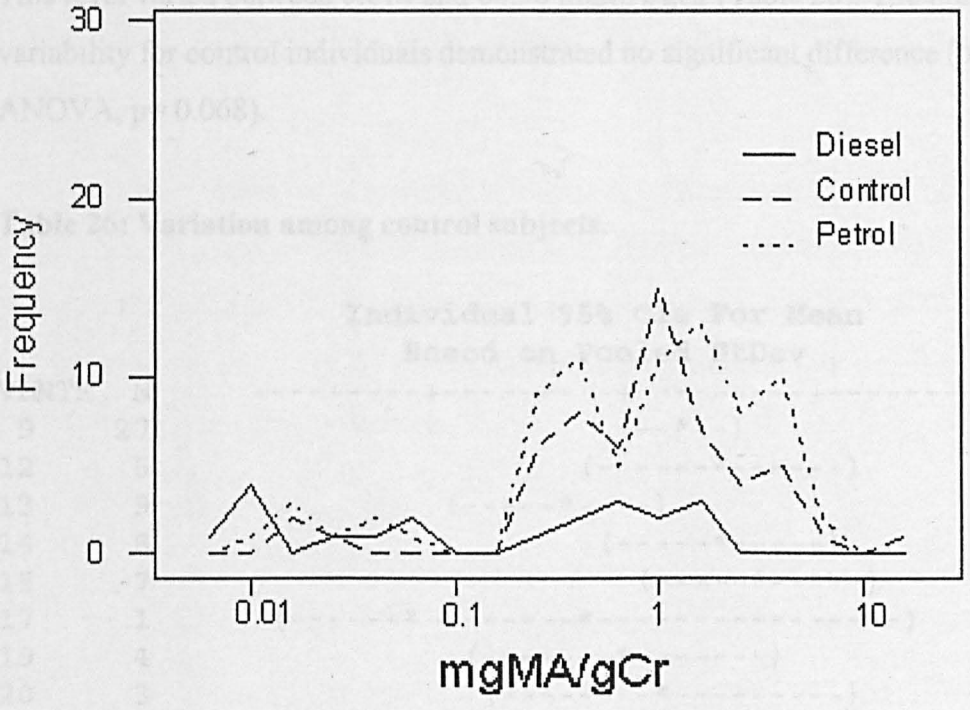


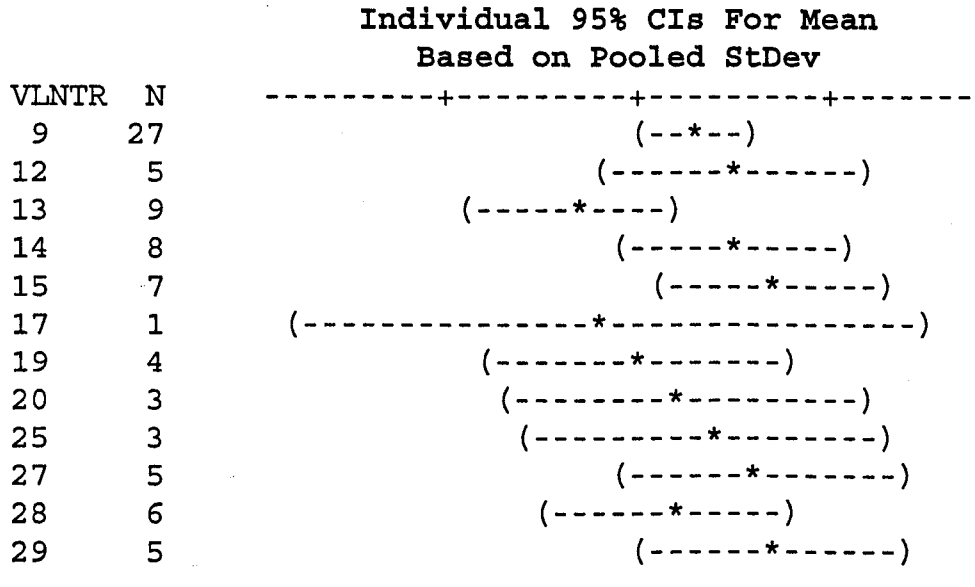
Figure 38: Urinary *t,t*-muconic acid levels for controls (n=56) and individuals after exposure (POST) to diesel (n=20) and petrol (77) fumes. Show only samples that above limit of detection. The x-axis is log mgMA/gCr.

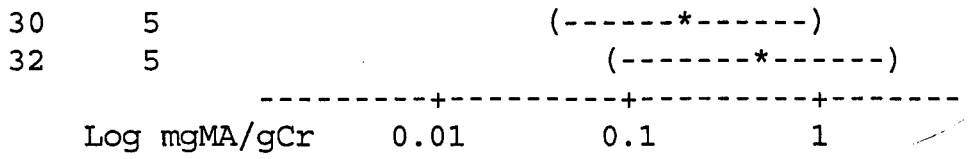
After excluding the non-detectable samples, the distribution of *t,t*-muconic acid for Pre (Figure 37) and Post (Figure 38) samples become normal compare to Figure 36, where two peaks observed. Control samples showed no difference in PRE and POST profiles. In general, the petrol curve was higher than the control curve for PRE and POST levels of *t,t*-muconic acid . Another observation can be noticed for POST levels, that a higher peak (or a group of peaks) was evident for control and individuals exposed to petrol fumes. The interindividual variation of urinary *t,t*-muconic acid levels and the influence of confounders might explain these profiles.

3.12.2 Control Group

Fourteen individuals were recruited to the control group. 120 urine sample were collected from control subjects of whom 72 had detectable *t,t*-muconic acid levels (44% of all control samples). The mean urinary *t,t*-muconic acid concentration of all control samples was 0.096 mgMA/gCr. Following removal of the samples which had *t,t*-muconic acid levels below the limit of detection the mean was 0.361 mgMA/gCr. This level varied between 0.014 and 64.36 mgMA/gCr (Table 26). The intra-subject variability for control individuals demonstrated no significant difference (one-way ANOVA, p= 0.068).

Table 26: Variation among control subjects.





3.12.3 Exposed Groups

Urine samples after were collected at various time points after a journey by car (POST), fuelled either by petrol or diesel. This variation in the timing of POST samples ranged from 2 h to 21 h. Because the mean of *t,t*-muconic acid half life urine is 5h ±2.3 h, samples taken 2 h to 7 h after the journey were assigned as POST1 (Boogaard and van Sittert, 1995). Samples taken between > 7 h - 21h after the journey were called POST2.

Twenty-one pairs of samples for PRE (mean=0.082 mgMA/gCr) and POST1 (mean = 0.294 mgMA/gCr) were collected. Forty-nine pairs of samples for PRE (mean=0.064 mgMA/gCr) and POST2 (mean=0.0.112 mgMA/gCr) were collected. When both samples that obtained from individuals who exposed to petrol and diesel were combined, POST1 (n=21, paired t-test, p=0.009) and POST2 (n=49, paired t-test, p=0.047) *t,t*-muconic acid levels were significantly different from PRE samples. No significant difference was found between POST1 and POST2 (n=8, paired t-test, p=0.681).

However, there was a significant increase in *t,t*-muconic acid concentration in POST1 urines compared to PRE samples following exposure to petrol (n=20, paired t-test, p=0.008)(Figure 39). Probably because of the sample size of POST2 samples following petrol exposure, no significant statistical elevation was found compared to PRE (n=8, paired t-test, p=0.062).

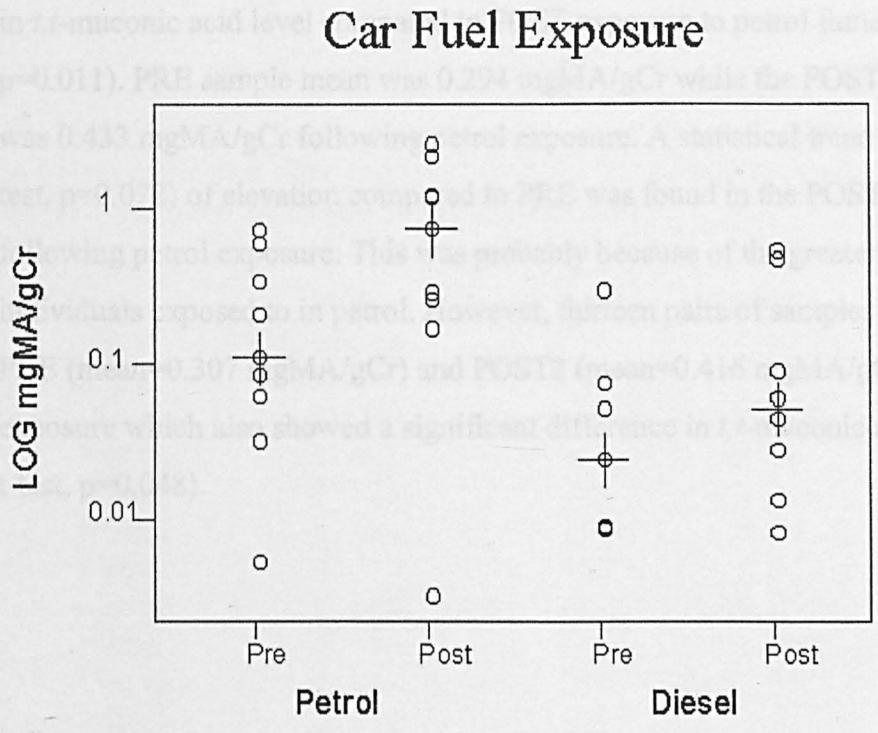


Figure 39: Exposure for petrol and diesel cars samples show the difference between PRE and POST (mean of POST 1 and POST 2) petrol samples (n=9) compared to that of diesel cars (n=7). Plus (+) symbol indicate the median among each group.

Also, there was no significant difference between POST2 and PRE urine samples for individuals exposed to diesel fumes (n=23, paired t-test, p=0.474) (Figure 39). Only one urine sample was obtained for POST1 for exposed to diesel fumes. POST samples were calculated when combining POST 1 and POST 2. In this case the difference between PRE and POST urinary *t,t*-muconic acid level was significant for urinary *t,t*-muconic acid following petrol exposure (n=37, paired t-test, p= 0.001) but not following diesel exposure (n=25, paired t-test, p= 0.463). The means for urinary *t,t*-muconic acid level following exposure to petrol were 0.254 and 0.69 mgMA/gCr for PRE and POST samples, respectively. *t,t*-Muconic acid level in petrol and diesel samples was plotted to show PRE and POST variation (Figure 39).

The number of samples per participant varied between 1 and 10 except for one individual who provided 18 samples. The mean of all samples for the same subject was used to test any difference between exposure to petrol and diesel for PRE and POST samples. Sixteen pairs (PRE and POST) of means representing different individuals, were tested and the PRE samples were shown to be statistically different

in *t,t*-muconic acid level compared to POST exposure to petrol fumes (paired t-test, p=0.011). PRE sample mean was 0.294 mgMA/gCr while the POST sample mean was 0.433 mgMA/gCr following petrol exposure. A statistical trend (n=7, paired t-test, p=0.072) of elevation compared to PRE was found in the POST1 samples following petrol exposure. This was probably because of the greater sample size of individuals exposed to in petrol. However, thirteen pairs of samples were recruited for PRE (mean=0.307 mgMA/gCr) and POST2 (mean=0.416 mgMA/gCr) petrol exposure which also showed a significant difference in *t,t*-muconic acid levels (paired t-test, p=0.048).

Table 27: Levels of urinary mgMA/gCr from volunteers before (PRE) exposure to petrol while and after exposure (POST). POST was derived by combining POST1 (between 2-7h) and POST2 (>7 to 21h).

Fuel	PRE (before)	POST1 (2-7h)	POST2 (>7-21h)	POST (2-21h)
Petrol	0.136	--	0.192	0.192
Petrol	1.825	0.201	--	0.201
Petrol	0.029	0.088	--	0.088
Petrol	0.124	0.190	--	0.190
Petrol	0.079	0.987	0.026	0.506
Petrol	0.452	--	0.096	0.096
Petrol	0.243	--	0.281	0.281
Petrol	0.140	2.570	--	2.570
Petrol	0.490	0.658	--	0.658
Petrol	0.016	--	0.003	0.003
Petrol	0.909	--	1.022	1.022
Petrol	0.717	1.016	0.115	0.566
Petrol	0.049	0.127	--	0.127
Petrol	0.080	0.127	--	0.127
Petrol	0.006	1.228	2.725	1.976
Petrol	0.005	0.138	0.239	0.188
Petrol	0.679	1.343	0.006	0.675
Petrol	0.006	0.006	0.261	0.134
Petrol	1.428	2.769	--	2.769
Petrol	0.073	0.273	4.266	2.269
Petrol	0.413	--	1.982	1.982
Petrol	0.007	--	0.023	0.023
Petrol	0.007	--	0.011	0.011
Petrol	0.168	--	0.424	0.424
Petrol	0.007	--	0.010	0.010
Petrol	0.014	--	0.576	0.576
Petrol	1.028	--	5.201	5.201
Petrol	2.747	--	4.773	4.773
Petrol	1.812	--	3.820	3.820
Petrol	0.009	--	0.025	0.025
Petrol	0.693	--	0.715	0.715
Petrol	0.569	1.405	0.937	1.171
Petrol	0.030	--	0.164	0.164
Petrol	0.807	0.447	--	0.447
Petrol	0.004	2.811	--	2.811
Petrol	0.005	0.003	--	0.003
Petrol	0.201	2.078	--	2.078

(--) Not determined.

Table 28 : Levels of urinary mgMA/gCr from volunteers before (PRE) exposure to diesel while and after exposure (POST). POST was derived by combining POST1 (between 2-7h) and POST2 (>7 to 21h).

Fuel	PRE (before)	POST1 (2-7h)	POST2 (>7-21h)	POST (2-21h)
Diesel	--	--	0.302	0.302
Diesel	0.106	--	0.521	0.521
Diesel	0.093	--	0.493	0.493
Diesel	0.071	--	0.500	0.500
Diesel	0.123	--	0.521	0.521
Diesel	0.005	--	0.479	0.479
Diesel	0.292	--	0.375	0.375
Diesel	0.375	--	0.500	0.500
Diesel	0.012	--	0.005	0.005
Diesel	0.007	--	0.007	0.007
Diesel	0.008	--	0.019	0.019
Diesel	0.007	0.006	--	0.006
Diesel	0.010	--	0.004	0.004
Diesel	0.005	--	0.017	0.017
Diesel	0.406	--	0.382	0.382
Diesel	0.009	--	0.006	0.006
Diesel	0.055	--	0.004	0.004
Diesel	0.015	--	0.005	0.005
Diesel	--	--	0.515	0.515
Diesel	0.005	--	0.003	0.003
Diesel	1.134	--	0.256	0.256
Diesel	0.006	--	0.004	0.004
Diesel	0.378	--	0.411	0.411
Diesel	0.014	--	0.012	0.012
Diesel	0.005	--	0.596	0.596
Diesel	0.007	--	0.004	0.004
Diesel	0.010	--	0.793	0.793

(--) Not determined.

3.12.4 Interindividual Variation

The number of urine samples for volunteers number 2, 4 and 6 who drove either petrol or diesel fuelled cars were 18, 10 and 6 pairs (PRE and POST) of samples, respectively (Table 29). A significant increase (n= 13, p= 0.035) in urinary *t,t*-muconic acid level was found when the mean of POST 1 was compared to the mean of PRE sample from volunteer 2. No difference was found between PRE and POST 2 (paired t-test, n= 11, p= 0.745) or PRE and POST (n= 18, p= 0.085) for volunteer 2. The difference between the means of PRE (0.083 mgMA/gCr) and POST 2 (0.28 mgMA/gCr) in volunteer 4 was significant (n= 10, p= 0.003). A diesel exposed volunteer 6 showed no significant difference (n= 6, p= 0.267) between PRE and POST levels of urinary *t,t*-muconic acid.

Table 29: Interindividual variations in urinary *t,t*-muconic acid levels corrected with creatinine.

			Mean (mgMA/gCr)				
Vl	Fuel	N	Pre	Post1	Post2	Post	p-value*
2	Petrol	13	0.085	0.296	-	-	0.035
2	Petrol	11	0.085	-	0.116	-	0.745
2	Petrol	18	0.106	-	-	0.250	0.085
4	Petrol	10	0.083	-	0.280	-	0.003
6	Diesel	6	0.023	-	0.012	-	0.267

N = number of paired samples (PRE and POST)per volunteer.
* = paired t-test
Vl = Volunteer Code.

3.12.5 Phase II

Seventy-seven samples were collected in phase II in the study. Unlike phase I, no samples were recruited from diesel exposed subjects. Petrol and control samples demonstrated a parametric distribution for *t,t*-muconic acid levels after a logarithmic conversion.

3.12.5.1 Interindividual variability

The difference in *t,t*-muconic acid excretion among the participants reflects the diversity levels between individuals (Figure 40). Volunteer number 36 showed an extremely low *t,t*-muconic acid levels.

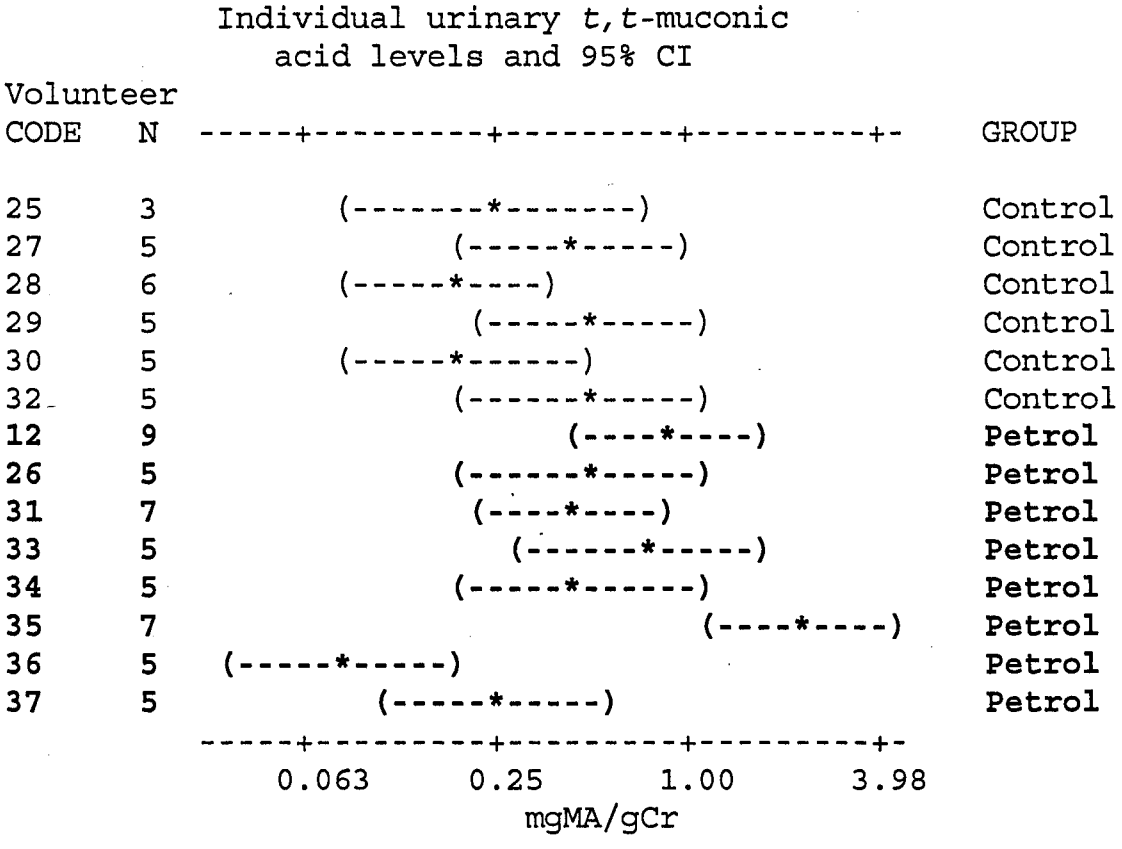


Figure 40: Urinary *t,t*-muconic acid levels (and 95% CI) for control and petrol exposed volunteers. N= stand for number of samples. Group= type of exposure. Bold lines indicate petrol samples exposure. *= mean.

The urinary *t,t*-muconic acid mean for all petrol exposed individuals (n= 48, mean = 0.763 mgMA/gCr) was significantly different (two-sample t-test, p= 0.026) from the control samples (n= 29, mean = 0.603 mgMA/gCr). However, no significant difference was seen when these groups of samples were differentiated for PRE and POST samples (one-way ANOVA, p= 0.277). When subject number 36 was excluded, a significant difference (one-way ANOVA, p= 0.031) was found in *t,t*-muconic acid levels between POST petrol samples (n= 29, mean = 0.863 mgMA/gCr) and POST control samples (n=22, mean = 0.579 mgMA/gCr).

3.12.6 Potential Confounders

The potential confounding factors that tested in the study are smoking, age, petrol source, alcohol consumption, the year of car production, car refuelling during driving, car window status during driving, use of air-conditioning, food preservatives consumption and traffic status (see the questionnaire).

3.12.6.1 Smoking

Control subjects showed no significant difference in urinary *t,t*-muconic acid level (one-way ANOVA, p= 0.677) between smokers (n= 4, mean = 0.22 mgMA/gCr) and non-smokers (n= 11, mean = 0.25 mgMA/gCr). Also, smoking on the day of sampling was not significant. The small number of smokers may have influenced these results. Passive smoking was not distinguished from active smoking in this study.

3.12.6.2 Age and Petrol Station

Age showed no significant correlation with *t,t*-muconic acid levels in urine (Pearson correlation, p>0.05). Furthermore, none of the volunteers lived in the neighbourhood of a petrol station or garage. Therefore, these two factors did not influence the study.

3.12.6.3 Alcohol

As for smoking, alcohol either habitually or on the day of sampling had no significant influence on urinary *t,t*-muconic acid excretion. Questions about the type of alcoholic drink consumed (beer, wine, and spirit) were asked. Because it is illegal to drive under the influence of alcohol. Alcohol consumption did not affect *t,t*-muconic acid levels.

3.12.6.4 Year of Car Production

No differences were found between this factor and any of BTEX levels that investigated, except when new diesel and new petrol cars were tested for benzene. In new petrol vehicles, higher levels of ambient benzene inside the cabin and *t,t*-muconic acid levels were found compared to samples from new diesel cars (Table 30). On the other hand, no significant difference in ambient toluene levels was found in samples form new petrol and diesel cars. Only one sample was taken from an old diesel car, which was insufficient for statistical analysis. No difference was found between ambient benzene, ambient toluene and urinary *t,t*-muconic acid levels for old and new petrol cars.

Table 30: Ambient benzene, toluene, EX (ethylbenzene+xylenes) and urinary *t,t*-muconic acid levels following exposure to petrol and diesel in old and new car cabins.

	Year of Car	Petrol (N) Mean	Diesel (N) Mean	p-value*
Benzene (ppb)	New	(15) 6.76	(19) 2.31	0.0290
	Old	(21) 9.55	(1) 3.53	-
Toluene (ppb)	New	(15)12.00	(19) 20.10	NS
	Old	(21)19.70	(1) 54.82	-
EX (ppb)	New	(12) 9.50	(19) 10.60	NS
	Old	(7)12.20	(0) -	-
POST2 (mgMA/gCr)	New	(13) 0.64	(25) 0.29	0.0330
	Old	(12) 0.42	(1) 0.75	-
POST (mgMA/gCr)	New	(15) 0.70	(26) 0.28	0.0074
	Old	(21) 0.60	(1) 0.75	-

*2-sample t-test.
NS: not significant

3.12.6.5 Refuelling Cars

Refuelling cars during the driving period also had no effect on *t,t*-muconic acid level in individuals exposed to petrol, whether POST 1 or POST 2 samples were tested (Pearson correlation, $p>0.05$). There was no difference between *t,t*-muconic acid levels in urine for petrol exposed individuals whether the car was refuelled during the journey, or not (Figure 41). However, *t,t*-muconic acid levels in POST 2

did correlated with refuelling during the journey for diesel exposed individuals. POST 1 samples were not available for diesel exposures.

