Biomonitoring of Exposure to Air Pollutants: Early Biomarkers of Exposure and Effect

by

Massimiliano Mascelloni

Submitted to the University of Birmingham in partial fulfilment for the degree of Doctor of Philosophy

Division of Environmental Health and Risk Management
School of Geography, Earth and Environmental Studies
College of Life and Environmental Sciences
University of Birmingham
Edgbaston, Birmingham
B15 2TT
United Kingdom

June 2015

UNIVERSITY^{OF}BIRMINGHAM

University of Birmingham Research Archive

e-theses repository

ABSTRACT

Exposure to air pollutants is a well known issue both in indoor and outdoor environments. However, the scientific community has only recently started to focus on indoor exposure. People spend a large portion of the day in indoor microenvironments, and although the exposure levels found indoors are often lower than outdoors, indoor exposure has a relevant contribution towards the total exposure.

With this work, the main biomarkers of exposure to VOCs and PAHs were analysed in different populations groups, focusing mainly on general population, with an aim to evaluate the exposure in everyday life by analysing biomarkers of exposure and effect.

We also tried to build and validate a physiologically relevant model for toxicological studies, in order to advance the knowledge on biomarkers and biomarker discovery.

We analysed airborne personal exposure to VOCs and PAHs and urinary BTEX, cigarette smoke exposure and oxidative stress biomarkers of a population of non-smoker volunteers from Birmingham, UK. The three main categories were: a control group that lived and worked in buildings that were refurbished or built more than one year before the study; a group of volunteers that lived or worked in buildings that were refurbished or built less than one year before the sampling and a group of occupationally exposed subjects that were working in traffic or using tools powered by petrol engines. The second group underwent a second sampling after one year, in order to measure differences between the first and second sampling. The third group was sampled at the end of the working week and again after the weekend, in order to measure differences between the end of the working week and the beginning of the new one.

In addition to this group of subjects, a subset of a previous study with high exposures to environmental tobacco smoke (ETS) was added (Harrison et al., 2009), together with a subset

of samples from schoolchildren in Saudi Arabia, living next to a high-traffic ring road and to an oil refinery (Trasande et al., 2015).

Results showed a significant correlation between urinary cotinine (ETS biomarker) and urinary VOCs, suggesting a contribution of ETS towards exposure even at low concentration, and a generalized variance of exposure biomarkers even in general population. Urinary VOCs were found suitable for exposure assessment in general population, although the correlations with oxidative stress biomarkers were weak.

Analyses of PAH (both airborne and urinary) in schoolchildren from Saudi Arabia did not reveal significant correlation between urinary PAHs and oxidative stress.

The results from this last study suggested that oxidative stress in that population might be related to some other compound or behaviour that was not measured.

In addition to these studies, we designed a custom exposure system to expose cells to controlled atmospheres, to evaluate the immediate effects of exposure to low concentrations of benzene. This system was designed to develop a physiologically relevant model of exposure that could be applied in future toxicological studies. The results obtained with this system proved the validity of the model for benzene exposure, where we observed ROS generation and DNA damage in the cells after benzene exposure.

ACKNOWLEDGEMENTS

Table of contents

List of tables

List of figures

XIX

Abbreviations

8-OH-dGMP	8-hydroxy-2'-deoxyguanosine mono phosphate
8oxoAde	8-oxo-dihydroadenine
8oxoDA	8-oxo-2'-deoxyadenine
8-oxo-dG	8-oxo-2'-deoxyguanosine
8OHGua	8-hydroxyguanine
OHC	5-hydroxycitosine
5OHU	5-hydroxyuracil
BC	Black Carbon
BMA	Benzyl mercapturic acid
BQ	Benzoquinone
BTEX	Benzene, Toluene, Ethylbenzene, o- m- p-Xylene
CA	Catechol
CI	Chemical Ionization
CYP2E1	Cytochrome P450 2E1
DCF	Dichlorofluorescein
DNA	Deoxyribonucleic acid
DSB	Double Strand Breaks
EI	Electron Impact Ionization
ELISA	Enzyme-Linked Immunosorbent Assay
ETS	Environmental Tobacco Smoke
FBS	Foetal Bovine Serum
FIXAT	FIngerprints of eXposure to Air Toxics
GCMS	Gas Chromatography- Mass Spectrometry

GPS	Global Positioning System
H2DCF-DA	2',7'-Dichlorodihydrofluorescein diacetate
HPLC/MS	High Performance Liquid Chromatography- Mass Spectrometry
HPLC/ECD	High Pressure Liquid Chromatography - Electro Chemical Detection
HQ	Hydroquinone
HS-GCMS	Headspace Gas Chromatography- Mass Spectrometry
HS-SPME	Headspace Solid Phase Microextraction
IL-6	Interleukin-6
IL-8	Interleukin-8
KS	Kolmogorov-Smirnov
KW	Kruskal-Wallis
LC-MS	Liquid Chromatography- Mass Spectrometry
LMPA	Low Melting Point Agarose
LOD	Limit of Detection
LOQ	Limit of Quantification
MATCH	Measurement and Modelling of Exposure to Air Toxics Concentrations for Health Effects Studies
MBMA	o-Methylbenzyl-mercapturic acid
MFC	Mass Flow Controllers
N/A	Not Applicable
n.d.	Not Detected
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NCI	Negative Chemical Ionization

NHEJ	Non-homologous End-joining
NMPA	Normal Melting Point Agarose
PAH	Polycyclic Aromatic Hydrocarbon
PH	Phenol
PMA	Phenylmercapturic Acid
PPM	Parts Per Million
PTFE	Polytetrafluoroethylene
ROS	Reactive Oxygen Species
SIM	Single Ion Monitoring
SPE	Solid Phase Microextraction
SPMA	S-phenylmercapturic Acid
SPME- GCMS	Solid Phase Microextraction-Gas Chromatography-Mass Spectrometry
STD	Standard
TD	Tail DNA
TTMA	Trans Trans Muconic Acid
VOC	Volatile Organic Compound

I. Introduction

This chapter presents an overview on exposure, metabolism, measurement and effects of VOC and exposure.

I.1 Exposure to VOCs and PAHs

Exposure to volatile organic compounds (VOCs) and polycyclic aromatic hydrocarbons (PAHs) is a well-known issue, and there is an increasing interest from the scientific world towards the estimation of the exposure in houses and workplaces for general population (Wolkoff, 1995).

Typically the exposure to VOCs is studied in populations or categories that are exposed to occupational levels, therefore high ambient concentrations. However, as mentioned above, exposures linked to indoor microenvironments are gaining importance towards the overall exposure of general population, and not only certain categories of occupational exposure.

The microenvironments that mostly contribute to exposure in everyday life are the home or the workplace (Delgado-Saborit et al., 2011). People spend the largest part of the day indoors (e.g. working, cooking, eating, sleeping) while outdoor activities are mostly limited to commuting, with some exceptions in general population. It has been reported in several international studies that people spend 80-93% of their time indoors, 1-7% in enclosed transit, and 2-7% of the time outdoors (Jenkins et al., 1992; Thatcher and Layton, 1995). Furthermore, it has been reported that within the time spent indoors, the large majority of it (60-70%) is spent in the house (Thatcher and Layton, 1995; Hinwood et al., 2003), making it one of the most influential microenvironments for exposure evaluation.

In addition to the home microenvironment, the workplace has also been reported to be an important microenvironment for exposure evaluation (Harrison et al., 2002).

Most of the measurements conducted in the field of PAHs and VOCs exposure have been performed by air sampling, at fixed site or in the breathing zone with personal or passive

samplers (Manini et al., 2006; Harrison et al., 2009; Scheepers et al., 2010). While this methodology gives a good global idea of the exposure, it does not take in account all the possible variables that can contribute to differences in exposure patterns, metabolism and absorption in a person (e.g. level of activity, age, fitness).

Air sampling itself gives an idea on the level of pollution in the microenvironment, and it is possible to calculate the exposure from this. Unfortunately, it does not take in account the ratio of absorption and the metabolization of the pollutants.

A good way to assess the exposure and the interaction with the environment is to analyse the metabolites of the pollutants that the subjects can get in contact with in their everyday life or in the workplace (Hu et al., 2006; Manini et al., 2006; Campo et al., 2011). In such investigations, the main subject of analysis is the human urine, which contains most of the metabolites that are excreted after being processed in the body. All of the analysed metabolites are relevant trackers of exposure to various pollutants, and their analysis and quantification allows making a more accurate estimation of the exposure of the subject. Moreover, due to the relatively easy recovery of the metabolites and the non-invasivene nature of sampling, urine analysis has been used widely for biomarker studies. Nowadays, there are a reasonable number of studies on the high level exposures and environmental exposures to PAHs and to VOCs using urine sampling (Qu et al., 2003; Fustinoni et al., 2010).

I.2   VOC and PAH sources

VOCs and PAHs are ubiquitous compounds that are found in both outdoor and indoor air. The most common sources of both VOCs and PAHs are related to combustion, including traffic, environmental tobacco smoke (ETS) and wood/incense burning. The contribution of outdoor air towards the overall exposure to both VOCs and PAHs is always present, and it represents one of the primary sources of pollutants since outdoor air can infiltrate indoor environments through ventilation (de Blas et al., 2012). However, household items, consumer

products, building materials and even furniture, or activities such as DIY are known sources of VOCs. Use of printers and photocopiers is also a source of PAHs. All of these sources can contribute towards an increase in the concentration of the pollutants in indoor microenvironments, creating in some cases significant changes in the concentration of certain airborne pollutants (Delgado-Saborit et al., 2011; Delgado Saborit et al., 2009; Edwards et al., 2001).

Building materials and furniture, including carpets, wall papers, linoleum and glues are known sources of VOCs such as formaldehyde, toluene, xylenes (Missia et al., 2010; Shin and Jo, 2014; Bolden et al., 2015). Together with the contribution of the outdoor air towards the indoor concentration, indoor sources can contribute significantly towards the overall exposure to VOCs. In some cases, the indoor concentrations can reach significantly high levels, causing serious adverse reactions, known as "sick building syndrome". Typically the sick building syndrome is associated to high indoor VOC levels, and the symptoms are dizziness, nausea, headaches or asthma (Wolkoff, 1995). These episodes, however, are generally related to spot exposures or poor building design, which in some cases can cause a significant increase in indoors VOCs concentrations causing adverse effects on the building's occupants (Missia et al., 2010).

I.3   Biomarkers, biomonitoring and metabolism

Biomarker discovery and the consequent biomarker monitoring are gaining importance in the exposure field as these methods represent a way to estimate and establish an early assessment on the level of exposure.

Biomarkers like proteins adducts (Qu et al., 2003; Rappaport et al., 2005) have been used for high level exposures, as the pollutants tend to conjugate with transport proteins like albumin in the bloodstream once absorbed by lungs. These methodologies have a good reliability for high level exposures and allow analysing the pollutants in a form which is similar

to the original as they are sampled before they get completely processed in the body, making it easier and more immediate to track a certain pollutant.

On the other hand, they require blood samples (invasive sampling) and have a low sensitivity; furthermore the half-life of the pollutants in the bloodstream could negatively affect the final result and the estimation. A more effective way to analyse the exposure is to track the exposure from the results of the metabolization, i.e. after excretion in urine. In this case, typical metabolites arising from different pollutants, known as biomarkers, can be found.

One of the most diffused pollutants in the VOC mixture is benzene which is known to generate not only albumin adducts when present in high amounts, but also trans-trans-muconic acid (TTMA), S-phenylmercapturic acid (SPMA), phenol (PH), quinones such as benzoquinone (BQ) and hydroquinone (HQ), and catechol (CA) via its metabolism (Melikian et al., 1999; Kim et al., 2006; Manini et al., 2010).

Some authors (Kerzic et al., 2010; Lim et al., 2011; Lin et al., 2007) have focussed on quinones, which showed a good reliability and a good metabolite to track the exposure. Quinones are one of the principal metabolites of benzene, arising from the metabolization in the liver, which consists in the addition of two oxygen atoms to two of the carbons of the benzene ring.
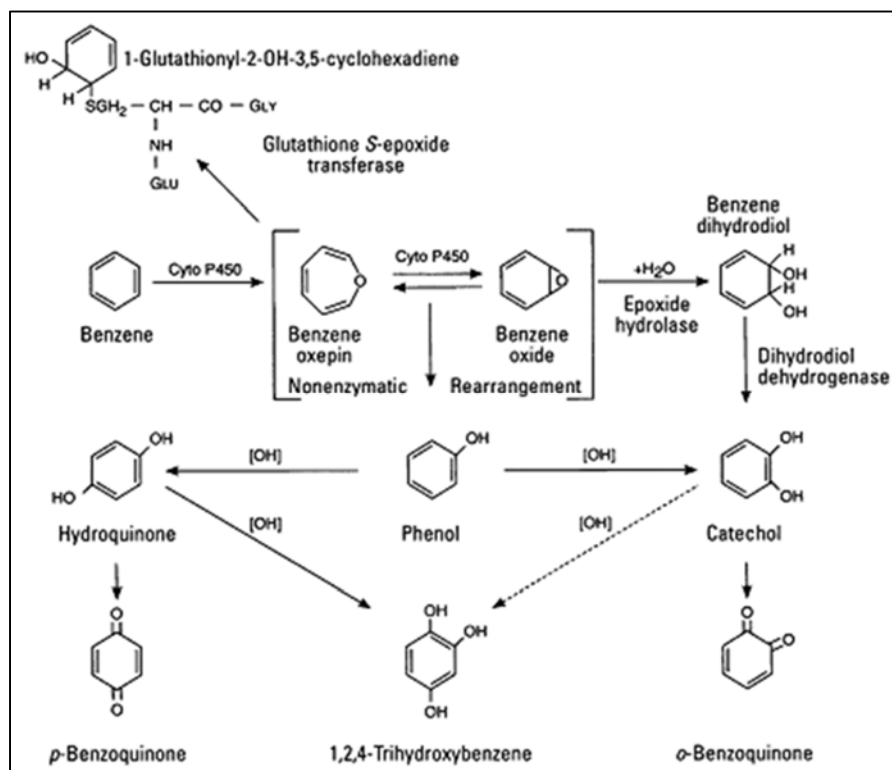
Figure 1: Schematics of benzene metabolism, Snyder and Hedli (1996).

As explained in Figure 1, benzene is metabolized in the mitochondria by Cytochrome P 450, which acts by adding one oxygen atom, which tends to rearrange as phenol or benzene dihydrodiol. Then, both of these metabolites can undergo to an enzymatic reaction (benzene dihydrodiol) or a chemical reaction with OH ending in catechol which rearranges in 1,2-benzoquinone. Another pathway of metabolization is from phenol, reacting with OH and forming hydroquinone, which rearranges in 1,4-Benzoquinone. Both benzoquinone species have a dynamic equilibrium between the reduced form (hydroquinone) and the oxidised form (benzoquinone), which is pH dependent. At the cellular pH (7.5) the prevalence is for the reduced form, because of the nicotinamide adenine dinucleotide phosphate (NADPH) contribution to the reduction of the benzoquinone in hydroquinone, although in an acidic pH the reduction does not occur (Boersma et al., 1994; Snyder and Hedli, 1996).
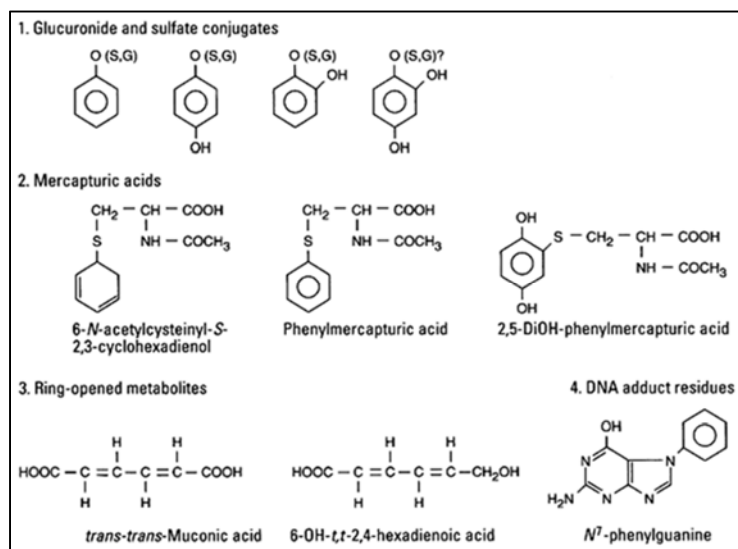
Figure 2: Main metabolites of benzene, Snyder and Hedli (1996).

These biomarkers can be monitored and quantified to evaluate the exposure to benzene in humans from urine samples, coupling the non-invasive sampling with the accuracy of the measurement of the metabolites.

Many studies have been performed on every metabolite, showing a good reproducibility for hydroquinones, which have been used as biomarkers in several cases (Qu et al., 2003; Waidyanatha et al., 2004; Kim et al., 2006). In general, all the studies on the metabolites, have been performed on subjects that were exposed to high levels of VOCs. There is a lack of knowledge about the low exposures of the general population and none of the previous studies has taken in account the exposure arising from the contribution of sources such as consumer products and building materials in indoor air.

A study on this kind of exposure, coupled with personal air sampling will help to better understand and evaluate the exposure to VOCs and PAHs in general population. Furthermore, such a study would help finding suitable biomarkers to analyse and evaluate low level exposures.

6

I.4 VOC exposure, DNA damage and toxicological models

In light of the contribution of indoor air towards the total exposure, this kind of exposure should be studied extensively. Indoor exposure to VOCs has been reported to cause a number of symptoms ranging from irritation of the respiratory system to sick building syndrome (Brinke et al., 2004; Wang et al., 2013). In addition, VOCs are also reported to contribute to oxidative stress induction via production of reactive metabolites and hence have the potential to damage DNA (Knaapen et al., 2006; Lu et al., 2007; Saint-Georges et al., 2008; Snyder and Hedli, 1996; Sørensen et al., 2003a; Wang et al., 2013). Exposure to VOCs can also lead to an inflammatory response in the lungs that can then result in oxidative stress. Some compounds, for example styrene, have been reported to be directly responsible of leading to oxidative stress by induction of inflammatory response (Bönisch et al., 2012; Röder-Stolinski et al., 2008). Inflammatory response has been suggested to be an important source of oxidative stress in a review by Knaapen et al. (2006a). An association between chronic inflammation and the development of cancer has been observed in many cases. In fact, chronic inflammatory bowel diseases are associated to the development of colon carcinoma (Collins et al., 1987), as well as chronic lung inflammatory diseases (i.e. sarcoidosis and fibrosis) are associated with a higher risk of cancer development (Schottenfeld and Beebe-Dimmer, 2006). In the review, the author suggests that the inflammatory process, involving interleukin production, which triggers the neutrophils response with production of reactive oxygen species (ROS), could trigger DNA damage. The author, however, specifies that this has been observed in rat, and while it still needs to be proven in humans, the proposed process represents a possible pathway of lung localized carcinogenicity in case of chronic exposure. The inflammatory response can be triggered by bacteria or foreign particles that enter the lung, as well as VOCs, PAHs and particulate matter, as mentioned previously. Indoor concentrations of the pollutants have the potential to trigger inflammatory response (Pariselli et al., 2009; Bönisch et al., 2012), making

7

indoor air crucial in terms of pollution-induced diseases and studies. The pathway described in the review starts with a non-specific response of lung cells through expression of cytokines. Resident macrophages in the lungs produce chemokines and cytokines (interleukin 8 is one of them, generally considered a marker for inflammatory response), together with the lung epithelial cells. This cytokine and chemokine production triggers the process of adherence, extravasation and migration of neutrophils that will reach the lung epithelium and produce a broad spectrum of oxidants, mainly resulting in production of H_2O_2 and its conversion in HOCl in order to eliminate the inflammation trigger (Klebanoff, 2005). In case of PAHs, it has been observed that the presence of ROS can activate the compounds by transforming them in their chemically active form of hydroxides. ROS generation arising from inflammatory response has been linked to DNA damage and higher levels of 8-oxo-dG. However, H_2O_2 is not directly responsible for DNA damage, but one of the ions deriving from HOCl formation, $\bullet$OH is thought to be the responsible for DNA lesions, due to its high reactivity (Pryor, 1988).

For benzene related DNA damage, together with the inflammatory response, another mechanism of action has been proposed through the known reactive benzene metabolite *p*-benzoquinone. One of *p*-benzoquinone cellular targets is topoisomerase-II, which is inhibited, generating DNA double strand breaks (DSB) which are then repaired by homologous recombination (HR) or non-homologous end-joining (NHEJ). NHEJ is thought to be more error prone than HR, however, generally, DSB damage can lead to DNA aberrations by base deletions or translocations due to genome instability. Furthermore, the metabolism of benzene through redox cycling and generation of ROS is proposed as another main source of DNA damage. When hydroquinone or catechol are oxidised in presence of oxygen, superoxide (O_2^-) is formed, which is then transformed in H_2O_2 by superoxide dismutase. H_2O_2 is not responsible for DNA damage directly, but it increases the oxidative potential of the cell, and in presence of transition metals ion or copper, hydroxyl radical ($\bullet$OH) is formed, which reacts with lipids,

8

proteins and DNA. •OH is responsible for the formation of 8-oxo-dG and other nucleobase alterations due to oxidation (Halliwell and Aruoma, 1991). H_2O_2 production is also linked to inflammatory response of which, as stated above, VOC exposure has been reported to be a trigger.

Therefore, low level VOCs, PAHs and their metabolites have a potential role in DNA damage due to their inflammation triggering effect, and their metabolization in the cells via redox reactions (Moller, Folkmann et al. 2008). VOCs and PAHs can also react directly with the DNA forming adducts with the nucleobases.

In the case of nucleobases adducts the nucleobases are modified by the reactive species that are produced during the metabolization of the exogenous compounds. In both cases the nucleobases are not anymore coupling with the complementary one on the other strand of DNA, perturbing the double helix. This perturbation is sensed by the cell which activates the DNA repair systems which will cut out the non-pairing nucleobases and substitute them with new ones (Loft, Danielsen et al. 2008). The increase of the ratio of damage with the consequent repair can generate mutations in the DNA, that can lead to irreversible DNA damage and either apoptosis or carcinogenicity as the repair system is anyway error-prone. The nucleobase that is more prone to modifications is Guanine, which is often modified in 8-oxo-dG, but also the other nucleobases are modified by reactive species like ·OH or ·O. Other common markers for guanine are 8-hydroxyguanine (8OHGua), 8-oxo-dihydroguanine (8oxoGua). The other nucleobases have also their typical modifications, such as 8-oxo-dihydroadenine (8oxoAde), 8-oxo-2'-deoxyadenine (8oxoDA), 5-hydroxyuracil (5OHU), 5-hydroxycitosine (5OHC) (Cooke, Evans et al. 2003; Cooke, Henderson et al. 2009; Ziech, Franco et al. 2010). The nucleobases that are excised from the DNA are then excreted from the cells in the bloodstream and then in the urine.

Measurement of these biomarker of oxidative stress is a field of study that in the last decade started to have an important role in understanding and evaluating the level of DNA damage generated by various agents (pathogenic, inflammatory, cancers, diabetes, degenerative diseases, environmental exposure) (Cooke, Evans et al. 2003).

Exposure to benzene and its correlation with the DNA damage has been extensively studied, especially for occupational exposures, proving that benzene and its metabolites are involved in DNA damage through reactive metabolic intermediates (Snyder and Hedli, 1996; Hartwig, 2010) and ROS generation (Barreto et al., 2009). Because of the detailed knowledge about its metabolism, benzene can serve as an ideal positive control test compound for the development of new toxicological assays and tests on environmental exposure. Furthermore, in several studies of human exposure to benzene at medium and low concentrations (0.001 ppm – 0.03 ppm), a correlation was found between levels of urinary benzene and benzene metabolites and urinary 8-oxo-2'-deoxyguanosine (8-oxo-dG) a biomarker that is widely considered to be associated with ROS related DNA damage and repair (Andreoli et al., 2012; Manini et al., 2010; Bagryantseva et al., 2010).

A recent review (Hartwig, 2010) summarised the possible mechanisms through which benzene and its metabolites can cause DNA damage. *In vivo*, after absorption, benzene is metabolized by cytochrome P450 2E1 (CYP2E1) in the liver to produce metabolites including: phenol, catechol, hydroquinone and 1,2,4 trihydroxybenzene. Then these closed ring metabolites undergo further metabolism in the bone marrow to form *o*- and *p*-benzoquinone. *P*-benzoquinone is thought to be the toxic metabolite responsible for myelotoxicity due to its high reactivity, and in fact the metabolite is known to form adducts with proteins and DNA (Rappaport et al., 2005; Linhart et al., 2011). Bone marrow, due to the high rate of cell mitosis and a lower DNA repair capacity (Buschfort-Papewalis et al., 2002) is a sensitive tissue for DNA damage, resulting in possible health effects sooner than other tissues.

However, all of the phenolic benzene metabolites are chemically active in the cells, increasing the oxidative DNA damage, directly or through further metabolization. Therefore, although the main target tissues and the metabolism of benzene have been studied and understood, it is important to develop models to assess the toxicity caused by VOC exposure to other metabolically competent tissues including the lung epithelium, which is the principal route of exposure to benzene and other VOCs. Even more, such models could be useful to study other VOCs, as currently there is still little evidence of any linkage between exposure and DNA damage for other VOC species, although it has been suggested from studies in animal models and *in-vitro* studies (Sarma et al., 2011; Singh et al., 2009).

Currently there are only few detailed studies on the development of *in vitro* models to examine the effects of such pollutants on the lung. Pariselli et al. (2009) developed a method to expose lung cells to various concentrations of benzene and toluene, evaluating some of the effects caused by the pollutants on lung cells growing on permeable supports that allow exposure of cells to gas mixtures, modelling a real life situation of exposure.

The CULTEX system to expose cells to controlled atmospheres has been developed and used mostly for studies on cigarette smoke, nanoparticles and gases such as NOx and O_3 (Ritter et al., 2001). Similar *in-vitro* methods were developed, although none of them used an active exposure to the pollutants. Sarma et al. (2011) exposed cells to VOCs by adding the compounds to the growth media, which is not a physiologically accurate model. In another case, the cells were exposed to controlled atmospheres containing VOCs, where the VOCs were injected in liquid form and the exposure was performed by evaporation of the compounds (Mögel et al., 2011). In this case, the exposure was more physiologically accurate, however the methodology is limited for future applications due to the pollutants that have to be added to the exposure chamber via injection. An exposure system using gaseous exposure such as the

CULTEX system, allows the possibility to expose cells to different pollutants and complex mixtures of pollutants.

Although interesting results on inflammatory response triggering by VOC exposure and cell viability were reported from Pariselli et al. (2009), no further studies on DNA damage or ROS production were reported on the effect of VOCs on lung epithelium models.

I.5 Half-life and excretion of unchanged VOCs

Airborne VOCs are absorbed through the lungs in different percentages and depending on the compound the lung retained dose changes, as Janasik et al. (2008) confirmed from previous studies (Kostrzewski, 1993; Gromiec and Piotrowski, 1984), and they are then transported in the bloodstream. Once the compounds are in the body they are metabolized at different ratios, depending on the mitochondrial genetic subgroup, level of exercise, amount of body fat (Fustinoni et al., 2005; Verdina et al., 2001; Rappaport et al., 2013), while a small fraction is excreted unchanged in the urine. The urinary VOCs pass in the urine by permeation by passive diffusion through the kidney glomerular tuft, and therefore are to be considered equilibrated with the blood concentration, which is regulated by their coefficient of solubility in water (Janasik et al., 2008; Fustinoni et al., 2000).

The excreted unchanged VOCs represent a very small fraction of the total VOC uptake, with calculated values ranging from 0.0011% to 0.0032% (Janasik et al., 2008; Ikeda, 1999; Fustinoni et al., 2007). Only a few studies on excretion and metabolization kinetics and half-lives ($T_{1/2}$) of VOCs have been performed, however, the results are consistent with each other.

As Fustinoni et al. (2009) reported, the uptake (and therefore the urinary excretion) of toluene is very rapid, and the urinary concentration rises quickly during the exposure. Right after the exposure (where the highest concentration C_0 is recorded) the urinary concentration of the VOC (toluene in the specific case) starts to drop quickly within 1-2 hours after the exposure reaching a C_1 which is approximately the 20-30% of C_0. After this step ($T'_{1/2}$) a

second, slower decrease in urinary concentration is observed, for the following 20-22 hours ($T''_{1/2}$) until the urinary VOC concentration reaches the baseline level (Fustinoni et al., 2009; Bois et al., 1996; Janasik et al., 2008).

A common procedure used for biomonitoring of the VOC exposure is the analysis of the metabolites (the most common are s-phenyl-mercapturic acid, trans-trans muconic acid for benzene, but also quinones have been used as biomarkers of exposure, o-creosol and hippuric acid for toluene) (Rappaport et al., 2010; Fustinoni et al., 2007; Janasik et al., 2008; Waidyanatha et al., 2001).

As reported by Waidyanatha et al. (2001) together with other authors, low level exposure raised a concern regarding the validity of these biomarkers due to their natural occurrence from other metabolic pathways during normal biological processes. For example protein catabolism or metabolism of benzoic acid (which occurs naturally in some fruit), produce hippuric acid, that can invalidate the evaluation of the exposure to toluene in low ambient concentrations. Generally, when analysed for low concentrations, the levels of the naturally occurring metabolites can represent a major problem for the correct evaluation of the exposure, due to the high and variable baseline level (Fustinoni et al., 2000; Waidyanatha et al., 2001).

A univocal way of determining the exposure is to measure the levels of the unchanged VOCs in the urine, using SPME-GCMS, which allows high sensitivity and specificity, which recently has been adopted by a growing number of researchers (Waidyanatha et al., 2001; Fustinoni et al., 1999; Janasik et al., 2008).

Table 1: Summary of previous works on excretion kinetics of VOCs, all of the studies are focused on occupational or voluntary exposures to high air concentrations in spot exposures.

Author and year	Paper title	Topics covered	Airborne VOC concentration
Bois et al. 1996	Population toxicokinetics of benzene	Benzene metabolization, benzene in exhaled air and blood with excretion kinetics	1.7 and 10ppm
Fustinoni et al. 2009	Self-collected urine sampling to study the kinetics of urinary toluene (and o-cresol) and define the best sampling time for biomonitoring.	Comparison between o-cresol and urinary toluene with excretion kinetics	16ppm (mean)
Janasik et al. 2008	Excretion of unchanged volatile organic compounds (toluene, ethylbenzene, xylene and mesitylene) in urine as result of experimental human volunteer exposure	Kinetics of urinary excretion of toluene, ethylbenzene, xylenes and mesitylene	26ppm (toluene) 100mg/m^3 for each compound
Pierce et al. 2004	Exponential modeling, washout curve reconstruction, and estimation of half-life of toluene and its metabolites	Excretion and washout curve modelling from different databases	Various, ranging from low level (not declared), to 50ppm and 80ppm

All of the previous works on the kinetics of the excretion of the unchanged VOCs have been performed with spot exposures to high airborne concentrations for obvious reasons of simplicity of calculation and estimation of the excretion rate. The airborne concentrations and the kind of measurements performed for the excretion curve calculation are summarised in Table 1.

All the excretion kinetics graphics and data are coherent in identifying two phases of excretion, a fast and a slow phase (T'$_{1/2}$ and T''$_{1/2}$) with the first one lasting between 2 and 4 hours, and the second one lasting up to 20-24 hours.
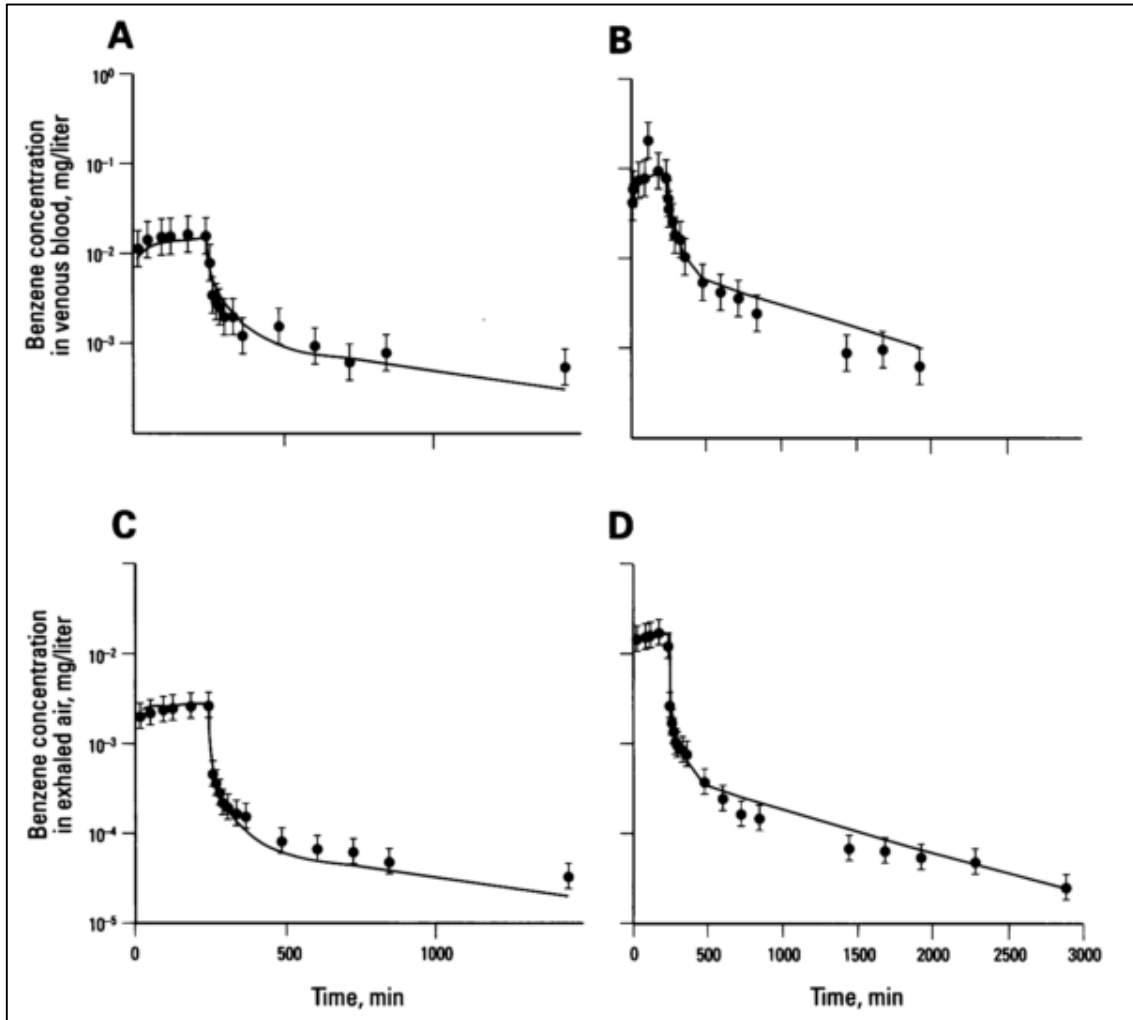
Figure 3: Time-course study on benzene excretion levels in blood and exhaled air, Bois et al. (1996).

Bois et al. (1996) (Figure 3) reported concentrations of benzene in blood and exhaled air in a time span of 50 hours after a single controlled exposure event. A and B graphics show the blood concentration of benzene, A is related to an exposure of 10ppm, while B is related to an exposure of 1.7 ppm, both exposures were of 4 hours. C and D refer to the concentration of benzene measured in the exhaled air, which is a measure of the evaporation of benzene from the bloodstream, and one of the excretion systems suggested by the author. The two phase pattern is evident, since the urinary excretion and the blood concentration are very strongly correlated, we can extrapolate that also the urinary concentrations will follow the same pattern.

I.6 Metabolites extraction methodologies

To analyse urinary biomarkers it is very important to have an extraction methodology suitable for the compounds of interest. There are several different extraction methodologies that have been used to extract metabolites from human urine. The factor that most influences the choice of one method or another, is the target compound or group of compounds.

The principal extraction methods can be divided in two main kinds based on the approach, liquid phase extraction and solid phase extraction, plus some exceptions on headspace extraction. Headspace extraction is a relatively limited method of extraction, as it allows extracting only the substances with a high vapour pressure (i.e. benzene). Liquid phase extraction methodologies rely on the higher affinity of the metabolites for non-polar than for polar solvents. The extraction efficiency can be enhanced by acidification of the solution and addition of salts. The limitation of this methodology is that it is not very reproducible, as the extraction efficiency can vary between different samples. Also in most cases there is the need of concentrating the organic phase, as the extraction is often performed in excess of solvent, and the evaporation step can lead to sample losses and inter-sample variability.

Solid phase extraction is performed using columns that are packed with a stationary phase that has a high affinity for the compounds that have to be extracted. Different stationary phases have different properties, and therefore, different affinities for different families of compounds. The extraction in this case is generally more reproducible and it shows a better recovery of the single compounds. Moreover, it allows the cleaning of the sample as once the compounds are adsorbed to the stationary phase they can be washed with different stringency, without eluting them. In this kind extraction, the recovery is dependent on the stationary phase that is used and it can be changed for different target compounds.

The liquid phase extraction is a less refined kind of extraction, with a generally smaller recovery ratio and it does not allow a sample cleaning, but it is faster and cheaper than the solid phase extraction. This methodology tends to be more consistent with hydrophobic compounds.

In Table 2 are reported some examples of extractions of different compounds from urine or synthetic urine samples.

Table 2: Summary of the urine metabolites and extraction methods.

Reference	Extracted compounds	Extraction method
Leinonen et al., 2006	Metabolites of: fluoxymesterone, 4-chloro-dehydromthyltestosterone, stanozolol, danazol and methyltestosterone	Liquid-Liquid: dihexylether or 1-octanol, polypropilene fiber helped, salting out method
Rodila et al., 2006	Drug and metabolite: drug ABT-869, metabolite A-849529	Liquid-Liquid: 1:11 hexanes/ethyl acetate solution, introduction of little salts and acid
Hu et al., 2006	Pyrene metabolite (OHP)	Liquid-Liquid: DMSO
Sabatini et al., 2008	Benzyl mercapturic acid (BMA), phenylmercapturic acid (PMA), o-methylbenzyl-mercapturic acid (MBMA)	Solid Phase Extraction: Evolute ABN cartridges (Argonaut technologies Ltd.)
Gaikwad et al., 2009	Estrogen metabolites, conjugates and DNA adducts	Solid Phase Extraction: Phenyl Solid phase extraction cartridges (Varian)
De Alwis et al., 2009.	Dialkil phosphate metabolites	Solid Phase Extraction: Elut PPL SPE cartridges (Varian)
Paci et al., 2007	SPMA (s-phenylmercapturic acid)	Solid Phase Extraction: Sep-Pak plus cartridges (C-18) (Waters)
Jacob et al., 2007	Naphtholes, hydroxyphenantrenes, hydroxyfluorenes, hydroxypyrenes	Liquid-Liquid extraction: 90:10 pentane/ethyl acetate
Kim et al., 2006	Muconic acid, s-phenylmercapturic acid, phenol, catechol, hydroquinone.	Liquid-Liquid extraction: Ethyl acetate, two consecutive extractions.
Waidyanatha et al., 2004	Muconic acid, s-phenylmercapturic acid, phenol, catechol, hydroquinone.	Liquid-Liquid extraction: Ethyl acetate, two consecutive extractions.

I.7   Metabolites analysis and related issues

Using urine analysis takes in account all the routes of exposure, including also the food pathway. Depending on how the food is cooked and its provenience, the levels of pollutants, and therefore their metabolites can vary (Falco, Bocio et al. 2005; Ibanez, Agudo et al. 2005; Marti-Cid, Llobet et al. 2008; Cirillo, Montuori et al. 2010; Xia, Duan et al. 2010). Hence it becomes important to keep track of food consumption and cooking method. It is also important to keep track of the lifestyle of subjects, as medicines, and cigarette smoke can also influence the excretion of benzene metabolites (Lovreglio, Barbieri et al. 2011). Monitoring these variables, the evaluation of the total exposure to the pollutants becomes more significant.

The most used techniques to analyse the majority of the metabolites are HPLC/MS, GCMS, HS-GCMS, due to the various physiochemical properties of the compounds. In fact, there is a large use of GCMS and HS-GCMS for volatile metabolites and HPLC/MS for the less volatile compounds. The headspace GCMS is used mostly for unmetabolized benzene and other compounds that have a high vapour pressure. This methodology reduces the extraction times and improves the reproducibility but it is strictly connected to the volatility of the compounds to analyse (Fustinoni, Giampiccolo et al. 1999).

Benzene, due to its known carcinogenicity, is one of the most extensively studied and monitored pollutants. As previously mentioned, all of the metabolites of benzene have been measured in previous studies, but most of them have given good results only for high levels of exposure. It has been reported that the levels of hydroquinone can vary from 0.066 µg/ml for people not exposed to benzene, up to 50.6 µg/ml for people exposed to high levels of benzene (>31 ppm) (Waidyanatha, Rothman et al. 2004; Kim, Vermeulen et al. 2006).

Also phenol and SPMA have shown to be good biomarker candidates for quantification as they show a good reliability and response in case of low VOCs concentration (Qu, Melikian

et al. 2000). All these compounds are generated from the benzene metabolism, which involves a passage through phenol for almost all of the final metabolites.

The best and most used metabolites of assess the exposure to low concentrations of benzene are the phenol and S-phenylmercapturic acid (SPMA). All of the metabolites, however, have some limitations for low level exposures, due to the fact that they can arise from other metabolic pathways that are not related to benzene exposure. The baseline levels of the metabolites represent a confounding factor that makes the exposure evaluation of general population difficult. Another way of assessing low level exposures is to measure urinary benzene which has not been metabolized, in order to avoid contamination of the metabolites arising from unrelated metabolic pathways (Weisel, 2010). Urinary benzene (together with urinary toluene, ethylbenzene, o- m- p-xylene and naphthalene) are analysed by headspace solid phase microextraction (HS-SPME), which allows very low detection limits (in the order of 10-20 ng/L), but it requires a specialized equipment to perform the extractions. On the other hand, the monitoring of the metabolites doesn't require special instrument, although the limits of detection are higher, due to the contribution to the production of the same compounds arising from other unrelated metabolic pathways of different substances.

I.8   Oxidative stress and DNA damage biomarker analysis

There are three main ways to measure the level of DNA damage caused by oxidative stress biomarkers in urine. The most commonly measured biomarker is 8-oxo-dG, as previously mentioned in Section I.4. A commonly used techniques is HPLC/ECD (Electro Chemical Detection) (Sørensen et al., 2003c), but also other methodologies are used, like the ELISA (Enzyme-Linked Immunosorbent Assay) that provides faster measurements and is produced as a kit for the measurements (Cooke, Lunec et al. 2002; Wu, Chiou et al. 2004). Other analytical methods have been developed using GC-MS, derivatizing the biomarkers after

extraction from urine (Lin, Jenner et al. 2004), enhancing the sensitivity and the throughput of the analysis. Each methodology has different pros and cons, the HPLC methods are often requiring an extensive clean-up of the sample, as urine is a very complex and variable matrix, and the methodology can result long and time consuming. Immunoassay or ELISA are quick methodologies and have a comparable sensitivity as the HPLC methods. However, these assays they can react with non specific molecules that present the same epitopes as oligomers of 8-OH-dGMP (8-hydroxy-2'-deoxyguanosine mono phosphate) in the case of 8-oxo-dG. Furthermore, the discovery of new biomarkers using these techniques is not possible due to the kind of sample analysis that are used. GC-MS analysis allows to obtain separation together with the possibility of identifying the compounds. Methods were developed to have an efficient extraction of the excreted nucleobases from urine and subsequent analysis in GC-MS, this methodology is reported to be efficient and sensitive, and it also gives qualitative information on the analysed compounds. The weak point of this method is the derivatization procedure that the markers have to undergo. A derivatization step is always critical for reactive molecules such as 8-oxo-dG. Furthermore, the addition of a derivatization step in the sample preparation increases the uncertainty of the measurement, especially for low concentrations. Trimethylsilylation is the typical derivatization that is used with GC-MS samples, but the normal procedure is performed at high temperatures and in contact with air. These conditions have demonstrated to create artifactual 8-oxo-dG due to the reactivity of the analytes. An ad-hoc methodology was then developed to prevent artifacts and therefore higher readings in DNA damage levels using GCMS technique (Jenner, England et al. 1998). The latest technique used for 8-oxo-dG measurement is LC-MS, which allows to analyse the molecule without the need of a derivatization step, simplifying the sample preparation procedure, and allowing to have reliable measurement in a broad range of concentrations (Lam et al., 2012). The main problem

of this methodology is that it requires a specific instrument that is not always available in laboratories that do not perform biological or liquid analyses on a regular basis.