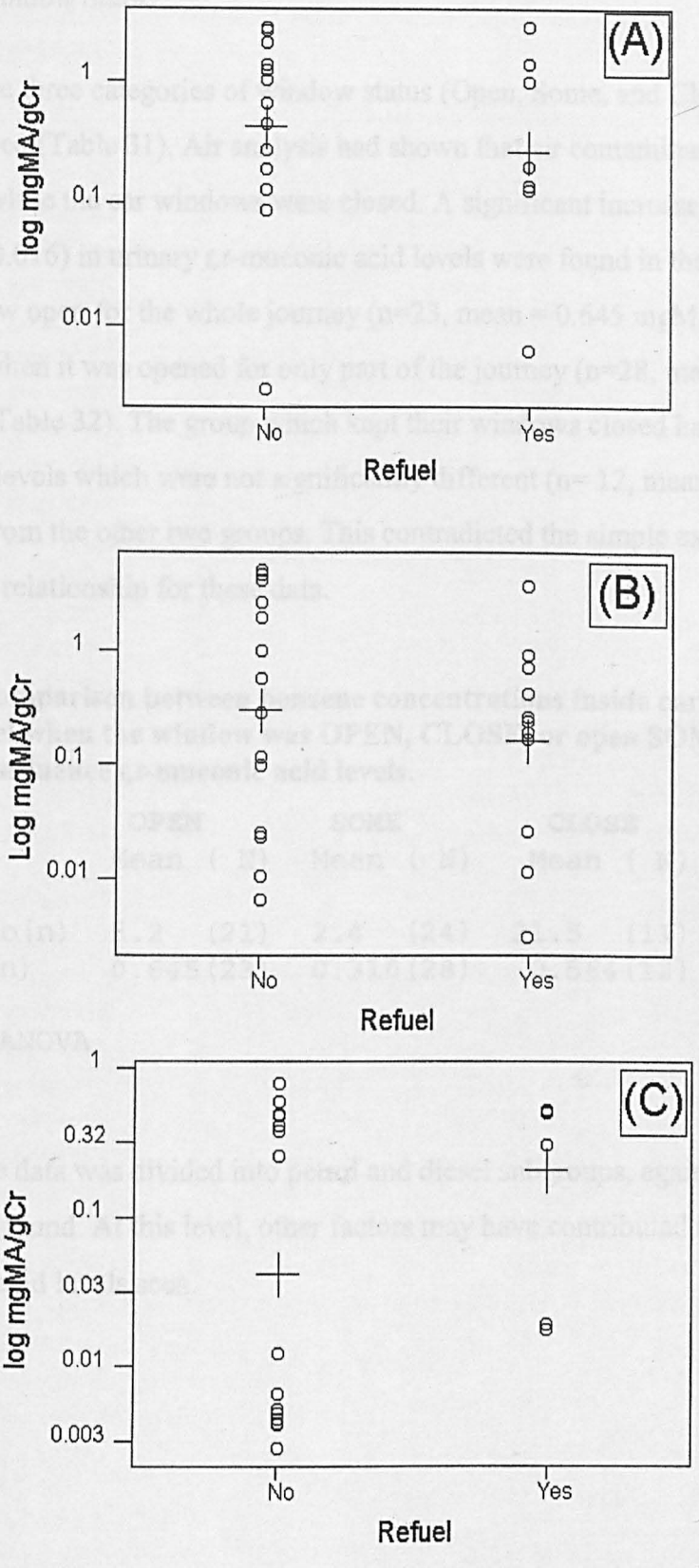


Figure 41: The influence of refuelling during the journey of urinary *t,t*-muconic acid levels fir individuals exposed to petrol (A: Post 1 samples, B: Post 2 samples) or diesel (C: Post2 samples)

3.12.6.6 Window Status

There are three categories of window status (Open, Some, and Close) regardless of fuel type used (Table 31). Air analysis had shown that air contaminants accumulated while the car windows were closed. A significant increase (one way ANOVA, p= 0.016) in urinary *t,t*-muconic acid levels were found in the group that had the window open for the whole journey (n=23, mean = 0.645 mgMA/gCr) compared to when it was opened for only part of the journey (n=28, mean = 0.310 mgMA/gCr) (Table 32). The group which kept their windows closed had urinary *t,t*-muconic acid levels which were not significantly different (n= 12, mean = 0.564 mgMA/gCr) from the other two groups. This contradicted the simple explanation of a dose-response relationship for these data.

Table 31: A comparison between benzene concentrations inside car fuelled with petrol or diesel when the window was OPEN, CLOSE, or open SOME of the time and the influence *t,t*-muconic acid levels.

	OPEN Mean (N)	SOME Mean (N)	CLOSE Mean (N)	p-value*
Benzene ppb(n)	5.2 (21)	2.4 (24)	21.5 (11)	<0.001
mgMA/gCr (n)	0.645(23)	0.310(28)	0.564(12)	0.016

* one-way ANOVA

When the data was divided into petrol and diesel subgroups, again no significant difference was found. At this level, other factors may have contributed to the variation in *t,t*-muconic acid levels seen.

Table 32: The influence of window status and fuel type of urinary *t,t*-muconic acid levels (mgMA/gCr).

Window	Fuel	Vl	POST1	POST2	POST
Open	petrol	2	0.006	0.261	0.134
Open	petrol	2	0.127	-	0.127
Open	petrol	2	0.127	-	0.127
Open	petrol	2	0.201	-	0.201
Open	petrol	3	0.273	4.266	2.269
Open	petrol	11	0.447	-	0.447
Open	petrol	2	1.016	0.115	0.566
Open	petrol	2	1.343	0.006	0.675
Open	petrol	16	2.078	-	2.078
Open	petrol	3	2.769	-	2.769
Open	petrol	11	2.811	-	2.811
Open	petrol	2	-	0.192	0.192
Open	petrol	4	-	0.01	0.01
Open	petrol	4	-	0.023	0.023
Open	petrol	4	-	1.982	1.982
Open	petrol	4	-	3.82	3.82
Open	petrol	4	-	4.773	4.773
Open	petrol	4	-	5.201	5.201
Open	petrol	10	-	0.164	0.164
Open	Diesel	1	-	0.493	0.493
Open	Diesel	1	-	0.500	0.500
Open	Diesel	5	-	0.019	0.019
Open	Diesel	6	-	0.004	0.004
Some	petrol	15	0.003	-	0.003
Some	petrol	2	0.088	-	0.088
Some	petrol	2	0.987	0.026	0.506
Some	petrol	9	1.405	0.937	1.171
Some	petrol	2	-	0.003	0.003
Some	petrol	2	-	0.096	0.096
Some	petrol	2	-	0.281	0.281
Some	petrol	2	-	1.022	1.022
Some	petrol	4	-	0.025	0.025
Some	petrol	7	-	0.715	0.715
Some	Diesel	5	0.006	-	0.006
Some	Diesel	1	-	0.302	0.302
Some	Diesel	1	-	0.375	0.375
Some	Diesel	1	-	0.479	0.479
Some	Diesel	1	-	0.500	0.500
Some	Diesel	1	-	0.521	0.521
Some	Diesel	5	-	0.005	0.005
Some	Diesel	5	-	0.007	0.007
Some	Diesel	6	-	0.005	0.005
Some	Diesel	6	-	0.017	0.017
Some	Diesel	21	-	0.003	0.003
Some	Diesel	21	-	0.256	0.256

(continue ...)

Window	Fuel	Vl	POST1	POST2	POST
Some	Diesel	22	-	0.004	0.004
Some	Diesel	22	-	0.411	0.411
Some	Diesel	23	-	0.012	0.012
Some	Diesel	23	-	0.596	0.596
Some	Diesel	24	-	0.004	0.004
Some	Diesel	24	-	0.793	0.793
Close	**petrol**	**2**	**0.138**	**0.239**	**0.188**
Close	**petrol**	**2**	**0.190**	**-**	**0.19**
Close	**petrol**	**2**	**0.658**	**-**	**0.658**
Close	**petrol**	**2**	**1.228**	**2.725**	**1.976**
Close	**petrol**	**2**	**2.570**	**-**	**2.57**
Close	**petrol**	**4**	**-**	**0.011**	**0.011**
Close	**petrol**	**4**	**-**	**0.424**	**0.424**
Close	**petrol**	**4**	**-**	**0.576**	**0.576**
Close	Diesel	1	-	0.521	0.521
Close	Diesel	6	-	0.004	0.004
Close	Diesel	6	-	0.382	0.382
Close	Diesel	18	-	0.515	0.515

(-) Not determined.
Vl: Volunteer Code.
POST1: post exposure urine sample (2 and 7h after start of journey), POST2:after-urine sample (>7 and 21h) after start of journey). POST: an average of POST 1 and POST 2.
Bold: petrol samples, Normal: Diesel samples.

3.12.6.7 Air-conditioning

Urinary *t,t*-muconic acid levels from diesel-exposed individuals showed a significant difference between the three status of air conditioning (operated for the whole journey (YES), part of the journey (SOME), and not operated (NO) (Table 33). Only one sample was obtained for POST 1 for diesel-exposed individuals. There was a significant difference in *t,t*-muconic acid level (one-way ANOVA, p=0.029) was found between the three air-conditioning groups (YES (n=9) = 0.30 mgMA/gCr, SOME (n=11) = 0.49 and No (n=5) = 0.13).

Table 33: Air toluene in diesel fuelled cars and *t,t*-muconic acid levels: the influence of air conditioning (ON, SOME times, OFF).

	ON	SOME	OFF	p-value
Toluene ppb(n)	11.6 (9)	15.5 (4)	28.4 (6)	0.019
mgMA/gCr (n)	0.35 (9)	0.49(11)	0.13(29)	0.029

A highly significant difference (ANOVA, p= 0.008) was found between individuals who operated the air-conditioning for part or all of the journey (n= 20, mean = 0.394 mgMA/gCr) compare to those that did not use it at all (n= 6, mean = 0.124 mgMA/gCr). No relationship found in petrol samples (Table 34).

Table 34: The influence of air conditioning and fuel type on urinary *t,t*-muconic acid levels (mgMA/gCr).

Aircond	Fuel	Vl	POST1	POST2	POST
On	petrol	2	-	0.003	0.003
On	petrol	2	0.088	-	0.088
On	petrol	2	-	0.096	0.096
On	petrol	2	0.127	-	0.127
On	petrol	2	0.127	-	0.127
On	petrol	2	0.138	0.239	0.188
On	petrol	2	0.190	-	0.190
On	petrol	2	-	0.192	0.192
On	petrol	2	0.201	-	0.201
On	petrol	2	-	0.281	0.281
On	petrol	2	0.987	0.026	0.506
On	petrol	2	1.016	0.115	0.566
On	petrol	2	0.658	-	0.658
On	petrol	2	1.343	0.006	0.675
On	petrol	2	-	1.022	1.022
On	petrol	2	1.228	2.725	1.976
On	petrol	2	2.570	-	2.570
On	petrol	3	0.273	4.266	2.269
On	petrol	3	2.769	-	2.769
On	petrol	7	-	0.715	0.715
On	petrol	9	1.405	0.937	1.171
On	petrol	11	0.447	-	0.447
On	petrol	11	2.811	-	2.811
On	petrol	15	0.003	-	0.003
On	petrol	16	2.078	-	2.078
On	Diesel	18	-	0.515	0.515
On	Diesel	21	-	0.003	0.003
On	Diesel	21	-	0.256	0.256
On	Diesel	22	-	0.004	0.004
On	Diesel	22	-	0.411	0.411
On	Diesel	23	-	0.012	0.012
On	Diesel	23	-	0.596	0.596
On	Diesel	24	-	0.004	0.004
On	Diesel	24	-	0.793	0.793
Some	petrol	4	-	0.011	0.011
Some	petrol	10	-	0.164	0.164

(continue ...)

Aircond	Fuel	Vl	POST1	POST2	POST
Some	Diesel	1	-	0.302	0.302
Some	Diesel	1	-	0.375	0.375
Some	Diesel	1	-	0.479	0.479
Some	Diesel	1	-	0.493	0.493
Some	Diesel	1	-	0.500	0.500
Some	Diesel	1	-	0.500	0.500
Some	Diesel	1	-	0.521	0.521
Some	Diesel	1	-	0.521	0.521
Some	Diesel	6	-	0.004	0.004
Some	Diesel	6	-	0.004	0.004
Some	Diesel	6	-	0.382	0.382
Off	**petrol**	**2**	**0.006**	**0.261**	**0.134**
Off	**petrol**	**4**	**-**	**0.010**	**0.010**
Off	**petrol**	**4**	**-**	**0.023**	**0.023**
Off	**petrol**	**4**	**-**	**0.025**	**0.025**
Off	**petrol**	**4**	**-**	**0.424**	**0.424**
Off	**petrol**	**4**	**-**	**0.576**	**0.576**
Off	**petrol**	**4**	**-**	**1.982**	**1.982**
Off	**petrol**	**4**	**-**	**3.820**	**3.820**
Off	**petrol**	**4**	**-**	**4.773**	**4.773**
Off	**petrol**	**4**	**-**	**5.201**	**5.201**
Off	Diesel	5	-	0.005	0.005
Off	Diesel	5	0.006	-	0.006
Off	Diesel	5	-	0.007	0.007
Off	Diesel	5	-	0.019	0.019
Off	Diesel	6	-	0.005	0.005
Off	Diesel	6	-	0.017	0.017

(-) Not determined.
Vl: Volunteer Code.
POST1: post exposure urine sample (2 and 7h after start of journey), POST2:after-urine sample (>7 and 21h) after start of journey). POST: an average of POST 1 and POST 2.
Bold: petrol samples, Normal: Diesel samples.

3.12.6.8 Preservatives

Sorbic acid is present in some preserved foods. None of the volunteers answered positively to sorbic acid consumption. As in Table 35, Although the literature suggests that consumption of preservatives may contribute to *t,t*-muconic acid levels in urine. Food preservatives had a significant effect (ANOVA, p=0.047) on *t,t*-muconic acid excretion in individuals exposed to petrol when preservatives – not specifically sorbic acid- was mentioned to be consumed on the day of sampling in the questionnaire. The mean *t,t*-muconic acid was 0.942 mgMA/gCr (n=8) for individuals who consumed preservatives compared to 0.364 mgMA/gCr (n=7) when they did not. Preservatives did not significantly influence *t,t*-muconic acid levels in

diesel-exposed individuals. There was no significant influence on *t,t*-muconic acid in individuals who habitually consumed food with added preservatives.

Table 35: The influence of preservatives consumption and fuel type on urinary *t,t*-muconic acid levels (mgMA/gCr).

Preserve	Habit	Fuel	Vl	PRE	POST 1	POST 2	POST
No	-	petrol	2	0.049	0.127	-	0.127
No	-	petrol	2	0.679	1.343	0.006	0.675
No	No	petrol	3	0.073	0.273	4.266	2.269
No	No	petrol	4	1.028	-	5.201	5.201
No	No	petrol	4	2.747	-	4.773	4.773
No	No	petrol	4	1.812	-	3.820	3.820
No	No	petrol	4	0.009	-	0.025	0.025
No	No	petrol	7	0.693	-	0.715	0.715
No	No	Diesel	1	0.106	-	0.521	0.521
No	No	Diesel	1	0.093	-	0.493	0.493
No	No	Diesel	1	0.071	-	0.500	0.500
No	No	Diesel	1	0.123	-	0.521	0.521
No	No	Diesel	1	0.005	-	0.479	0.479
No	No	Diesel	1	0.292	-	0.375	0.375
No	No	Diesel	1	0.375	-	0.500	0.500
No	-	Diesel	1	-	-	0.302	0.302
No	No	Diesel	5	0.007	0.006	-	0.006
No	No	Diesel	6	0.010	-	0.004	0.004
No	No	Diesel	6	0.005	-	0.017	0.017
No	No	Diesel	6	0.406	-	0.382	0.382
No	No	Diesel	6	0.055	-	0.004	0.004
No	No	Diesel	6	0.015	-	0.005	0.005
No	-	Diesel	21	0.005	-	0.003	0.003
No	-	Diesel	21	1.134	-	0.256	0.256
No	-	Diesel	22	0.006	-	0.004	0.004
No	-	Diesel	22	0.378	-	0.411	0.411
No	-	Diesel	23	0.014	-	0.012	0.012
No	-	Diesel	23	0.005	-	0.596	0.596
No	-	Diesel	24	0.007	-	0.004	0.004
No	-	Diesel	24	0.010	-	0.793	0.793
Yes	No	petrol	2	0.717	1.016	0.115	0.566
Yes	-	petrol	2	0.080	0.127	-	0.127
Yes	-	petrol	2	0.909	-	1.022	1.022
Yes	No	petrol	4	0.007	-	0.023	0.023
Yes	No	petrol	4	0.007	-	0.011	0.011
Yes	No	petrol	4	0.168	-	0.424	0.424
Yes	No	petrol	4	0.007	-	0.010	0.010

(continue ...)

Preserve	Habit	Fuel	Vl	PRE	POST 1	POST 2	POST
-	Yes	petrol	2	0.140	2.570	-	2.570
-	Yes	petrol	2	0.452	-	0.096	0.096
-	Yes	petrol	2	0.243	-	0.281	0.281
-	Yes	petrol	2	0.016	-	0.003	0.003
-	-	petrol	2	0.006	0.006	0.261	0.134
-	-	petrol	2	0.029	0.088	-	0.088
-	-	petrol	2	0.005	0.138	0.239	0.188
-	-	petrol	2	0.124	0.190	-	0.190
-	-	petrol	2	1.825	0.201	-	0.201
-	-	petrol	2	0.490	0.658	-	0.658
-	-	petrol	2	0.079	0.987	0.026	0.507
-	-	petrol	2	0.006	1.228	2.725	1.976
-	-	petrol	2	0.136	-	0.192	0.192
-	No	petrol	3	1.428	2.769	-	2.769
-	No	petrol	4	0.014	-	0.576	0.576
-	Yes	petrol	4	0.413	-	1.982	1.982
-	-	petrol	9	0.569	1.405	0.937	1.171
-	No	petrol	10	0.030	-	0.164	0.164
-	No	petrol	11	0.807	0.447	-	0.447
-	-	petrol	11	0.004	2.811	-	2.811
-	-	petrol	15	0.005	0.003	-	0.003
-	No	petrol	16	0.201	2.078	-	2.078
-	No	Diesel	5	0.012	-	0.005	0.005
-	No	Diesel	5	0.007	-	0.007	0.007
-	No	Diesel	5	0.008	-	0.019	0.019
-	No	Diesel	6	0.009	-	0.006	0.006
-	Yes	Diesel	18	-	-	0.515	0.515

(-) Not determined.
Vl: Volunteer Code.
PRE: Pre-exposure urine sample, POST1: post exposure urine sample (2 and 7h after start of journey), POST2:after-urine sample (>7 and 21h) after start of journey). POST: an average of POST 1 and POST 2.
Preserve : preservative was consumed on the day of sampling.
Habit : preservatives were habitually consumed.
Bold: petrol samples, Normal: Diesel samples.

3.12.6.9 Traffic Status

There was no significant correlation between the traffic status and *t,t*-muconic acid level in petrol-exposed individuals (Pearson correlation, n=14, p=0.292) or diesel-exposed individuals (Pearson correlation, n=17, p=0.064) samples. This indicated that traffic status does not inluence exposure inside the car cabin to petrol or diesel fumes.

3.12.7 *t,t*-Muconic Acid Distribution in 48 Hours-profile

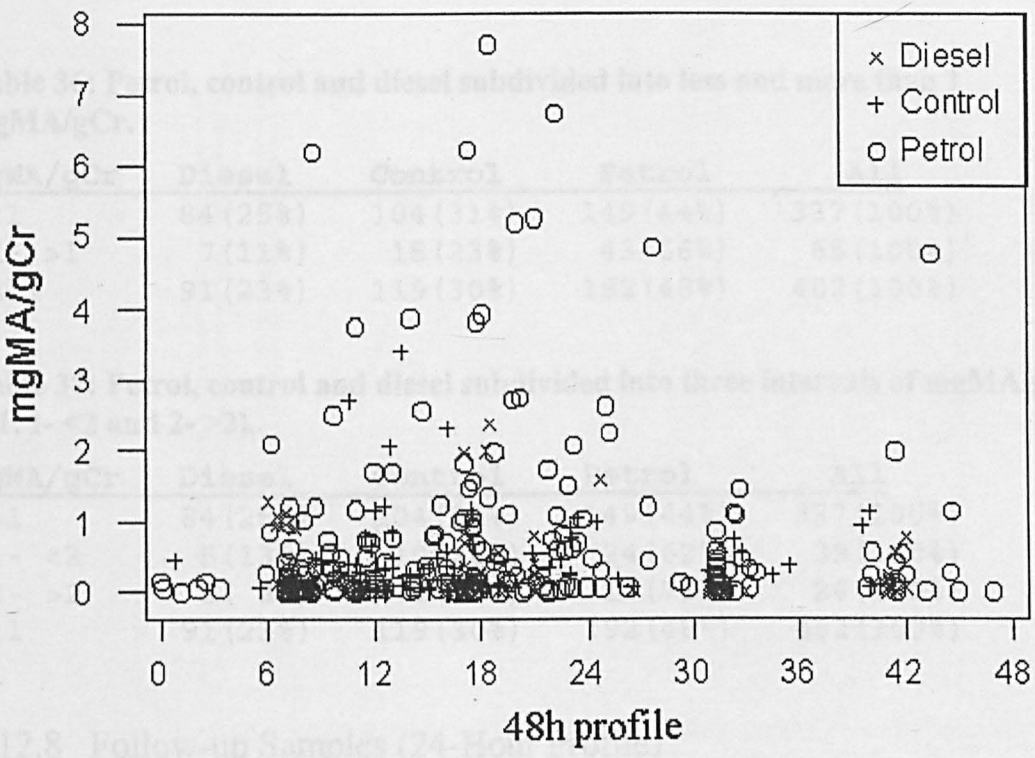


Figure 42: The distribution of urinary *t,t*-muconic acid for all the control samples and those who exposed to petrol and diesel fumes (n=402).

Figure 42 shows the urinary *t,t*-muconic acid distribution for all control samples and following exposure to petrol or diesel (n=402). Some of the urines form individuals exposed to petrol exceeded 3.5 mgMA/gCr level. A study of *t,t*-muconic acid levels following environmental levels of exposure to petrol corroborated these data (Crebelli.et al., 2001). The maximum *t,t*-muconic acid levels were in petrol-exposed individuals at about 6:00 pm which is after the rush hour at Newcastle upon Tyne City.

The *t,t*-muconic acid level of the urine was 68.362 mgMA/gCr. This sample was unique because the volunteer 15 had smoked more than 11 cigarettes and drank more than 2 pints of beer prior to sampling. This sample was excluded from the study.

When the *t,t*-muconic acid levels of the three groups (diesel, control and petrol) were sub-divided into less one mgMA/gCr or one mgMA/gCr and greater, there was a significantly higher level for individuals who exposed to petrol compared to the other

groups (Multiple Chi^2, p=0.003)(Table 36). With separate samples that reached the 2 mgMA/gCr and over, a significant increase (Multiple Chi^2, p=0.015)(Table 37).

Table 36: Petrol, control and diesel subdivided into less and more than 1 mgMA/gCr.

mgMA/gCr	Diesel	Control	Petrol	All
<1	84(25%)	104(31%)	149(44%)	337(100%)
1- >1	7(11%)	15(23%)	43(66%)	65(100%)
All	91(23%)	119(30%)	192(48%)	402(100%)

Table 37: Petrol, control and diesel subdivided into three intervals of mgMA/gCr (<1, 1- <2 and 2- >2).

mgMA/gCr	Diesel	Control	Petrol	All
>1	84(25%)	104(31%)	149(44%)	337(100%)
1- <2	5(13%)	10(26%)	24(62%)	39(100%)
2- >2	2(8%)	5(19%)	19(73%)	26(100%)
All	91(23%)	119(30%)	192(48%)	402(100%)

3.12.8 Follow-up Samples (24-Hour Profile)

The follow-up samples were in two types. Some PRE and POST samples in phase I were taken in sequence for more than one day. In phase II, 24 hours samples were collected from control and petrol exposed individuals.

3.12.8.1 More Than One Day Follow-up

The samples collected as PRE -POST in successive days in phase I samples. Two petrol exposed volunteers, one diesel exposed volunteer, and one control were presented next. Volunteers (2) and (4) are from the petrol exposed group. A detectable *t,t*-muconic acid was found for (2) and the first period for (4). However, no detectable levels were found for the second period for (4) as presented in the following section. Control and diesel exposed volunteers demonstrated no detectable levels in their samples that might related to POST samples. One sample from the control volunteer was detected but represents a PRE sample.

Five days samples (15 - 20/5/99) were collected from volunteer (2). Good relation was found with the driving events (Figure 43). With using public transportation (metro and bus), no detectable *t,t*-muconic acid was observed. In 17/5/99 with only public transportation utilisation, undetermined factor seems to

cause a high concentration of *t,t*-muconic acid (6.219 mgMA/gCr) in PRE sample. No refuelling took place in that period.