I.9   Aim of the study

The aim of this study was to evaluate the impact and contribution of different behaviours and microenvironments towards the total exposure of general population. We concentrated not only on the markers of personal exposure, but also on the biomarkers of early effect, such as oxidative stress.

The thesis was oriented towards measuring and identifying as many biomarkers as possible, in order to identify a fingerprint of exposure to air toxics from urine samples. We tried to evaluate the best methods of metabolites and biomarkers extraction from urine. Due to the extremely low level of exposure and high inter-individual variations of general population, the task of identification and measurement of most metabolites and biomarkers proved to be difficult. As a result, the focus was shifted to analysis of VOCs and the association with the marker for oxidative stress. Specific objectives included:

- Assessment of the differences in urinary VOC concentrations between low-exposure and high-exposure (occupationally exposed and exposed to high levels of ETS) populations.

- Assessment of the correlation between personal exposure and a widely accepted oxidative stress biomarker (8-oxo-dG)

- Identification of typical behaviours and microenvironments that potentially contribute to the total exposure

Since analysis of urinary PAH metabolites for low exposure populations was found to be difficult to quantify, additional analyses were undertaken to characterize the impact of high level exposure on urinary PAH metabolites. This study was conducted in collaboration with

the King Abdulaziz University in Saudi Arabia. The objective of this study was to identify the contribution of high levels of exposure towards the biomarkers of oxidative stress.

While analysis of biomarkers provides important information about exposure, it is difficult to quantify and properly evaluate the biological effect of exposure to different concentrations of air studies without a metabolically comparable model. With this in mind, a cell exposure chamber was designed in order to reproduce a model of a metabolically competent tissue (lung epithelium A549 cells) for toxicological studies. The objectives of this study were to:

- Test the feasibility of the methodology in order to reproduce *in-vitro* a response comparable to the one observed *in-vivo*.

- Test the early effects of exposure to VOCs on cellular metabolism

- Establish a new methodology to study singular pollutants and mixtures on a physiologically accurate model

- Establish a methodology for future in-depth studies on the metabolism of pollutants

The thesis chapters are organized as follows:

Chapter 2 discusses method development for analysis of urinary PAH, VOCs metabolites and oxidative stress biomarkers.

Chapter 3 details the methodology for collection and analysis of personal exposure and urine samples analysed in the current study.

Chapter 4 discusses urinary VOC and oxidative stress marker concentrations in different subject groups, and underlying causes and confounders.

Chapter 5 presents results on chemical characterization and oxidative stress biomarker for a population of children in a highly polluted city in Saudi Arabia.

Chapter 6 describes a cell exposure study focused on effects of VOC exposure.

Chapter 7 and 8 describe the conclusions from the current study and future directions, respectively.

II.   Method development

This chapter describes in detail the different approaches tested to extract and analyse the various metabolites in the urine samples.

II.1   Benzoquinone and hydroquinone stability in aqueous solutions

Due to our instrument availability we focussed mostly on quinones for benzene exposure assessment. Our initial goal was to analyse benzoquinone, if possible, as this metabolite has better chromatographic properties. Hydroquinone was considered as a secondary target because of the difficulties to separate and quantify it by GC-MS.

Tests were performed to assess the stability of benzoquinone in urine. Benzoquinone is reported to be one of the main compounds responsible of DNA toxicity arising from benzene exposure (Hartwig, 2010; Snyder and Hedli, 1996), therefore we investigated the possibility to measure the compound in the urine. This would have given us the possibility of determining how much of the benzene is actually metabolised by using as a biomarker the compound responsible of the effects. Benzoquinone has been analysed in other works as a protein (albumin) adduct in blood (Lin et al., 2007; Rappaport et al., 2005), we explored the possibility of analysing the compound in urine.

Benzoquinone was dissolved in ethanol and spiked in different concentrations in synthetic urine. Extractions were performed with various methodologies (liquid extraction and SPE) to assess the best way to extract the compound. Results suggested that under any conditions the benzoquinone had poor stability, converting in hydroquinone. To confirm this hypothesis, 0.1 mg of benzoquinone were dissolved in various solvents with different grades of polarity and available hydrogens, then the relative amount of hydroquinone formed from benzoquinone was measured (Table 3). When dissolved in ethanol, more than 26% of benzoquinone rearranged in hydroquinone, due to the available hydrogen atom of the ethanol molecule, a similar behaviour was observed in water, but could not be measured directly by GC-MS. This behaviour has been described in the literature (Cheng et al., 2009; Boersma et

24

al., 1994), our tests confirmed that under physiological conditions there is little to no occurrence of benzoquinone, which converts to hydroquinone.

Table 3: Summary of benzoquinone (BQ) solubility in different solvents and amount of hydroquinone (HQ) measured by GC-MS analysis. (+ is an arbitrary measurement of solubility of benzoquinone in the solvent, +++ indicates the highest solubility and + indicates the lowest)

Solvent	Boiling point	BQ solubility	HQ amount
Dichloromethane	39.6 C	+++	5.12%
Ethyl acetate	77.1 C	+++	7.45%
Pentane	36.1 C	+	7.02%
Ethanol	78.0 C	++	26.73%

II.2   Hydroquinone/benzoquinone liquid extraction

Hydroquinone and benzoquinone (together with other metabolites) were extracted from synthetic urine using different liquid extraction approaches to evaluate and maximise the extraction efficiency. All of the method development was performed using synthetic urine, described in annex X.1

Liquid extraction was performed adding 5 volumes of ethyl acetate to 1 ml of urine previously spiked with 10 µl of internal standards (final concentration 1ng/µl) and 100 µl HCl 0.1M, sample was shaken for 2 min and then the organic layer was collected for analysis.

This main procedure for extraction was tested also with some modifications:

- Extraction performed in native conditions (no acid addition)

- 0.5g of NaCl was added to the urine to maximise the recovery of hydrophobic compounds in the organic phase

- Extraction performed with dichloromethane

- Extraction performed with smaller amounts of organic solvent (1ml)

25

II.3   Hydroquinone/benzoquinone solid phase extraction

Metabolites were extracted also using solid phase extraction columns. Two different columns were used: Bond Elut C-18 and Bond Elut Plexa (Agilent Technologies, USA).

The sample preparation was performed by diluting 1ml of urine with 2ml of 1% formic acid prior loading it on the column, which was previously conditioned with 1ml methanol. The column was mounted on a vacuum manifold that allowed to have the sample flowing through the SPE column via aspiration. The diluted sample was then loaded on the column and the flow was regulated to be approximately 1ml/min. The loaded column was then washed with 5% methanol and the extraction was performed with 2 washes of 500 µl of methanol.

The same procedure was used with both columns.

Different elution solvents were tested to maximise the recovery of the desired compounds:

- Elution with acetonitrile

- Elution with ethyl acetate

- Elution with pentane

- Elution with CH_2Cl_2

The best overall results however were obtained using the manufacturer's recommendations for acid loading to extract hydrophobic compounds, briefly:

- From each vial 0.3 ml of synthetic or real urine, containing the standards, were diluted in 0.9ml of 1% formic acid

- The SPE columns were preconditioned with 0.5 ml Methanol followed by 0.5 ml of water

- The samples were loaded in the SPE columns with an approximate flow rate of 1 ml/min and washed with 1 ml Methanol 5%

- The elution was performed with 1 ml Methanol 100%

The Agilent Bond Elut PLEXA SPE columns showed the best overall recovery rate, while the C-18 columns were not able to retain some of the compounds.

II.4 Benzoquinone and hydroquinone calibration curve and quantification

Lim et al. (2011) published a study of quinones in human urine, where the reported concentrations were in the order of 3×10^{-8} Mol/L with minimum standard values of 1×10^{-8}.

Based on the information reported on the paper, we estimated 6pg/µl as an average concentration value. To test the feasibility of an assay to measure benzoquinone and hydroquinone by GC-MS, we tried to set a calibration curve with the compounds directly injected in the GC-MS from an organic solvent and from an aqueous solution (synthetic urine) after liquid extraction. To perform the experiment, the solutions were prepared as follows:

STOCK SOLUTIONS – NATURAL STANDARDS in water

Hydroquinone 5.252 mg

Benzoquinone 5.016 mg

These Natural Standards were dissolved in 5ml of water, obtaining the stock solution of Natural STDS (Ns_Stock_Water approximated concentration: 1mg/ml).

NATURAL STANDARDS in ethyl acetate

Hydroquinone 5.095 mg

Benzoquinone 5.118 mg

These Natural Standards were dissolved in 5ml of ethyl acetate, obtaining the stock solution of Natural STDS (NS_Stock_EtAc approximated concentration: 1mg/ml).

Table 4: standard stock solutions concentration.

Name of Natural Stock	BQ Concentration	HQ concentration	Solvent
NS_Stock_Water	1.00320 mg/mL	1.0504 mg/mL	Water
NS_Stock_EtAc	1.02360 mg/mL	1.0190 mg/mL	Ethyl Acetate

The two stock solutions were then diluted to obtain the following intermediate solutions:

Table 5: Standard intermediate solutions with BQ and HQ concentrations.

Name of Intermediate	BQ Concentration (approximated)	HQ concentration (approximated)	Solvent
A_Water	10,000 pg/µl	10,000 pg/µl	Water
B_Water	100,000 pg/µl	100,000 pg/µl	Water
C_Water	1,000,000 pg/µl (NS_Stock_Water)	1,000,000 pg/µl (NS_Stock_Water)	Water
A_EtAc	10,000 pg/µl	10,000 pg/µl	Ethyl Acetate
B_EtAc	100,000 pg/µl	100,000 pg/µl	Ethyl Acetate
C_EtAc	1,000,000 pg/µl (NS_Stock_EtAc)	1,000,000 pg/µl (NS_Stock_EtAc)	Ethyl Acetate

STOCK SOLUTION – INTERNAL STANDARD

IS Stock solution E_EtAc: The Internal STDS in ethyl acetate were prepared dissolving 0.604mg of benzoquinone D4 in 5ml of ethyl acetate, obtaining a final concentration of 0.1208mg/ml approximated to 0.1mg/ml.IS Stock solution E_Water: The Internal STDS in Water were prepared dissolving Benzoquinone D4 0.561mg in 5ml of water, obtaining a final concentration of 0.1122mg/mLapproximated to 0.1mg/ml.

IS Stock Solution F: For a second set of liquid extractions a new internal standard has been made with a higher concentration in water as after preliminary analyses, it was almost impossible to detect the deuterated standards:

Benzoquinone D4 5.069 mg

In 5ml of water obtaining the stock solution of Internal STDS (1.0138 mg/mL, approximated to 1mg/ml).

Table 6: internal standard stock solutions concentration.

Name of IS Stock	BQD4 Concentration	pg/µl Conc. (approximated)	Solvent
E_Water	0.1122 mg/mL	100,000 pg/µl	Water
F_Water	1.0138 mg/mL	1,000,000 pg/µl	Water
E_EtAc	0.1208 mg/mL	100,000 pg/µl	Ethyl Acetate

WORKING STANDARDS

The Stock and Intermediate solutions have been left at room temperature for 2 hours to allow

the compounds to reach equilibrium due to the reaction of self rearrangement of the BQ/HQ.

All the standard solutions were prepared in triplicate.

The dilutions used for the creation of the standard solutions for the calibration curve and for

the extractions (1ml of final volume) are summarised in the following tables:

Table 7: detail of the dilutions and volumes of the various standard solutions spiked in each solvent to produce the working solutions.

Vial name	Solvent	Volume IS + intermediate name	Volume Natural STD + intermediate name
BLK_EtAc	Ethyl acetate	0	0
IS ONLY_EtAc	Ethyl acetate	10μl E_EtAc	0
100 pg/μl_EtAc	Ethyl acetate	10μl E_EtAc	10μl A_EtAc
1,000 pg/μl_EtAc	Ethyl acetate	10μl E_EtAc	10μl B_EtAc
5,000 pg/μl_EtAc	Ethyl acetate	10μl E_EtAc	5μl C_EtAc
10,000 pg/μl_EtAc	Ethyl acetate	10μl E_EtAc	10μl C_EtAc
20,000 pg/μl_EtAc	Ethyl acetate	10μl E_EtAc	20μl C_EtAc
BLK_Water	Synthetic urine	0	0
IS ONLY_Water	Synthetic urine	10μl E_Water	0
100 pg/μl_Water	Synthetic urine	10μl E_Water	10μl A_Water
1,000 pg/μl_Water	Synthetic urine	10μl E_Water	10μl B_ Water
5,000 pg/μl_Water	Synthetic urine	10μl E_Water	5μl C_ Water
10,000 pg/μl_Water	Synthetic urine	10μl E_Water	10μl C_ Water
20,000 pg/μl_Water	Synthetic urine	10μl E_Water	20μl C_ Water

Table 8: standard concentrations in ethyl acetate working solutions.

Vial Name	BQ Concentration	HQ concentration	IS (BQ d4) Concentration	Solvent
BLK_EtAc	0	0	1,000 pg/μl	Ethyl Acetate
IS ONLY_EtAc	0	0	1,000 pg/μl	Ethyl Acetate
100 pg/μl_EtAc	100 pg/μl	100 pg/μl	1,000 pg/μl	Ethyl Acetate
1,000 pg/μl_EtAc	1,000 pg/μl	1,000 pg/μl	1,000 pg/μl	Ethyl Acetate
5,000 pg/μl_EtAc	5,000 pg/μl	5,000 pg/μl	1,000 pg/μl	Ethyl Acetate
10,000 pg/μl_EtAc	10,000 pg/μl	10,000 pg/μl	1,000 pg/μl	Ethyl Acetate
20,000 pg/μl_EtAc	20,000 pg/μl	20,000 pg/μl	1,000 pg/μl	Ethyl Acetate

Table 9: standard concentrations in the synthetic urine working solutions.

Vial Name	BQ Concentration	HQ concentration	IS (BQ d4) Concentration	Solvent
BLK_Water	0	0	1,000 pg/μl	Synthetic Urine
IS ONLY_Water	0	0	1,000 pg/μl	Synthetic Urine
100 pg/μl_Water	100 pg/μl	100 pg/μl	1,000 pg/μl	Synthetic Urine
1,000 pg/μl_Water	1,000 pg/μl	1,000 pg/μl	1,000 pg/μl	Synthetic Urine
5,000 pg/μl_Water	5,000 pg/μl	5,000 pg/μl	1,000 pg/μl	Synthetic Urine
10,000 pg/μl_Water	10,000 pg/μl	10,000 pg/μl	1,000 pg/μl	Synthetic Urine
20,000 pg/μl_Water	20,000 pg/μl	20,000 pg/μl	1,000 pg/μl	Synthetic Urine

In the second slot of extractions, instead of the IS Stock solution E, due to the impossibility of detecting the peak, the Internal Stock solution F was used (see Table 6 for information on concentrations), having a 10-fold increase in Internal STD concentration.

The working solutions in ethyl acetate described in Table 7 were injected directly in the GCMS to evaluate the response and the linearity of the standards without the extraction process.

The standard points for liquid extraction testing were processed as follows:

After preparing the working solutions described in Table 9, the samples (i.e. working solutions) were left for 1 hour at room temperature to equilibrate prior analysis.

Liquid extraction was performed in 2 times, adding each time 500 µl of ethyl acetate, shaking for 1 minute and recovering the organic layer once it was settled. Both extractions were combined in a vial (final volume expected to be 1 mL).

For the GCMS analysis 50µl of the extracted organic phase were transferred in a vial with a glass insert, containing 2.5µl of P-Terphenyl D14 (20 000pg/µl, obtaining a final concentration of 1000 pg/µl).

The direct injections of the standards gave good results in terms of accuracy of the curve for benzoquinone, which proved to be linear between 100 and 20,000 pg/µl. The calibration curve for hydroquinone proved to be problematic due to the poor peak shape of the compound. The benzoquinone peak was sharp and detectable in all the concentrations, also was the Benzoquinone D4 peak, as showed in the table below:

Table 10: GC-MS responses of directly injected BQ and HQ standards dissolved in ethyl acetate.

Sample name	Benzoquinone D4 (1,000 pg/µl)	Hydroquinone D4 (naturally occurring)	Benzoquinone	Hydroquinone	P-terphenyl D14 (1,000 pg/µl)
BLK	-	-	-	-	1,143,599
IS ONLY	209,966	20,233	3,225	-	839,674
100 pg/µl	220,503	12,627	39,374	-	1,224,327
1,000 pg/µl	170,774	8,792	284,685	32,684	780,733
5,000 pg/µl	196,405	37,011	1,734,266	692,035	1,054,394
10,000 pg/µl	226,007	96,766	3,736,630	2,038,451	1,075,130
20,000 pg/µl	227,530	135,592	8,359,599	4,971,429	1,152,567

The internal standard benzoquinone D4 response was constant in all of the samples, while the hydroquinone D4 was less reproducible. The hydroquinone D4 in this case, was obtained from the self rearrangement of the benzoquinone in hydroquinone. Furthermore, the hydroquinone (both the Internal and the Natural STD) had a poor shaped peak in our system, making difficult the identification and the quantification of the peak in low quantities, as a precise retention time was impossible to determine for the hydroquinone peak. As showed in the figure below (Figure 4) in the maximum concentration (20,000pg/µl) the hydroquinone peak at 13.00 min presented a considerable tailing, which carried over 15.00 min. In lower concentrations it was possible to find the presence of the identifying ions (110, 81 for HQ and 114, 85 for HQD4) in that timeframe but it was impossible to locate a univocal peak that would identify the compound. It was evident a problem of tailing in high concentrations (20,000pg/µl), due to the tailing, it was impossible to identify it and integrate consistently due to the bad peak shape, especially at low concentrations. On the other hand, benzoquinone, showed a better response pattern and chromatographic outcome, as showed in Figure 5.
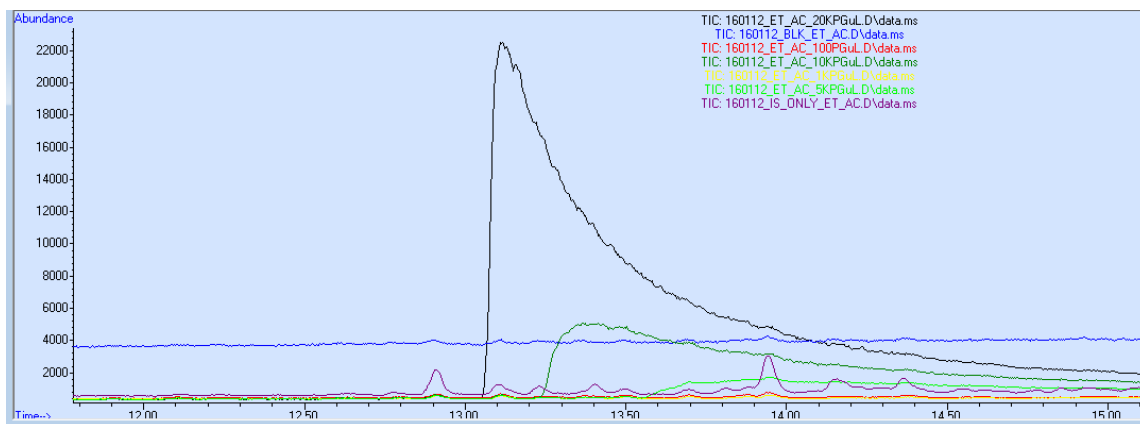
Figure 4: overlay of the Total Ion Chromatograms for every concentration, showing the peak of Hydroquinone.

-The black line represents a standard with a concentration of 20,000 pg/µl

- The blue line represents an ethyl acetate blank injection

- The red line represents a standard with a concentration of 100pg/µl

- The dark green line represents a standard with a concentration of 10,000 pg/µl

- The yellow line represents a standard with a concentration of 1,000 pg/µl

- The bright green line represents a standard with a concentration of 5,000 pg/µl

- The violet line represents an injection of only internal standard

Below is showed the peak of Benzoquinone, which was well shaped and reliable instead.
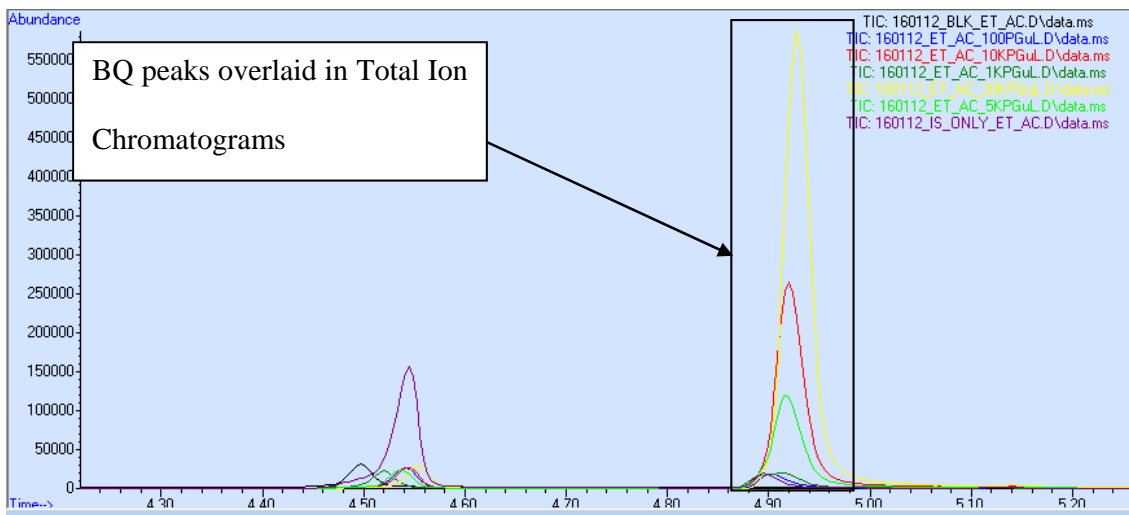
Figure 5: overlay of the Total Ion Chromatograms for every concentration of benzoquinone, showing the good trend of the standard points.