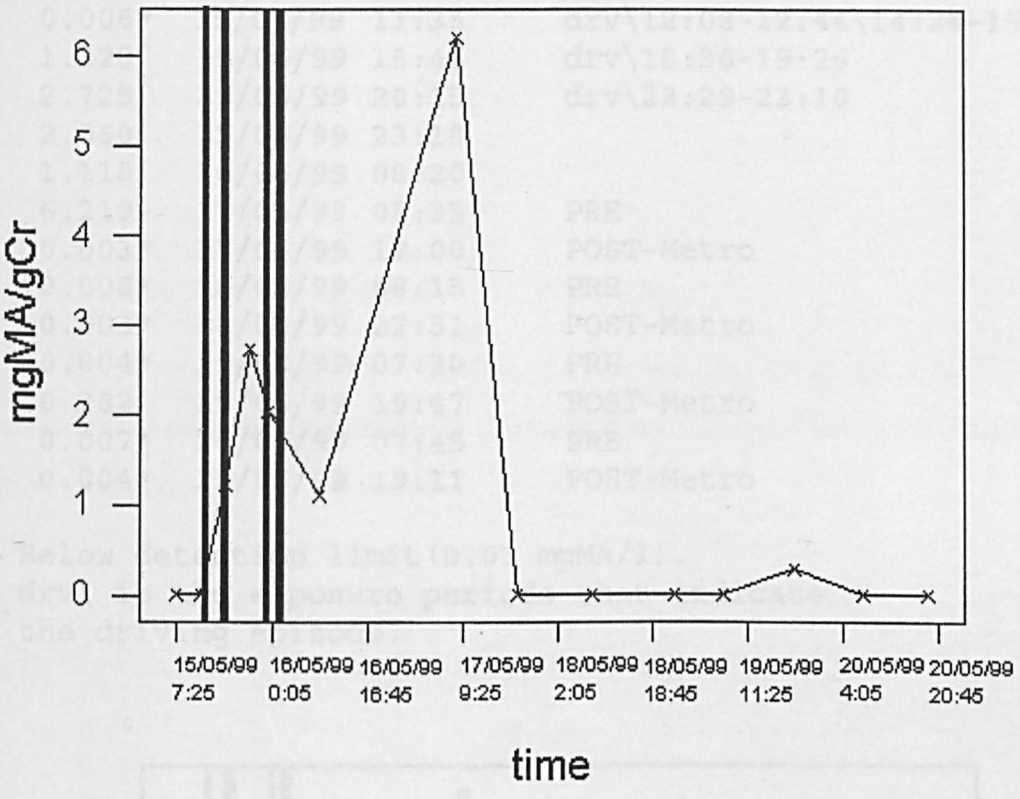


Figure 43: Volunteer (2) samples were taken in five successive days. The vertical dark lines represent the exposure events and the width indicates the exposure duration.

Two periods of successive days samples were obtained from volunteer 4 who was exposing to petrol. The first period for volunteer 4 was obtained from 10 - 13/8/99. The four days samples demonstrate an increase in POST after driving except in the last day when the samples were below detection limit. Refuelling took place in 11/08/99. The *t,t*-muconic acid concentrations in the first period agreed with the exposure in the first two days as seen in Figure 44.

Table 38: Volunteer (2) five days samples

mgMA/gCr	Day	Time	Note
0.005*	15/05/99	07:25	
0.006*^	15/05/99	11:35	drv\12:08-12:44\14:24-15:39
1.228^	15/05/99	15:45	drv\18:36-19:26
2.725^	15/05/99	20:15	drv\22:29-23:10
2.060	15/05/99	23:18	
1.118	16/05/99	08:20	
6.219	17/05/99	08:35	PRE
0.003*	17/05/99	19:00	POST-Metro
0.006*	18/05/99	08:15	PRE
0.003*	18/05/99	22:51	POST-Metro
0.004*	19/05/99	07:30	PRE
0.282	19/05/99	19:47	POST-Metro
0.007*	20/05/99	07:45	PRE
0.004*	20/05/99	19:11	POST-Metro

* Below detection limit(0.01 mgMA/l).
^ drv\ is the exposure periods that indicate the driving episode.

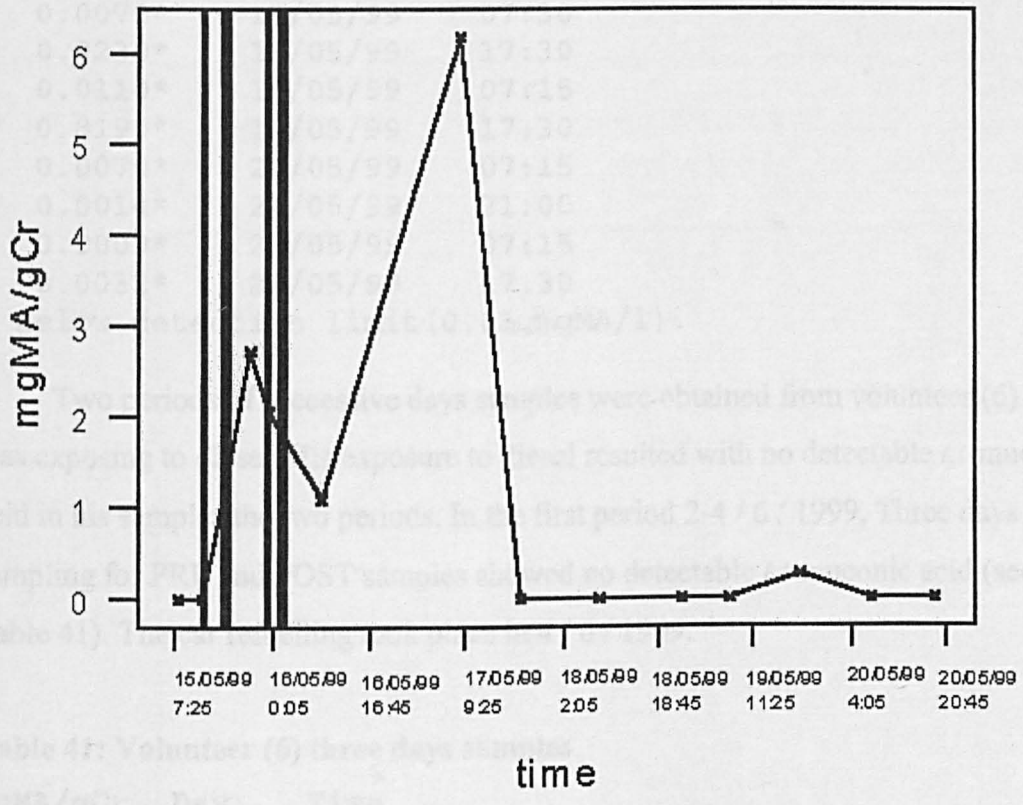


Figure 44: Volunteer (4) samples were taken in four successive days. The vertical dark lines represent the exposure events and the width indicates the exposure duration.

Table 39: Volunteer (4) three days samples.

mgMA/gCr	Day	Time (hh:mm)
2.747	10/8/99	07:30
4.773	10/8/99	19:30
1.812	11/8/99	07:20
3.820	11/8/99	17:45
0.009*	12/8/99	07:15
0.025*	12/8/99	18:00
0.014*	13/8/99	07:20

* Below detection limit (0.01 mgMA/l).

The second one (17 - 21/5/99) showed no detectable *t,t*-muconic acid. The car was refuelled in 20/05/99. Unlike the first period, no concentrations were detected in the second period.

Table 40: Volunteer (4) five days samples.

mgMA/gCr	Day	Time (hh:mm)
0.0090*	17/05/99	07:15
0.0170*	17/05/99	17:30
0.0070*	18/05/99	07:30
0.0230*	18/05/99	17:30
0.0110*	19/05/99	07:15
0.0190*	19/05/99	17:30
0.0070*	20/05/99	07:15
0.0014*	20/05/99	21:00
0.0009*	21/05/99	07:15
0.0032*	21/05/99	17:30

* Below detection limit (0.01 mgMA/l).

Two periods of successive days samples were obtained from volunteer (6) who was exposing to diesel. His exposure to diesel resulted with no detectable *t,t*-muconic acid in his samples the two periods. In the first period 2-4 / 6 / 1999, Three days sampling for PRE and POST samples showed no detectable *t,t*-muconic acid (see Table 41). The car refuelling took place in 4 / 6 / 1999.

Table 41: Volunteer (6) three days samples

mgMA/gCr	Day	Time
0.002*	2/6/99	07:00
0.028*	2/6/99	18:00
0.009*	3/6/99	07:00
0.003*	3/6/99	17:30
0.005*	4/6/99	07:30

0.017*	4/6/99	17:30

* Below detection limit (0.01 mgMA/l).

The second period for volunteer (6) that collected in 9-13/8/1999 also demonstrated no detectable *t,t*-muconic acid (see Table 42). No refuelling took place in this period.

Table 42: Volunteer (6) four days samples.

mgMA/gCr	Day	Time (hh:mm)
0.004*	09/8/99	07:30
0.030*	09/8/99	17:00
0.055*	10/8/99	07:30
0.004*	10/8/99	17:30
0.015*	11/8/99	07:30
0.006*	11/8/99	17:00
0.015*	12/8/99	07:30
0.005*	12/8/99	17:00
0.012*	13/8/99	07:00

* Below detection limit (0.01 mgMA/l).

Volunteer (9) is one of the control subjects from 9 -13 /8/99. The PRE and POST spot samples during five days were below detection limit for *t,t*-muconic acid except the last sample as in Table 43. The volunteer was not a driver.

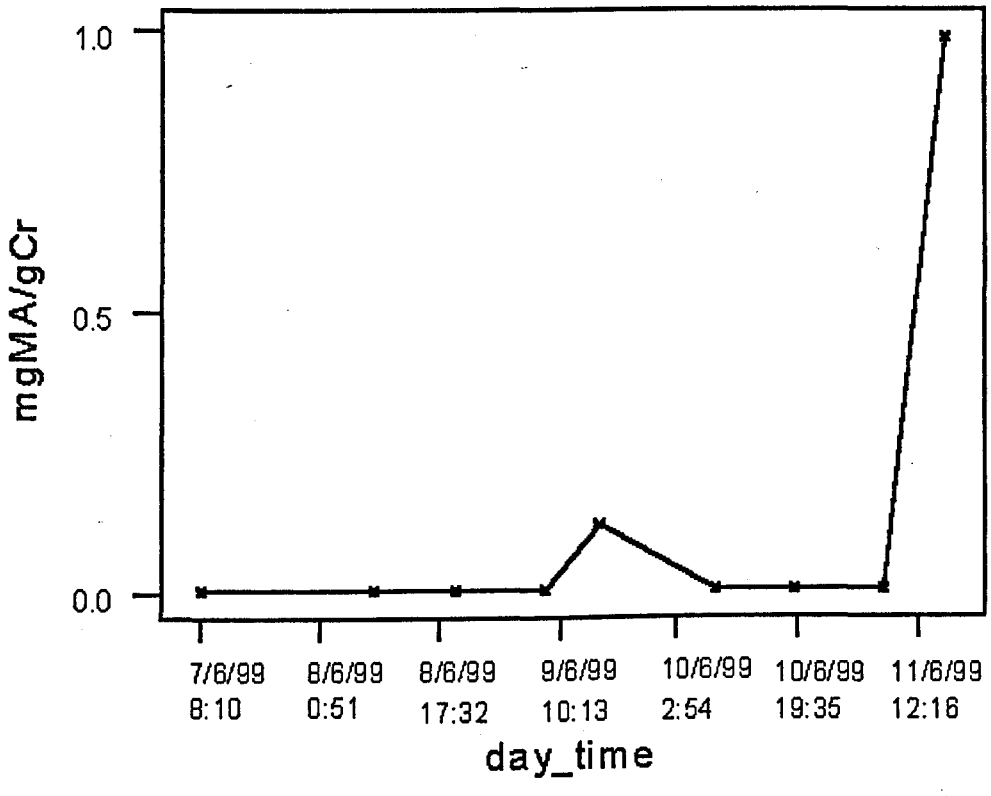


Figure 45: Volunteer (9) samples were taken on five successive days. Pseudo-peaks show the influence of creatinine over undetectable levels. Be aware that the y-axis has a small range.

Table 43: Volunteer (9) four days samples.

mgMA/gCr	Day	Time(hh:mm)
0.006*	07/6/99	08:10
0.004*	08/6/99	08:30
0.006*	08/6/99	19:55
0.005*	09/6/99	08:05
0.116*	09/6/99	16:00
0.004*	10/6/99	08:10
0.007*	10/6/99	19:00
0.004*	11/6/99	07:30
0.989	11/6/99	16:30

* Below detection limit(0.01 mgMA/l).

3.12.8.2 Twenty Four Hours Samples

Twenty four hours samples were recruited to determine the variability of the urinary *t,t*-muconic acid throughout the 24h period for petrol and control subjects. Each set of samples was called a "profile".

In the first set of samples, two volunteers (2 and 3) were tested for the 24h profiles. Later, an additional set of profiles was recruited for 24h for other volunteers (12, 25-37) to examine the finding in the first set.

Twenty profiles were obtained for petrol and control subjects. Some of these profiles have vertical bars, which indicate the petrol exposure periods. The width of these bars indicates the duration of the exposure. Also, in some profiles a horizontal dashed line represents by an imaginary detection border (horizontal dashed line) as in graph (A) in Figure 46.

Five profiles (A, B, C, D, and E) were obtained from volunteer (2) can be seen in Figure 46. Four of them (A, B, C, and D) were including exposure to petrol in the day of sampling. Profile (E) is a public transportation profile unlike the other

volunteer (2) profiles. Three out of four petrol profiles of volunteer (2) include one or more samples that exceeded 1 mgMA/gCr.

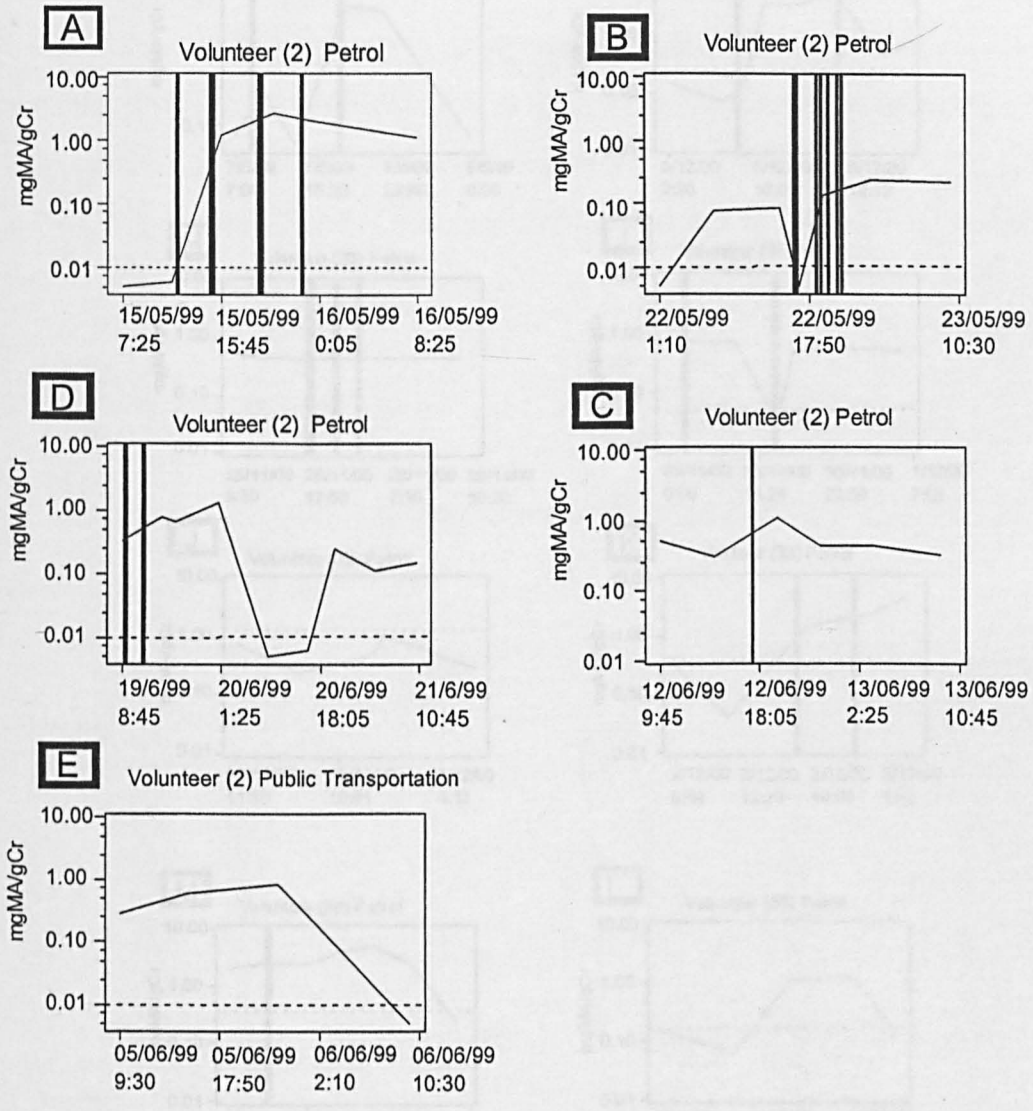


Figure 46: Volunteer (2) profiles. The detection limit for the HPLC is plotted as a horizontal dashed line.

Ten profiles also obtained from ten volunteers who were exposed to petrol. Seven of the profiles were presented accompanied with exposure periods. A slight elevation noticed mainly after the exposure bars. Volunteers (3), (12), (26), (31), (32), (33), (34), (35), (36), and (37) profiles were presented in F, G, H, J, K, L, M, N, and O graphs, respectively as in Figure 47. Half of the petrol profiles contain at least one sample that has 1 mgMA/gCr or more.

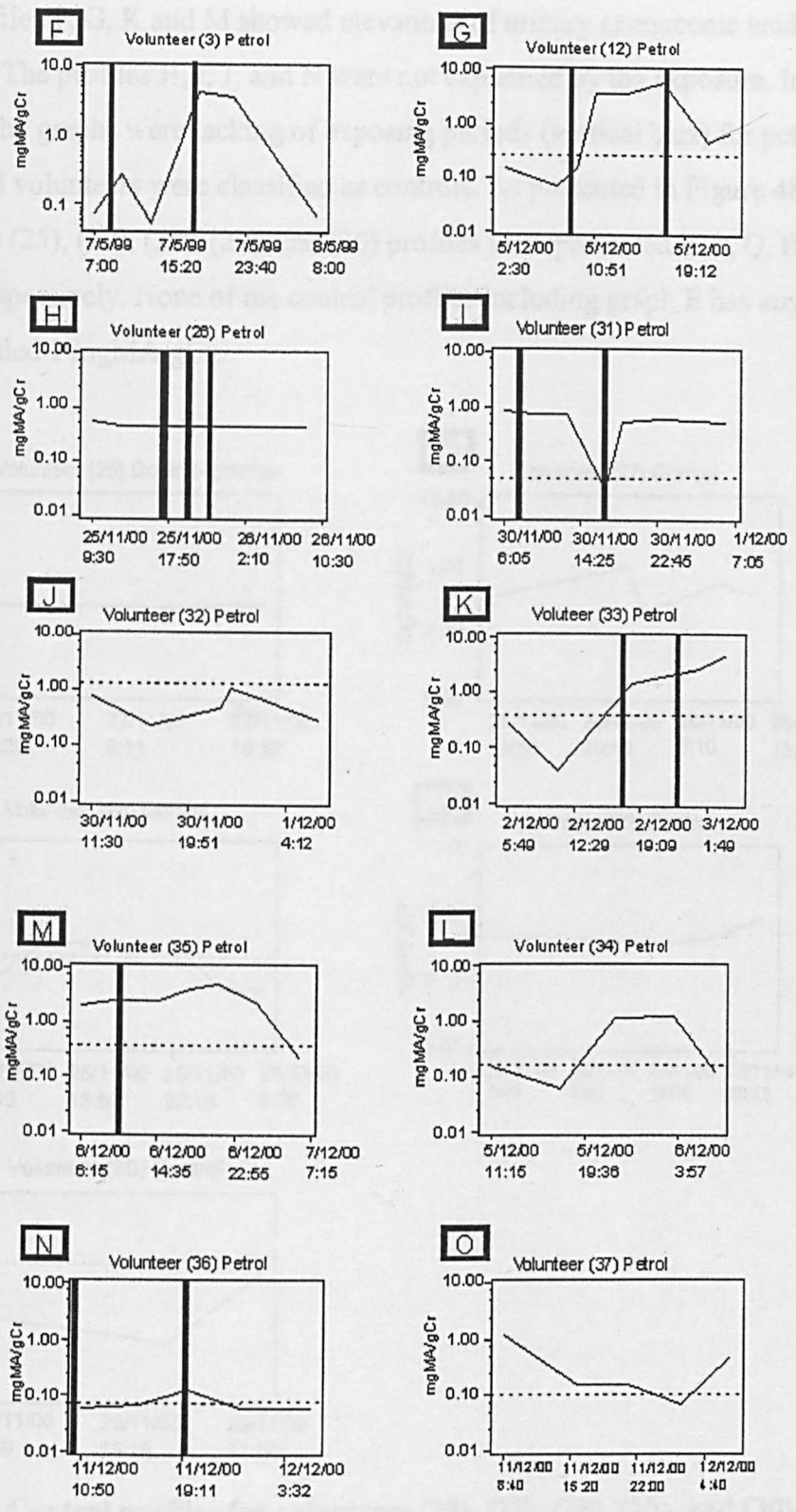


Figure 47: Petrol profiles for volunteers (3), (12), (26), (31), (32), (33), (34), (35), (36), and (37). The detection limit for the HPLC is plotted as a horizontal dashed line.

Profiles F, G, K and M showed elevations of urinary *t,t*-muconic acid after the exposure. The profiles H, I, J, and N were not explained by the exposure. In profiles J, L and O, the graphs were lacking of exposing periods (vertical bars) for petrol. Five unexposed volunteers were classified as controls. As presented in Figure 48, volunteers (25), (27), (28), (29), and (30) profiles were presented in P, Q, R, S, and T graphs, respectively. None of the control profiles including graph E has any sample that exceeded 1 mgMA/gCr.

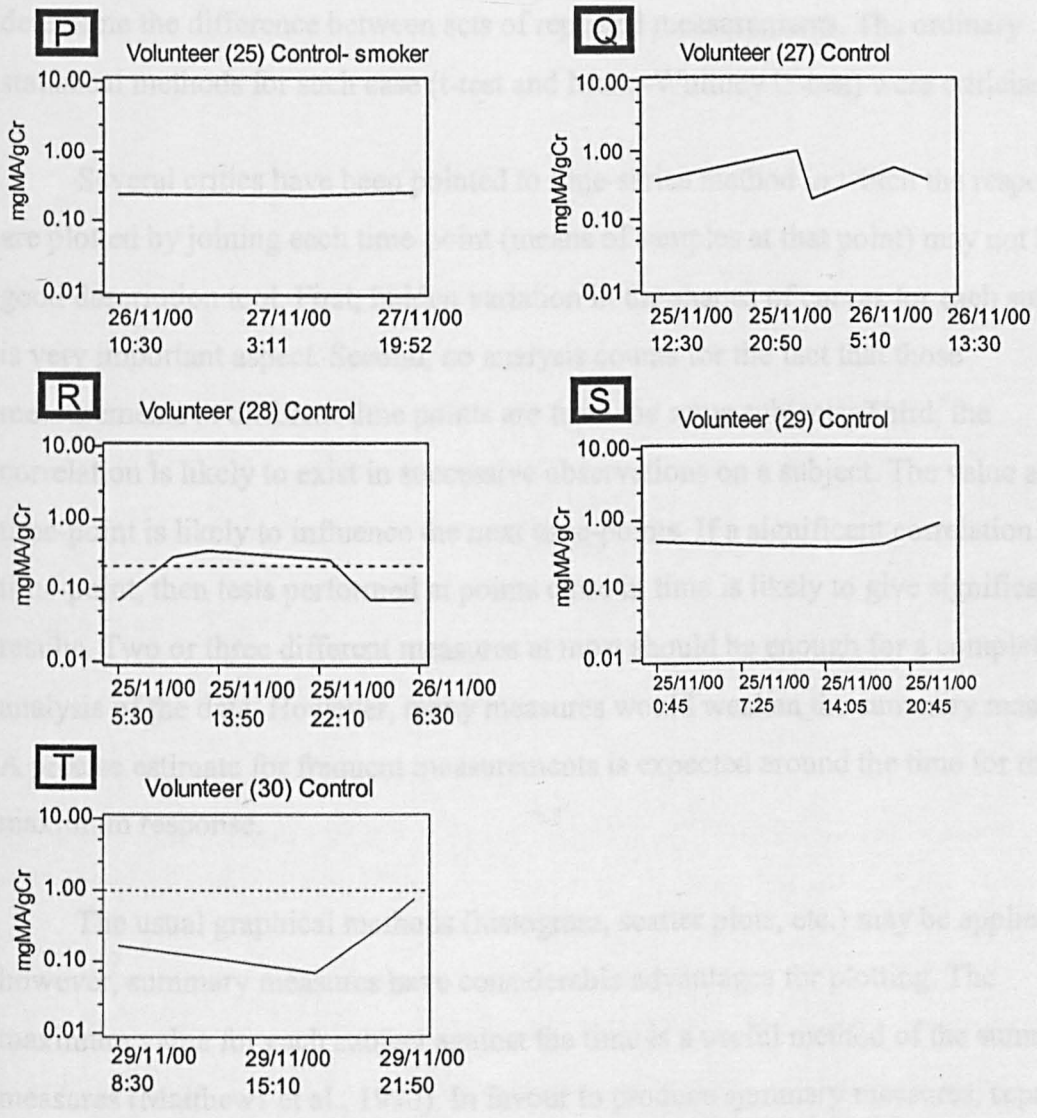


Figure 48: Control profiles for volunteers (25), (27), (28), (29), and (30). The detection limit for the HPLC is plotted as a horizontal dashed line.

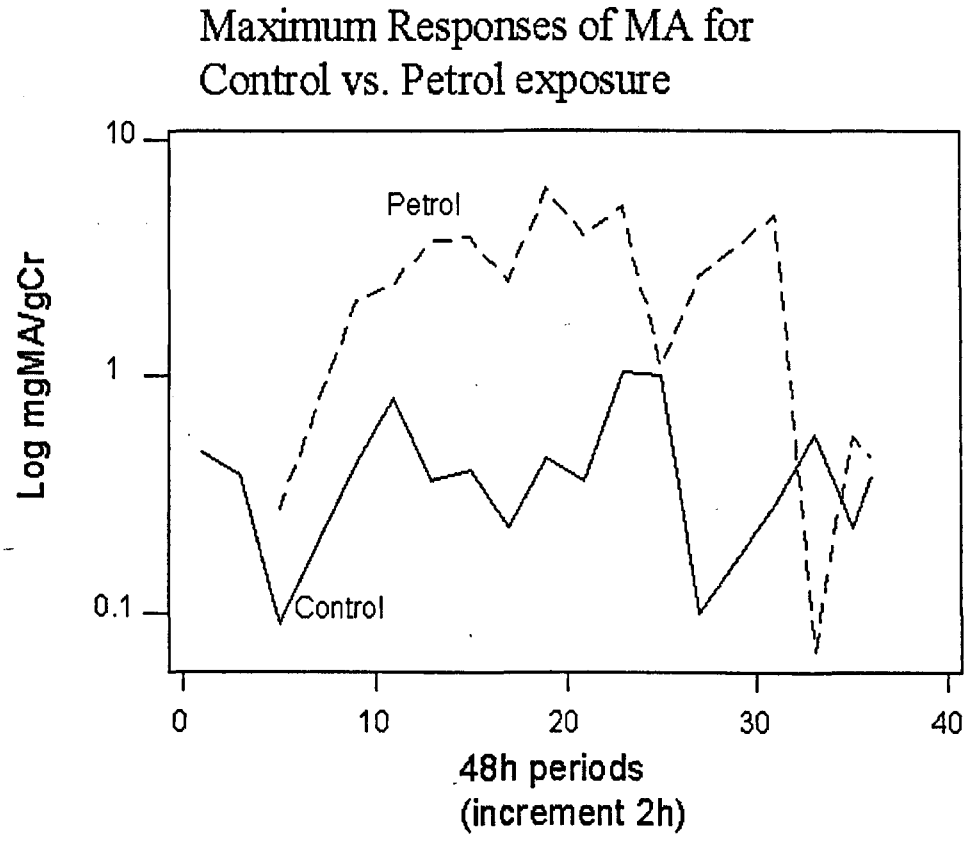
3.12.8.3 Summary Measures

"Summary Measures" is a statistical method that draws a profile for all profiles of all subjects against time (Matthews et al., 1990). A statistician suggested this method for our study. Most biological variables that change over time are smooth and continuous. When the difference between two variables in one time-point is not significant and the next time-point is significant, then a false difference may be expected. Therefore, separating significance tests is not a reasonable method to determine the difference between sets of repeated measurements. The ordinary statistical methods for such case (t-test and Mann-Whitney U-test) were criticised.

Several critics have been pointed to time-series method in which the responses are plotted by joining each time-point (means of samples at that point) may not be a good description tool. First, hidden variation in the shapes of curves for each subject is very important aspect. Second, no analysis counts for the fact that those measurements at different time points are from the same subjects. Third, the correlation is likely to exist in successive observations on a subject. The value at one time-point is likely to influence the next time-points. If a significant correlation at one time-point, then tests performed at points close in time is likely to give significant results. Two or three different measures at most should be enough for a complete analysis of the data. However, many measures would weaken the summary measures. A precise estimate for frequent measurements is expected around the time for the maximum response.

The usual graphical methods (histogram, scatter plots, etc.) may be applied, however, summary measures have considerable advantages for plotting. The maximum value for each subject against the time is a useful method of the summary measures (Matthews et al., 1990). In favour to produce summary measures, separate graphs for each subject should be plotted. Then combine each group measures together. If the peaked graph skew, then logarithm transformation will be recommended. An obvious distinguishes between maximum responses of *t,t*-muconic acid in petrol samples compared to the control (Figure 49). More than one peak observed in the graphs that indicate no significant one peak can be observed clearly unlike the principle of using summary measures. In summary measures, the study design depends on the follow up after expose all subjects to same dose starting in the

same time. This study hasn't been designed to start the exposures in the same time with the same dose. Therefore, multiple peak graphs obtained for the maximum



responses as in Figure 49.

Figure 49: Maximum responses of Log mgMA/gCr from 8 petrol subjects compare to 6 control subjects.

In some cases, minimum values may be applied in the summary measures (Matthews et al., 1990). The minimum responses have no significant difference between petrol and control samples as shown in Figure 50.

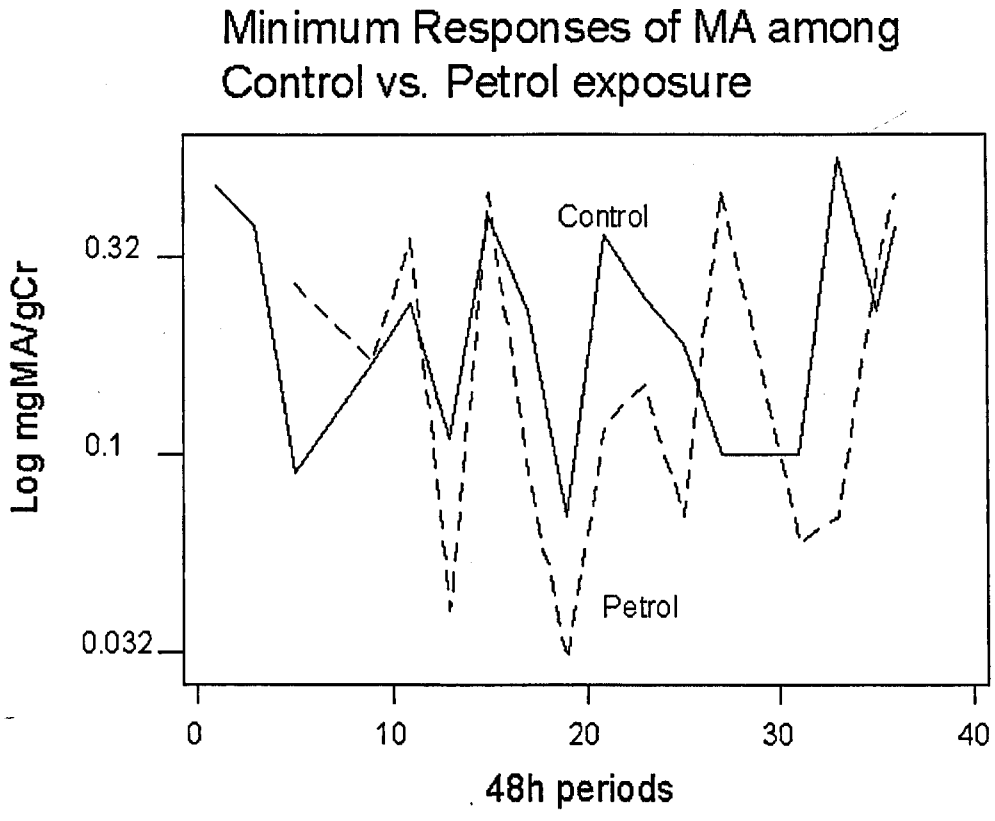


Figure 50: Minimum responses of Log mgMA/gCr from 8 petrol subjects compare to 6 control subjects.

Table 44: Maximum and minimum log mgMA/gCr for control and petrol samples to plot summery measures.

48h	NC	MinC	MaxC	NP	MinP	MaxP
1	1	-0.319	-0.319	*	*	*
3	*	*	*	1	-0.420	-0.420
5	1	-1.046	-1.046	1	-0.569	-0.567
9	2	-0.770	-0.377	3	-0.770	0.318
11	3	-0.620	-0.102	5	-0.456	0.398
13	3	-0.959	-0.444	4	-1.398	0.577
15	1	-0.398	-0.398	3	-0.337	0.588
17	1	-0.638	-0.638	3	-1.046	0.410
19	2	-1.155	-0.347	3	-1.523	0.796
21	1	-0.444	-0.443	5	-0.921	0.597
23	6	-0.602	0.021	3	-0.824	0.721
25	2	-0.721	0.004	5	-1.155	0.041
27	1	-1.000	-1.000	3	-0.328	0.420
31	2	-1.000	-0.538	4	-1.222	0.685
33	1	-0.252	-0.252	1	-1.155	-1.155
35	1	-0.638	-0.638	3	-0.538	-0.260
36	1	-0.420	-0.420	1	-0.337	-0.337

Note: (**48h**) 48 hours follow-up with 2h increment, (**NC**) number of samples per single period of time for control participants while (**NP**) for petrol instead, (**MinC**) minimum Log mgMA/gCr for control and (**MinP**) for petrol, (**MaxC**) maximum Log mgMA/gCr for control and (**MaxP**) for petrol.

3.12.9 BTEX in Petrol and Diesel Cars

A preliminary test was carried on to determine the level of contaminants inside a petrol car during a two-hour journey. An air sample was collected through 2 hours at a flow rate 4.25 L/min. Benzene concentration was 53.2 $\mu g/m^3$ (15.2 ppb). The toluene concentration was 213.8 $\mu g/m^3$ (55.2 ppb). Two parameters were determined to convert mass per volume ($\mu g/m^3$) to the volume per volume (ppb) concentrations; ambient temperature (20°C), atmospheric pressure (1010 mbar). 1987 is year of production of the tested car.

On the other hand, ethylbenzene and xylenes level was added to the study. Only benzene concentration demonstrated a significant difference between air samples that collected from petrol and diesel compartments. In Table 45, no difference of toluene (15-21 ppb) and the mixture of ethylbenzene, para-, meta-, and ortho-xylenes (10.5 ppb) between the mean of petrol and diesel samples. Benzene concentration means for petrol and diesel are 7.5 and 2.6 ppb, respectively. As a consequence, the difference is significant as well when benzene/toluene ratio was tested. Benzene concentration is 45% as much as toluene in petrol. On the other hand, diesel samples showed 10%.

Table 45: Matrix of BTEX concentration versus fuel type.

	Petrol Mean			Diesel Mean			
Pollutant	(N)	$\mu g/m^3$	ppb	(N)	$\mu g/m^3$	ppb	p-value^
Benzene	37	25.7	7.5	20	8.4	2.6	0.01
Toluene	37	57.0	14.9	20	81.1	21.1	0.24
EX	19	46.3	10.5	19	46.7	10.6	0.96
B/T	37	45%*		20	10%*		0.019

EX Ethylbenzene + Xylenes (p,m,o)
B/T benzene/toluene
* without units (ratio)
^ 2-sample t-test

Benzene concentration in air samples that collected from petrol cars is double that taken from diesel cars. This finding might explain the higher concentration of *t,t*-muconic acid in samples exposed to petrol compare to diesel. Another point worth to

be mentioned that toluene, which is a well-known inhibitor to benzene metabolisation increased proportionally in diesel.

Each air sample has been included in the analysis because it is reflecting the actual levels of the targeted contaminants in air (Table 46). Each one had been affected by many independent factors such as the trip distance, traffic density, etc. The correlation between BTEX components was determined as shown in Figure 51. All were correlated positively. Benzene (log ppb) correlated significantly ($p < 0.001$, Pearson correlation (r)= 0.608) with toluene (log ppb). The correlation was significant between benzene and the combination of ethylbenzene and xylenes (EX) ($p<0.001$, $r = 0.375$). The correlation between toluene and EX was also significant ($p<0.001$, $r = 0.625$).

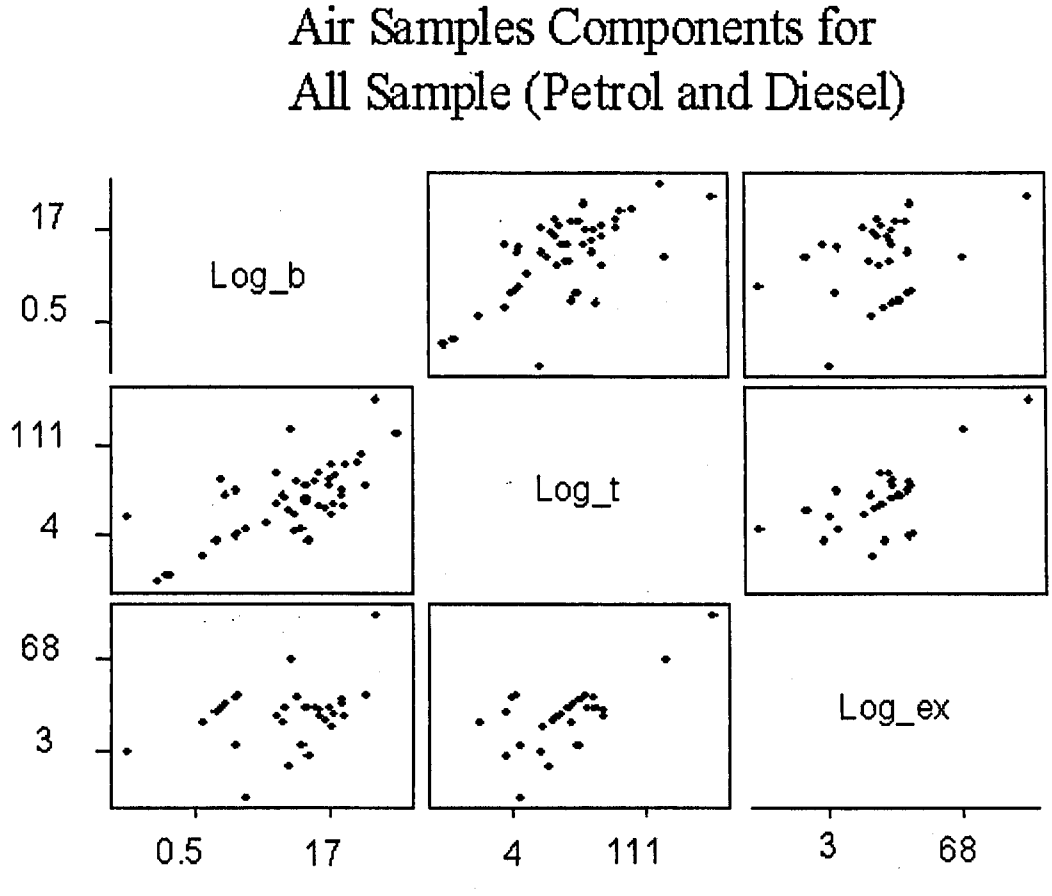


Figure 51: Matrix plot for the logarithm of benzene (Log_b), toluene (Log_t), and ethybenzene and xylenes (Log_ex). The unit is Log ppb.

Table 46: Data for BTEX concentrations in air samples collected from cars during driving. Seven records were omitted because of failing to collect air samples.

Fuel	Vl	Benzene (ppb)	Toluene (ppb)	EX (ppb)
petrol	2	0.19	0.79	-
petrol	2	0.23	0.98	-
petrol	2	1.84	5.12	0.75
petrol	2	6.94	4.96	-
petrol	2	6.99	8.79	-
petrol	2	7.13	29.58	20.78
petrol	2	9.31	24.71	-
petrol	2	9.16	14.65	-
petrol	2	9.55	3.50	3.04
petrol	2	11.13	29.86	14.06
petrol	2	12.73	39.77	13.50
petrol	2	17.20	25.44	14.68
petrol	2	17.35	32.26	-
petrol	2	20.30	38.57	-
petrol	2	36.37	60.82	-
petrol	2	39.83	80.75	-
petrol	2	60.05	579.69	303.72
petrol	2	102.57	171.54	-
petrol	3	5.85	10.24	2.12
petrol	3	18.31	54.82	-
petrol	4	0.61	1.95	9.02
petrol	4	0.83	3.53	12.27
petrol	4	1.42	21.04	4.19
petrol	4	1.53	4.56	22.53
petrol	4	12.56	12.33	10.70
petrol	4	15.26	11.09	9.63
petrol	4	17.80	8.71	7.56
petrol	4	23.35	18.65	16.19
petrol	4	24.61	12.39	10.75
petrol	4	45.27	24.79	21.51
petrol	7	4.86	18.15	8.88
petrol	9	9.16	14.65	-
petrol	10	3.06	6.36	-
petrol	11	9.05	15.65	-
petrol	11	26.48	55.51	-
petrol	15	0.24	1.00	-
petrol	16	18.31	54.82	-
Diesel	1	0.08	8.15	3.51
Diesel	5	4.07	39.57	11.04
Diesel	5	5.16	16.24	14.10
Diesel	5	8.33	4.99	4.33
Diesel	5	18.98	13.55	11.76
Diesel	6	1.05	18.38	16.72

(Continues ...)

Fuel	Vl	Benzene (ppb)	Toluene (ppb)	EX (ppb)
Diesel	6	1.40	4.25	20.66
Diesel	6	4.26	12.93	11.22
Diesel	6	6.08	188.13	70.91
Diesel	6	8.95	17.13	14.87
Diesel	6	24.40	22.18	19.25
Diesel	18	18.31	54.82	-
Diesel	21	0.97	32.54	14.69
Diesel	21	1.42	21.04	4.19
Diesel	22	0.97	32.54	14.69
Diesel	22	1.42	21.04	4.19
Diesel	23	0.97	32.54	14.69
Diesel	23	1.42	21.04	4.19
Diesel	24	0.97	32.54	14.69
Diesel	24	1.42	21.04	4.19

Vl: volunteer code.
EX: ethylbenzene and xylenes in ppb.
(-) unknown.

3.12.9.1 Smoking and Passive Smoking

None of the subjects had active passive smoking in the day of sampling. Only two subjects use to smoke one cigarette a day habitually but not in the day of sampling. On the other hand, no habitual passive smoking subjects were found among the samples other than those two subjects. Neither smoking nor passive smoking significantly influenced the study measurements.

3.12.9.2 Year of Car Production

After divide vehicles as new (1990-98) and old (1987-89), no differences were found between this factor and any of the aimed pollutants except when new diesel and new petrol cars tested for benzene (Table 47). Among new cars, petrol samples (n= 15, mean = 6.7 ppb) demonstrated higher level of benzene compare to the new diesel samples (n= 19, mean = 2.3 ppb, two sample t-test, p= 0.029) as in Figure 52.

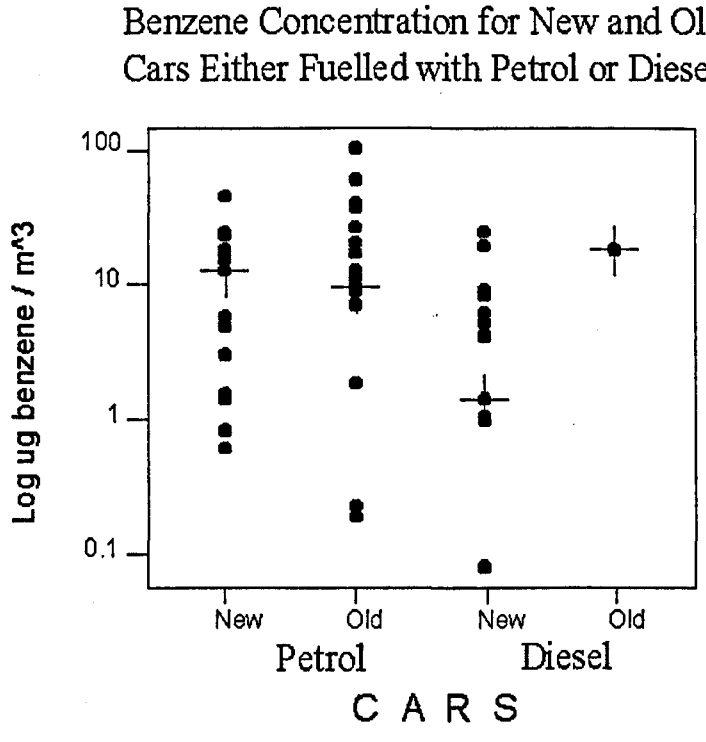


Figure 52: The distribution of benzene concentration in new (1990-98) and old (1987-89) in petrol and diesel cars that involved in the study. Plus sign (+) indicates the median. Only one sample was obtained from old diesel car.

No differences in the other contaminants (toluene (ANOVA), p=0.418, ethylbenzene and xylenes (ANOVA), p=0.878) were found between petrol and diesel,

and old and new cars samples. Benzene over toluene ratio also showed no significant differences among them (ANOVA, p=0.099).

Table 47: Old and new cars benzene and toluene level compare to fuel type.

Type	Fuel	Vl	Benzene (ppb)	Toluene (ppb)	b/t ratio
New	Diesel	1	0.08	8.15	0.01
New	Diesel	5	4.07	39.57	0.10
New	Diesel	5	5.16	16.24	0.32
New	Diesel	5	8.33	4.99	1.67
New	Diesel	5	18.98	13.55	1.40
New	Diesel	6	1.05	18.38	0.06
New	Diesel	6	1.40	4.25	0.33
New	Diesel	6	4.26	12.93	0.33
New	Diesel	6	6.08	188.13	0.03
New	Diesel	6	8.95	17.13	0.52
New	Diesel	6	24.40	22.18	1.10
New	Diesel	21	0.97	32.54	0.03
New	Diesel	21	1.42	21.04	0.07
New	Diesel	22	0.97	32.54	0.03
New	Diesel	22	1.42	21.04	0.07
New	Diesel	23	0.97	32.54	0.03
New	Diesel	23	1.42	21.04	0.07
New	Diesel	24	0.97	32.54	0.03
New	Diesel	24	1.42	21.04	0.07
New	**petrol**	**3**	**5.85**	**10.24**	**0.57**
New	**petrol**	**3**	**18.31**	**54.82**	**0.33**
New	**petrol**	**4**	**0.61**	**1.95**	**0.31**
New	**petrol**	**4**	**0.83**	**3.53**	**0.24**
New	**petrol**	**4**	**1.42**	**21.04**	**0.07**
New	**petrol**	**4**	**1.53**	**4.56**	**0.34**
New	**petrol**	**4**	**12.56**	**12.33**	**1.02**
New	**petrol**	**4**	**15.26**	**11.09**	**1.38**
New	**petrol**	**4**	**17.80**	**8.71**	**2.04**
New	**petrol**	**4**	**23.35**	**18.65**	**1.25**
New	**petrol**	**4**	**24.61**	**12.39**	**1.99**
New	**petrol**	**4**	**45.27**	**24.79**	**1.83**
New	**petrol**	**7**	**4.86**	**18.15**	**0.27**
New	**petrol**	**10**	**3.06**	**6.36**	**0.48**
New	**petrol**	**16**	**18.31**	**54.82**	**0.33**

(Continuos ...)

Type	Fuel	Vl	Benzene (ppb)	Toluene (ppb)	b/t ratio
Old	Diesel	18	18.31	54.82	0.33
Old	petrol	2	0.19	0.79	0.24
Old	petrol	2	0.23	0.98	0.23
Old	petrol	2	1.84	5.12	0.36
Old	petrol	2	6.94	4.96	1.40
Old	petrol	2	6.99	8.79	0.80
Old	petrol	2	7.13	29.58	0.24
Old	petrol	2	9.31	24.71	0.38
Old	petrol	2	9.16	14.65	0.63
Old	petrol	2	9.55	3.50	2.73
Old	petrol	2	11.13	29.86	0.37
Old	petrol	2	12.73	39.77	0.32
Old	petrol	2	17.20	25.44	0.68
Old	petrol	2	17.35	32.26	0.54
Old	petrol	2	20.30	38.57	0.53
Old	petrol	2	36.37	60.82	0.60
Old	petrol	2	39.83	80.75	0.49
Old	petrol	2	60.05	579.69	0.10
Old	petrol	2	102.57	171.54	0.60
Old	petrol	9	9.16	14.65	0.63
Old	petrol	11	9.05	15.65	0.58
Old	petrol	11	26.48	55.51	0.48

Type: New (1990-98) and old (1987-1989).
Vl: volunteer code.
b/t: benzene in ppb per toluene in ppb.
Seven records were omitted because of failing to collect air samples. Another one sample excluded because the car year of production was unknown.

3.12.9.3 Vehicle Refuel

There is no significant differences for any of pollutants of interest in air concentrations cars that were refuelled and those that were not (two-sample t-test, $p>0.5$). Benzene, toluene and ethylbenzene & xylenes p-values were 0.66, 0.87 and 0.73, respectively (Table 48). The air sample was taken from inside the car compartment, which is a distance away from the refuelling point. The relationship between this factor and personal exposure (*t,t*-muconic acid) will be tested later. The distribution of Log (benzene ppb) in the two groups of refuel can be seen in Figure 53.