- The yellow line represents a standard with a concentration of 20,000 pg/µl

- The red line represents a standard with a concentration of 10,000 pg/µl

- The bright green line represents a standard with a concentration of 5,000 pg/µl

- The dark green line represents a standard with a concentration of 1,000 pg/µl

- The black line represents a blank injection with only ethyl acetate

- The violet line represents an injection of only internal standard

Each response was then normalized against the P-Terphenyl D14, dividing the response of the compound by the response of the P-Terphenyl, obtaining the results showed in the following table:

Table 11: Normalized responses of the standard in each concentration.

Sample name	Normalized response (Compound/P-ter)			
	Benzoquinone D4	Hydroquinone D4	Benzoquinone	Hydroquinone
BLK	0.00000	0.00000	0.00000	0.00000
IS ONLY	0.25006	0.02410	0.00384	0.00000
100 pg/µl	0.18010	0.01031	0.03216	0.00000
1,000 pg/µl	0.21874	0.01126	0.36464	0.04186
5,000 pg/µl	0.18627	0.03510	1.64480	0.65633
10,000 pg/µl	0.21021	0.09000	3.47551	1.89600
20,000 pg/µl	0.19741	0.11764	7.25303	4.31335

After the normalization, it was possible to evaluate that the responses followed the trend of the standard points. The following table shows the ratio between the Natural Standard and the Internal Standard with the theoretical value that we should find, calculated by the ratio between the concentration of the Internal Standard by the concentration of the Natural Standard. The results were very reliable for benzoquinone, while the hydroquinone, due to the difficulties in the integration process, was giving problems of overestimation, when detectable.

Table 12: Normalized BQ and HQ NS/IS ratios compared with the theoretical value.

Sample name	(Natural STD/Internal STD)		
	Theoretical value Conc NS/ Conc IS	Benzoquinone Resp NS/Resp IS	Hydroquinone Resp NS/Resp IS
BLK	0	0.00	0.00
IS ONLY	0	0.02	0.00
100 pg/µl	0.1	0.18	0.00
1,000 pg/µl	1	1.67	3.72
5,000 pg/µl	5	8.83	18.70
10,000 pg/µl	10	16.53	21.07
20,000 pg/µl	20	36.74	36.66

The calibration curves for each of the directly compounds are synthesised in the following graphics:

33
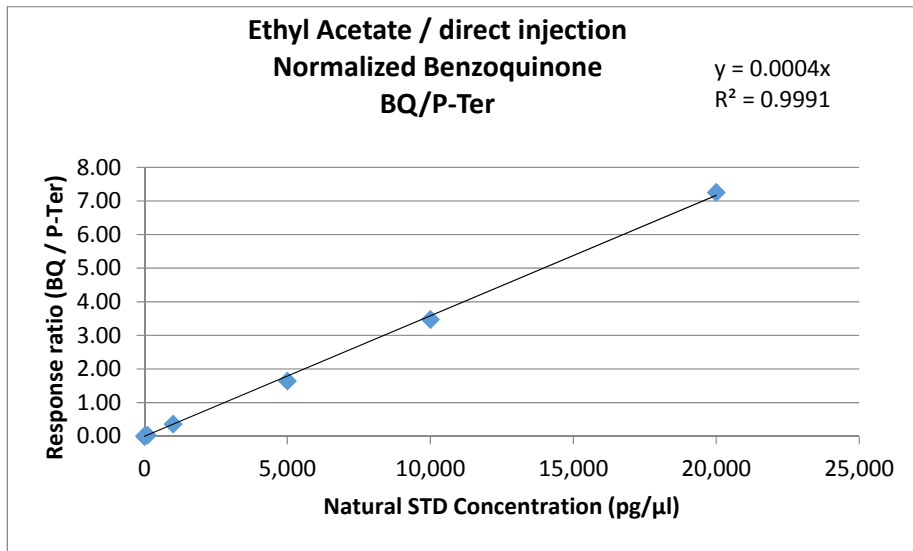
Figure 6: Response/concentration plot of benzoquinone, normalised against the recovery standard P-Terphenyl D14 in direct injection.
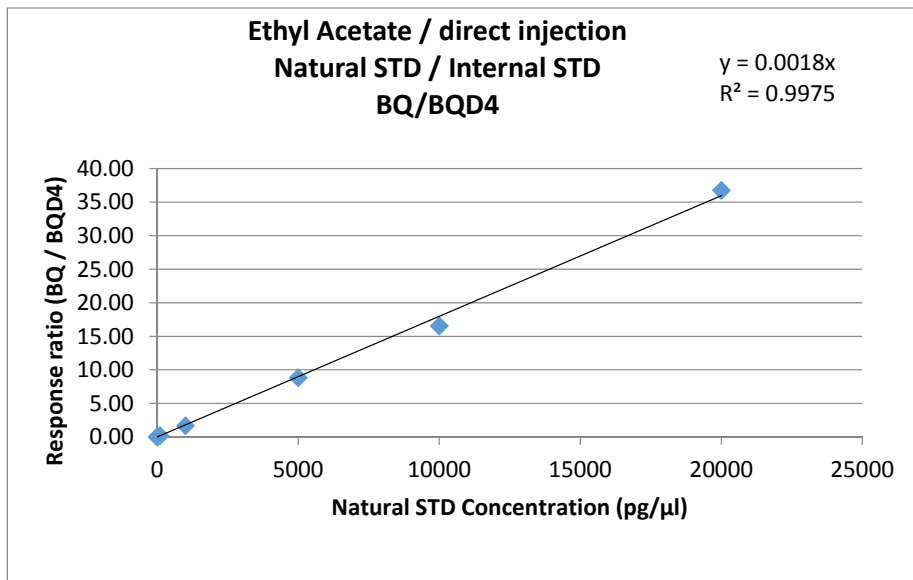
Figure 7: Benzoquinone D4/Benzoquinone response plot, which shows a good linear calibration curve obtained from direct injection of the standards.

HIDROQUINONE D4
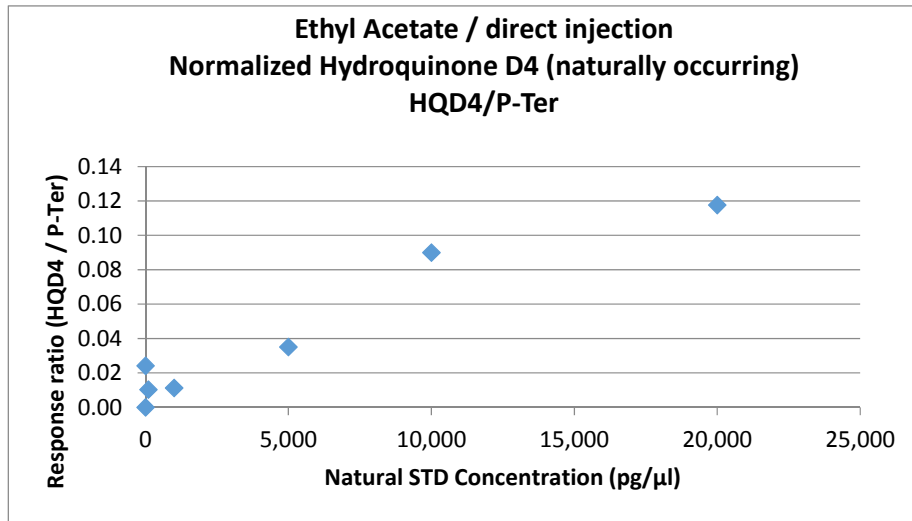
Figure 8: Graphical plot of the response/concentration of hydroquinone D4, the response is not constant although the amount of standard in the solution is the same for every sample.

HIDROQUINONE
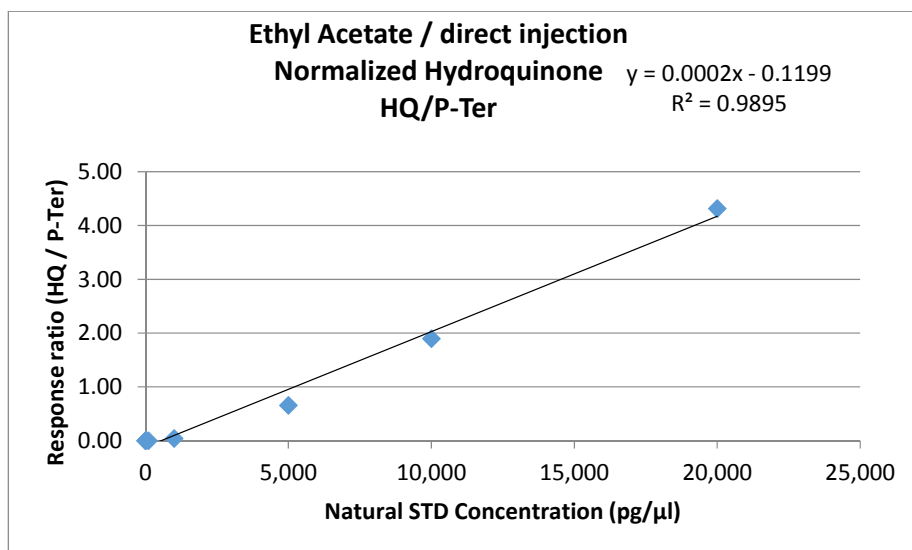
Figure 9: Graphical plot of the response/concentration of hydroquinone normalized against P-terphenyl D14.
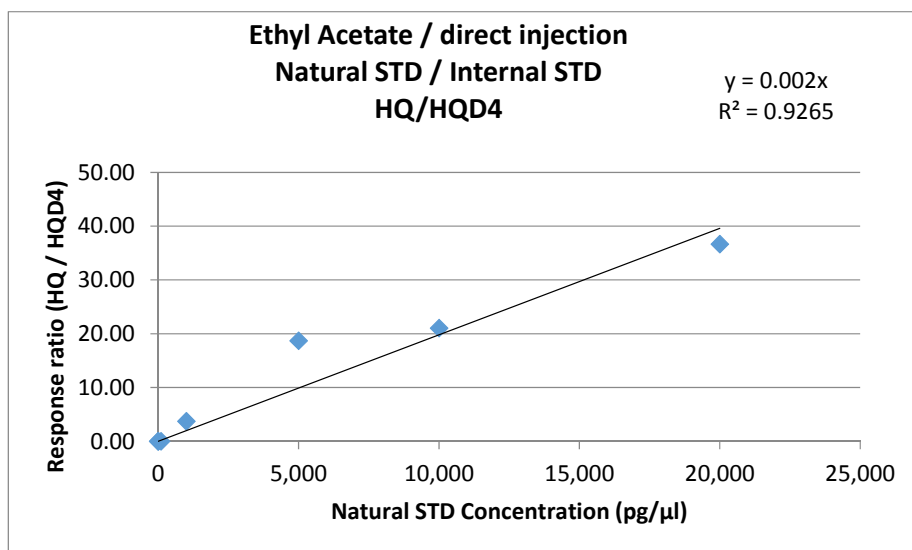
Figure 10: Graphical plot of the response ratio/concentration of hydroquinone D4/hydroquinone.

We used as internal standard the naturally occurring hydroquinone, arising from the self rearrangement of benzoquinone. The hydroquinone internal standard concentration proved to be affected by the natural standards concentration as it evident an increase of the response with the increase of the concentration of the natural standards.

36

To assess the reproducibility and the recovery ratio of the liquid extraction method, the same volumes and concentrations (Table 7) of standards of the ethyl acetate calibration curve (Table 8) were spiked in synthetic urine obtaining the concentrations listed in Table 9. Each standard point was prepared in triplicate and the average of the three values was used.

Due to problems with the internal standard arising from the solubilisation in water and the extraction process, it was difficult to identify the peak and integrate it, as showed in the figure below:
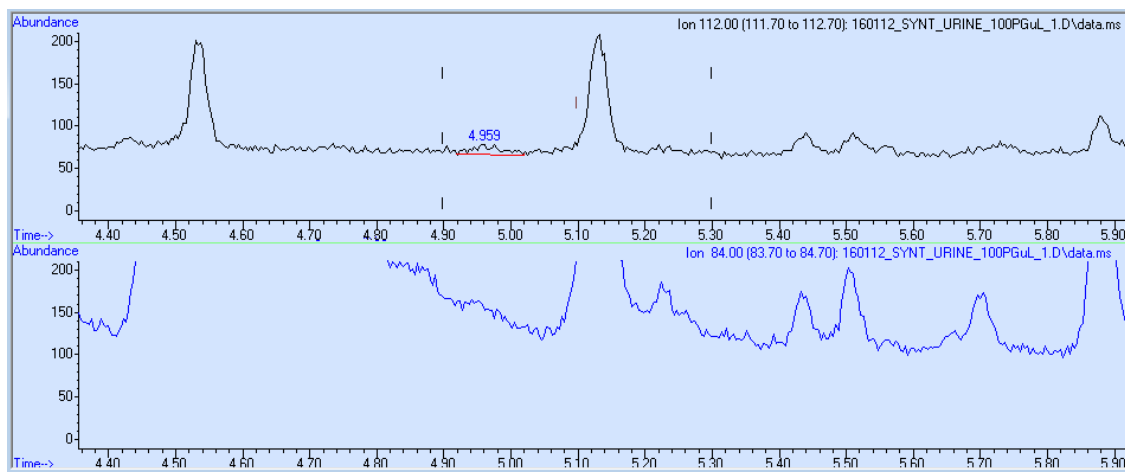
Figure 11: Benzoquinone D4 peak after liquid extraction (BQD4 concentration: 1,000pg/µl; Natural STD concentration: 100pg/µl). the peak can be hardly identified from the main ion (top) and the secondary ion (bottom), in some cases it is impossible to identify it (retention time of BQD4 is between 4.9 and 5 min, the peak at 5.15 was identified to be a contaminant peak).

With the increase of the Natural Standards (10 000 and 20 000 pg/µl) the Benzoquinone D4 response increases, showing that there is a concentration dependent cross contamination from the natural standards.
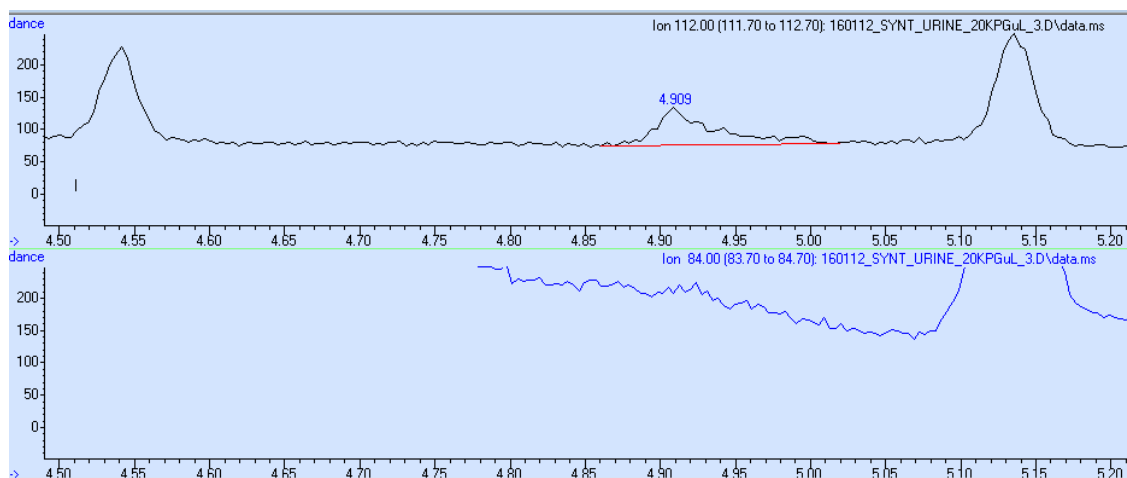
Figure 12: Benzoquinone D4 peak after liquid extraction with 20000 pg/µl of natural standards, showing a higher response than the same standard with lower concentration (Figure 11).

The average responses are synthetized in the table below. Two samples of real urine were also analysed, they were spiked only with the internal standards:

Table 13: Responses of standards and analytes after liquid extraction from synthetic and real urine.

Sample name	Compound name (responses)				
	BQ D4	HQ D4	BQ	HQ	P-terphenyl D14
BLK	-	-	-	-	1,400,421
IS ONLY	149	13,995	-	-	1,737,227
100 pg/µl AVG	271	11,763	37	26,737	1,795,643
1,000 pg/µl AVG	84	13,906	-	207,163	1,935,076
5,000 pg/µl AVG	288	20,112	21,918	1,670,264	2,330,709
10,000 pg/µl AVG	1,011	31,870	183,454	4,522,252	2,662,767
20,000 pg/µl AVG	1,805	42,512	517,040	9,604,019	2,149,031
URINE A01	236	113,431	570	124,386	2,613,857
URINE B10	204	37,250	402	3,162	2,276,109

The responses, were then normalized against the recovery standard P-Terphenyl D14, obtaining the results showed in the following table:

Table 14: Responses normalized against the recovery standard.

Sample name	Normalized response (Compound/P-ter)			
	Benzoquinone D4	Hydroquinone D4	Benzoquinone	Hydroquinone
BLK	0.00000	0.00000	0.00000	0.00000
IS ONLY	0.00009	0.00806	0.00000	0.00000
100 pg/µl AVG	0.00015	0.00655	0.00002	0.01489
1,000 pg/µl AVG	0.00004	0.00719	0.00000	0.10706
5,000 pg/µl AVG	0.00012	0.00863	0.00940	0.71663
10,000 pg/µl AVG	0.00038	0.01197	0.06890	1.69833
20,000 pg/µl AVG	0.00084	0.01978	0.24059	4.46900
URINE A01	0.00009	0.04340	0.00022	0.04759
URINE B10	0.00009	0.01637	0.00018	0.00139

The ratio between the natural standard and the internal standard was then calculated, and compared with the theoretical value of the ratio:

Table 15: Natural standard/Internal standard ratio, compared with the theoretical value.

Sample name	(Natural STD/Internal STD)		
	Theoretical value	Benzoquinone	Hydroquinone
BLK	0	N/A	N/A
IS ONLY	0	0.00	0.00
100pg/µl AVG	0.1	0.14	2.27
1,000pg/µl AVG	1	0.00	14.90
5,000pg/µl AVG	5	76.10	83.05
10,000pg/µl AVG	10	181.52	141.90
20,000pg/µl AVG	20	286.50	225.91
URINE A01	N/A	2.42	1.10
URINE B10	N/A	1.97	0.08