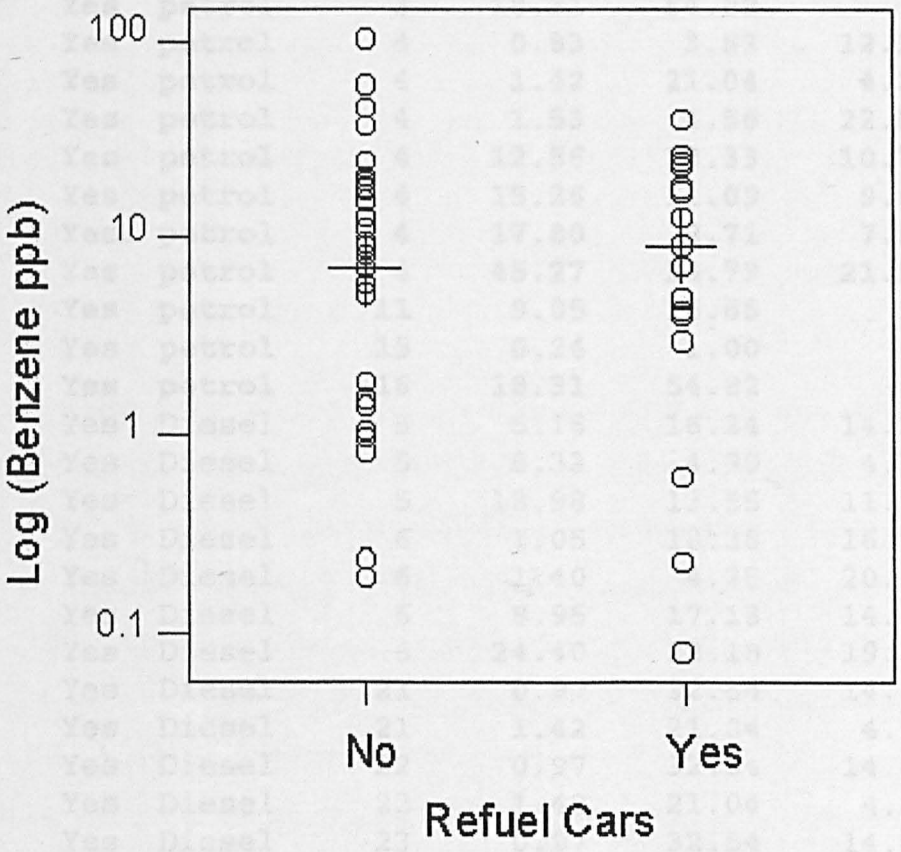


Figure 53: Ambient benzene concentrations in refuelled (Yes, n=18) and None refuelled (No, n=38) cars.

Table 48: Benzene level when the car was refuelled during the driving event.

Refuel	Fuel	Vl	Benzene (ppb)	Toluene (ppb)	EX (ppb)	b/t ratio
Yes	petrol	2	0.19	0.79	-	0.24
Yes	petrol	2	1.84	5.12	0.75	0.36
Yes	petrol	2	6.94	4.96	-	1.40
Yes	petrol	2	6.99	8.79	-	0.80
Yes	petrol	2	9.31	24.71	-	0.38
Yes	petrol	2	9.55	3.50	3.04	2.73
Yes	petrol	2	11.13	29.86	14.06	0.37
Yes	petrol	2	20.30	38.57	-	0.53
Yes	petrol	2	36.37	60.82	-	0.60
Yes	petrol	2	60.05	579.69	303.72	0.10
Yes	petrol	2	102.57	171.54	-	0.60
Yes	petrol	3	5.85	10.24	2.12	0.57
Yes	petrol	3	18.31	54.82	-	0.33
Yes	petrol	4	0.83	3.53	12.27	0.24
Yes	petrol	4	1.42	21.04	4.19	0.07
Yes	petrol	4	1.53	4.56	22.53	0.34
Yes	petrol	4	12.56	12.33	10.70	1.02
Yes	petrol	4	15.26	11.09	9.63	1.38
Yes	petrol	4	17.80	8.71	7.56	2.04
Yes	petrol	4	45.27	24.79	21.51	1.83
Yes	petrol	11	9.05	15.65	-	0.58
Yes	petrol	15	0.24	1.00	-	0.24
Yes	petrol	16	18.31	54.82	-	0.33
Yes	Diesel	5	5.16	16.24	14.10	0.32
Yes	Diesel	5	8.33	4.99	4.33	1.67
Yes	Diesel	5	18.98	13.55	11.76	1.40
Yes	Diesel	6	1.05	18.38	16.72	0.06
Yes	Diesel	6	1.40	4.25	20.66	0.33
Yes	Diesel	6	8.95	17.13	14.87	0.52
Yes	Diesel	6	24.40	22.18	19.25	1.10
Yes	Diesel	21	0.97	32.54	14.69	0.03
Yes	Diesel	21	1.42	21.04	4.19	0.07
Yes	Diesel	22	0.97	32.54	14.69	0.03
Yes	Diesel	22	1.42	21.04	4.19	0.07
Yes	Diesel	23	0.97	32.54	14.69	0.03
Yes	Diesel	23	1.42	21.04	4.19	0.07
Yes	Diesel	24	0.97	32.54	14.69	0.03
Yes	Diesel	24	1.42	21.04	4.19	0.07
No	petrol	2	0.23	0.98	-	0.23
No	petrol	2	7.13	29.58	20.78	0.24
No	petrol	2	9.16	14.65	-	0.63
No	petrol	2	12.73	39.77	13.50	0.32
No	petrol	2	17.20	25.44	14.68	0.68
No	petrol	2	17.35	32.26	-	0.54
No	petrol	2	39.83	80.75	-	0.49

(Continuos ...)

Refuel	Fuel	Vl	Benzene (ppb)	Toluene (ppb)	EX (ppb)	b/t ratio
No	petrol	4	0.61	1.95	9.02	0.31
No	petrol	4	23.35	18.65	16.19	1.25
No	petrol	4	24.61	12.39	10.75	1.99
No	petrol	7	4.86	18.15	8.88	0.27
No	petrol	9	9.16	14.65	-	0.63
No	petrol	10	3.06	6.36	-	0.48
No	petrol	11	26.48	55.51	-	0.48
No	Diesel	1	0.08	8.15	3.51	0.01
No	Diesel	5	4.07	39.57	11.04	0.10
No	Diesel	6	4.26	12.93	11.22	0.33
No	Diesel	18	18.31	54.82	-	0.33

VLNTR: volunteer code
EX: ethylbenzene and xylenes in ppb.
b/t: benzene in ppb per toluene in ppb.
(-): unknown.
Seven records were omitted because of failing to collect air samples. Another sample ignored for not mentioning the refuel question.

3.12.9.4 Window Status

The driver was asked in the questionnaire whether the window was open for the whole journey (was categorised YES), if window was closed (NO), or if the window was open for part of the journey (SOME). Data is presented in Table 48. Benzene measurements were related with the window status significantly (one-way ANOVA, $p<0.001$). There was a statistical significant difference between closed window (n= 11, mean = 21.5 ppb) and the other groups (95%CI) as demonstrated in Figure 41 for benzene level. No significant difference was found between the groups with the open window either in the whole journey (n= 21, mean = 5.7 ppb) or in part of it (n= 24, mean = 2.4 ppb). Neither toluene nor the mixture of ethylbenzene and xylenes were statistically different among the window status groups.

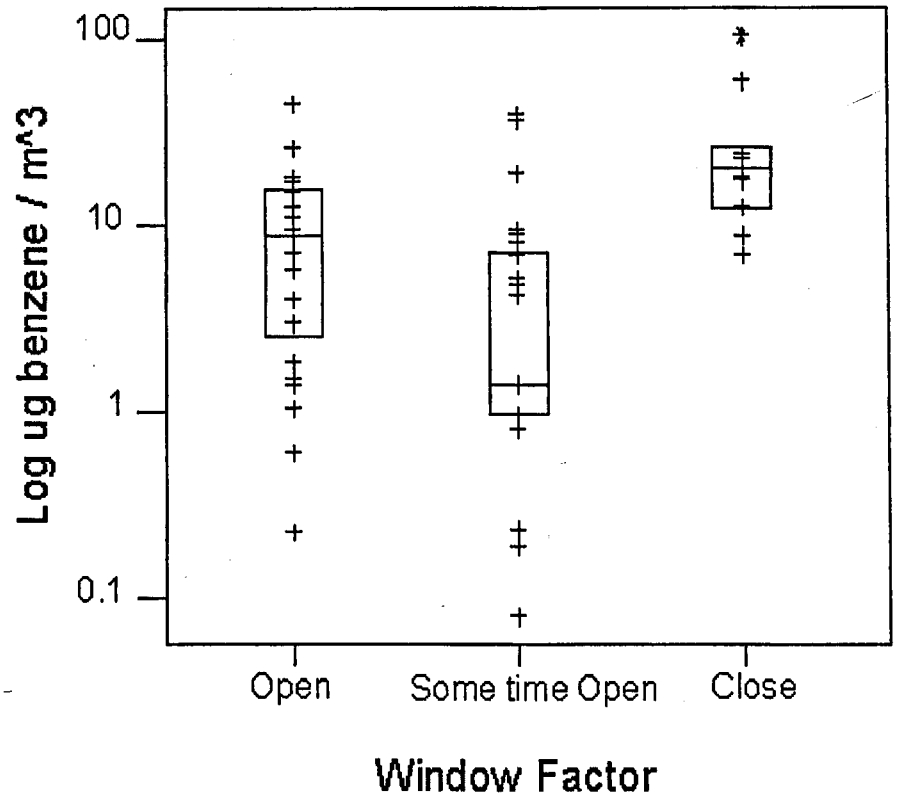


Figure 54: Window status compared to benzene level (log μg/m^3). The boxplot indicate the mean (middle line) and 95%CI (outer rectangular).

None of the other contaminants had a significant difference (one-way ANOVA). The differences that obtained in benzene levels between the three groups were still significant when only petrol air samples were considered (one-way ANOVA, Open n= 19 and mean = 6.3 ppb, Some times Open n= 10 and mean = 4.1 ppb, Close n= 8 and mean = 24.1 ppb, p= 0.04) and diesel samples (Open n= 2 and mean = 2.1 ppb, Some times Open n= 14 and mean = 1.7 ppb, Close n= 3 and mean = 15.9 ppb, p= 0.029). Due to the small sample size, the difference between closed and opened windows was insignificant. However, the means still aligned with the findings from the combined petrol and diesel results. Closing the vehicle windows make the car compartment a confined environment and the emitted pollutants accumulated in the car compartment unless ventilation was started by opening the car window.

Table 49: Window status compare to benzene, toluene and EX inside the car.

Window	Fuel	Vl	Benzene (ppb)	Toluene (ppb)	EX (ppb)	b/t ratio
Open	petrol	2	0.23	0.98	-	0.23
Open	petrol	2	1.84	5.12	0.75	0.36
Open	petrol	2	7.13	29.58	20.78	0.24
Open	petrol	2	9.55	3.50	3.04	2.73
Open	petrol	2	11.13	29.86	14.06	0.37
Open	petrol	2	17.20	25.44	14.68	0.68
Open	petrol	2	17.35	32.26	-	0.54
Open	petrol	3	5.85	10.24	2.12	0.57
Open	petrol	3	18.31	54.82	-	0.33
Open	petrol	4	0.61	1.95	9.02	0.31
Open	petrol	4	1.42	21.04	4.19	0.07
Open	petrol	4	1.53	4.56	22.53	0.34
Open	petrol	4	12.56	12.33	10.70	1.02
Open	petrol	4	15.26	11.09	9.63	1.38
Open	petrol	4	45.27	24.79	21.51	1.83
Open	petrol	10	3.06	6.36	-	0.48
Open	petrol	11	9.05	15.65	-	0.58
Open	petrol	11	26.48	55.51	-	0.48
Open	petrol	16	18.31	54.82	-	0.33
Open	Diesel	5	4.07	39.57	11.04	0.10
Open	Diesel	6	1.05	18.38	16.72	0.06
Some	petrol	2	0.19	0.79	-	0.24
Some	petrol	2	6.99	8.79	-	0.80
Some	petrol	2	9.31	24.71	-	0.38
Some	petrol	2	9.16	14.65	-	0.63
Some	petrol	2	36.37	60.82	-	0.60
Some	petrol	2	39.83	80.75	-	0.49
Some	petrol	4	0.83	3.53	12.27	0.24
Some	petrol	7	4.86	18.15	8.88	0.27
Some	petrol	9	9.16	14.65	-	0.63
Some	petrol	15	0.24	1.00	-	0.24
Some	Diesel	1	0.08	8.15	3.51	0.01
Some	Diesel	5	5.16	16.24	14.10	0.32
Some	Diesel	5	8.33	4.99	4.33	1.67
Some	Diesel	5	18.98	13.55	11.76	1.40
Some	Diesel	6	1.40	4.25	20.66	0.33
Some	Diesel	6	4.26	12.93	11.22	0.33
Some	Diesel	21	0.97	32.54	14.69	0.03
Some	Diesel	21	1.42	21.04	4.19	0.07
Some	Diesel	22	0.97	32.54	14.69	0.03
Some	Diesel	22	1.42	21.04	4.19	0.07
Some	Diesel	23	0.97	32.54	14.69	0.03
Some	Diesel	23	1.42	21.04	4.19	0.07
Some	Diesel	24	0.97	32.54	14.69	0.03
Some	Diesel	24	1.42	21.04	4.19	0.07

(Continuos ...)

Window	Fuel	Vl	Benzene (ppb)	Toluene (ppb)	EX (ppb)	b/t ratio
Close	**petrol**	**2**	**6.94**	**4.96**	**-**	**1.40**
Close	**petrol**	**2**	**12.73**	**39.77**	**13.50**	**0.32**
Close	**petrol**	**2**	**20.30**	**38.57**	**-**	**0.53**
Close	**petrol**	**2**	**60.05**	**579.69**	**303.72**	**0.10**
Close	**petrol**	**2**	**102.57**	**171.54**	**-**	**0.60**
Close	**petrol**	**4**	**17.80**	**8.71**	**7.56**	**2.04**
Close	**petrol**	**4**	**23.35**	**18.65**	**16.19**	**1.25**
Close	**petrol**	**4**	**24.61**	**12.39**	**10.75**	**1.99**
Close	Diesel	6	8.95	17.13	14.87	0.52
Close	Diesel	6	24.40	22.18	19.25	1.10
Close	Diesel	18	18.31	54.82	-	0.33

Vl : volunteer code
EX : ethylbenzene and xylenes in ppb.
b/t: benzene in ppb per toluene in ppb.
(-): unknown.
Seven records were omitted because of failing to collect air samples. Another sample ignored for not mentioning the refuel question.

3.12.9.5 Air-conditioning

There are three possible answers for a question about using air-conditioning system. If it was switched ON for the whole journey, then YES was chosen. NO should be chosen if it was switched OFF. If it was ON for part of the journey then SOME should be chosen. The results are shown in Table 50.

No significant differences were found in benzene level between the subgroups of air-condition factor. Also, no significant differences were found after separating petrol and diesel samples. Toluene differences only found in diesel cars. The air samples for diesel cars showed a significant differences between the air-conditioning groups when toluene was tested (one-way ANOVA, p=0.019). Toluene concentrations in NO (n=6, mean = 28.4 ppb), SOMETIME (n=4, mean = 15.5 ppb) and YES (n=9, mean = 11.6 ppb) groups significantly differed. This may affect the *t,t*-muconic acid excretion especially in diesel cars. Sample size may influence the failing to prove any differences could be existing in the other chemicals.

Table 50: Air-conditioning status and the level of BTEX inside the cars while driving.

AC	Fuel	Vl	Benzene (ppb)	Toluene (ppb)	EX (ppb)	b/t ratio
On	petrol	2	0.19	0.79	-	0.24
On	petrol	2	0.23	0.98	-	0.23
On	petrol	2	1.84	5.12	0.75	0.36
On	petrol	2	6.94	4.96	-	1.40
On	petrol	2	6.99	8.79	-	0.80
On	petrol	2	7.13	29.58	20.78	0.24
On	petrol	2	9.31	24.71	-	0.38
On	petrol	2	9.16	14.65	-	0.63
On	petrol	2	9.55	3.50	3.04	2.73
On	petrol	2	11.13	29.86	14.06	0.37
On	petrol	2	12.73	39.77	13.50	0.32
On	petrol	2	17.35	32.26	-	0.54
On	petrol	2	20.30	38.57	-	0.53
On	petrol	2	36.37	60.82	-	0.60
On	petrol	2	39.83	80.75	-	0.49
On	petrol	2	60.05	579.69	303.72	0.10
On	petrol	2	102.57	171.54	-	0.60
On	petrol	3	5.85	10.24	2.12	0.57
On	petrol	3	18.31	54.82	-	0.33

(Continuos ...)

AC	Fuel	Vl	Benzene (ppb)	Toluene (ppb)	EX (ppb)	b/t ratio
On	**petrol**	**7**	**4.86**	**18.15**	**8.88**	**0.27**
On	**petrol**	**9**	**9.16**	**14.65**	**-**	**0.63**
On	**petrol**	**11**	**9.05**	**15.65**	**-**	**0.58**
On	**petrol**	**11**	**26.48**	**55.51**	**-**	**0.48**
On	**petrol**	**15**	**0.24**	**1.00**	**-**	**0.24**
On	**petrol**	**16**	**18.31**	**54.82**	**-**	**0.33**
On	Diesel	18	18.31	54.82	-	0.33
On	Diesel	21	0.97	32.54	14.69	0.03
On	Diesel	21	1.42	21.04	4.19	0.07
On	Diesel	22	0.97	32.54	14.69	0.03
On	Diesel	22	1.42	21.04	4.19	0.07
On	Diesel	23	0.97	32.54	14.69	0.03
On	Diesel	23	1.42	21.04	4.19	0.07
On	Diesel	24	0.97	32.54	14.69	0.03
On	Diesel	24	1.42	21.04	4.19	0.07
Some	**petrol**	**4**	**24.61**	**12.39**	**10.75**	**1.99**
Some	**petrol**	**10**	**3.06**	**6.36**	**-**	**0.48**
Some	Diesel	1	0.08	8.15	3.51	0.01
Some	Diesel	6	1.05	18.38	16.72	0.06
Some	Diesel	6	8.95	17.13	14.87	0.52
Some	Diesel	6	24.40	22.18	19.25	1.10
Off	**petrol**	**2**	**17.20**	**25.44**	**14.68**	**0.68**
Off	**petrol**	**4**	**0.61**	**1.95**	**9.02**	**0.31**
Off	**petrol**	**4**	**0.83**	**3.53**	**12.27**	**0.24**
Off	**petrol**	**4**	**1.42**	**21.04**	**4.19**	**0.07**
Off	**petrol**	**4**	**1.53**	**4.56**	**22.53**	**0.34**
Off	**petrol**	**4**	**12.56**	**12.33**	**10.70**	**1.02**
Off	**petrol**	**4**	**15.26**	**11.09**	**9.63**	**1.38**
Off	**petrol**	**4**	**17.80**	**8.71**	**7.56**	**2.04**
Off	**petrol**	**4**	**23.35**	**18.65**	**16.19**	**1.25**
Off	**petrol**	**4**	**45.27**	**24.79**	**21.51**	**1.83**
Off	Diesel	5	4.07	39.57	11.04	0.10
Off	Diesel	5	5.16	16.24	14.10	0.32
Off	Diesel	5	8.33	4.99	4.33	1.67
Off	Diesel	5	18.98	13.55	11.76	1.40
Off	Diesel	6	1.40	4.25	20.66	0.33
Off	Diesel	6	4.26	12.93	11.22	0.33

AC : Air-condition system.
Vl : volunteer code
EX : ethylbenzene and xylenes in ppb.
b/t: benzene in ppb per toluene in ppb.
(-): unknown.
Seven records were omitted because of failing to collect air samples. Another sample ignored for not mentioning the refuel question.

3.12.9.6 Traffic Status

Heavy traffic is a factor of concern for environmental exposure. Driving through a heavy traffic was either occur (yes, n= 16) or not occur (no, n= 18). The results were presented in Table 52. In this study, none of the components of BTEX were elevated significantly in car driving in heavy traffic(2-sample t-test, p>0.05) compare to no heavy traffic. The same finding was reached even after the samples were subdivided into petrol and diesel cars (2-sample t-test, p>0.05), see Table 51.

Table 51: Traffic status versus BTEX for all samples, petrol and diesel samples (2-sample t-test, p-value).

Chemical	p - value		
Log ppb	All	Petrol	Diesel
benzene	0.944	0.337	0.295
toluene	0.097	0.263	0.983
ex*	0.546	0.380	0.939
b/t**	0.295	0.751	0.334

*ex= ethylbenzene & xylenes
**b/t= benzene ppb/ toluene ppb

Table 52: Traffic status and the level of benzene inside the cars while driving.

Queue	Fuel	Vl	Benzene (ppb)	Toluene (ppb)	EX (ppb)	b/t ratio
Yes	**petrol**	**2**	**1.84**	**5.12**	**0.75**	**0.36**
Yes	**petrol**	**2**	**9.55**	**3.50**	**3.04**	**2.73**
Yes	**petrol**	**2**	**11.13**	**29.86**	**14.06**	**0.37**
Yes	**petrol**	**2**	**12.73**	**39.77**	**13.50**	**0.32**
Yes	**petrol**	**4**	**0.61**	**1.95**	**9.02**	**0.31**
Yes	**petrol**	**4**	**0.83**	**3.53**	**12.27**	**0.24**
Yes	**petrol**	**4**	**1.42**	**21.04**	**4.19**	**0.07**
Yes	**petrol**	**4**	**1.53**	**4.56**	**22.53**	**0.34**
Yes	**petrol**	**4**	**17.80**	**8.71**	**7.56**	**2.04**
Yes	**petrol**	**4**	**23.35**	**18.65**	**16.19**	**1.25**
Yes	**petrol**	**4**	**24.61**	**12.39**	**10.75**	**1.99**
Yes	**petrol**	**4**	**45.27**	**24.79**	**21.51**	**1.83**
Yes	Diesel	1	0.08	8.15	3.51	0.01
Yes	Diesel	5	4.07	39.57	11.04	0.10
Yes	Diesel	5	5.16	16.24	14.10	0.32
Yes	Diesel	6	1.05	18.38	16.72	0.06
No	**petrol**	**2**	**7.13**	**29.58**	**20.78**	**0.24**
No	**petrol**	**2**	**17.20**	**25.44**	**14.68**	**0.68**
No	**petrol**	**4**	**12.56**	**12.33**	**10.70**	**1.02**
No	**petrol**	**4**	**15.26**	**11.09**	**9.63**	**1.38**
No	Diesel	5	8.33	4.99	4.33	1.67
No	Diesel	5	18.98	13.55	11.76	1.40
No	Diesel	6	1.40	4.25	20.66	0.33
No	Diesel	6	4.26	12.93	11.22	0.33
No	Diesel	6	8.95	17.13	14.87	0.52
No	Diesel	6	24.40	22.18	19.25	1.10
No	Diesel	21	0.97	32.54	14.69	0.03
No	Diesel	21	1.42	21.04	4.19	0.07
No	Diesel	22	0.97	32.54	14.69	0.03
No	Diesel	22	1.42	21.04	4.19	0.07
No	Diesel	23	0.97	32.54	14.69	0.03
No	Diesel	23	1.42	21.04	4.19	0.07
No	Diesel	24	0.97	32.54	14.69	0.03
No	Diesel	24	1.42	21.04	4.19	0.07

Queue: heavy traffic queue.
Vl : volunteer code
EX : ethylbenzene and xylenes in ppb.
b/t: benzene in ppb per toluene in ppb.
(-): unknown.
Seven records were omitted because of failing to collect air samples. Another 23 samples were removed for not mentioning the refuel question.

3.12.10 Urinary *t,t*-Muconic Acid and Air Sample

The relationship between benzene in air samples and urinary *t,t*-muconic acid was varied. In petrol samples, only POST1 were related significantly with benzene (R^2=30.2%, p=0.012) as shown in Figure 55 and toluene (R^2=21%, p=0.042) positively.