In previous experiments the urine sample B10 was found to have a higher concentration of hydroquinone and benzoquinone than the A01 sample, in contrast with what was obtained in this experiment. A graphical representation of the obtained data showed a definite increase of the internal standards with the increase of the concentration of the natural standards. In the following graphs are showed the standard curves after liquid extraction:
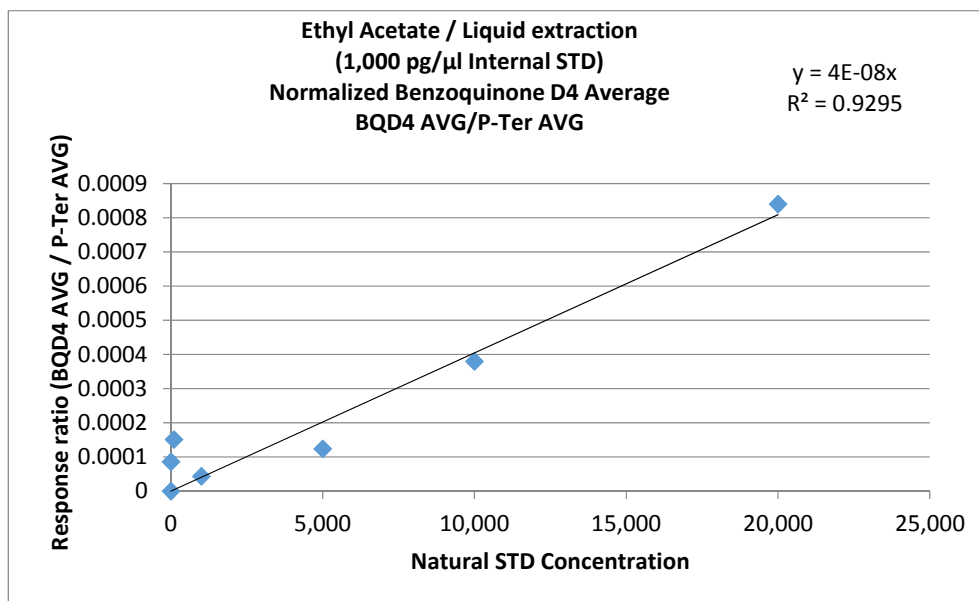
Figure 13: Plot of the response of internal standard benzoquinone D4 normalized against P-terphenyl D14, the trend line has been added to underline the increasing trend despite the samples containing always the same amount of standard (the trend line is forced through zero).
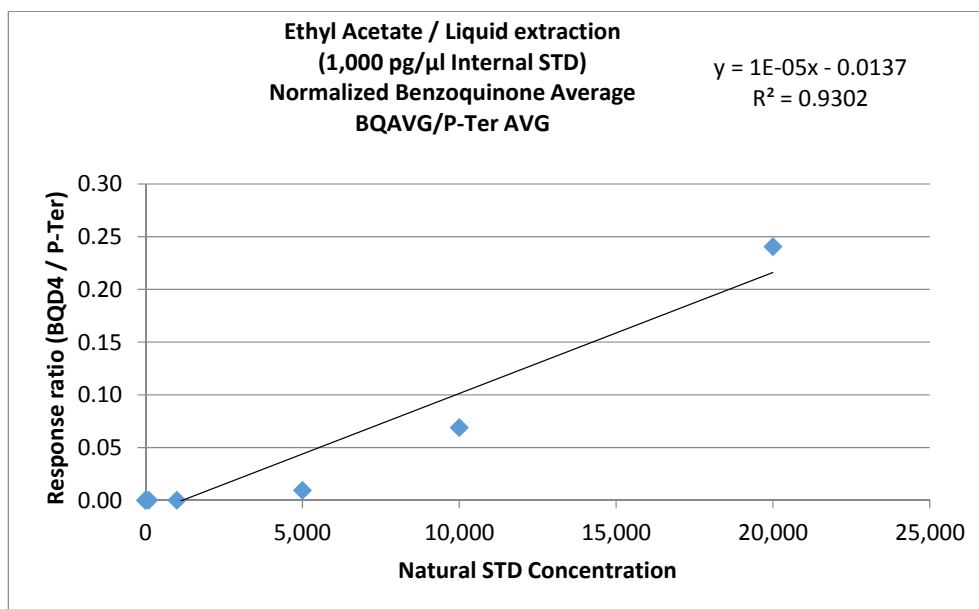
Figure 14: Plot of the response of natural standard benzoquinone normalized against the recovery standard P-terphenyl D14, the standard is almost not detectable until the 5000 pg/µl.
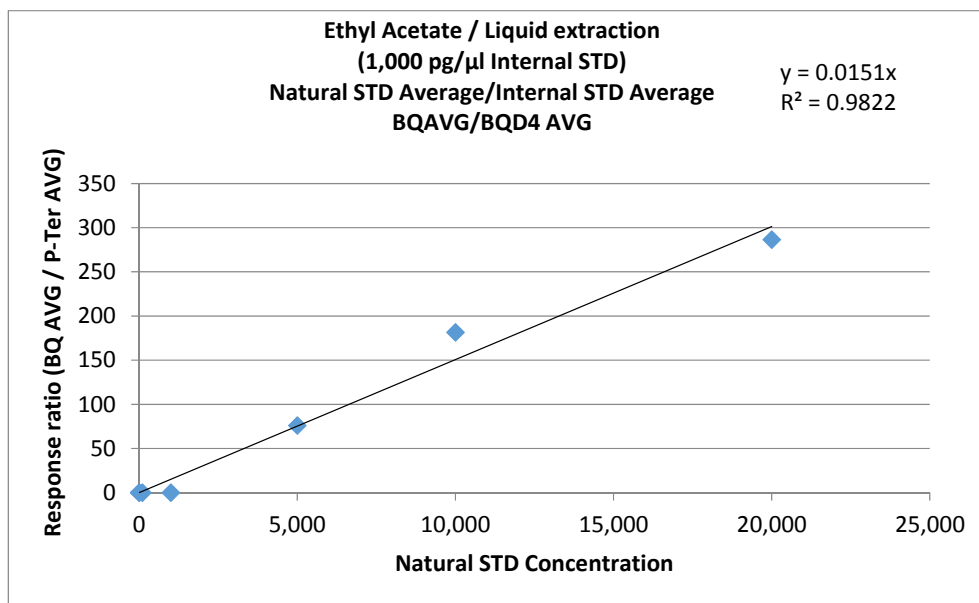
Figure 15: Graphical plot of benzoquinone natural standard/internal standard ratio. Despite a good fitting trend line (forced through zero), the behaviour of the internal standard prevented a reliable calibration.
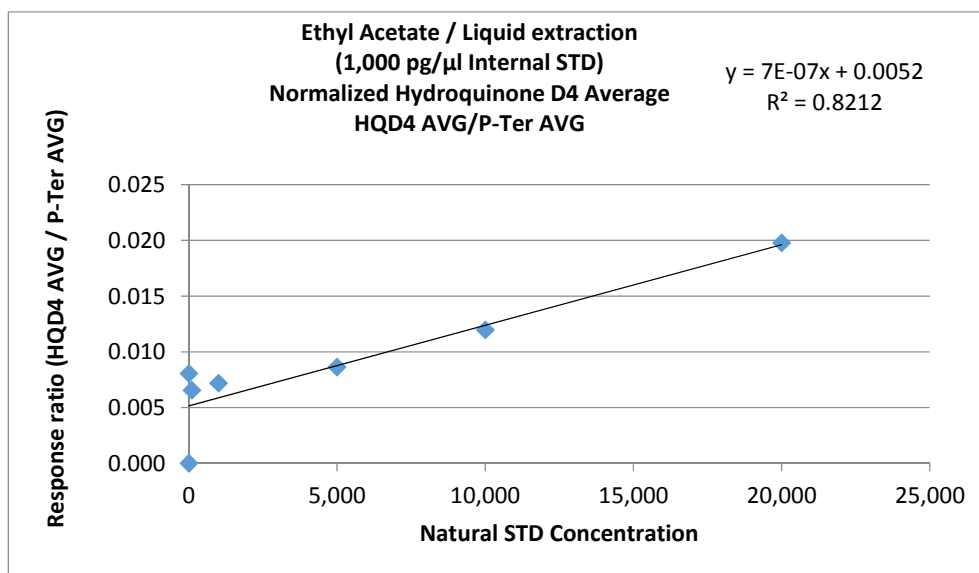
Figure 16: Graphical plot of internal standard hydroquinone D4 normalized against the recovery standard P-terphenyl D14, the trend line was added to underline the increasing trend with the increase of natural standard concentration.
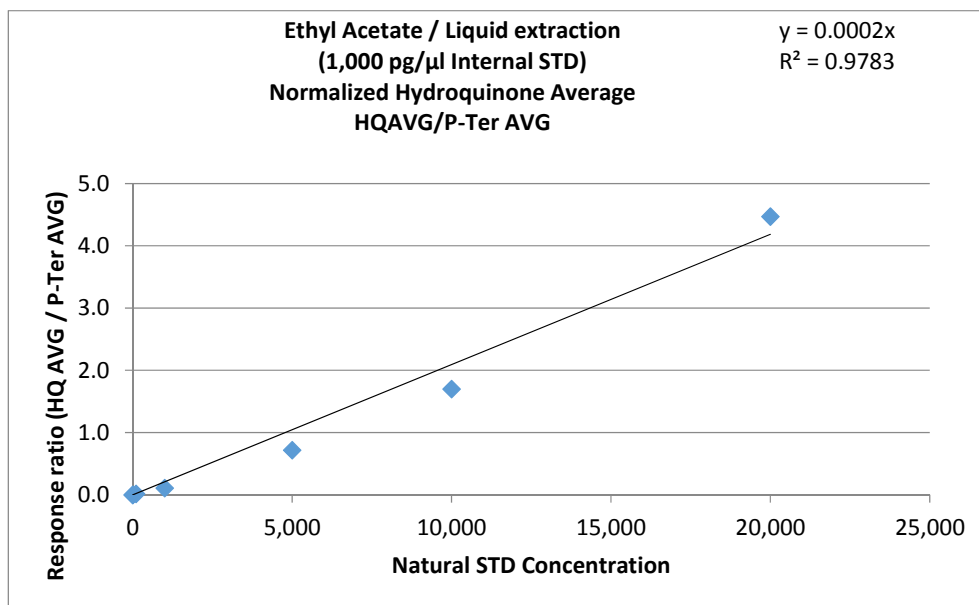
Figure 17: Graphical plot of natural standard hydroquinone normalized against recovery standard P-terphenyl D14, the trend line is forced through zero.
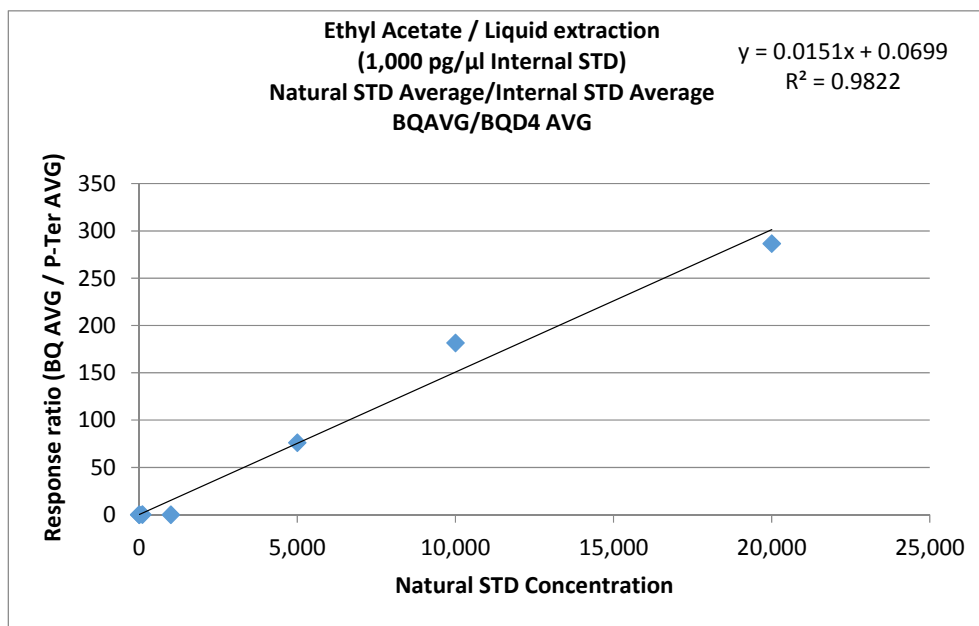
Figure 18: Calibration curve of hydroquinone, obtained with the ratio natural standard/internal standard. Despite the good R^2 value, the compound is almost not detectable the 5000 pg/µl.

Despite obtaining acceptable R² values, especially for hydroquinone, the problems arising from the standards being in a water solution prevented a reliable calibration curve. Furthermore, the standards are almost undetectable under 5000 pg/µl, while the urine samples of the study were taken from general population, low exposures, hence low levels of metabolites, were expected to be found in urine.

Due to the weak signal of the internal standard after the extraction, a new batch of samples was prepared, with an internal standard concentration of 1 000 000 pg/µl (1ug/ul) (F_Water, Table 6) and the same dilution procedure was performed, resulting in having an internal standard which was 10 times more concentrated. Together with the standard samples, the same 2 real urine samples were added to have a second measurement of the metabolites in the urine.

The results are synthesized in the tables below:

Table 16: Responses of the compounds after liquid extraction in ethyl acetate.

Sample name	Compound name (responses)				
	Benzoquinone D4	Hydroquinone D4	Benzoquinone	Hydroquinone	P-terphenyl D14
BLK	-	-	-	-	1,012,791
IS ONLY	-	22,722	-	131,072	1,128,581
100 pg/µl AVG	-	25,141	-	16,624	724,703
1,000 pg/µl AVG	111	27,339	-	202,612	677,441
5,000 pg/µl AVG	131	46,425	2,626	1,285,983	873,480
10,000 pg/µl AVG	171	52,990	18,712	3,591,054	893,129
20,000 pg/µl AVG	585	56,352	223,729	9,725,341	917,353
URINE A01	163	1,977,911	1,567	65,387	1,076,663
URINE B10	274	704,405	213	44,093	1,104,647

Table 17: Responses normalized against p-terphenyl D14.

Sample name	Normalized response (Compound/P-ter)			
	Benzoquinone D4	Hydroquinone D4	Benzoquinone	Hydroquinone
BLK	0.00000	0.00000	0.00000	0.00000
IS ONLY	0.00000	0.02013	0.00000	0.11614
100pg/µl AVG	0.00000	0.03469	0.00000	0.02294
1,000pg/µl AVG	0.00016	0.04036	0.00000	0.29908
5,000pg/µl AVG	0.00015	0.05315	0.00301	1.47225
10,000pg/µl AVG	0.00019	0.05933	0.02095	4.02076
20,000pg/µl AVG	0.00064	0.06143	0.24389	10.60152
URINE A01	0.00015	1.83708	0.00146	0.06073
URINE B10	0.00025	0.63767	0.00019	0.03992

Table 18: Response ratio natural standard/internal standard, compared with the theoretical value.

Sample name	(Natural STD/Internal STD)		
	Theoretical value	Benzoquinone	Hydroquinone
BLK	0	n.d.	n.d.
IS ONLY	0	n.d.	5.77
100pg/µl AVG	0.01	n.d.	0.66
1,000pg/µl AVG	0.1	0.00	7.41
5,000pg/µl AVG	0.5	19.99	27.70
10,000pg/µl AVG	1	109.43	67.77
20,000pg/µl AVG	2	382.44	172.58
URINE A01	N/A	9.61	0.03
URINE B10	N/A	0.78	0.06

Although the concentration of the Benzoquinone D4 even if the concentration was 10 times higher, no significant change was observed between the previous batch and the latest (Table 13 and Table 16), indicating that the measured concentration of the internal standard does not depend from the concentration in the sample. A similar behaviour was observed also for the hydroquinone D4, which showed an increased response, but did not reflect the 10 fold increase of the standard concentration. The responses of the natural standards changed even if the concentration of the standards was not changed. Furthermore, the real urine samples

showed a major difference of the internal standard response within the samples, together with natural standard/internal standard ratios that were not correlated between the two experiments (Table 19). A hypothesis that could be suggested is that some of the real urine components generate a matrix effect that is not linear or predictable in different samples. Moreover, the internal standard in the second batch of experiments was 10 times more concentrated, therefore, a drop of the natural standard/internal standard ratio would be expected, while instead the ratio for benzoquinone was found to be increased in the sample A01.

Table 19: Comparison of the two batches of real urine, the same two samples were analysed twice, obtaining different results. The second batch contained an internal standard concentration 10 times higher than the first.

	(Natural STD/Internal STD)	
Sample name	Benzoquinone	Hydroquinone
URINE A01 1st batch	2.42	1.10
URINE B10 1st batch	1.97	0.08
URINE A01 2nd batch	9.61	0.03
URINE B10 2nd batch	0.78	0.06

Recoveries from the liquid extraction were calculated, dividing the response of every compound after extraction by the response of the corresponding concentration of the same compound in direct injection. The recoveries for the benzoquinone D4 were found to be not significant in most cases, while recoveries of hydroquinone D4 were found to be generally high and in some cases, over 100%. These findings suggested that there was a major contribution to hydroquinone concentration arising from the self rearrangement of benzoquinone, triggered by the aqueous solution, despite the acidification step and the equilibration time allowed.

A variation of the recoveries was calculated using the responses from each compound, normalized against the recovery standard p-terphenyl D14. Recovery ratios for hydroquinone D4 were found to be generally under 65% for the standards containing 1,000 pg/µl of internal standard. In the samples with internal standard solution with a concentration of 10,000 pg/µl, all of the hydroquinone D4 recoveries were significantly higher, with most of them over 100%.

Since the responses of the second batch of analyses were compared with the responses of the directly injected standards, it is important to remember that in this case, the internal standard concentration was 10 times higher than in the directly injected standards, and it would be expectable to see high recoveries in the benzoquinone D4. No relevant differences were observed between the two sets of extractions for benzoquinone D4, while a major difference for hydroquinone D4 was observed, suggesting that the majority of the Benzoquinone in the urine rearranges in Hydroquinone. This self rearrangement is not happening with the ethyl acetate in the direct injections, which have proven to keep the compounds in the original form, lowering the rate of self rearrangement from a form to another.

Table 20: Calculated recoveries for the first batch of standards (internal standard concentration 1000 pg/µl) calculated on the response of directly injected standards/extracted standards ratio and on normalized directly injected standards/normalized extracted standards ratio.

Recoveries %									
Standard point (pg/µl)	Extracted STD response / Directly Injected STD response					Normalized response (Compound/P-ter)			
	BQD4	HQD4	BQ	HQ		BQD4	HQD4	BQ	HQ
BLK	n.d.	n.d.	n.d.	n.d.		n.d.	n.d.	n.d.	n.d.
IS ONLY	0%	69%	0%	n.d.		0%	33%	0%	n.d.
100	0%	93%	0%	n.d.		0%	64%	0%	n.d.
1,000	0%	158%	0%	634%		0%	64%	0%	256%
5,000	0%	54%	1%	241%		0%	25%	1%	109%
10,000	0%	33%	5%	222%		0%	13%	2%	90%
20,000	1%	31%	6%	193%		0%	17%	3%	104%

Table 21: Calculated recoveries for the second batch of standards (internal standard concentration 10000 pg/μl) calculated on the response of directly injected standards/extracted standards ratio and on normalized directly injected standards/normalized extracted standards ratio.

Standard point (pg/μl)	Recoveries % Extracted STD response / Directly Injected STD response					Recoveries % Normalized response (Compound/P-ter)			
	BQD4	HQD4	BQ	HQ		BQD4	HQD4	BQ	HQ
BLK	n.d.	n.d.	n.d.	n.d.		n.d.	n.d.	n.d.	n.d.
IS ONLY	0%	112%	0%	n.d.		0%	84%	0%	n.d.
100	0%	199%	0%	n.d.		0%	336%	0%	n.d.
1,000	0%	311%	0%	620%		0%	358%	0%	714%
5,000	0%	125%	0%	186%		0%	151%	0%	224%
10,000	0%	55%	1%	176%		0%	66%	1%	212%
20,000	0%	42%	3%	196%		0%	52%	3%	246%

The problems arising from the peak shape of hydroquinone represented a major issue as it was not possible to identify the peak at low concentrations and integrate it in a reliable way.

The rearrangement of benzoquinone in hydroquinone affected the results, especially in the real urine samples using benzoquinone D4 as an internal standard. The outcome of the recoveries underlined a problem of reliability of the methodology, as the recoveries of the natural standards were not constant over the different concentrations, and more importantly the recoveries of the internal standard (hydroquinone D4) seemed to be affected by the concentration of the natural standard. Furthermore, the extractions performed in different batches showed a poor inter experimental reproducibility, this made the measurement of the compounds in urine impossible in a reliable assay.

To investigate about a matrix effect in urine, another extraction experiment was performed, extracting the standards spiked in distilled water and not in synthetic urine, to check if the matrix where the hydroquinone and the benzoquinone affected the extraction and the results of the analyses.

The vials were prepared for liquid extraction using the same protocol described above, but spiking the standards in distilled water instead of synthetic urine.

A single high concentration standard vial was added, containing an unknown concentration of benzoquinone (close to the benzoquinone saturation point in aqueous solution).

All the vials were processed with liquid extraction using ethyl acetate with the same methodology described above in this section.

A 500 µl aliquot was then transferred to a GC vial and analysed by GCMS to check the reproducibility and the hydroquinone/benzoquinone ratios.

Since this was mostly a qualitative experiment, the recovery standard P-Terphenyl D-14 was not added to the samples.

Table 22: Responses of internal and natural standards extracted from water by liquid extraction.

Sample name and concentration (pg/µl)	Compound name (responses)			
	Benzoquinone D4	Hydroquinone D4	Benzoquinone	Hydroquinone
BLK	0	0	83821	226471
100	505908	748266	124069	244709
1,000	649195	888623	230492	527256
5,000	608435	783617	459520	674265
10,000	559937	635763	772526	878541
20,000	673335	844569	1674894	1893525
High CONC BQ STD	116384	5672	670357395	24755322

In Table 22 are reported the responses for each standard point extracted from water, it is possible to notice a difference in benzoquinone D4 and hydroquinone D4 which are almost constant at every concentration point, in opposition to the results obtained with the extractions from synthetic urine.

Since the P-terphenyl D14 was not added, the values could not be normalized. The natural standard/internal standard ratios for the standard points were then compared in the following table.

Table 23: Natural standard/internal standard ratio comparisons with the theoretical value.

Sample name and concentration (pg/µl)	Theoretical value	(Natural STD/Internal STD)	
		Benzoquinone	Hydroquinone
BLK	0	N/A	N/A
100	0,01	0,25	0,33
1,000	0,1	0,36	0,59
5,000	0,5	0,76	0,86
10,000	1	1,38	1,38
20,000	2	2,49	2,24
High CONC BQ STD	N/A	5759,88	4364,48

In this case, the ratio didn't seem to be affected by the different concentrations, and followed a good calibration curve, although there was an overestimation of the quantities, that became a considerable error at low concentrations.

Benzoquinone D4 showed a stable response throughout all of the samples when extracted from water. The response at low concentrations of benzoquinone was higher than the previous experiments.

The same pattern observed for benzoquinone was observed for hydroquinone, the internal standard was extracted consistently and the response did not increase with the increase of the concentration of the natural standard. Also in this case, the natural standards and the ratio between natural and internal standard gave an overestimation at low concentrations, mostly due to the poor peak shape and the difficulties in integration and localization of the peak.

These results proved that urine generated a matrix effect, affecting negatively the liquid extraction protocol. Furthermore, the pH of urine converted most of the benzoquinone in hydroquinone, which is the specie giving most chromatographic problems. These findings and the impossibility of obtaining a reliable chromatogram and integration for low hydroquinone

concentrations evidenced methodological problems with the liquid extraction methodology for our study, which would have medium to low levels of exposure, and therefore low concentrations of metabolites would be expected.

II.5   Quinones extraction by solid phase extraction (SPE)

Taking in account the results obtained with liquid extraction, we investigated another methodology to extract metabolites from urine, using solid phase extraction (SPE).

For the SPE extraction, Agilent Bond Elut PLEXA cartridges were used. The methodology used for the extraction was described in section II.3, briefly:

- The standard vials were prepared with the same procedure of the previous extraction experiments, each point in triplicate, with an internal standard concentration of 10000 pg/µl for Benzoquinone D4, but in this case 10000 pg/µl of hydroquinone D6 was added to the internal standard solution
- From each vial 0.3 ml of synthetic or real urine were diluted in 0.9ml of 1% formic acid
- The SPE columns were preconditioned with 0.5 ml Methanol followed by 0.5 ml of water
- The samples were loaded in the SPE columns with an approximate flow rate of 1 ml/min and washed with 1 ml Methanol 5%
- The elution was performed with 1 ml Methanol 100%

50 µl from the eluted solution were then transferred in a GC vial with a glass insert, and then spiked with 2.5 µl of P-terphenyl D14 solution.

Each standard point was prepared in triplicate and analysed, then the three values were averaged.

In order to rule out a possible interference caused by phenol, another batch of synthetic urine not containing phenol was prepared. One of the three samples of each standard point was extracted from a version of the synthetic urine containing phenol, which did not seem to affect negatively the results. The average responses from the extractions are listed in the following table:

Table 24: Responses of standards extracted using SPE, the average of each point is obtained including the synthetic urine containing phenol.

Sample name and standard concentration (pg/µl)	compound name (responses)				
	BQ D4	HQ D4	BQ	HQ	P-terphenyl D14
BLK	n.d.	26344	n.d.	13858	374509
IS ONLY	n.d.	502089	n.d.	7309	513587
100	n.d.	610941	n.d.	13992	549034
1,000	n.d.	705405	n.d.	82914	593573
5,000	n.d.	654035	n.d.	321661	558979
10,000	n.d.	769472	n.d.	726560	601605
20,000	n.d.	650121	9444	1223305	546976
URINE A01	n.d.	452276	n.d.	78887	697060
URINE B10	n.d.	398997	n.d.	28100	552757

The responses were then normalized against the recovery standard P-terphenyl D14:

Table 25: Normalized responses of natural and internal standards against the recovery standard P-terphenyl D14.