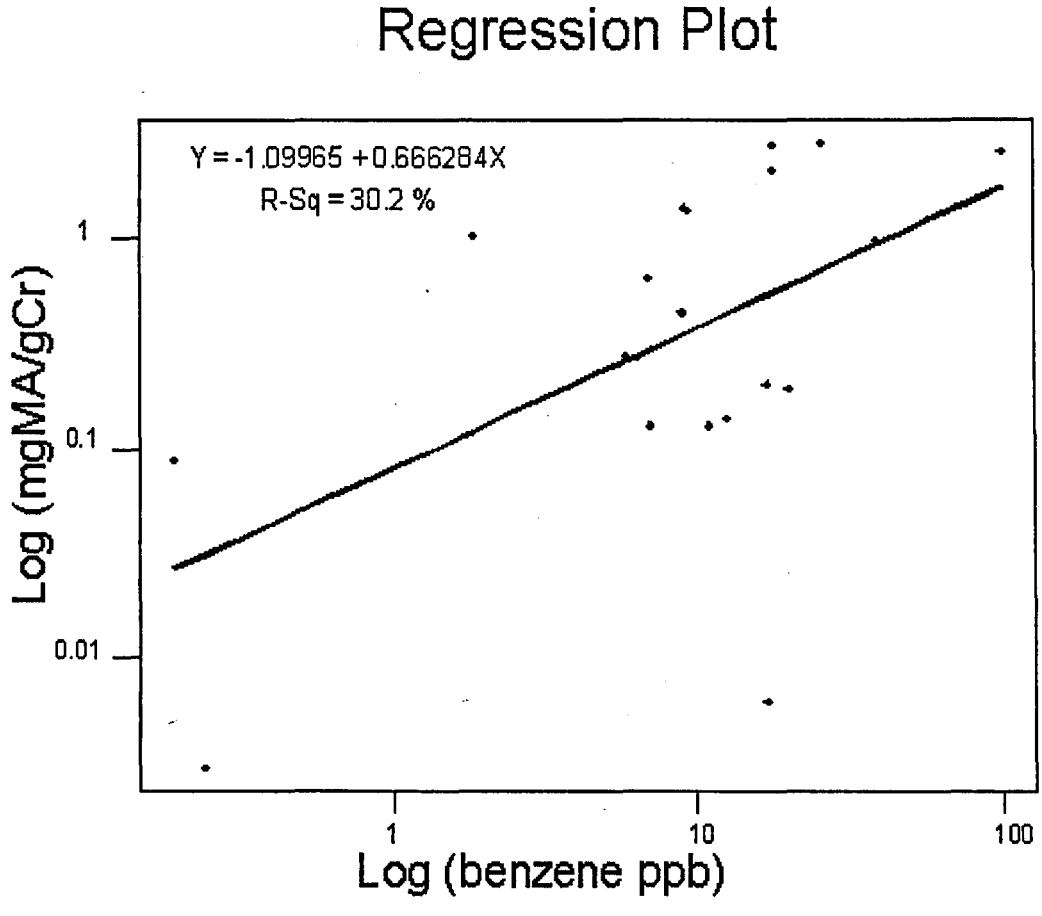


Figure 55: Ambient benzene concentration plotted against urinary *t,t*-muconic acid that determined in samples that were taken between 2 and 7h after the PRE samples in petrol group.

On the other hand, benzene /toluene ratio correlated negatively with POST2 (n=24, R^2=25%, p=0.011) while failed to correlate with POST1 (n=18, p=0.414) or POST (n=36, p=0.227) samples (Table 53). In diesel samples, a negative correlation

between the xylenes with ethylbenzene and urinary *t,t*-muconic acid in POST2 (n=18, R^2=47%, p=0.002).

Table 53:.Benzene and toluene versus urinary *t,t*-muconic acid concentration from subjects exposed to petrol or diesel cars emission.

Fuel	Vl	Benzene (ppb)	Toluene (ppb)	Post1 [	Post2 mgMA/gCr	Post]
petrol	2	0.19	0.79	0.09	-	0.09
petrol	2	0.23	0.98	-	0.19	0.19
petrol	2	1.84	5.12	1.01	0.12	0.56
petrol	2	6.94	4.96	0.66	-	0.66
petrol	2	6.99	8.79	-	0.28	0.28
petrol	2	7.13	29.58	0.13	-	0.13
petrol	2	9.16	14.65	-	0.00	0.00
petrol	2	9.31	24.71	-	1.02	1.02
petrol	2	9.55	3.50	1.34	0.01	0.67
petrol	2	11.13	29.86	0.13	-	0.13
petrol	2	12.73	39.77	0.14	0.24	0.19
petrol	2	17.20	25.44	0.01	0.26	0.13
petrol	2	17.35	32.26	0.20	-	0.20
petrol	2	20.30	38.57	0.19	-	0.19
petrol	2	36.37	60.81	-	0.10	0.10
petrol	2	39.83	80.74	1.00	0.03	0.51
petrol	2	60.05	579.70	1.23	2.72	1.98
petrol	2	102.57	171.55	2.56	-	2.57
petrol	3	5.85	10.24	0.28	4.27	2.27
petrol	3	18.31	54.82	2.77	-	2.77
petrol	4	0.61	1.95	-	3.82	3.82
petrol	4	0.83	3.53	-	0.03	0.03
petrol	4	1.42	21.04	-	5.20	5.20
petrol	4	1.53	4.56	-	4.78	4.78
petrol	4	12.56	12.33	-	1.98	1.98
petrol	4	15.26	11.09	-	0.02	0.02
petrol	4	17.80	8.71	-	0.58	0.58
petrol	4	23.35	18.65	-	0.42	0.42
petrol	4	24.61	12.39	-	0.01	0.01
petrol	4	45.27	24.79	-	0.01	0.01
petrol	7	4.86	18.15	-	0.71	0.71
petrol	9	9.16	14.65	1.40	0.94	1.17
petrol	10	3.06	6.36	-	0.16	0.16
petrol	11	9.05	15.65	0.46	-	0.45
petrol	11	26.48	55.51	2.81	-	2.81
petrol	15	0.24	1.00	0.00	-	0.00
petrol	16	18.31	54.82	2.07	-	2.08
Diesel	1	0.08	8.15	-	0.30	0.30
Diesel	5	4.07	39.57	-	0.02	0.02
Diesel	5	5.16	16.24	0.01	-	0.01

Diesel	5	8.33	4.99	-	0.01	0.01
Diesel	5	18.98	13.55	-	0.00	0.00
Diesel	6	1.05	18.38	-	0.00	0.00

(Contineous ...)

Fuel	Vl	Benzene (ppb)	Toluene (ppb)	Post1 [	Post2 mgMA/gCr	Post]
Diesel	6	1.40	4.25	-	0.01	0.01
Diesel	6	4.26	12.93	-	0.02	0.02
Diesel	6	6.08	188.15	-	0.01	0.01
Diesel	6	8.95	17.13	-	0.38	0.38
Diesel	6	24.40	22.18	-	0.00	0.00
Diesel	18	18.31	54.82	-	0.52	0.52
Diesel	21	0.97	32.54	-	0.00	0.00
Diesel	21	1.42	21.04	-	0.26	0.26
Diesel	22	0.97	32.54	-	0.00	0.00
Diesel	22	1.42	21.04	-	0.41	0.41
Diesel	23	0.97	32.54	-	0.01	0.01
Diesel	23	1.42	21.04	-	0.60	0.60
Diesel	24	0.97	32.54	-	0.00	0.00
Diesel	24	1.42	21.04	-	0.79	0.79

Vl : volunteer code
(-): not determined.

SECTION (IV):

GENERAL DISCUSSION

SECTION (IV): GENERAL DISCUSSION

The impact of chemicals on health is one of the crucial issues of concern to the world. Environmental exposure to organic compounds imposes new challenges in terms of health and related issues. Carcinogens such as benzene have brought a great deal of public concern regarding the risk to human health. Environmental low-level exposure to benzene may occur through contaminated air, water, and soil and from the food chain. Air exposure sources are mainly from tobacco smoke or petrol emissions.

Animal studies strongly supported benzene toxicity. Sister chromatid exchange (lymphocytes in bone marrow), micronuclei (bone marrow and peripheral blood erythrocytes (polychromatic and normochromatic)), cell mutation (lymphocytes in spleen and lung), and DNA synthesis inhibitation (bone marrow) were reported in several animal studies (Luke et al., 1988b, Luke et al., 1988a, Lutz and Schlatter, 1977, Mullin et al., 1995, Plappert et al., 1994a, Sabourin et al., 1990, Tice et al., 1980, Tice et al., 1982, Ward et al., 1992).

A retrospective cohort study (Yin et al., 1987b, Yin et al., 1987a, Yin et al., 1989) was conducted among 28460 benzene-exposed workers from 233 factories in 12 Chinese cities. Control group includes 28257 unexposed workers. Thirty cases of leukaemia were found in the benzene-exposed group and four in the control. The standardised mortality ratio (SMR) was 5.7 ($p<0.01$). The average latency for leukaemia in the study was 11.4 years. Furthermore, a leukaemia case was reported by (Rinsky et al., 1987) who exposed for cumulatively in a year to 100 ppb and for 2 years latency period. This case could be extremely susceptible to leukaemia than the other cases whose exposure varied between 10 and 640ppm. It is important to consider other factors that possibly influence the risk on the susceptible group. One of them is the benzene absorption rate, which is inversely proportional to exposure concentration (Sabourin et al., 1987). Also, age factor (elder and children), health

status, and alcohol consumption are may underestimated in the epidemiological studies for benzene exposure.

In 1989, legal restriction was imposed on the use of benzene in the UK to ensure that all products were less than 0.1% by mass (Courage and Duarte-Davidson, 1999). However, petrol was an exempted product and the upper limit for benzene concentration was set at 5%. The average benzene percentage in petrol in the UK is 2%. The UK government aims to reach an ambient air quality benzene standard of 5 ppb by the year 2005 (Courage and Duarte-Davidson, 1999). To help achieve this, from 1st January 1993, cars fuelled by petrol had to be fitted with a three-way catalytic converter and carbon canister. The purpose of the three-way catalytic converter was to meet the exhaust emission limit and the carbon canister aimed to control evaporative hydrocarbon emission.

Environmental exposure to benzene could be expected to have a greater effect on vulnerable groups (elders, children, diseased people, etc) than healthy adults. For example, a child's breathing zone is generally lower than an adult is. When walking along the roadside, a child's breathing level will be closer to car exhaust emissions. This means that children may inhale a higher quantity of emitted gases, such as benzene.

An aim of this study was to develop a new method to determine a biomarker for low-level benzene exposure. Another aim was to validate a method to determine BTEX at low-levels from inside car cabin. To evaluate these methods for environmental application, a monitoring study was conducted. At the beginning of the study, taxi companies in Newcastle upon Tyne were aimed. Unfortunately, none of them responded positively after discussing the nature of the study. Therefore, samples were recruited from friends and staff in the Department of Environmental and Occupational Medicine. At the beginning, air samplers produced noise. An insulated box reduced the noise significantly. Although, restriction was aimed on smokers, as smoking is one of the main expected confounders, few were unintentionally obtained and discovered by the questionnaires. The small sample size is due to the nature of the study, which requires collecting urine samples before and after the period of exposure. Also, exposed samples sometimes accompanied with collecting air samples. The air samples duration was variable between exposed subjects due to driving patterns.

Sometimes, subjects drove for long continuous period. Others drove several short journeys in the day of sampling.

8-Hydroxy-2'-deoxyguanosine (8OhdG) as a marker of oxidative damage which can be produced by DNA damage. Although, it was not a specific marker, determination of urinary 8OhdG levels to correlate it with BTEX and *t,t*-muconic acid levels were aimed. The 8OhdG assay is based on HPLC-ECD (Shignaga et al., 1989). All steps of the assay were successfully reproduced except the SPE extraction. The brand of SPE kit might be reason behind the extraction failure. Therefore, the assay was excluded from this study.

Several biomarkers for benzene were mentioned previously (see Chapter 3). Although, the *S*-phenylmercapturic acid is widely used, *t,t*-muconic acid gets some advantages over it. First, *t,t*-muconic acid was selected as a marker of effect for one of the benzene toxic pathways unlike *S*-phenylmercapturic acid. The ring opening reaction to the reactive *t,t*-muconaldehyde is considered to be a toxic pathway (Yardley-Jones et al., 1991). Another study (Henschler et al., 1991) investigated the genotoxic and cell mutation properties of *t,t*-muconaldehyde, a putative toxic metabolite of benzene and a probable precursor of the *t,t*-muconic acid. *t,t*-Muconaldehyde showed direct mutagenic activity in tester TA100 using their assay. Further study (Latriano et al., 1989) demonstrated a DNA adduct that formed by *t,t*-muconaldehyde with [^{14}C]- deoxyguanosine monophosphate in vitro. Second, the correlation between low benzene exposure (<1ppm) and *t,t*-muconic acid is more stronger (r=0.64) than the correlation with *S*-phenylmercapturic acid (r=0.56) (Carere and Crebelli, 1998). Also, *t,t*-muconic acid has a strong correlation with blood benzene (r=0.84) (Carere and Crebelli, 1998, Aitio et al., 1997). Third, *S*-phenylmercapturic acid analysis is either based on GC-MS or a competitive enzyme-linked immunosorbant assay (ELISA). GC-MS is much complicated than *t,t*-muconic acid assay (HPLC). ELISA is less sensitive for levels that less than 1ppm when compare to GC-MS assay (Aston et al., 2002). Therefore, *t,t*-muconic acid is more sensitive for environmental levels (<1 ppm) compare to *S*-phenylmercapturic acid, which is more suitable for occupational levels (>1ppm).

Using butanol solvent instead of the ordinary solid phase extraction developed a new urinary t, t-muconic acid extraction technique. In comparison with solid phase

extraction, butanol extraction has some advantages. One of the advantages of using solvent in extracting *t,t*-muconic acid over the solid phase extraction cartridge (SAX) is in concentrating samples. SAX method dilutes *t,t*-muconic acid three times after the extraction step. However, the butanol extraction allows the analyst to view lower concentrations. After the evaporation step, the resuspension of the residues will determine the reconcentration. Reconcentrated samples may produce an overlap with other peaks. Therefore, further extraction by other method may resolve this problem in future. This prospect may lead to a promising improvement for this technique.

The consumption of drying gas, due to the evaporation duration of the solvent and the high flow rate of the gas, is a disadvantage of the solvent extraction. In solvent extraction, butanol evaporation varied between 75min and 110min with a 5ml/min flow rate of nitrogen gas.

This method was validated in this study and satisfactory precision and accuracy were obtained. In this study, the limit of detection was 0.01mg/l, which was the same as that mentioned by Boogaard and van Sittert (1995). A GC-MS assay for *t,t*-muconic acid analysis also reported a limit of detection of 0.01 mg/l (Ruppert et al., 1995).

The background level for *t,t*-muconic acid in urine was 0.143 mgMA/gCr, which was close to the maximum mean (Lee et al., 1993a). The *t,t*-muconic acid range for the control samples varied between 0.017 and 0.558 mgMA/gCr which corroborated that determined by Ruppert et al. (1997). The range of means of urinary *t,t*-muconic acid background levels from seven studies was between 0.033 and 0.14 mgMA /gCr. The overall range in these studies was 0.01 - 0.59 mgMA/gCr (Lee et al., 1993a; Ghittori et al., 1995; Ruppert et al., 1995; Ong et al., 1995; Ruppert et al., 1997; Javelaud et al., 1998; Crebelli.et al., 2001). The number of non-smoking control subjects in these studies was varies between 10 and 84. The amount of *t,t*-muconic acid found in control urine may be explained by environmental levels of exposure to benzene arising from petrol-cars emissions, or from consumption of sorbic acid (a food preservatives). Sorbic acid has been found to enhance *t,t*-muconic acid excretion in human urine (Ducos et al., 1992). Although, sorbic acid is not very popular in the food industry, it is difficult to identify sorbic acid in foodstuffs, especially when listed

under another name. In this study, none of the subjects answered about its consumption positively.

Phase (I) samples consists of two urine samples; one before the exposure (Pre) and another after the exposure (Post). The main purpose of the phase (I) group is to determine the change of *t,t*-muconic acid level in urine after the exposure. A questionnaire was also applied to control the potential factors that could influence the results of the study. The time difference between the collection of post and pre urines varied widely. With considering the half-life (5.0h ± 2.3h) for urinary *t,t*-muconic acid excretion after exposure (Boogaard and van Sittert, 1995), samples were segregated into two categories POST1 and POST2. POST1 samples were collected from 3 to 7 h from the start of driving and POST2 samples were collected 7 and 21 h after the start of driving. Because some samples had more than one post-urine sample, the average of the *t,t*-muconic acid levels was considered.

In this study, urinary *t,t*-muconic acid levels after exposure to petrol emissions inside the car was two-fold higher than pre-exposure levels. Another investigation of nineteen refinery workers demonstrated a similar elevation in urinary *t,t*-muconic acid (Lee et al., 1993a). Similarly, Crebille (2001) studied traffic police and showed that urinary *t,t*-muconic acid levels were 0.081mgAM/gCr pre-exposure and 0.116mgMA/gCr post-exposure. However, in the diesel group, no difference was found between levels before and after exposure. Seventy percent of the petrol-exposed volunteers who were healthy non-smokers had at least one urine sample in the day of sampling equal to or above 1 mgMA/gCr. All of the control samples were below 1 mgMA/gCr.

Phase (II) samples on the other hand characterised with collecting urine sample for 24h. The purpose of phase (II) samples is to determine the pattern of t,t-muconic acid levels during a 24h profile to investigate the relationship between exposure status and the marker level.

At times, the level of *t,t*-muconic acid from some volunteers was not elevated following confirmed exposure to benzene. For example, after one day of exposure to petrol emissions, volunteer 4 showed the highest *t,t*-muconic acid level of the study. However, after two days and after another exposure, the levels of *t,t*-muconic acid

were below the limit of detection. This was noticed in another study in which Boogaard and van Sittert (1995) investigated the profile of urinary *t,t*-muconic acid excretion for two workers and related it to air benzene concentrations.

It is true that this level is below the minimal risk level for benzene (50 ppb) (Hibbs et al., 1997). However, with considering the nature of carcinogens (based on the single cell mutation) and synergistic factors, the risk possibly increased on the susceptible group. Therefore, the method may help in investaging other factors have an influence on them in future studies.

To detect low-levels of benzene, toluene, ethylbenzene, and xylenes (BTEX) within car cabins a method was validated. It was based on gas chromatography coupled with mass spectrometer detection (GC-MS). All air samples collected from inside petrol and diesel cars were analysed

The level of ambient benzene in UK atmosphere (outside cars) in 1999 was reported varying between 0.31 and 1.9 ppb (Courage and Duarte-Davidson, 1999). In a road study carried in Newcastle upon Tyne, the level was 3.2 ppb which in the mean for several days of monitoring.

In petrol cars, air benzene and toluene means in this study were 7.5 and 15 ppb, respectively, while the average concentration of ethylbenzene and total xylenes mixtures was 10.5 ppb. The ambient benzene level was less than what was reported in other studies published before 1994 (Eikmann et al., 1992; Neumeier, 1993). The level of benzene in these studies varied between 13-57 ppb.

The BTEX concentrations determined in this study reflected the reduction in car emission due to improvements in automobile design since 1992and the implementation of the new environmental regulations (Wallace, 1996).

The concentration of toluene in diesel samples was ten times the concentration of benzene. The ratio of benzene to toluene (B/T) for air samples was 45% and 10% taken from petrol and diesel fuelled cars, respectively. This ratio in petrol air samples is twice that in diesel samples.

The relationship between benzene exposure at low level and *t,t*-muconic acid excretion is also one of this study objectives. When all samples were included, there was no difference in *t,t*-muconic acid levels between individuals exposed to petrol and control samples. However, after excluding non-detectable samples, the mean *t,t*-muconic acid level in petrol car drivers was about double that of control subjects. There was a three-fold increase in the urinary *t,t*-muconic level of individuals that had driven petrol-fuelled cars compared to those who used diesel. Because benzene and toluene compete for the same CYP2E1 binding site, it could be predicted that the higher the toluene exposure, the lower the excretion of benzene metabolites (Sammett and Lee, 1979). The prediction was supported during this study because *t,t*-muconic acid levels were lower in samples collected after a relatively high exposure to toluene (BTEX analysis).

In this study, the urinary *t,t*-muconic acid level in POST 1 samples (collected between 2 to 7 hours after the start of exposure to petrol emissions) correlated positively with air benzene and toluene concentrations taken from inside the car.

The relationships between ambient levels of BTEX and t,t-muconic acid (post-urine) have been investigated in three studies (Ghittori et al., 1995; Ong et al., 1996; Javelaud et al., 1998). The coefficient of determination (R^2) was varied between 0.46 and 0.58. Although this research showed a significant relationship between benzene concentrations inside petrol-fuelled cars and *t,t*-muconic acid excretion, the association was weaker ($R^2 = 0.30$) than that found by the mentioned studies.

Several factors may help explaining this inconsistency. The studies cited in the literature were conducted for occupational levels of exposure, whereas this study investigated the environmental exposure. In this study, benzene exposures varied greatly because of the nature of the study. The number of volunteers was low (n= 19) compared to other studies (n: 30-145), and this would have also influenced variability in the data. On the other hand, no relationship was found between benzene level and the difference between post and pre level on t,t-muconic acid. The sample size might play a major role in failing approach another study finding (Ong et al., 1995) which showed a stronger relationship with benzene levels (R2= 0.80, n= 64) compare to post samples only.

Removing benzene completely from petrol fuel might not be a realistic goal because it is generated via internal combustion processes besides existing in petrol. However, the toxic effects of benzene might be reduced if a structurally similar compound is added to the fuel. The target organ of toluene toxicity (central nervous system) is not the same as for benzene (bone marrow). Also, the threshold limit for toluene is very high (TWA= 100ppm) and there is no recognised carcinogenic risk for toluene.

Potential confounders as well as the sample size of any similar environmental study must be considered carefully as well as the sample size of the study. Because the sampling was done in parallel with a comprehensive questionnaire, satisfactory results were obtained in this study. For any future study, it should be noted that confounders that might influence one society might not affect others. For example, methods of tobacco consumption (smoking, chewing, or sniffing) might seriously bias results. If chewing is popular in one society, then this method of consumption should be considered in that study.

Probably, because of the small sample size of this study, no association was determined between *t,t*-muconic acid excretion and the potential confounders. This study tried to exclude tobacco smokers since smoking can easily bias exposure from automobile emissions. Although smoking was not reported on the day of sampling, four frequent smokers participated in the study. There was no difference in *t,t*-muconic acid levels between non-smokers and these four subjects. Age, sex, or living nearby a petrol station had no significant influence on urinary *t,t*-muconic acid levels in this study. It has been claimed that external traffic conditions and refuelling during a journey could elevate benzene levels inside the car (Wixtrom and Brown, 1992). However, in this study, no difference was found for subjects who drove through heavy traffic during the sampling period. Also, no difference was found between subjects who refuelled their cars during the sampling period. The year of car manufacture was also investigated, however, no significant difference was found between new and old petrol vehicles with respect to air benzene concentration or urinary *t,t*-muconic acid excretion.

Opening the vehicle window during the journey for petrol and diesel groups was divided into three categories: OPEN, CLOSE or open in SOME part of the journey.

There was a relationship between the benzene concentration when the window was closed and *t,t*-muconic acid level in urine. The lowest mean for air benzene concentration (2.4 ppb) and *t,t*-muconic acid 0.310 mgMA/gCr was associated with the SOME category. Two factors could have influenced these values: the air-dynamics within the car cabin (window status and air-conditioning status) and the year of vehicle manufacture. The mixing process of air pollutants inside and outside the vehicle would be affected by the air-dynamic factor. If the level of contaminants outside the vehicle were higher than inside, then the outside environment would be an additional source of the pollution. On the contrary, if the opposite situation occurred, then dilution could be expected when the window was open. In late 1998, the concentration of ambient benzene and toluene in Newcastle upon Tyne city was 3.16 and 3.14 ppb, respectively (personal communication), but this varied thought the day.

Operating air-condition in diesel cars associated with decrease of toluene level and increase of *t,t*-muconic acid level. This may be explained the toluene inhibition role in benzene metabolism as discussed early. This factor did not appear to influence the urinary *t,t*-muconic acid levels in subjects who expose to petrol-fuelled cars.

The results confirm the validity of *t,t*-muconic acid as a sensitive marker for low-level exposure to benzene (ppb). This technique may help in future to assess the impact of petrol type, either based on the brand of petrol (BP, TEXACO, etc) or chemical constituents (unleaded, premium, etc.).

In conclusion, this study has validated a method to extract *t,t*-muconic acid from urine using butanol instead of the traditional solid phase extraction technique. The *t,t*-muconic acid assay had the required degree of sensitivity to detect *t,t*-muconic acid in subjects who were exposed to low benzene concentrations. The results indicated that this method could aid in risk assessment of benzene toxicity at environmental levels of exposure. The relatively high benzene concentration (21.5 ppb) found inside the cabins of petrol-fuelled cars when the window was closed gives reason for concern, as this was about seven-fold higher than ambient levels in Newcastle upon Tyne City. This may be of importance when considering the risk to health to "at risk" groups, e.g. young, old and diseased populations.

Future Work

This research has suggested several areas for future study:

1- Investigate the relationship between benzene and *t,t*-muconic acid with larger sample size, to test the confounders role on the exposure (ie. benzene/ toluene ratio).

2- Determine the relationship between *t,t*-muconic acid and 8-hydroxydeoxyquanosine levels in lymphocytes and urine as oxidative product of DNA damage from benzene exposure.

3- Evaluate S-phenylmercapturic acid and *t,t*-muconic acid as biomarkers of low level benzene exposure, and determine the most reliable for environmental exposure (Corti and Snyder, 1998).

4- Determine 1,3-butadiene toxicity as another petrol emission product from vehicle besides BTEX (Darrall et al., 1998).

5- To study the impact of a hot climate on benzene exposure and toxicity.

APPENDICES

Appendix I: Urine Data

Table A

Raw data of urinary *t,t*-muconic acid from individuals exposed to diesel-car emission.