Sample name and standard concentration (pg/µl)	Normalized response (Compound/P-ter)			
	Benzoquinone D4	Hydroquinone D4	Benzoquinone	Hydroquinone
BLK	n.d.	0.07	n.d.	0.04
IS ONLY	n.d.	0.98	n.d.	0.01
100	n.d.	1.11	n.d.	0.03
1,000	n.d.	1.19	n.d.	0.14
5,000	n.d.	1.17	n.d.	0.58
10,000	n.d.	1.28	n.d.	1.21
20,000	n.d.	1.19	0.02	2.24
URINE A01	n.d.	0.65	n.d.	0.11
URINE B10	n.d.	0.72	n.d.	0.05

The data was processed with the same procedure of previous experiments for comparison, obtaining the ratio between the normalized natural standard average and the normalized internal standard average:

Table 26: Normalized natural standard/internal standard ratio, compared with the theoretical value.

Sample name and standard concentration (pg/µl)	(Natural STD/Internal STD)		
	theoretical value	Benzoquinone	Hydroquinone
BLK	0	N/A	0,53
IS ONLY	0	N/A	0,01
100	0,01	N/A	0,02
1,000	0,1	N/A	0,12
5,000	0,5	N/A	0,49
10,000	1	N/A	0,94
20,000	2	N/A	1,88
URINE A01	N/A	N/A	0,17
URINE B10	N/A	N/A	0,07

The recovery of benzoquinone is zero for the internal standard, while in the natural standards, benzoquinone was found only in the standard solution with a concentration of 20,000 pg/µl.

Although the calibration curve was found to have a good R^2 value, the chromatographic problems of hydroquinone were still present, making it difficult to integrate consistently hydroquinone at low concentrations.
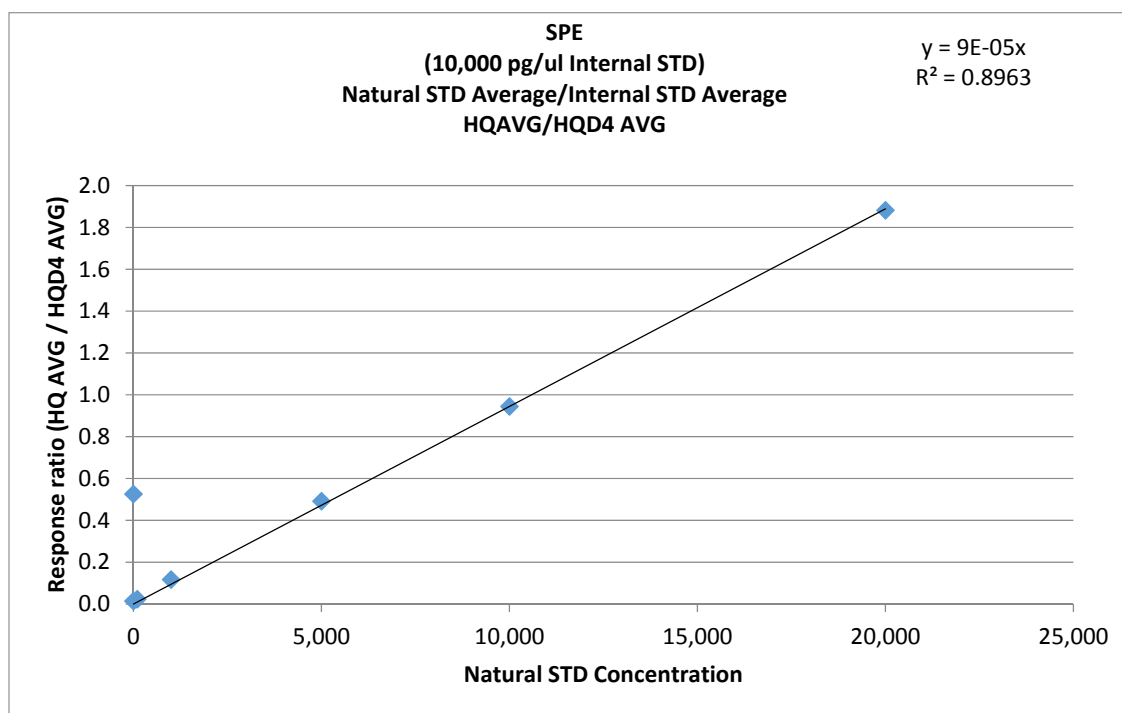
Figure 19: Plot of natural standard/internal standard response ratio for hydroquinone after SPE. R² value is affected by one of the blanks where a hydroquinone peak was detected.

In the graphic above is showed the fitting of the standard points obtained from the ratio between natural standard response and internal standard response.

The fitting of the points is improved compared to the liquid extraction, and the R^2 is higher because of a hydroquinone peak was found in one of the blanks.

Due to the results obtained with the SPE procedure, an extended assay with more real samples was performed, to validate the reliability and feasibility of the methodology.

A calibration curve was prepared in triplicate with the same procedure described above, with standard points from 100 to 20,000 pg/µl in synthetic urine. The extractions were performed with the same procedure of SPE previously described, and the analyses were performed by GC-MS. In this batch of extractions, 5 samples of urine were analysed, including the urine sample B10 to have a replicate to evaluate. The samples of this batch were obtained from a pilot study performed in collaboration with the Arizona State University (ASU), with

subjects on different commuting routes and different means of commute, with an expected medium to low range exposure to VOCs mostly due to road traffic pollution.

The outcome of the analyses underlined the chromatographic problems with the peak shape of hydroquinone, which presented an extended tailing, preventing the possibility of reproducible integration of the peak in the standard solutions. Furthermore, in the matrix complexity of the urine, generated a high background noise, which affected the correct quantification of the hydroquinone, as showed in Figure 20 for the internal standards and in Figure 21 for the natural standards.
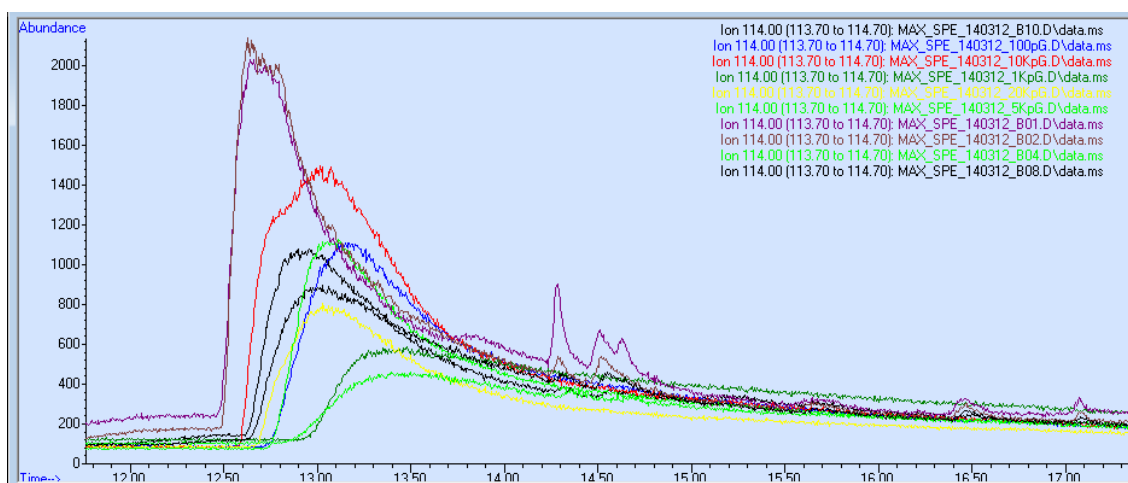
Figure 20: Overlay of the ion 114 (hydroquinone D6 internal standard of all the runs: 10.000pg/ml) the R.T. is variable and also the peak shape not reliable, the peaks of samples B01 and B02 are sharper than the peaks of the standard solutions but the tailing of the peaks prevents a reproducible integration and quantification.
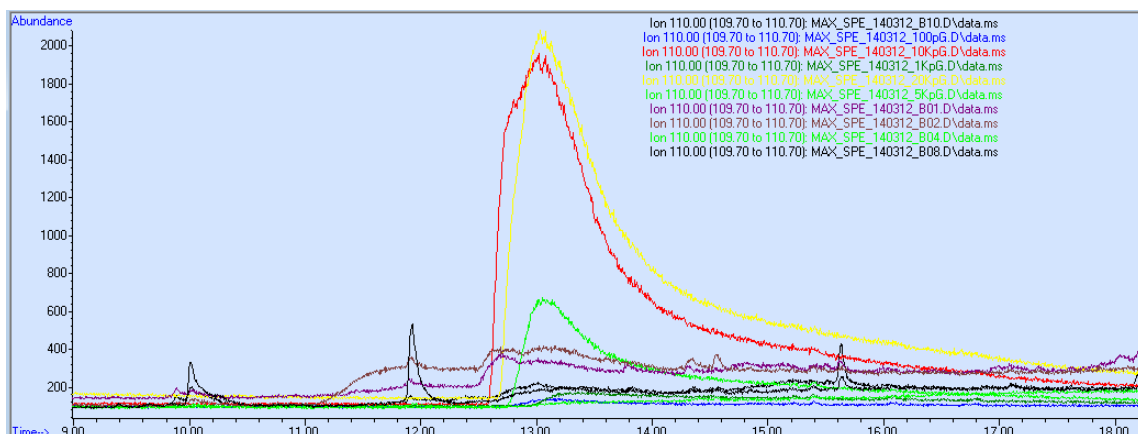
Figure 21: Overlay of ion 110 (hydroquinone) for all the standards and samples, the high concentrations (blue and yellow) are easily quantifiable, but in the low concentrations it is not possible to identify a definite peak, affecting negatively the quantification.

The obtained data was analysed with the same method, the responses were normalized against the recovery standard P-terphenyl D14 and synthesized in the following table.

Table 27: Responses of internal standards and natural standards, normalized against the recovery standard P-terphenyl D14.

sample name and concentration (pg/µl)	Normalized response (Compound/P-ter)			
	Benzoquinone D4	Hydroquinone D4	Benzoquinone	Hydroquinone
100	n.d.	0.67	n.d.	0.02
1,000	n.d.	0.55	n.d.	0.09
5,000	n.d.	0.64	n.d.	0.30
10,000	n.d.	0.60	n.d.	0.94
20,000	n.d.	0.48	n.d.	1.08
URINE B01	n.d.	0.47	n.d.	0.06
URINE B02	n.d.	0.62	n.d.	0.06
URINE B04	n.d.	0.47	n.d.	n.d
URINE B08	n.d.	0.61	n.d.	0.02
URINE B10	n.d.	0.55	n.d.	0.01

The natural standard/internal standard ratio was then calculated and compared with the theoretical value.

Table 28: Natural standard/internal standard ratio, compared with the theoretical values.

sample name and concentration (pg/µl)	(Natural STD/Internal STD)		
	Theoretical value	Benzoquinone	Hydroquinone
100	0,01	N/A	0,03
1,000	0,1	N/A	0,17
5,000	0,5	N/A	0,46
10,000	1	N/A	1,56
20,000	2	N/A	2,27
URINE B01	N/A	N/A	0,12
URINE B02	N/A	N/A	0,10
URINE B04	N/A	N/A	0,00
URINE B08	N/A	N/A	0,04
URINE B10	N/A	N/A	0,03

The sample B10 of urine was analysed previously (Table 26 for SPE, Table 15 for liquid extraction), showing a higher concentration according to the natural standard/internal standard ratio. It is also important to point out that depending on the integration of the peak, the results of the quantification can vary of a significant amount, therefore, especially at low concentrations, the quantification of hydroquinone was not considered to be reliable and feasible. This is underlined in Table 29 where the concentrations were calculated using the responses obtained.

Table 29: Concentrations calculated using the responses of the standard points and the samples, calculated using the calibration curve obtained in this batch of extractions. The concentrations are generally overestimated, especially at low concentrations.

Sample name and concentration (pg/µl)	Hydroquinone concentration (pg/µl)
100	334
1,000	1544
5,000	4956
10,000	15604
20,000	18062
URINE B01	962
URINE B02	1015
URINE B04	n.d.
URINE B08	364
URINE B10	246

Due to the poor peak shape and the tailing of hydroquinone peak it was difficult to have an exact and reliable integration, hence the quantification of the hydroquinone could not be validated, due to the high variability. Benzoquinone was almost never detected, this is possibly due to the adsorption of the compound to the column which in some way promotes the rearrangement of benzoquinone in hydroquinone. Considering the concentration ranges expected in our study, although the extraction proved to be reliable and reproducible, this methodology could not be used, due to the chromatographic issues arising from hydroquinone.

II.6   Extraction efficiency evaluation

SPE with Bond Elut Plexa columns was chosen within the different SPE cartridges due to the higher efficiency of extraction over a broad range of compounds. Extraction efficiency was evaluated by the calculation of the recovery of the compounds. Recovery was calculated on the ratio between the responses of the compounds extracted from urine over the directly injected compounds. Urine from a non exposed subject was scanned to confirm the absence of the compounds of interest. The urine was then spiked with internal standards (2000 pg/µl final concentration) and different levels of natural standards (2, 20, 200, 2000 pg/µl final concentration). The extraction was performed following the acid loading protocol described above, diluting the urine with 1% formic acid. 2µl of the eluate were then injected in the GC-MS for analysis. The control was performed by diluting the standards directly in dichloromethane and injecting 2 µl of the solution in the GC-MS. To assess the stability of the compounds in solution, two batches of standards were used, a fresh batch and a batch that was stored at -20°C for two weeks. The standard solutions were made by dissolving the quinones in methanol. A summary of the results is reported in Table 30.

Phenanthraquinone was not reported in the table because they were not detectable under 200 pg/µl in all of the analyses. Analysing the results of the table it is possible to see that most of the recoveries were acceptable (over 60%), but most of the compounds showed a variable

rate of recovery. For example anthraquinone, which showed a good recovery rate, in some case presented a recovery of only 37%, while in other cases the recovery value was over 100%. The high value can be due to a standard spiking error, due to pipetting inconsistency, the low values could indicate a possible variability of the extraction methodology, although they could be arising from instrumental differences due to the conditions of the GC-MS system. More importantly, the two batches of internal standards (one stored for two weeks at -20°C and one freshly prepared), showed a definite decay of the signal due to the storage. Using as an example anthraquinone d8, the oldest batch recoveries ranged from 9.22% to 13.96%, while the freshly prepared batch recoveries ranged from 43.53% to 61.24%, evidencing an important decay of the signal due to the storage. This behaviour was observed in all the internal standards. Overall, the recoveries were satisfactory, although for some compounds the difference of recovery between the different standard points was rather big.

Table 30: Summary of recoveries of quinones extracted from urine using SPE.

Extraction batch	Concentration	RECOVERY %											
		1,4-naphthoquinone d6	1,4-Naphthoquinone	2,6-di-tert-butyl-1,4-benzoquinone	2methyl-1,4-naphthoquinone d8	2-methyl-1,4-naphthoquinone	Acenaphthoquinone	Anthraquinone d8	Anthraquinone	2-methyl-anthraquinone	2,3-dimethyl-anthraquinone	Benz[a]anthracenequinone-7,12-dione	5,12-naphthacenequinone
direct injection from DCM	2 pg/µl	100.00%	100.00%	n.d.	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	n.d.
	20 pg/µl	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%
	200 pg/µl	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%
	2000 pg/µl	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%
spiking in urine and SPE ISTD batch stored at -20°C for 2 weeks	Blank	0.09%	5.17%	n.d.	59.22%	61.40%	22.75%	11.80%	64.08%	133.06%	133.04%	125.09%	n.d.
	2 pg/µl	0.08%	2.72%	n.d.	55.85%	103.84%	22.07%	10.51%	93.19%	185.22%	199.48%	188.75%	n.d.
	20 pg/µl	0.09%	0.36%	55.55%	65.42%	98.41%	7.64%	11.45%	96.35%	134.17%	136.70%	108.38%	107.58%
	200 pg/µl	0.15%	0.06%	36.85%	65.88%	70.74%	3.77%	13.96%	79.90%	85.40%	69.74%	50.65%	49.47%
	2000 pg/µl	0.11%	0.98%	25.36%	60.50%	64.57%	3.46%	9.22%	39.98%	38.84%	36.10%	36.61%	22.31%
spiking in urine and SPE ISTD batch prepared fresh	Blank	0.38%	5.32%	n.d.	74.93%	30.46%	28.35%	47.69%	37.63%	44.71%	40.63%	46.41%	n.d.
	2 pg/µl	0.31%	4.25%	n.d.	74.82%	88.35%	28.55%	43.53%	80.87%	101.05%	94.54%	93.18%	n.d.
	20 pg/µl	1.96%	1.09%	49.50%	86.75%	110.73%	12.19%	60.77%	118.89%	132.38%	133.20%	142.27%	106.86%
	200 pg/µl	1.91%	0.12%	67.39%	90.74%	104.96%	5.87%	61.24%	112.67%	111.24%	92.01%	75.54%	62.17%
	2000 pg/µl	0.76%	2.14%	36.30%	82.10%	80.24%	5.47%	54.01%	61.09%	61.44%	61.41%	71.90%	66.43%

II.7 Quinones GC-MS analysis

Hydroquinone and benzoquinone were analysed using a GC-MS Agilent 5973 (Agilent technologies, USA). For the analyses an ultra inert liner and column (HP5-MS, ultra inert, 30m, i.d. 0.25mm, film thickness 0.25μm, Agilent technologies) were used, to improve the peak shape and maximise the sensitivity.

The MS analysis of quinones was performed by EI (electronic ionization) and NCI (negative chemical ionization) using methane as ionization gas. The NCI gave better results in terms of sensitivity, and was used also to scan for other quinones that might occur from the metabolization of PAHs or arise from direct exposure: anthraquinone; 1,2-naphthoquinone; 1,4-naphthoquinone; methyl-1,4-naphthoquinone; 2-methyl-1,4-naphthoquinone; 1,4-acenaphthenequinone; 9,10-phenanthrenequinone; 2,6-di-tert-butyl-1,4-benzoquinone; 2,3-dimethyl-anthraquinone; 2-methyl-anthraquinone; benz[a]anthracene-7,12-dione; 5,12-naphthacenequinone. The MS analysis was performed in SIM (single ion monitoring) mode, to enhance the sensitivity for each compound. The GC separation was carried out with the injection port held at 250°C and the oven temperature was ramped with a constant gradient for the oven from 60°C to 300°C at 4°C/min. The ions scanned were: 122 (methyl-1,4-benzoquinone), 158 (1,4-naphthoquinone and 1,2-naphthoquinone), 164 (1,4-naphthoquinone d6), 172 (2-methyl-1,4-naphthoquinone), 180 (2-methyl-1,4-naphthoquinone d8), 182 (acenphthoquinone), 208 (anthraquinone and phenanthraquinone), 216 (anthraquinone d8), 220 (2,6-di-tert-butyl-1,4-benzoquinone), 222 (2-methyl-anthraquinone), 236 (2,3-dimethyl-anthraquinone), 258 (benz[a]anthracene-7,12-dione and 5,12-naphthacenequinone).

II.8 Hydroquinone analysis by LC-MS/MS

An LC-MS/MS system was made available for a short period, and an analysis of hydroquinone concentration in synthetic urine was attempted. Unfortunately, the time slot was

too short to develop a method and tune the instrument in order to obtain an optimized chromatogram. To identify the hydroquinone, the transition m/z 110/81 was selected, as the ion m/z 81 was identified as the collision induced fragment of hydroquinone (MW 110).

As shown in Figure 22, even without an accurate tuning of the instrument, the chromatographic problems arising from hydroquinone were not present, and it was possible to identify the peak at low concentrations such as 10 pg/ml.

The chromatographic separation was performed using a C-18 column at a flowrate of 1ml/min. The elution was performed with methanol using a gradient from 20% to 100% in 20min. Unfortunately, due to the limited time available, the method could not be refined further. These results underlined the better suitability of LC-MS methods for the analyses of polar metabolites such as hydroquinone, which had been difficult to analyse by GC-MS due to its physiochemical characteristics.
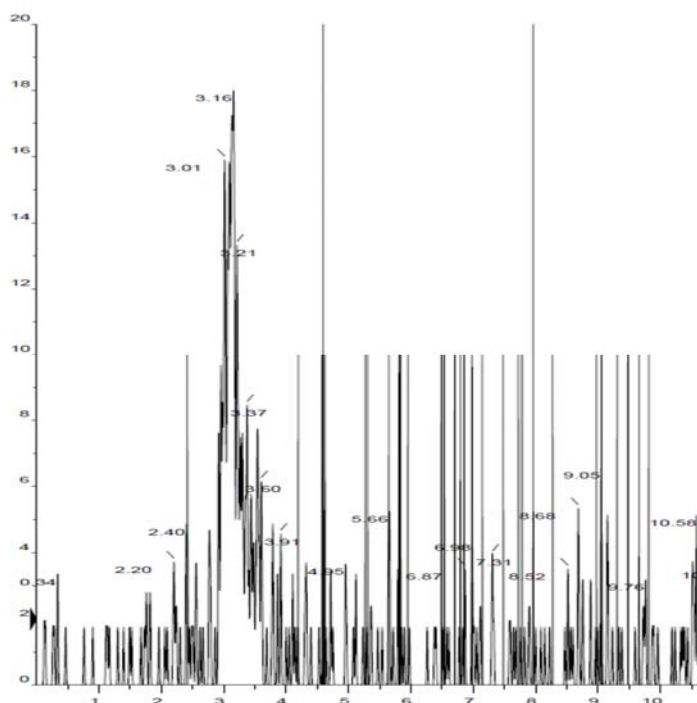
Figure 22: Hydroquinone chromatogram (10 pg/ml) from LC-MS/MS. m/z 110/81 transition, the hydroquinone peak is identified between 3 and 3.5 min, on the Y axis are indicated the counts per second.

Due to the limited time and the impossibility of developing a refined method for hydroquinone analyses, the methodology was not considered further, and other biomarker of exposure were selected.

II.9   Limit of detection

The instrumental LOD (limit of detection) was calculated by dividing the standard concentration by 1/3 of the signal to noise ratio at the same concentration (Equation 1). The LOQ (limit of quantification) was calculated by dividing the standard concentration by 1/5 of the signal to noise ratio at the same concentration (Equation 2). The results are summarised in Table 31.

Equation 1

$$LOD = \frac{Std\ Concentration}{\frac{1}{3}\ Signal/noise\ ratio}$$

Equation 2

$$LOQ = \frac{Std\ Concentration}{\frac{1}{5}\ Signal/noise\ ratio}$$

Table 31: LOD and LOQ of each of the quinones analysed in NCI.

	Concentration pg/µl	Signal/noise	LOD (pg/µl)	LOQ (pg/µl)
9,10-phenanthrenequinone	1058	3810	0.83	1.39
acenaphthenequinone	20	974	0.06	0.10
2-methyl-1,4-naphthoquinone	1002	128978	0.02	0.04
1,2-naphthoquinone	1009	n.d.	N/A	N/A
1,4-naphthoquinone	955	84885	0.03	0.06
anthraquinone	1030	56069	0.05	0.09
2,6-di-tert-butyl-1,4-benzoquinone	1076	634	5	8
2,3-dimethyl-anthraquinone	1036	152324	0.02	0.03
2-methyl-anthraquinone	990	51057	0.06	0.1
benz[a]anthracene-7,12-dione	1048	341562	0.01	0.01
5,12-naphthacenequinone	1005	602	5	8
methyl-1,4-benzoquinone	955	443	6	11

II.10 Outcomes of method development for quinones in urine

Extraction efficiency of the quinones from urine was evaluated through analysis by GC-MS. High concentrations of quinones could be easily detected, even though the chromatographic separation was not excellent. Benzoquinone was proven unstable in solvents containing available hydrogens, reducing in hydroquinone (Roginsky et al., 1999), therefore using the SPE method we could not detect benzoquinone. In addition, the observed matrix effect generated by urine prevented the use of liquid extraction (liquid extraction from synthetic urine: Table 15; liquid extraction from water: Table 23). Hydroquinone has proven to be a difficult biomarker to analyse using GC-MS. Due to its chemically active nature it tends to interact with the column, preventing the formation of a sharp peak making impossible the quantification due to the formation of a tailing peak in chromatograms affecting the quantification, especially at low concentrations. Furthermore, hydroquinone appeared to be thermally unstable, preventing detection of low concentrations in EI-GC-MS chromatography. CI-GC-MS provided better results in terms of sensitivity, however the chromatographic issues prevented a reliable quantification. Derivatization was considered, but at low concentrations in

a complex matrix such as urine, adding a derivatization step would have increased quantification variability, invalidating the assay. Without the availability of an LC-MS system, the analysis of hydroquinone in urine as a biomarker of exposure to benzene was abandoned.

The other species were analysed using CI-GC-MS, building a reliable and reproducible quantification method, but despite the low LODs (Table 31), urine samples from the higher exposure group did not provide quantifiable concentrations of any of the species.

II.11 8-oxo-dG analysis by GC-MS

A GC-MS methodology to analyse 8-oxo-dG was tested, following the protocol described by Lin et al. (2004). The methodology consisted of extraction of the 8-oxo-dG from the urine by SPE, derivatization of the extracted compounds by sylanization and GC-MS analysis. In the paper describing the methodology the urine volume is adjusted according to the creatinine concentration (3200µl for creatinine concentrations under 5 mM, 1600 µl for creatinine concentrations up to 10 mM, and 800 µl for creatinine concentrations above 10 mM). For the development of the methodology a constant volume of 1000 µl was used. Tests were performed using both synthetic and real urine, that were spiked with the standards (8-oxo-dG and [18 O]8-oxo-dG for internal standard).

The extraction was performed using Oasis HLB cartridge 3 cc/60mg 30 µm (Waters, UK). The procedure used was as follows:

1 volume of formic acid (10%) (Sigma Aldrich, UK) was added to 10 volumes of urine from a control subject. Acidified samples were incubated for 1h at 4°C, they were then centrifuged at 10,000G for 10min at 4°C.

Supernatant was collected and the extraction with Oasis HLB vac cartridges was performed with the following steps:

-Vac cartridges were preconditioned with 5ml methanol and 8 ml of 20 mM formic acid.

-Sample was diluted with 20 mM formic acid to a final volume of 5.8 ml and the sample was loaded on the cartridge.

-The diluted urine was passed through the column at a flowrate of 2ml/min.

-The column was washed with 2 ml of 20 mM formic acid were loaded to wash the sample

-Elution was performed with 1.5 ml of 17.5% methanol in 20 mM formic acid

-After each step the cartridges were completely dried using vacuum flow

8-oxo-dG is reported to be stable in 20mM formic acid for at least 5h at 4°C.

The collected fraction was then transferred in an autosampler vial and frozen at -80, and then the sample was freeze-dried.

Freeze-dried sample vials were purged with nitrogen and immediately sealed with caps with PTFE septa to prevent oxygen contamination. Acetonitrile/ethanethiol (20 µl: 3:1, v/v) was added to the vial to solubilize the urinary residue.

80µl of bis(trimethylsilyl)trifluoroacetamide (containing 1% trimethylchlorosilane) (Sigma Aldrich, UK) were added to the vial for derivatization at room temperature for at least 6h, which are reported to be enough for the derivatization of all the compounds. The samples were then analysed for 8-oxo-dG

GCMS analysis:

The analyses were performed directly after the derivatization step with no further sample preparation, injecting also the derivatization matrix.

Injection port was kept at 250°C, GC-MS interface was kept at 290°C.

The separation was made with the following column temperature gradient:

-2min at 80°C

-Increase up to 300°C at the rate of 15°C/min

-10 min at 300°C

Ion source maintained at 230°C

The selective ions were 383 and 643 for 8-oxo-dG, and 385 and 645 for [18 O]8-oxo-dG.

The procedure proved to be complicated and prone to errors and problems: injection of the derivatization matrix in the GC-MS system resulted in formation of ghost peaks due to the unreacted sylanes that caused chromatographic problems. The freeze-drying procedure couldn't be performed properly due to the presence of methanol which caused the sample to thaw prematurely, also the freeze-drying facilities were not located in the same laboratory and the transfer of the samples between the facilities affected negatively the procedure. After several replications, it was not possible to obtain a reliable extraction procedure. Alterations to the main procedure were tested: elution from the SPE column with 100% methanol followed by drying under nitrogen flow, evaporation of the organic phase under nitrogen flow prior the freeze-drying step. None of the procedures gave satisfactory results.

The derivatization step was checked by diluting the 8-oxo-dG in water in high concentrations (1 high concentration standard and 2 consecutive dilutions, respectively 1/3 and 1/4 of the original high concentration standard) and freeze-drying without the extraction step, then the derivatization protocol was applied. In this case we were able to identify the 8-oxo-dG peak, due to the extremely high concentration, as showed in Figure 23, although the response is obviously unrealistic. The main problem we encountered by using this methodology, was to keep the instrument clean from contaminations. As showed in Figure 24, after a small number of injections, parasite peaks started to build up in the chromatograms, due to the injection of the derivatization matrix. This contamination was highly interfering with the measurements, because one of the main ions of the contaminants was ion 383, which we used to quantify 8-oxo-dG. The contamination could be quenched by trimming the GC column, extensively cleaning the injection syringe, and changing the injector liner, but these procedures were both time consuming and they affected the results by changing the retention times of the ions. A sample from a volunteering smoker was analysed, expecting higher levels of 8-oxo-

dG, to test the methodology on a real sample. Even after a cleaning of the instrument, the parasite peaks were still present, and were interfering with the measurements (Figure 25). In this case it was possible to identify the 8-oxo-dG peak, but we were not able to quantify the 8-oxo-dG due to the impossibility of calculating a reliable and reproducible calibration curve.
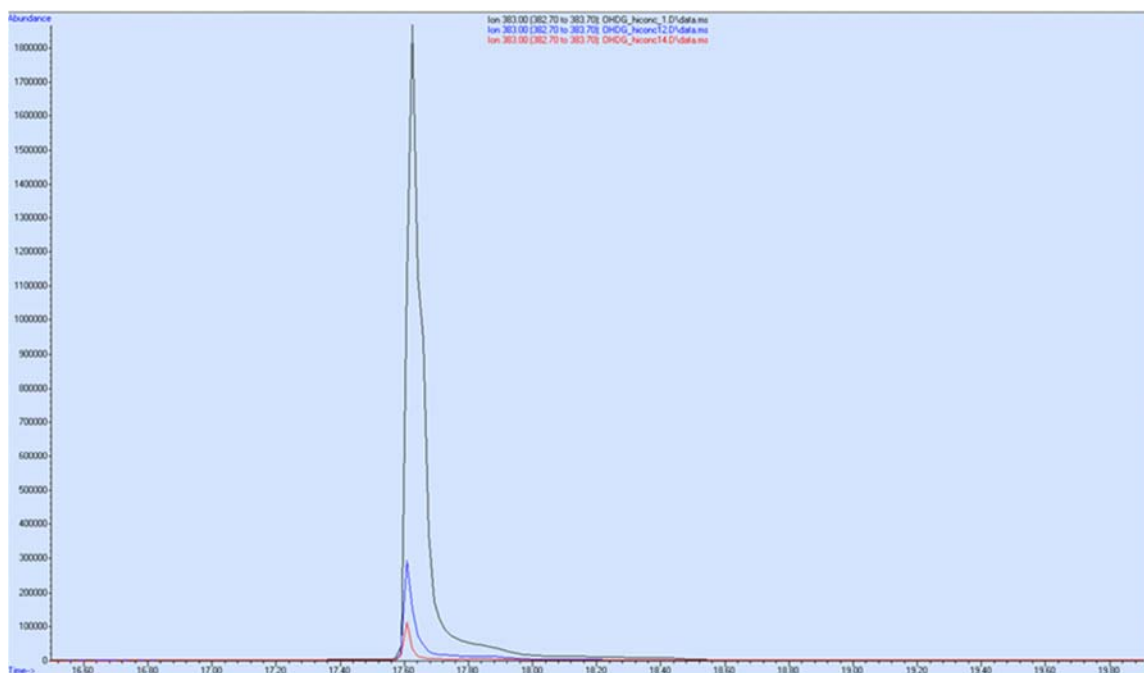
Figure 23: Overlay of three ion chromatograms (m/z 383) high concentration 8-oxo-dG standards, the concentration was not measured, as the chromatographic run was used to determine the retention time of the substance. The used concentrations were the product of two sequential dilutions 1:2.
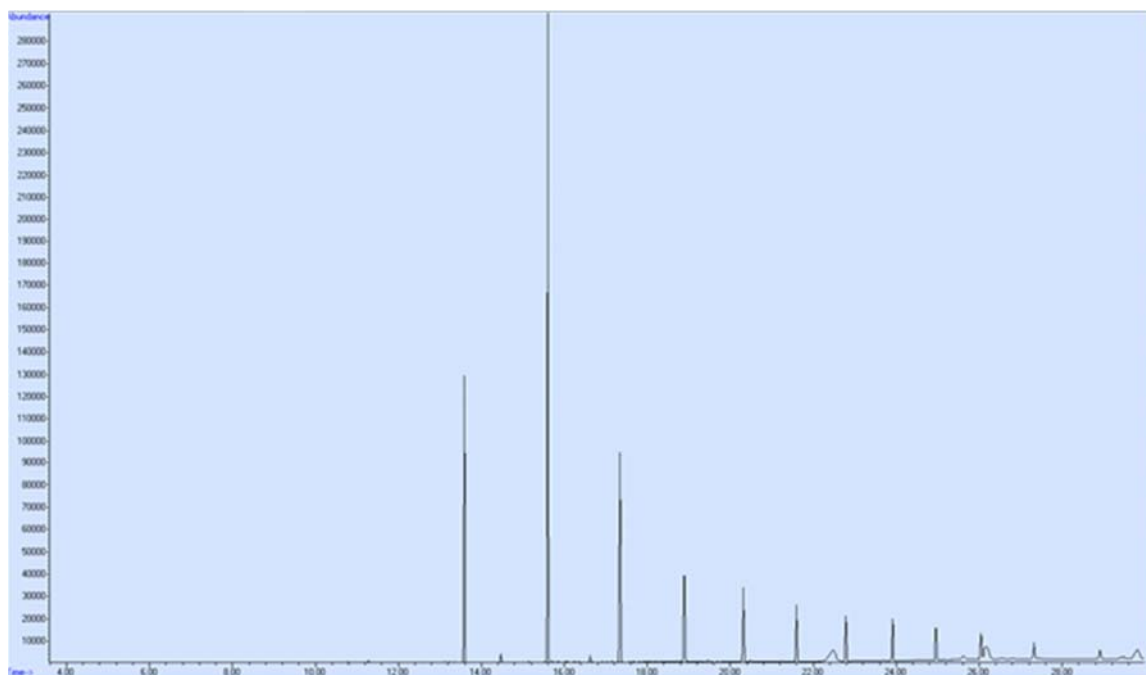
Figure 24: Blank ion chromatogram (methanol only) of m/z 383 after a small number of injections of derivatized 8-oxo-dG and derivatization matrix.
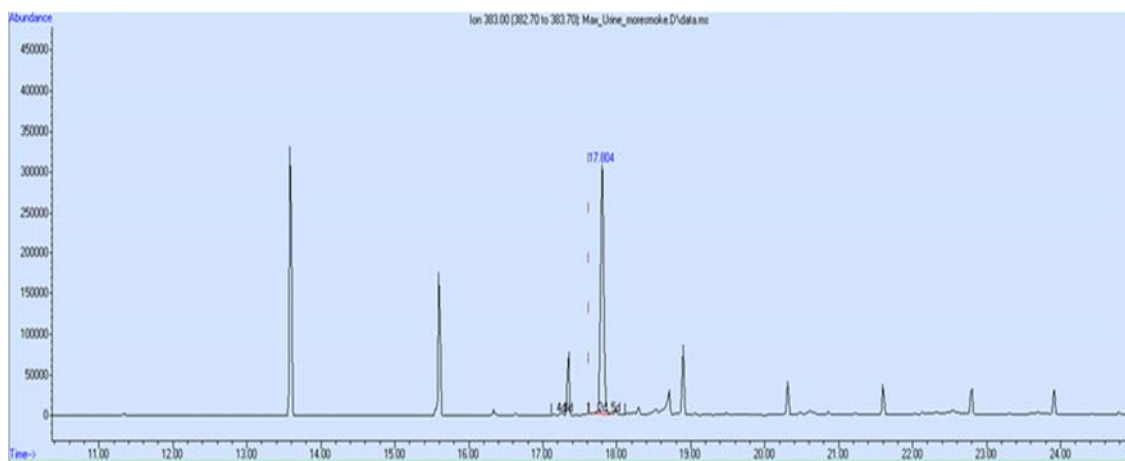
Figure 25: Ion chromatogram of ion m/z 383, sample obtained from a volunteering smoker, the 8-oxo-dG peak is identified, however, the parasite peaks were a major confounding factor.

The derivatization procedure, proved to be a not reliable and reproducible method. The retention time of 8-oxo-dG was found, but the response and the peak shape were not

satisfactory for analyses of low concentrations. Furthermore, the analysis procedure proved to be unreliable and error prone with our facilities. After considering the problems with the methodology it was decided to analyse the samples in an external laboratory with a simpler method, using SPE extraction and analysing the 8-oxo-dG by LC-MS.

III. Methodologies

This chapter describes the various sampling campaigns and the analytical procedures for urine samples from volunteers. The sampling campaigns were completed in collaboration with Ms. Barbara Macias Hernandez under the FIXAT project.
Part of the sampling was carried out in collaboration with a private highway maintenance company and a landscape gardening company. Some of the chemical analyses were completed in collaboration with Prof. Silvia Fustinoni, University of Milan. This study was funded by CEFIC and Wellcome Trust.

The sampling campaign and the FIXAT project were divided in two main parts: one consisted in biomarker discovery and biomonitoring, on which this work is based on, while the other one consisted in the characterization of the personal exposures and modelling of the lung dose for such exposures. The latter was performed by Mrs. Barbara Macias Hernandez, and will be included in her PhD thesis. The personal exposure data was however available for comparisons and correlation analyses with the urinary biomarkers, however no further analyses could be performed with the personal exposure data set. All of the urine analyses of FIXAT project were performed in first person, except the 8-oxo-dG and the creatinine measurements, which were performed in external laboratories.

The analytical procedures were a significant part of the learning process involved in the project. The analyses performed in Milan were performed in first person by the author, who gratefully acknowledges the department of occupational health for the training opportunity.

In the same way, the unmetabolized VOCs from MATCH project were performed in first person. Furthermore, another training opportunity was given by the University of Leicester for 8-oxo-dG analyses, where only a part of the analyses were performed. Due to unforeseen circumstances, the analyses were later performed in fullness by an external laboratory.

Chemical analyses of the samples collected during the campaigns was conducted at the University of Birmingham (UoB) (UK) and University of Milan (UoM) (Italy). Urinary hydroxy PAH (OH-PAH) were analysed for MATCH project in university of California, San

Francisco (UCSF). Jeddah schoolchildren urinary PAHs were analysed by the Institute and Outpatient Clinic of Occupational, Social and Environmental Medicine of the University of Erlangen-Nuremberg (UoE) (Germany).8-oxo-dG was analysed in an external laboratory at Department of Public Health, Chung Shan Medical University (CSMU), Taichung, Taiwan. Table 32 summarizes the type of analysis conducted for each set of samples.

Table 32: Summary of the chemical analyses.

Project	Sample type	Sample collection	Sampler	Airborne VOC and PAH	Urinary VOC and cotinine	Creatinine	8-oxo-dG	Urinary OH-PAH
FIXAT	Personal exposure VOC	2011-2013	Sorbent tubes	UoB			CSMU	
	Urine		Mid-stream urine sample		UoM	UoB	CSMU	
MATCH	Personal exposure VOC	2005-2007	Sorbent tubes	UoB			CSMU	
	Urine		Mid-stream urine sample		UoM	UoB	CSMU	UCSF
Independent Measurements	Urine	2011-2013	Mid-stream urine sample		UoM	UoB	CSMU	
Jeddah schoolchildren	Urine	2013	Mid-stream urine sample			UoE / CSMU	CSMU	UoE

III.1 FIXAT and MATCH studies

Samples analysed in this study were collected under the FIXAT (FIngerprints of eXposure to Air Toxics) project. The study included 44 subjects divided in three main groups: a control group, a home/workplace exposed group, and an occupationally exposed group. All of the groups were formed by non-smokers (declared), with no distinction of sex or ethnicity, with an age range from 18 to 45 years old, with a majority of subjects in the 26-35 range. The objective of FIXAT study was to compare and characterise the exposure to building materials, furniture and consumer products in new and recently refurbished buildings, and further compare the exposure levels with occupationally exposed subjects.

71

MATCH project was a previous project that took place across the smoking ban in UK (2005-2007). The project aimed to identify the microenvironments that were the main contributors towards the overall exposure to VOCs and PAHs, together with the contribution of ETS (Aquilina et al., 2010; Delgado-Saborit et al., 2011; Harrison et al., 2009). Due to the differences in the volunteers recruitment and the indoor smoke regulations, the volunteers from MATCH project were exposed to higher concentrations of VOCs and PAHs. Furthermore, the urine samples that were chosen for the analysis along with the FIXAT ones, were the ones belonging to volunteers that had the highest personal exposures (data obtained by MATCH database) and had given the consent for further analyses. MATCH project focused on personal exposure to VOCs and PAHs, together with biological monitoring of selected biomarkers: urinary cotinine and hydroxycotinine for ETS exposure, urinary hydroxy PAHs (1-hydroxypyrene and 1-hydroxyphenanthrene). The analyses performed along with FIXAT samples, added the measurements of unmetabolized VOCs and urinary cotinine for the samples that were not analysed for that biomarker.

When added to the FIXAT samples, the MATCH samples provided a high level exposure group that was not classified as occupationally exposed, representing a fourth subgroup with a different kind of exposure, characterized mostly by ETS exposure.

III.2 Questionnaires and volunteers

The suitable volunteers for the study were selected within the recruited subjects using questionnaires. A screening questionnaire was produced to assess the suitability of the volunteers that applied for the experiments (see template in annex, section X.2). Each volunteer was selected through the screening questionnaire, where questions about the household and the volunteer's job were asked. In the screening questionnaire, questions on everyday activities and smoking habits were also included to assess the baseline level of exposure of the subject,

in order to rule out volunteers that were not suitable for the sampling campaign. Based on the answers to the questionnaire, the subjects were first divided in two groups- suitable and not suitable for the sampling campaign, and subsequently in one of the three groups as described below.

The subjects of the control group (N=17) were subjects living in a house or work in a building that was built or refurbished more than one year before the sampling. Also these subjects needed not to have jobs or perform regularly any other activity that would put them in contact with VOCs or PAHs in high concentrations. A set of questionnaires was prepared to collect information on each subject on all of the possible variables of each sampling event.

The subjects of the home/workplace exposed group (N=14) were subjects that at the time of the sampling were living or working in a building that was built or refurbished recently (<1 year before the sampling).

For groups 1 and 2, subjects were screened based on their occupations exposure to VOCs and PAHs in high concentrations. Any potential subjects with jobs/activities that would lead to high VOC/PAH exposure were not included in the study group.

The subjects of the occupationally exposed group (N=14) were recruited among workers that are exposed to VOCs and PAHs in their daily work activities. In our case, the volunteers were recruited from a highway maintenance company that kindly accepted to collaborate and from a landscape gardening company. In both cases the volunteers worked in an expected medium-high VOC and PAH concentration environment. The volunteers from the highway maintenance company were working on the roadside, while the landscape gardeners were using petrol engine powered tools.

The questionnaires were then analysed and the obtained information was used in conjunction with the data obtained for the personal exposure.

III.3 Personal exposure and sampling

Figure 26: Sampling briefcase, containing a VOC pump (top), connected to a sorbent tube and a PAH pump (bottom, black) connected to a quartz filter and the micro aethalometer (bottom, blue).