Row	VLNTR	Date	Time	mgMA/l	mgCr/l	DL	smk
1	18	27/9/98	00:45	0.599	1.164	0	s
2	18	28/9/98	02:30	0.010	1.083	1	s
3	1	30/1/99	06:30	0.010	0.094	1	n
4	1	30/1/99	19:00	0.010	0.184	1	n
5	1	13/3/99	06:00	0.020	0.215	0	n
6	1	13/3/99	18:20	0.038	0.255	0	n
7	1	20/3/99	06:30	0.022	0.312	0	n
8	1	20/3/99	18:30	0.028	0.153	0	n
9	6	19/5/99	07:15	0.010	1.387	1	n
10	6	19/5/99	17:30	0.010	1.200	1	n
11	6	18/5/99	08:15	0.010	0.761	1	n
12	6	18/5/99	17:30	0.010	1.939	1	n
13	1	22/5/99	06:30	0.258	2.098	0	n
14	1	22/5/99	19:00	0.070	1.486	0	n
15	1	23/5/99	09:00	0.010	2.212	1	n
16	1	23/5/99	18:00	0.071	1.360	0	n
17	6	24/5/99	07:00	0.010	2.025	1	n
18	1	30/5/99	(08:00)	0.010	2.185	1	n
19	1	30/5/99	16:00	0.010	1.362	1	n
20	6	2/6/99	07:00	0.010	4.058	1	n
21	6	2/6/99	18:00	0.010	0.357	1	n
22	6	3/6/99	07:00	0.010	1.166	1	n
23	6	3/6/99	17:30	0.010	3.412	1	n
24	6	4/6/99	07:30	0.010	1.993	1	n
25	1	4/6/99	09:00	0.010	3.431	1	n
26	1	4/6/99	21:00	0.010	2.017	1	n
27	6	4/6/99	17:30	0.010	0.573	1	n
28	6	8/6/99	07:30	1.360	1.507	0	n
29	6	8/6/99	17:05	1.442	1.756	0	n
30	6	9/6/99	07:30	0.010	1.719	1	n
31	21	6/7/99	11:00	0.010	2.186	1	n
32	21	6/7/99	19:20	0.010	3.576	1	n
33	22	6/7/99	11:00	0.010	1.541	1	n
34	22	6/7/99	19:20	0.010	2.280	1	n
35	23	6/7/99	19:20	0.010	0.817	1	n
36	24	6/7/99	19:20	0.010	2.409	1	n
37	6	9/6/99	17:00	0.124	0.498	0	n
38	6	10/6/99	07:00	0.533	1.314	0	n
39	6	10/6/99	17:00	0.321	0.843	0	n
40	6	5/7/99	18:00	0.010	1.773	1	n

(Continue ...)

Row	VLNTR	Date	Time	mgMA/l	mgCr/l	DL	smk
41	6	5/7/99	08:30	0.010	1.123	1	n
42	23	6/7/99	11:15	0.010	0.737	1	n
43	24	6/7/99	11:15	0.010	1.516	1	n
44	21	20/7/99	08:30	0.010	0.363	1	n
45	21	20/7/99	21:00	0.495	1.934	0	n
46	22	20/7/99	08:30	0.895	2.367	0	n
47	22	20/7/99	21:00	0.942	2.294	0	n
48	24	20/7/99	08:00	0.010	0.997	1	n
49	24	20/7/99	21:00	1.343	1.694	0	n
50	1	26/7/99	18:00	0.536	1.775	0	n
51	1	27/7/99	07:30	0.812	1.265	0	n
52	1	27/7/99	18:00	1.302	1.705	0	n
53	6	9/8/99	07:30	0.010	2.231	1	n
54	6	9/8/99	17:00	0.010	0.334	1	n
55	6	10/8/99	07:30	0.010	2.409	1	n
56	6	10/8/99	17:30	0.010	0.180	1	n
57	6	11/8/99	07:30	0.010	2.242	1	n
58	6	11/8/99	17:00	0.010	0.659	1	n
59	6	12/8/99	07:30	0.010	1.543	1	n
60	6	12/8/99	17:00	0.010	0.661	1	n
61	6	13/8/99	07:00	0.010	1.942	1	n
62	6	13/8/99	17:00	0.010	0.815	1	n

Vlnr: Volunteer code, Date: Date of sampling, Time: Time of urine collection, ml: urine volume in millilitre, gCr/l: gram of creatinine per litre, DL: Detection limit; 0=detectable 1=undetectable, mgMA/l: milligram *t,t*-muconic acid per urine litre, mgMA/gCr: milligram *t,t*-muconic acid per gram creatinine, grp: group of volunteers; n= control, p= petrol, d= diesel, smk: smoking status; n= non-smoker, s= smoker.

Table B

Raw data of urinary *t,t*-muconic acid from individuals who did not exposed to car emissions deliberately.

Row	VLNTR	Date	Time	ml	mgMA/l	mgCr/l	DL	smk
1	13	20/4/98	13:50	*	0.411	1.162	0	s
2	13	3/4/98	12:30	*	0.394	2.951	0	s
3	13	15/4/98	15:00	*	0.236	1.912	0	s
4	13	17/4/98	12:30	*	0.198	2.660	0	s

```
   5      13  21/4/98    11:10          *     0.228    3.402
0      s
   6       2  28/4/98    14:00          *     0.145    1.615
0      n
   7       2                                   0.373    1.506
0      n
   8      14  3/4/98     16:10          *     4.525    2.040
0      n
   9       2  3/4/99     14:45          *     0.899    2.534
0      n
```

(Continue ...)

Row	VLNTR		Date	Time		ml	mgMA/l	mgCr/l	DL	smk
10		9					1.119	1.007	0	n
11		9					0.425	0.885	0	n
12		9					0.256	0.959	0	n
13		14	20/4/98	13:30		*	5.106	1.450	0	n
14		14	21/6/98	13:30		*	4.321	3.677	0	n
15		9					0.010	1.508	1	n
16		12					0.380	2.120	0	n
17		2	21/4/98	16:30		*	0.963	2.071	0	n
18		2	24/4/98	00:00		*	0.434	2.826	0	n
19		14	22/4/98	12:30		*	0.984	2.876	0	n
20		9					2.021	5.142	0	n
21		2	30/3/98	17:30		*	0.567	2.737	0	n
22		12					3.948	2.335	0	n
23		9					0.010	0.295	1	n
24		15	29/4/98	15:00		*	0.010	2.412	1	s
25		19					0.236	1.380	0	n
26		9					0.010	0.115	1	n
27		15	16/8/98	16:00		*	13.938	0.204	0	n
28		9	16/3/98	10:35		*	5.995	2.270	0	n
29		11	22/5/98				2.147	2.659	0	p
30		19	2/4/98	12:36		*	0.869	1.187	0	n
31		15	22/4/98	15:30		*	3.031	4.300	0	s
32		12					1.116	1.639	1	n
33		19	31/3/98	11:05		*	0.010	0.205	1	n

34		11	27/5/98			3.208	1.141	
0	p							
35		15	30/4/98	11:50	*	1.788	1.300	
1	s							
36		15	23/4/98	16:30	*	0.264	1.461	
0	s							
37		15	28/4/98	12:55	*	2.306	0.591	
0	s							
38		12				1.331	1.135	
0	n							
39		15	15/4/98	16:10	*	0.885	3.001	
0	s							
40		2				4.115	2.229	
0	n							
41		19	1/4/98	11:10	*	1.254	1.158	
0	n							
42		11	27/5/98			0.010	2.355	
1	p							
43		13	31/3/98	11:55	*	0.010	1.567	
1	s							
44		14	30/3/98	16:45	*	0.937	2.764	
0	n							
45		14	31/3/98	17:30	*	0.910	3.510	
0	n							
46		9				1.120	3.689	
0	n							
47		12				1.014	3.635	
0	n							
48		2	2/4/98	17:40	*	1.157	3.007	
0	n							
49		9				0.010	0.463	
1	n							
50		14	16/3/98			0.878	1.512	
0	n							
51		11	22/5/98			0.927	2.074	
0	p							
52		2	1/4/98	14:00	*	0.010	2.705	
1	n							
53		19	31/3/98	17:05	*	0.023	2.260	
0	n							
54		2	31/3/98	11:15	*	0.010	1.998	
1	n							
55		13	1/4/98	15:00	*	0.344	3.587	
0	s							
56		14	1/4/98	17:30	*	0.713	3.056	
0	n							
57		2	14/5/99	11:42	*	*	*	
2								
58		2	17/5/99	08:35	*	20.380	3.277	
0	n							

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  59       2  17/5/99   19:00        300     0.010   2.751
1      n
  60       2  18/5/99   08:15        200     0.010   1.363
1      n
  61       2  18/5/99   22:51        240     0.010   2.531
1      n
  62       2  19/5/99   07:30        280     0.010   1.872
1      n
  63       2  19/5/99   19:47        180     0.732   2.032
0      n
  64       2  20/5/99   07:45        450     0.010   1.047
1      n
  65       5  17/5/99                        0.421   1.802
0      d
  66       5  17/5/99                        0.010   1.526
1      d
```

(Continue ...)

Row	VLNTR		Date	Time	ml	mgMA/l	mgCr/l	DL	smk
67		5	18/5/99			0.010	1.637	1	d
68		5	18/5/99			0.010	0.668	1	d
69		5	19/5/99			0.010	1.403	1	d
70		5	19/5/99			0.010	1.506	1	d
71		5	20/5/99			0.010	1.060	1	d
72		5	20/5/99			0.010	1.200	1	d
73		5	21/5/99			0.010	0.422	1	d
74		2	20/5/99	19:11	280	0.010	2.045	1	n
75		2	25/5/99	08:29	310	0.010	1.074	1	n
76		2	25/5/99	18:45	280	0.406	1.992	0	n
77		2	5/6/99	09:30	380	0.828	2.928	0	n
78		2	5/6/99	15:12	230	1.767	2.880	0	n
79		2	5/6/99	22:38	350	1.918	2.291	0	n
80		2	6/6/99	09:40	370	0.010	2.088	1	n
81		9	7/6/99	08:10	*	0.010	1.699	1	n
82		9	8/6/99	08:30	*	0.010	2.719	1	n
83		5	7/6/99			0.010	2.261	1	d
84		5	7/6/99			0.220	2.350	0	d
85		5	8/6/99			0.210	2.168	0	d
86		5	8/6/99			0.010	2.054	1	d
87		5	10/6/99			0.010	1.725	1	d
88		5	10/6/99			0.010	2.232	1	d
89		5	11/6/99			0.010	1.210	1	d
90		9	8/6/99	19:55	*	0.010	1.755	1	n

91		9	9/6/99	08:05		*	0.010	1.955
1	n							
92		9	9/6/99	16:00		*	0.010	0.086
1	n							
93		9	10/6/99	08:10		*	0.010	2.691
1	n							
94		9	10/6/99	19:00		*	0.010	1.343
1	n							
95		9	11/6/99	07:30		*	0.010	2.604
1	n							
96		9	11/6/99	16:30		*	0.010	1.152
1	n							
97		9	16/6/99				0.010	1.443
1	n							
98		9	16/6/99	17:35		*	1.705	1.440
1	n							
99		9	17/6/99	08:45		*	0.834	1.701
0	n							
100		9	17/6/99	19:00		*	0.010	1.016
1	n							
101		5	11/6/99				0.010	0.524
1	d							
102		5	16/6/99				0.010	2.014
1	d							
103		5	16/6/99				0.010	1.340
1	d							
104		5	17/6/99				0.010	1.488
1	d							
105		5	17/6/99				0.010	1.747
1	d							
106		5	18/6/99				0.010	1.355
1	d							
107		17	7/7/99	08:00		*	0.010	1.517
1	n							
108		5	18/6/99				0.010	1.846
1	d							
109		20	6/7/99	17:30		*	0.207	0.302
0	n							
110		2	23/6/99	12:31		*	0.010	1.553
1	n							
111		2	23/6/99	22:47		*	0.259	2.464
0	n							
112		9	24/6/99	08:30		*	0.010	2.403
1	n							
113		9	24/6/99	16:00		*	1.610	2.768
0	n							
114		9	25/6/99	08:15		*	1.517	2.089
0	n							
115		9	25/6/99	15:30		*	1.496	1.606
0	n							

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 116      20  7/7/99    08:00          *     0.010    1.976
1      n
 117      20  7/7/99    17:00          *     0.010    0.806
1      n
 118      23  20/7/99                        0.010    1.925
1      d
 119      23  20/7/99                        0.010    0.410
1      d
 120      25  26/11/00  10:30        850     0.508    2.121
0      s
 121      25  26/11/00  23:00        450     0.943    3.782
0      s
 122      25  27/11/00  10:30        400     1.550    6.655
0      s
```

(Continue ...)

Row	VLNTR	Date	Time	ml	mgMA/l	mgCr/l	DL smk
123	27	25/11/00	12:30	50	1.756	4.863	0 n
124	27	26/11/00	00:30	50	4.518	4.484	0 n
125	27	26/11/00	01:45	100	0.942	4.835	0 n
126	27	26/11/00	08:45	100	2.515	4.503	0 n
127	27	26/11/00	11:30	50	2.000	5.252	0 n
128	28	25/11/00	05:30	800	0.010	1.498	1 n
129	28	25/11/00	10:05	650	0.010	0.341	1 n
130	28	25/11/00	13:00	200	0.727	2.048	0 n
131	28	25/11/00	23:00	300	0.809	3.053	0 n
132	28	26/11/00	02:30	550	0.010	1.356	1 n
133	28	26/11/00	06:00	400	0.010	1.394	1 n
134	29	25/11/00	00:45	125	0.800	1.678	0 s
135	29	25/11/00	09:00	250	1.003	2.379	0 s
136	29	25/11/00	16:00	120	0.960	2.398	0 s
137	29	25/11/00	18:45	175	0.542	1.213	0 s
138	29	25/11/00	23:00	200	1.490	1.649	0 s
139	30	29/11/00	08:30	700	0.146	0.844	0 n
140	30	29/11/00	13:30	*	0.010	0.948	1 n
141	30	29/11/00	18:15	*	0.010	1.431	1 n
142	30	29/11/00	21:45	450	0.010	0.275	1 n
143	30	29/11/00	23:10	450	0.010	0.133	1 n
144	32	30/11/00	11:30	300	0.373	0.474	0 n
145	32	30/11/00	16:30	400	0.010	0.436	1 n
146	32	30/11/00	22:30	400	0.191	0.408	0 n

147	32	30/11/00	23:30	200	0.010	0.095	1 n
148	32	1/12/00	07:00	300	0.010	0.351	1 n

Vlnr: Volunteer code, Date: Date of sampling, Time: Time of urine collection, ml: urine volume in millilitre, gCr/l: gram of creatinine per litre, DL: Detection limit; 0=detectable 1=undetectable, mgMA/l: milligram *t,t*-muconic acid per urine litre, mgMA/gCr: milligram *t,t*-muconic acid per gram creatinine, grp: group of volunteers; n= control, p= petrol, d= diesel, smk: smoking status; n= non-smoker, s= smoker.

Table C

Raw data of urinary *t,t*-muconic acid from individuals who exposed to petrol-car emission.

Row	VLNTR	Date	Time	ml	mgMA/l	mgCr/l	DL	smk
1	2	13/4/98	14:05	*	0.331	2.361	0	n
2	2	15/3/98	02:00	*	0.214	2.227	0	n
3	2	7/4/98	20:30	*	0.221	1.144	0	n
4	2	7/4/98	22:14	*	1.858	3.163	0	n
5	2	14/3/98	10:33	*	0.698	1.543	0	n
6	10	1/3/98	21:45	*	0.209	1.278	0	n
7	2	21/2/98	22:28	*	0.123	1.203	0	n

(Continue ...)

Row	VLNTR	Date	Time	ml	mgMA/l	mgCr/l	DL	smk
8	2	7/2/98	16:30	*	0.113	1.283	0	n
9	2	29/1/98	08:00	*	0.108	0.795	0	n
10	10	1/3/98	10:53	*	0.092	3.107	0	n
11	2	21/2/98	20:20	*	0.107	1.357	0	n
12	2	30/1/98	08:36	*	3.359	1.840	0	n
13	2	29/1/98	21:30	*	0.259	1.349	0	n
14	2	21/2/98	16:00	*	0.914	0.927	0	n
15	2	30/1/98	17:15	*	0.452	2.253	0	n
16	2	7/2/98	08:41	*	0.035	1.196	1	n
17	2	14/2/98	17:00	*	0.168	0.888	0	n
18	2	28/3/98	07:30	*	0.010	3.527	1	n
19	2	13/4/98	19:45	*	6.670	2.595	0	n
20	2	7/4/98	07:10	*	0.397	1.636	0	n
21	2	7/4/98	14:55	*	0.954	3.396	0	n
22	15	15/7/98	12:35	*	0.010	1.837	1	s
23	15	15/7/98	18:00	*	0.010	3.022	1	s
24	9	24/6/98	20:55	*	2.095	2.236	0	n
25	2	9/5/98	09:50	*	1.446	2.951	0	n
26	9	24/6/98	15:45	*	0.969	0.690	0	n
27	2	24/6/98	21:49	*	0.010	4.277	1	n
28	9	24/6/98	07:00	*	1.149	2.020	0	n
29	2	9/5/98	16:00	*	1.868	2.840	0	n
30	2	24/6/98	07:45	*	0.053	3.282	0	n
31	2	24/6/98	17:37	*	0.010	3.451	1	n

32	3	29/8/98	07:00	*	0.447	0.313	0
33	3	29/8/98	11:30	*	0.495	0.179	0
34	16	29/8/98	07:00	*	0.010	0.050	1
35	16	29/8/98	11:30	*	1.417	0.682	1
36	2	14/11/98	23:45	*	1.756	1.719	0
37	2	14/11/98	17:27	*	1.425	1.568	0
38	7	27/11/98	10:00	*	0.210	0.707	0
39	7	27/11/98	18:05	*	0.215	0.429	0
40	7	29/11/98	11:30	*	0.626	0.903	0
41	7	29/11/98	22:00	*	1.065	1.489	0
42	2	5/12/98	11:45	*	2.491	3.476	0
43	2	5/12/98	16:52	*	2.141	2.140	0
44	2	5/12/98	22:57	*	0.015	0.127	0
45	2	17/12/98	07:00	*	0.010	0.205	1
46	2	17/12/98	12:00	*	0.026	0.207	0
47	2	18/12/98	08:25	*	0.022	0.277	0
48	2	18/12/98	21:45	*	0.034	0.263	0
49	3	7/5/99	07:00	435	0.041	0.563	0
50	3	7/5/99	10:00	285	0.050	0.185	0
51	3	7/5/99	13:15	350	0.055	1.076	0
52	3	7/5/99	18:30	230	2.971	0.696	0
53	3	7/5/99	22:15	100	3.638	0.977	0
54	3	8/5/99	07:30	180	0.101	1.515	0
55	2	15/5/99	23:18	240	3.725	1.808	0
56	2	15/5/99	07:25	450	0.010	1.960	1

```
  57      2  15/5/99   11:35    240   0.010   1.768    1
n
  58      2  15/5/99   15:45    220   1.574   1.282    0
n
  59      2  15/5/99   20:15    170   3.644   1.337    0
n
  60      2  16/5/99   08:20    340   2.519   2.253    0
n
  61      4  17/5/99   07:15      *   0.010   0.844    1
n
  62      4  17/5/99   17:30      *   0.010   0.462    1
n
  63      4  18/5/99   07:30      *   0.010   1.157    1
n
  64      4  18/5/99   17:30      *   0.010   0.339    1
n
```

(Continue ...)

Row	VLNTR	Date	Time	ml	mgMA/l	mgCr/l	DL smk
65	4	19/5/99	07:15	*	0.010	0.703	1 n
66	4	19/5/99	17:30	*	0.010	0.422	1 n
67	4	20/5/99	07:15	*	0.010	1.147	1 n
68	4	20/5/99	21:00	*	0.010	0.695	1 n
69	4	21/5/99	07:15	*	0.010	1.074	1 n
70	2	22/5/99	01:10	280	0.010	1.659	1 n
71	2	22/5/99	07:15	450	0.088	0.881	0 n
72	2	22/5/99	14:30	300	0.140	1.247	0 n
73	2	22/5/99	16:40	130	0.010	1.520	1 n
74	2	22/5/99	19:25	100	0.319	1.812	0 n
75	2	23/5/99	00:45	230	0.465	1.520	0 n
76	2	23/5/99	09:30	460	0.290	1.027	0 n
77	4	21/5/99	17:30	*	0.010	0.315	1 n
78	4	24/5/99	07:45	*	0.674	1.886	0 n
79	4	24/5/99	17:30	*	0.950	0.608	0 n
80	4	25/5/99	07:15	*	0.423	1.214	0 n
81	4	25/5/99	18:40	*	0.337	0.295	0 n
82	4	26/5/99	07:15	*	0.603	0.621	0 n
83	4	26/5/99	18:45	*	0.518	0.262	0 n
84	4	27/5/99	07:15	*	0.434	2.019	0 n
85	4	27/5/99	17:45	*	0.455	0.841	0 n
86	4	28/5/99	07:15	*	0.010	2.279	1 n
87	4	28/5/99	17:00	*	1.357	1.293	0 n
88	4	3/6/99	08:10	*	0.010	1.346	1 n

89	4	3/6/99	17:15	*	0.010	0.968	1
90	4	4/6/99	07:15	*	0.010	1.480	1
91	4	4/6/99	17:30	*	0.089	0.216	1
92	4	7/6/99	07:15	*	0.722	1.138	0
93	4	7/6/99	17:30	*	1.558	1.454	0
94	4	9/6/99	07:00	*	0.010	0.742	1
95	4	8/6/99	17:30	*	1.173	0.592	0
96	4	8/6/99	07:15	*	0.545	1.320	0
97	4	9/8/99	17:15	*	0.467	0.317	0
98	4	10/6/99	07:15	*	1.187	0.970	0
99	4	10/6/99	17:30	*	0.270	0.292	0
100	4	11/6/99	07:15	*	1.302	1.524	0
101	4	11/6/99	18:00	*	0.010	0.352	1
102	4	14/6/99	07:15	*	0.010	1.344	1
103	4	14/6/99	18:00	*	0.010	0.475	1
104	4	15/6/99	07:15	*	0.010	0.849	1
105	4	15/6/99	17:30	*	0.010	0.491	1
106	4	16/6/99	07:20	*	0.010	0.717	1
107	4	16/6/99	17:30	*	0.010	0.949	1
108	4	17/6/99	07:15	*	0.010	0.628	1
109	4	17/6/99	17:30	*	0.010	1.124	1
110	2	12/6/99	00:05	*	0.010	2.651	1
111	2	12/6/99	09:45	350	0.010	2.365	1
112	2	12/6/99	14:15	170	0.839	2.678	0
113	2	12/6/99	19:30	150	2.156	1.913	0

```
 114       2  12/6/99    23:10     180    0.010    1.589     1
n
 115       2  13/6/99    03:30     200    0.721    1.643     0
n
 116       2  13/6/99    09:10     200    0.768    2.348     0
n
 117       2  19/6/99    08:45     630    0.265    0.774     0
n
 118       2  19/6/99    15:20     270    1.656    2.034     0
n
 119       2  19/6/99    17:45     130    1.107    1.630     0
n
 120       2  20/6/99    00:45     150    0.010    1.522     1
n
 121       2  20/6/99    09:10     340    0.010    1.543     1
n
```

(Continue ...)

Row	VLNTR	Date	Time	ml	mgMA/l	mgCr/l	DL smk
122	2	20/6/99	09:10	340	0.010	1.920	1 n
123	2	20/6/99	15:45	340	0.010	1.601	1 n
124	2	20/6/99	20:30	340	0.413	1.582	0 n
125	2	21/6/99	03:15	350	0.152	1.311	1 n
126	2	21/6/99	10:15	200	0.291	1.862	0 n
127	4	18/6/99	17:30	*	0.010	0.511	1 n
128	4	21/6/99	07:15	*	0.010	0.919	1 n
129	4	21/6/99	20:00	*	0.010	0.436	1 n
130	4	22/6/99	07:15	*	0.010	1.057	1 n
131	4	9/8/99	08:00	*	2.114	2.056	0 n
132	4	9/8/99	20:00	*	0.900	0.173	0 n
133	4	10/8/99	07:30	*	0.010	1.061	1 n
134	4	10/8/99	19:30	*	1.077	0.226	0 n
135	4	11/8/99	07:20	*	0.010	0.970	1 n
136	4	11/8/99	17:45	*	2.184	0.572	0 n
137	4	12/8/99	07:15	*	0.010	1.125	1 n
138	4	12/8/99	18:00	*	0.010	0.397	1 n
139	4	13/8/99	07:20	*	0.010	0.730	1 n
140	4	13/8/99	17:45	*	0.010	0.660	1 n
141	26	25/11/00	09:30	295	2.436	4.332	0 n
142	26	25/11/00	12:30	150	1.297	2.825	0 n
143	26	25/11/00	18:45	440	0.897	1.972	0 n
144	26	26/11/00	00:15	260	1.209	2.579	0 n
145	26	26/11/00	08:45	205	2.192	4.768	0 n

146	31	30/11/00	06:05	100	3.081	3.574	0
n							
147	31	30/11/00	09:20	55	2.983	3.972	0
n							
148	31	30/11/00	13:00	90	2.025	2.635	0
n							
149	31	30/11/00	17:05	100	0.010	3.053	1
n							
150	31	30/11/00	19:00	60	1.276	2.342	0
n							
151	31	30/11/00	23:10	110	1.551	2.531	0
n							
152	31	1/12/00	06:06	70	1.432	2.607	0
n							
153	33	2/12/00	05:49	650	0.010	0.370	1
n							
154	33	2/12/00	10:43	150	0.010	2.683	1
n							
155	33	2/12/00	18:22	413	3.279	2.417	0
n							
156	33	3/12/00	01:05	414	4.181	1.593	0
n							
157	33	3/12/00	03:44	415	2.985	0.616	0
n							
158	34	5/12/00	11:15	416	0.010	0.720	1
n							
159	34	5/12/00	17:20	417	0.010	1.621	1
n							
160	34	5/12/00	22:15	418	1.563	1.422	0
n							
161	34	6/12/00	03:30	419	0.783	0.654	0
n							
162	34	6/12/00	07:15	250	0.010	0.872	1
n							
163	35	6/12/00	06:15	100	4.651	2.237	0
n							
164	35	6/12/00	09:45	125	4.745	1.896	0
n							
165	35	6/12/00	14:40	280	3.490	1.356	0
n							
166	35	6/12/00	18:05	260	3.858	0.976	0
n							
167	35	6/12/00	21:10	350	3.192	0.607	0
n							
168	35	7/12/00	01:15	340	1.835	0.825	0
n							
169	35	7/12/00	06:00	250	0.010	0.446	1
n							
170	12	5/12/00	02:30	225	0.010	0.664	1
n							

```
 171      12  5/12/00    07:25     300    0.010    1.431     1
n
 172      12  5/12/00    09:10     200    0.010    0.711     1
n
 173      12  5/12/00    11:00     250    1.935    0.512     0
n
 174      12  5/12/00    14:00     275    3.305    0.853     0
n
 175      12  5/12/00    17:15     250    6.456    1.033     0
n
 176      12  5/12/00    21:25     300    0.239    0.540     0
n
 177      12  5/12/00    22:40     275    0.140    0.237     0
n
 178      12  5/12/00    23:30     350    0.010    0.142     1
n
```

(Continue ...)

Row	VLNTR	Date	Time	ml	mgMA/l	mgCr/l	DL	smk
179	36	11/12/00	10:50	210	0.010	1.545	1	n
180	36	11/12/00	15:35	200	0.010	1.384	1	n
181	36	11/12/00	19:35	80	0.345	2.948	0	n
182	36	11/12/00	23:55	280	0.010	1.716	1	n
183	36	12/12/00	05:45	300	0.010	1.716	1	n
184	37	11/12/00	08:40	430	0.794	0.664	0	n
185	37	11/12/00	15:55	180	0.281	1.905	0	n
186	37	11/12/00	21:05	100	0.445	2.977	0	n
187	37	12/12/00	02:20	200	0.010	1.346	1	n
188	37	12/12/00	07:15	500	0.010	0.209	1	n

Vlnr: Volunteer code, Date: Date of sampling, Time: Time of urine collection, ml: urine volume in millilitre, gCr/l: gram of creatinine per litre, DL: Detection limit; 0=detectable 1=undetectable, mgMA/l: milligram *t,t*-muconic acid per urine litre, mgMA/gCr: milligram *t,t*-muconic acid per gram creatinine, grp: group of volunteers; n= control, p= petrol, d= diesel, smk: smoking status; n= non-smoker, s= smoker.

Appendix II: Air Sample, Urine and Questionnaire Data

Table D

The raw data for each volunteer health status.

vltr	age(y)	sex	lnghlth	Lngthstat	Medicn	mdduratn
1	27	1	0	0	0	0
2	33	1	0	0	0	0
3	54	2	0	0	0	0
4	57	1	0	0	1	1
5	44	1	1	1	2	3
6	28	1	0	0	0	0
7	31	1	0	0	0	0
9	33	1	0	0	0	0
10	27	1	0	0	0	0
11	32	1	0	0	0	0
15	23	1	0	0	0	0
16	53	1	0	0	0	0
18	19	1	0	0	0	0
21	16	1	0	0	0	0
22	31	1	0	0	0	0
23	67	1	0	0	1	2
24	16	1	0	0	0	0

Vl: volunteer code,
age(y): age of volunteer in years.
sex: gender; 1=male, 2=female.
lnghlth: long-term health problems; 0=no, 1=yes.
lngthstat: long-term health problems description; 0= none, 1= asthma (very rare), epilepsy (controlled).
medicn: name & quantity of the taken medicine; 0= none, 1= 75mg aspirin per day, 2= 100mg phenytion.
mdduratn: the medicine up take period; 0= no, 1= 18 months, 2= 8 years, 3= 24 years.

Table E

Raw data for smoking and drinking alcohol status for the volunteers.

Vl	smk	cigrt	psmk	beer	wine	sprt	prsrv	smk2	cigrtpr	cigrtin	ps2	drnk2	prsv2	sorbic
1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0-1	0	0	0	0	0	0-1	0
3	0	0	-	-	-	-	0	0	0	0	0	0	0	0
4	0	0	0	1	1	1	0-1	0	0	0	0	0	0-1	0
5	0	0	0	0	0	0	0	0	0	0	0	0	0	0
6	0	0	0	1	1	-	0	0	0	0	0	0	0	0
7	0	0	0	0	0	0	0	0	0	0	0	0	0	0
9	0	0	0	0	0	0	-	0	-	-	0	0	-	-
10	0	0	0	0	0	0	0	0	-	-	0	0	-	-
11	0	0	0	0-1	0	0	0	0	-	-	0	0-1	-	-
15	1	1	0	2	1	0	-	0	-	-	0	0	-	-
16	0	0	-	-	-	-	0	0	-	-	0	0	-	-
18	1	1	1	0	0	0	1	1	1	1	0	0	-	-
21	0	0	0	0	0	0	-	0	0	0	0	0	0	0
22	0	0	0	0	0	0	-	0	0	0	0	0	0	0
23	0	0	0	0	0	0	-	0	0	0	0	0	0	0
24	0	0	0	0	0	0	-	0	0	0	0	0	0	0

Vl: volunteer code.
smk: habitual smoking; 0=not smokes, 1=smokes,
cigrt: habitual smoking for cigarettes; 0=no cigarettes, 1=1 to 10 cigarette a day.
ps: passive smoking; 0=no passive smoking, 1= expose to passive smoking.
beer: no. of pints of beer a week; 0=none, 1= less than 12 pints,
wine: no. of glasses of wine a week; 0=none, 1= less than 12 pints.
sprt: no. of measures of spirit a week; 0=none, 1= less than 12 pints.
prsrv: eating long life dairy products; 0=yes, 1=no.
ps2: passive smoking in the day of sampling; 0=no, 1=no.
drnk2: drinking alcohol in the day of sampling; 0=no, 1=yes.
prsv2: consume diet consist of preservative; 0=no, 1=yes.
sorbic: does the diet contained sorbic acid; 0=no, 1=yes.
(-) Not available.

Table F

Raw data related to the car, fuel, and time of driving.

Vl	Mnfctr	model		fl	year	typ	supplr	crrprd	Drmin	dstnce	Wrkhr	rfl
1	Mercedes	VAW	308D	2	1994	3	-	3	60-600	45-90	9-12	0-1
2	Rover	Montago	p.6	p	1987	1	1,2,4,5,6,12,14,16	-	58-418	32-126	-	0-1
3	Alfa Romeo	1.55	2L	p	1997	1	14	-	50-190	≤250	-	0
4	Rover	200	2p.4Si	p	1997	1	1	-	60-205	45-124	7.4-11	0-1
5	Ford	Escort	p.8TD	D	1997	3	-	-	110-360	55-278	9	0-1
6	Ford	Escort	p.8TD	D	1997	3	-	-	60-140	36-85	8-10	0-1
7	Fiat	Punto		p	1998	1	3	-	213	174	-	1
9	Rover	Montago	p.6	p	1987	1	12	-	418	323	-	1
10	Vauxhall	Astra	p.6GI	p	1997	1	4	-	337	226	-	1
11	Ford	Escort		p	1988	1	4	-	-	-	-	0-1
15	Rover	-		p		1	1	-	180	200	-	0
16	Alfa Romeo	1.55	2L	p	1997	1	14	-	180	200	-	0
18	Peugoet	405	p.8	D	1988	3	-	-	375	130	9	1
21	Ford	Transit	Minibs	D	1995	3	-	-	265-457	205-289	-	0
22	Ford	Transit	Minibs	D	1995	3	-	-	265-457	205-289	-	0
23	Ford	Transit	Minibs	D	1995	3	-	-	265-457	205-289	-	0
24	Ford	Transit	Minibs	D	1995	3	-	-	265-457	205-289	-	0

Vl: volunteer code.
mnfctr: vehicle manufacturer.
model: vehicle model.
year: year of production.
fl: fuel type; p=petrol, D=diesel.
typ: type of fuel; 1=unleaded, 2=4star(premium), 3=diesel.
supplr: fuel supplier; 1=ASDA, 3=ESSO, 4=FINA, 5=IMPERIAL, 6=JET, 12=TEXACO, 14=TOTAL, 16=other supplier.
crrprd: period in the career.
Drmin: driving duration period (min).
dstnce: the driven distance (miles).
wrkhr: working hours.
rfl: refuelling the vehicle; 0=no, 1=yes.
(-): Not available.

Table G
Raw data for *t,t*-muconic acid levels in urine from exposed individuals before and after the exposure.

VI	PRE	POST1	m1d	POST2	m2d	POST
1	0.27	-	-	0.52	0.04	0.52
1	0.27	-	-	0.49	0.15	0.49
1	0.27	-	-	0.50	0.18	0.50
1	0.27	-	-	0.52	0.04	0.52
1	0.27	-	-	0.48	0.04	0.48
1	0.29	-	-	0.38	0.01	0.38
1	0.38	-	-	0.50	0.00	0.50
1	-	-	-	0.30	0.30	0.30
2	0.14	-	-	0.19	0.06	0.19
2	1.83	0.20	-1.62	-	-	0.20
2	0.03	0.09	0.06	-	-	0.09
2	0.12	0.19	0.07	-	-	0.19
2	0.08	0.99	0.91	0.03	-0.05	0.51
2	0.45	-	-	0.10	-0.36	0.10
2	0.24	-	-	0.28	0.04	0.28
2	0.14	2.57	2.43	-	-	2.57
2	0.49	0.66	0.17	-	-	0.66
2	0.02	-	-	0.00	-0.01	0.00
2	0.91	-	-	1.02	0.11	1.02
2	0.72	1.02	0.30	0.12	-0.60	0.57
2	0.05	0.13	0.08	-	-	0.13
2	0.08	0.13	0.05	-	-	0.13
2	0.01	1.23	1.22	2.72	2.72	1.98
2	0.01	0.14	0.13	0.24	0.23	0.19
2	0.68	1.34	0.66	0.01	-0.67	0.67
2	0.01	0.01	0.00	0.26	0.25	0.13
3	1.43	2.77	1.34	-	-	2.77
3	0.07	0.27	0.20	4.27	4.19	2.27
4	0.17	-	-	0.42	0.26	0.42
4	0.01	-	-	0.02	0.02	0.02
4	0.01	-	-	0.01	0.00	0.01
4	0.01	-	-	0.01	0.00	0.01
4	1.03	-	-	5.20	4.17	5.20
4	2.75	-	-	4.77	2.03	4.77
4	1.81	-	-	3.82	2.01	3.82
4	0.01	-	-	0.03	0.02	0.03
4	0.41	-	-	1.98	1.57	1.98
4	0.01	-	-	0.58	0.56	0.58
5	0.01	-	-	0.00	-0.01	0.00
5	0.01	-	-	0.01	0.00	0.01
5	0.01	-	-	0.02	0.01	0.02
5	0.01	0.01	0.00	-	-	0.01

(Continue ...)

VI	PRE	POST1	m1d	POST2	m2d	POST
6	0.01	-	-	0.00	-0.01	0.00
6	0.01	-	-	0.02	0.01	0.02
6	0.41	-	-	0.38	-0.02	0.38
6	0.01	-	-	0.01	0.00	0.01
6	0.06	-	-	0.00	-0.05	0.00
6	0.02	-	-	0.01	-0.01	0.01
7	0.69	-	-	0.72	0.02	0.72
9	0.57	1.41	0.84	0.94	0.37	1.17
10	0.03	-	-	0.16	0.13	0.16
11	0.81	0.45	-0.36	-	-	0.45
11	0.01	2.81	2.80	-	-	2.80
15	0.01	0.01	0.00	-	-	0.01
16	0.20	2.08	1.88	-	-	2.08
18	-	-	-	0.52	-	0.52
21	0.01	-	-	0.01	0.00	0.01
21	1.13	-	-	0.26	-0.88	0.26
22	0.01	-	-	0.01	0.00	0.00
22	0.38	-	-	0.41	0.03	0.41
23	0.01	-	-	0.01	0.00	0.01
23	0.01	-	-	0.60	0.59	0.60
24	0.01	-	-	0.01	0.00	0.01
24	0.01	-	-	0.79	0.78	0.79

PRE: urinary *t,t*-muconic acid corrected with creatinine before the exposure (mgMA/gCr).
POST1: POST 1 samples (mgMA/gCr).
m1d: difference mgMA/gCr between PRE and POST1.
POST2: POST 2 samples (mgMA/gCr).
m2d: difference mgMA/gCr between PRE and POST1.
POST: the mean of POST 1 & 2 (mgMA/gCr).

Table H

Benzene, toluene, and ethyl benzene and xylenes inside the cars.

Vl	fuel	b	t	ex	b_ppb	t_ppb	ex_ppb	b/t
1	Diesel	0.19	24.2	12.01	0.08	8.15	3.51	0.01
2	petrol	0.61	3.06	-	0.23	0.98	-	0.23
2	petrol	46.03	100.94	-	17.35	32.26	-	0.54
2	petrol	0.49	2.44	-	0.19	0.79	-	0.24
2	petrol	53.94	120.92	-	20.3	38.57	-	0.53
2	petrol	104.64	250.2	-	39.83	80.75	-	0.49
2	petrol	95.31	188	-	36.37	60.82	-	0.60
2	petrol	18.49	27.41	-	6.99	8.79	-	0.80
2	petrol	283.2	558.64	-	102.57	171.54	-	0.60
2	petrol	18.29	15.4	-	6.94	4.96	-	1.40
2	petrol	24.15	45.57	-	9.16	14.65	-	0.63
2	petrol	24.14	75.57	-	9.31	24.71	-	0.38
2	petrol	5.17	16.91	2.87	1.84	5.12	0.75	0.36
2	petrol	19.98	97.82	79.16	7.13	29.58	20.78	0.24
2	petrol	31.19	98.72	53.57	11.13	29.86	14.06	0.37
2	petrol	152.75	1739.25	1049.91	60.05	579.69	303.72	0.10
2	petrol	34.12	125.74	49.17	12.73	39.77	13.5	0.32
2	petrol	25.05	10.84	10.84	9.55	3.5	3.04	2.73
2	petrol	45.5	79.39	52.79	17.2	25.44	14.68	0.68
3	petrol	47.26	166.89	-	18.31	54.82	-	0.33
3	petrol	14.88	30.73	7.33	5.85	10.24	2.12	0.57

4	petrol	61.44	57.89	57.89	23.35	18.65	16.19	1.25
4	petrol	40.11	34.38	34.38	15.26	11.09	9.63	1.38
4	petrol	63.88	37.93	37.93	24.61	12.39	10.75	1.99
4	petrol	116.64	75.34	75.34	45.27	24.79	21.51	1.83
4	petrol	3.67	63.9	14.67	1.42	21.04	4.19	0.07
4	petrol	4	14.04	80	1.53	4.56	22.53	0.34
4	petrol	1.63	6.13	32.59	0.61	1.95	9.02	0.31
4	petrol	2.18	10.89	43.56	0.83	3.53	12.27	0.24
4	petrol	32.77	37.93	37.93	12.56	12.33	10.7	1.02
4	petrol	46.45	26.83	26.83	17.8	8.71	7.56	2.04
5	Diesel	49.86	41.98	41.98	18.98	13.55	11.76	1.40
5	Diesel	21.62	15.28	15.28	8.33	4.99	4.33	1.67
5	Diesel	10.65	122.17	39.29	4.07	39.57	11.04	0.10
5	Diesel	13.47	50	50	5.16	16.24	14.1	0.32
6	Diesel	64.11	68.75	68.75	24.4	22.18	19.25	1.10
6	Diesel	10.98	39.29	39.29	4.26	12.93	11.22	0.33
6	Diesel	23.43	52.88	52.88	8.95	17.13	14.87	0.52
6	Diesel	15.71	573.25	248.97	6.08	188.13	70.91	0.03
6	Diesel	2.75	56.65	59.37	1.05	18.38	16.72	0.06
6	Diesel	3.67	13.09	73.33	1.4	4.25	20.66	0.33
7	petrol	13.63	60	33.83	4.86	18.15	8.88	0.27

(Continue ...)

Vl	fuel	b	t	ex	b_ppb	t_ppb	ex_ppb	b/t
9	petrol	24.15	45.57	-	9.16	14.65	-	0.63
10	petrol	8.6	21.09	-	3.06	6.36	-	0.48
11	petrol	23.78	48.5	-	9.05	15.65	-	0.58
11	petrol	69.67	172.26	-	26.48	55.51	-	0.48
15	petrol	0.61	3.06	-	0.24	1	-	0.24
16	petrol	47.26	166.89	-	18.31	54.82	-	0.33
18	Diesel	47.26	166.89	-	18.31	54.82	-	0.33
21	Diesel	2.52	99.15	51.57	0.97	32.54	14.69	0.03
21	Diesel	3.67	63.9	14.67	1.42	21.04	4.19	0.07
22	Diesel	2.52	99.15	51.57	0.97	32.54	14.69	0.03
22	Diesel	3.67	63.9	14.67	1.42	21.04	4.19	0.07
23	Diesel	2.52	99.15	51.57	0.97	32.54	14.69	0.03
23	Diesel	3.67	63.9	14.67	1.42	21.04	4.19	0.07
24	Diesel	2.52	99.15	51.57	0.97	32.54	14.69	0.03
24	Diesel	3.67	63.9	14.67	1.42	21.04	4.19	0.07

Vl: Volunteer code.
b: μg benzene per cubic metre.
t: μg toluene per cubic metre.
ex: μg ethylbenzene & xylenes per cubic metre.
b_ppb: benzene part per billion.
t_ppb: toluene part per billion.
ex_ppb: ethylbenzene & xylenes part per billion.
b/t: (benzene ppb / toluene ppb) ratio
(-): Not available.

Appendix III: Questionnaire Form.

Serial Number: .....

LAST NAME: ..... FIRST:.....
COMPANY: .....
COMPANY ADDRESS: .....
.....
DATE : ...../...../199... (DAY/MONTH /YEAR)

Please note that all the information given on this form is protected by the DATA PROTECTION ACT.

Serial Number: .....

.Please tick ✓ the appropriate answer ☐

1- Date of Birth : ..... / ..... / 19 ... ... (day/month /year)

2- 2- Sex :male ☐ female ☐

3- Habits:

a- Do you smoke : Yes ☐ No ☐

b- if yes, would you specify and how many a day?

I- cigarettes	none ☐	1 to 10 ☐	11 to 20 ☐	more than 20 ☐
II- cigars	none ☐	1 to 10 ☐	11 to 20 ☐	more than 20 ☐
III- pipes	none ☐	1 to 10 ☐	11 to 20 ☐	more than 20 ☐

c- Do others smoke in front of you (Passive smoking; i.e. friends, partners) :

I- cigarettes	none ☐	1 to 10 ☐	11 to 20 ☐	more than 20 ☐
II- cigars	none ☐	1 to 10 ☐	11 to 20 ☐	more than 20 ☐
III- pipes	none ☐	1 to 10 ☐	11 to 20 ☐	more than 20 ☐

d- alcohol per week:

i- pints of beer :	none ☐	less than 12 ☐	12 24 ☐	more than 24 ☐
ii- glasses of wine :	none ☐	less than 12 ☐	12 to 24 ☐	more than 24 ☐
iii- measures of spirits :	none ☐	less than 12 ☐	12 to 24 ☐	more than 24 ☐

e- Is there a petrol station or a garage nearby your house : yes ☐ no ☐

4- Health Status:

a- Do you currently have any **short-term** health problems, other than a cold or flu? yes ☐ no ☐

i- If YES, please state nature of problem (i.e. headaches, tiredness)

.....

b- Do you have any **long-term** health problems, such as Asthma:? yes ☐ no ☐

i- If YES, please state nature of problem: .....

.....

c- If you are ON MEDICATION, please specify:

.....

d- If YES, how long have you been taking this medication?

.....

5- Vehicle:

a- Make and year of production:.....(19.....)

b- Fuelled with: petrol ☐* diesel ☐ electricity ☐

* if petrol fuel is ticked, please specify the fuel type and supplier usually used:

i- Type : Unleaded ☐ 4 Star (premium) ☐

ii- Supplier : ASDA ☐ BP ☐ ESSO ☐ FINA ☐

IMPERIAL ☐ JET ☐ MOBILE ☐ Q8 ☐

SAVE ☐ SHELL ☐ SPOT ☐ TEXACO ☐

TESCO ☐ TOTAL☐ UK petroleum ☐

please specify: .....

c- Is the vehicle equipped with partition that separates the driver from the passengers : yes ☐ no ☐

6- Carrier:

a- How long you have been in your current carrier ?

less than 6 months ☐ 6 to 12 months ☐more than 12 months☐

b- If you have been in the current carrier for less than 6 months, then what was your previous carrier? .....

Consent Agreement:

I agree to participate in this study and have understand that any results produced will be ANONYMOUSLY published, or with my further consent.

SIGNATURE: ..... date : .....

Serial Number: .....

THE DAY OF SAMPLING

Date : ..... / ..... /199.....(day/month /year)

6- Air Sampling :

Start (hr : min)	Flow rate (l/min)	End (hr : min)	Flow rate (l/min)

7- Driving:

a- What is the distance that have been travelled (millimetre) during sampling:

miles, or km

i- Start ....

ii- Finish. ....

b- When did you work today : Start..... : ..... Finish ..... : .....

c- Refuel vehicle: ..... litres, or ..... gallons

d- Have you driven with the window open: yes ☐ sometime ☐ no ☐

e- Did you have the air-conditioning ON : yes ☐ sometime ☐ no ☐

f- Have you got the air-condition on : recirculation mode☐ fresh-air mode ☐

g- Have you been in heavy traffic : 1- Yes☐ 0- No☐

8- Smoking

a- Have you had smoked today? yes ☐ no ☐

b- If yes, then how many you have smoked before work?

I- cigarettes none ☐ 1 to 10 ☐ 11 to 20 ☐ more than 20☐

II- cigars none ☐ 1 to 10 ☐ 11 to 20 ☐ more than 20☐

III- pipes none ☐ 1 to 10 ☐ 11 to 20 ☐ more than 20☐

c- If yes, then how many you have smoked during work?

I- cigarettes none ☐ 1 to 10 ☐ 11 to 20 ☐ more than 20☐

II- cigars none ☐ 1 to 10 ☐ 11 to 20 ☐ more than 20☐

III- pipes none ☐ 1 to 10 ☐ 11 to 20 ☐ more than 20☐

d- If yes, then how many you have smoked after work?

I- cigarettes none ☐ 1 to 10 ☐ 11 to 20☐ more than 20☐

II- cigars none ☐ 1 to 10 ☐ 11 to 20 ☐ more than 20☐

III- pipes none ☐ 1 to 10 ☐ 11 to 20 ☐ more than 20☐

e- How many cigarettes were smoked in front of you ?

	none	0 to 5	6 to 10	more than 10
i- before work	☐	☐	☐	☐
ii- during work	☐	☐	☐	☐
iii- after work	☐	☐	☐	☐

f- Have the others had smoked in front of you (Passive smoking; i.e. friends, partners) : yes ☐ no ☐

g- If yes, then how many have been smoked in front of you?

I- cigarettes	none ☐	1 to 10 ☐	11 to 20 ☐	more than 20☐
II- cigars	none ☐	1 to 10 ☐	11 to 20 ☐	more than 20☐
III- pipes	none ☐	1 to 10 ☐	11 to 20 ☐	more than 20☐

9- Diet and Drinks:

a- Do eat long life dairy products (i.e. contains preservatives): yes ☐ no ☐

b- If YES, does the preservative name exist in the following list: yes ☐ no ☐

Sorbic acid (differ from ascorbic acid)
Sorbistat
Preservastat
2,4-Hexadienoic acid
2-Propenyl acrylic acid

c- Have you had any alcoholic drinks today? yes ☐ no ☐

d- If YES, please specify:

i- pints of beer : none ☐ 1☐ 2 ☐ more than 2 ☐

ii- glasses of wine : none ☐ 1☐ 2 ☐ more than 2 ☐

iii- measures of spirits: none ☐ 1 ☐ 2 ☐ more than 2 ☐

10- Urine Samples:

a- Time of collecting the first sample (before drive)..... : ..... (pm / am)

b- Time of collecting the second sample ..... : ..... (pm / am)

c- Time of collecting the third sample ..... : ..... (pm / am)

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