Personal exposure sampling was performed based on the methodology used in MATCH project (Harrison et al., 2009), with minor modifications and the addition of more sampling devices. Volunteers were provided with personal air monitors in a suitcase or a backpack, depending on the volunteer preferences, consisting of: one VOC sorbent tube and one quartz filter connected to a pump for active VOC and PAH sampling and one BC (black carbon) microaethalometer. In some cases, we were able to provide an accelerometer to log the physical activity of the subject. The equipment was delivered in the morning, before the volunteer left the house, so the subject would be able to perform the everyday tasks as normal. The sampling lasted 24 hours and the following morning the researchers collected the equipment and a mid-stream sample of the first morning urine.

At the time of the sampling, the volunteer was provided with a set of questionnaires to track their daily activities and dietary information. These included:

1.   An activity diary (see template in annex, section X.3), where the subject could indicate each activity performed and the time when it was performed, with additional information on the level of exercise involved.

2.   A travelling questionnaire (see template in annex, section X.8), to track the commuting of the subject, in order to track the commuting journeys and the condition of the commute (i.e. commuting in traffic, car's air conditioned on or off, window opened or closed).

3.   A dietary questionnaire (see template in annex, section X.6), where volunteers could indicate any food and drink they had during the sampling day. Together with the food, they were asked to indicate if they were taking any self-medication or prescribed drugs (see template in annex, section X.5). Another section was dedicated to the intake of caffeinated drinks.

Volunteers were also given several forms for ETS exposure (see template in annex, section X.7), where they could indicate the time and the details of the exposure in case that they were exposed to ETS during the sampling day.

III.4 Urine sampling and storage

After the 24h sampling event a urine sample was collected. The subjects were instructed to collect a first morning mid-stream urine sample, which was then collected as soon as possible by the researchers and placed in refrigerated carrying boxes. If the subject's house was not easily reachable, or the sample was going to be collected far from office hours, the subjects were provided with instant ice packs to refrigerate the urine before the collection. The sample was then centrifuged for 10min at 10.000G and the supernatant was divided in 2ml aliquots and transferred to a -80°C freezer. A similar procedure was used for the urine sampling of both MATCH project and Jeddah schoolchildren (Chapter V, Trasande et al., 2015), however, in these cases, no centrifugation was performed and the samples were transferred to a -80°C freezer after the sampling. In Jeddah schoolchildren, three urine samples were collected, over

three days for two sites (nearby an oil refinery and nearby a busy ring road) and over another three days in the control site (rural background). Unfortunately, only part of these samples were made available (only one of the three days) and the control samples were not made available.

III.5 Sample analyses and quality control

All of the samples were checked for quality control by analysing replicates. The standard protocol for the analyses included a replicate of a sample and a mid-range standard every 5 analysed samples, in order to assess the reproducibility of the analyses and to check the conditions of the instrument. When the samples were analysed in external laboratories, an adequate number of duplicates were provided, in order to have a double blind control. When the difference between duplicate measurements more than 10%, the analyses were not considered valid and were repeated. Variations in the mid-range standard would suggest problems with the instrument, therefore, when variations in the measurement of the standard were detected, the instrument was then stopped and checked for problems.

Together with the quality control, a calibration curve was performed at the beginning of the sample sequence and one was performed at the end. In case of long queues, a third calibration curve was included in the middle of the sequence. To avoid carryover contaminations, after each calibration curve and mid-range standard, a blank run was performed, which provided further information on the condition of the instrument. These quality control steps were used for unmetabolized VOCs and airborne VOCs. The external laboratories, however, applied similar controls, which were double checked by the double blind replicates. The creatinine measurements were performed in multiple laboratories, and the results were found to be identical.

III.6 VOCs and PAHs analysis from personal exposure measurement

VOCs and PAHs were sampled using the methodology described previously in section III.3 and in MATCH project with some minor modifications (Delgado Saborit et al., 2009; Harrison et al., 2009; Delgado-Saborit et al., 2013).

VOCs from sorbent tubes were analysed using a thermal desorption autosampler, connected to a GC-MS. The analytical procedure was kept unchanged from MATCH project, however, more compounds were analysed. The compound list, with retention times and quantifying ion of each compound is presented in Table 33.

Table 33: Analysed VOCs, with retention time and quantifier ion.

Compound	Retention time	Quantifier ion
Benzene	7.003	78
Toluene	9.666	91
Ethylbenzene	12.412	91
p-xylene	12.666	91
m-xylene	12.874	91
pyridine	14.229	79
o-xylene	14.276	91
cumene	13.927	105
dipentene	14.767	68
n-propylbenzene	15.054	91
styrene	16.576	104
p-isopropyltoluene	16.955	119
1,3,5-trimethylbenzene	16.154	105
1,2,4-trimethylbenzene	17.297	105
1,2,3-trimethylbenzene	18.930	105
Benzaldehyde	24.095	77
Octaldehyde	17.532	43
2-ethyl-1-hexanol	23.048	57
3-vinylpyridine	27.217	105
Naphthalene	29.312	128

PAHs measurements were performed using the methodology described by Delgado-Saborit et al. (2013).

FIXAT project personal exposure samples were analysed by Mrs Barbara Macias Hernandez, for the following PAHs: naphthalene, acenaphthylene, acenaphthene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benzo(a)anthracene, chrysene, benzo(b)fluoranthene, benzo(k)fluoranthene, benzo(a)pyrene, indeno(1,2,3-cd)pyrene, dibenz(a,h)anthracene, benzo(ghi)perylene, coronene, benzo(e)pyrene.

The analysed airborne quinones derived from PAHs were: 1,4-naphthoquinone, 2,6-di-tert-butyl-1,4-benzoquinone, 2-methyl-1,4-naphthoquinone, acenaphthenequinone, 9,10 anthraquinone, 2-methyl-anthraquinone, 2,3-dimethyl-anthraquinone, benz(a)anthracene 7,12-dione, 5,12-naphthacene-quinone.

MATCH project data was obtained for urinary concentrations of: 2-naphthol, 1-hydroxyfluorene, 2-hydroxyfluorene, 1-hydroxyphenanthrene, 2-hydroxyphenanthrene, 1-hydroxypyrene.

III.7 Unmetabolized BTEX and naphthalene in urine

Unmetabolized BTEX (benzene, toluene, ethylbenzene, o- m- p-xylene) and naphthalene were analysed in urine. The analyses were carried out at the University of Milan thanks to a collaboration with Prof. Silvia Fustinoni. Unmetabolized BTEX concentrations were analysed using the Headspace SPME-GC-MS following the procedure described in Fustinoni et al. (1999). Briefly, 0.6ml of urine were poured in a 2ml crimp cap vial (GERSTEL GmbH & Co.KG, Germany) containing 300mg NaCl and 1µl of internal standard solution (Benzene d6, Toluene d8, M-xylene d10, Naphthalene d10 in methanol) was added to the sample. The vial was immediately sealed by crimping, using silicone-PTFE septa caps (GERSTEL GmbH & Co.KG, Germany). The vials and NaCl were baked overnight at 60°C to

avoid contamination before use. The vials were then loaded on the instrument for the Headspace SPME GC-MS analysis. The analysis was performed by using a Gerstel MPS2 autosampler (GERSTEL GmbH & Co.KG, Germany) equipped on an Agilent 6890 (Agilent Technologies, CA, United States). The measurement was performed with a PDMS 100 µm SPME fibre, sampling the headspace of the sample for 5 minutes at room temperature, followed by desorption for 3 minutes by inserting the fibre in the chromatographic injection port. GC-MS separation was performed using helium as carrier gas at a constant flowrate of 1ml/min, the injector temperature was held at 250°C and the GC oven temperature was held for 3 minutes at 40°C, ramped to 70°C at 3°C/min, held for 1 minute and then ramped up to 250°C at 20°C/min. The final temperature was held for 5 minutes. Signals from the ions were acquired using the single ion monitoring mode (SIM), m/z ratios acquired were: 78 for benzene, 84 for benzene d6, 91 for toluene, 98 for toluene d8, 106 for ethylbenzene, M+P_xylene, 115 for M-xylene d10, o-xylene, 128 for naphthalene and 136 for naphthalene d10. Analyses were performed using Electron Impact (EI) ionization with the ion source kept at 250°C using an energy of 70eV. The column used was a DB1 (60m, 0.25mm i.d., 1µm film thickness) J&W scientific (Agilent Technologies, CA, United States). According to the methodology described, LOQs were of 15 ng/L for Benzene, Toluene, O-, M-, P-Xylene and 8 ng/L for Ethylbenzene (Fustinoni et al. 2010; Fustinoni et al. 2010). Quality control points and samples replicates were inserted in the analytical sequence every 10 samples to validate the readings. All the data obtained was then normalized against creatinine concentration.

III.8 8-oxo-dG measurement

8-oxo-dG has been measured in an external laboratory (CSMU, Taiwan) using LC-MS methodology (Lam et al., 2012): briefly: 0.5ml of all urine samples were processed by SPE extraction after being spiked with 3pMol of internal standard (8-[15N5]oxo-dG). The samples were then diluted 1:1 with deionized water and spun at 16,000G for 1 minute at room

temperature to precipitate impurities. SPE cartridges Env+Isolute (1ml, 50mg, Biotage, Uppsala, Sweden) were then preconditioned with 1ml methanol and equilibrated with 1ml deionized water. Samples were loaded on the cartridges and the flow was kept at approximate 1ml/min. The cartridges were then flushed twice with 1ml 2% methanol to wash the interferences. The cartridges were vacuumed dry to prevent any water to contaminate the eluate. The elution was performed with 20% (v/v) acetonitrile in methanol and then the eluate was dried under nitrogen flow and stored at -20°C until analysis, which was performed by LC-MS. All the data obtained was normalized against creatinine concentration.

III.9 Creatinine concentration measurement

Creatinine concentration was calculated with Jaffe's colorimetric method (Kroll et al., 1986).

III.10 Urinary 1-hydroxypyrene and hydroxyphenanthrenes measurement

Urinary 1-hydroxypyrene and 1- 2- 3- 4- 9-hydroxyphenanthrenes were measured in external laboratories, MATCH samples were analysed by the UCSF (USA) by LC-MS/MS (Aquilina et al., 2010), while Jeddah schoolchildren samples were analysed by the UoE (Germany) by fluorescence liquid chromatography following the procedure described by Hemat et al. (2012).

III.11 Urinary cotinine measurement

Urinary cotinine was measured by LC-MS/MS analysis, with the methodology described by Fustinoni et al. (2013). Urine was spiked with an internal standard (cotinine d3) and diluted 1:2 with HPLC grade water and injected in a LC-MS/MS. The separation was performed by a C-18 reverse phase column, and the precursor ion was 177 m/z (180 m/z for the internal standard), while the quantitation ion was 80 m/z. As described in the paper, the LOD was 0.1 µg/L for urinary cotinine, but in our case we used different cut off values for the different groups due to

the distribution of the measurements (urinary cotinine <1µg/L for non-ETS exposed, <50µg/L for ETS exposed, >50µg/L for active smokers).

III.12 Peak integration

Chromatograms from GC-MS were analysed using Agilent Chem Station. The responses obtained by peak integration were inserted in a spreadsheet that was then analysed with Microsoft Excel 2013. All the responses refer to the area of the integrated peak.

III.13 Data analysis

The obtained data was analysed with SPSS Statistics V.21 and Microsoft Excel 2013.

III.14 Subject recruitment and group assignment

Subjects' urine analysed in this study included urine samples of 41 volunteers from MATCH (measurement and modelling of exposure to air toxics concentrations for health effects studies) project (Delgado Saborit et al., 2009) and 44 from FIXAT project. Further 24 samples, marked as IM (independent measurements), were obtained from a pilot study on indoor and outdoor measurement, and some independent measurement campaigns in London and Birmingham (UK) with different routes and modes of transport. Due to the high variability of the exposure and subjects' behaviour, this group was not included in the statistical analyses. FIXAT group was further divided in three subgroups: a control group, a group formed of volunteers living or working in a new or recently refurbished building, and an occupationally exposed volunteer group. The three groups were composed as described in section III.1 based on the information gathered from the screening questionnaires. The volunteers living or working in recently built/refurbished buildings received two sampling visits, one at the beginning of the project, and one 1 year later, in order to measure any difference on the exposure arising from building materials and any other material that was used in the building or refurbishment of the house or workplace. The three main groups were divided by exposure level: the exposure levels were

81

firstly estimated using screening questionnaires in FIXAT project, and then measured by personal exposure sampling with sorbent tubes. In MATCH group the samples were chosen within the higher VOC exposure, which was measured by the analysis of the personal exposure data in the same study (data available from Saborit et al. 2009). The IM group was a pilot study involving different subjects that were not screened before, but contained several smokers, which placed the expected exposure levels variable between low and high. MATCH samples were collected between 2005 and 2007, while the sampling of the other groups was performed between 2011 and 2014. In the FIXAT project the control volunteers were selected within the general population using a screening questionnaire asking for smoking habits, exposure to environmental tobacco smoke (ETS), occupation, commuting habits and a description of the house. All of the subjects that showed a possible high level exposure behaviour or storing solvents/fuels in the house were evaluated and in case of effective exposure, were excluded from the control group. The second group from FIXAT project (exposed subgroup) is formed of subjects with the same requisites as the control group, but living in a recently built or refurbished house (<1 year). The occupationally exposed group volunteers were selected within workers that use petrol powered tools or work in the traffic, as for that reason they are exposed to higher level of VOCs compared to the general population. The subjects from the pilot study were not screened with the questionnaires, so the group included general population, subjects exposed to ETS, commuters on busy roads. The subjects from MATCH samples were chosen within the higher VOC concentrations in the personal exposure within the sample pool of the study.

III.15 Exposure System
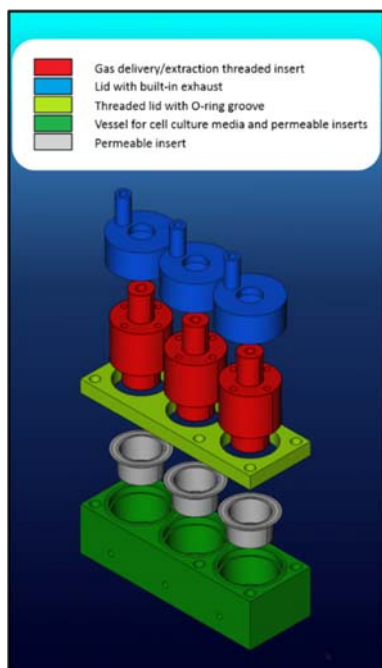
Figure 27: Exploded representation of the exposure vessel with all the part of the assembly.
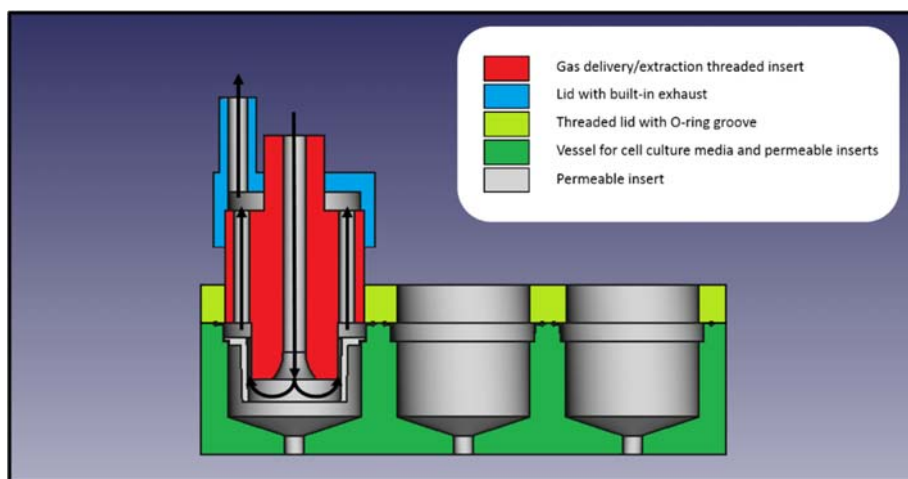
Figure 28: Cross section of the assembled exposure vessel, arrows indicate the gas flow. In use, the two vessels to the right are also fitted with the gas delivery system and permeable insert.

An exposure vessel was custom designed based on the CULTEXs system (Vitrocell, Germany) described by Pariselli et al. (2009). The system consisted of three wells milled into a polymethyl methacrylate (PMMA) block, suitable to fit a permeable 6-well insert (Corning Inc., NL) into each one, and 6ml of media under the inserts (Figure 27, Figure 28 and Figure 29). The capacity to circulate cell culture media was included by adding two 5mm holes at the

bottom and on the side of each well. These could be capped if the circulation of the media was not needed.
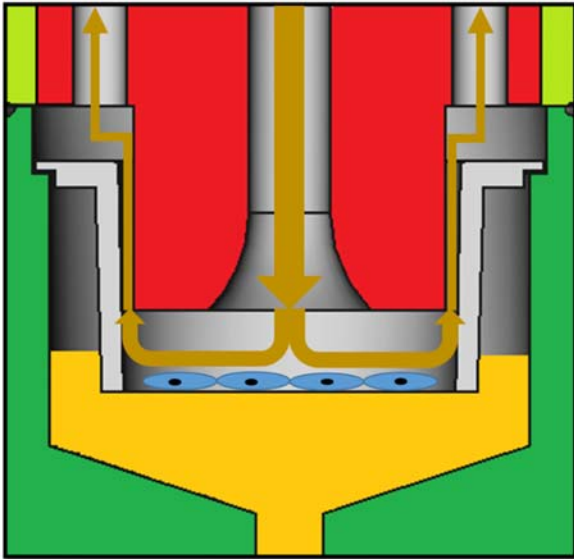
Figure 29: Detailed cross-section of the gas-cell interface, with the level of the cell culture media indicated in yellow.

Figure 30: Assembled exposure vessel in working order, hosted inside the GC oven.

Figure 31: Exposure vessel assembly. 1: GC oven; 2: heater/bubbler; 3: MFC controller and MFCs; 4: Computer controlling MFCs and gas flows.

The lid was constructed with threaded holes to fit gas delivery inserts, and the wells were sealed using nitrile O-rings. The gas delivery tubes were designed with a trumpet shape in order to reduce the linear velocity of the delivered gas to reduce mechanical stress to the cells, and to allow maximum mixing and even delivery of the gas along each insert (Figure 28). Each tube was threaded externally to screw into the lid allowing fine adjustments of the distance between the nozzle and the cell layer. The tubes were provided with holes to allow the delivered gas to flow out to the exhaust, providing a continuous flow. The vessel was kept in a gas chromatograph (GC) oven at 37°C during the exposure to maintain the cells at an optimal temperature. The vessel was kept in an incubator at 37°C between the exposures. The gas delivery to the cells was regulated by mass flow controllers (MFCs) (Brooks Instrument, NL). One 500 sccm (standard cubic centimeters/minute) MFC was used to regulate the synthetic air (BOC gases, UK) flow, and a 100 sccm MFC was used to regulate the benzene flow (1ppm balanced in nitrogen, BOC gases, UK). A total flow rate of 30 ml/min was used, split between the three wells, with a theoretical flow rate of 10 ml/min in each well.

The synthetic air passed through a bubbler heated at 37°C to humidify the air to prevent desiccation of cells, and the pollutant delivery (benzene 1ppm balanced in nitrogen, BOC, UK) was regulated by a second MFC. The two gases were then mixed in a glass mixing chamber hosted inside the GC oven, filtered through a 0.45µm filter and delivered to the cells. The system worked with positive pressure of the exposure gases to ensure sterility.

III.16 Cell Culture

A549, epithelial lung carcinoma cells (HPA, 86012804) have been previously used as a model for lung epithelium exposure (Pariselli et al., 2009; Saint-Georges et al., 2008; Shang et al., 2013). Furthermore, this cell line has been reported to express CYP2E1, the cytochrome P450 involved in the metabolism of benzene (Foster et al., 1998; Medinsky et al., 1994; Snyder and Hedli, 1996; Sørensen et al., 2003a; Mögel et al., 2011), and therefore represents a suitable model of study for benzene and VOC toxicology.

A549 cells were cultured in 6-well plates using RPMI 1640 medium (2.5 ml), containing 25mM HEPES and $NaHCO_3$ to maintain the pH constant during the exposure period (CO_2 was not contained in the synthetic air). The medium was further supplemented with glutamine (2mM), penicillin (100 U/ml), streptomycin (100 µg/ml), amphotericin B (0.25 µg/ml) and 10% foetal bovine serum (FBS). Cells were passaged regularly using a standard trypsin-EDTA protocol into 6-well plates once they reached approximately 90% confluence. The standard protocol for trypsinization consisted in washing the cells with phosphate buffer solution (PBS), followed by a 5 minute incubation at 37°C in 0.5 ml per well of 0.25% trypsin-EDTA solution. After the trypsin digested the adhesion proteins, the cells were recovered by pipetting, diluted and seeded in new plates. For the exposure experiments, 0.3×10^6 cells were seeded into 1.5 ml RPMI 1640 on the collagen coated Transwell inserts with pores of 1µm (Corning, NL) and 2 to 2.5

ml RPMI 1640 were added at the bottom of the well, and the plate was placed in an incubator. Once the cells reached 90% confluence (24–36 hours) they were serum starved for 12 hours by exchanging the medium containing FBS with FBS free medium, to synchronize the cell cycle prior exposure (Campisi et al., 1984). After the exposure, the cells were washed with PBS for 5 minutes before trypsinization.

III.17 Synthetic Air and Benzene Exposure

For each exposure experiment, 3 Transwell inserts cells from the same 6-well plate were exposed for 2 hours to synthetic air (controls). Afterwards, the exposure vessel was then rinsed, washed with 70% ethanol and allowed to dry. Subsequently, the remaining 3 Transwell inserts were exposed to one of three different concentrations of benzene (0.03 ppm; 0.1 ppm; 0.3 ppm), which were reported to have cytotoxic effects (Pariselli et al., 2009). A first exposure experiment was performed with 3 technical replicates simultaneously exposed to each benzene concentration accompanied by an air-exposed control. The obtained results were then confirmed by an independent exposure experiment replicate. The low (0.03 ppm) and high (0.3 ppm) benzene concentrations were further tested by a third independent exposure experiment replicate.

Each exposure experiment was accompanied by a set of incubator controls, which consisted in a 6-well plate left for 2 hours in the incubator, where 3 wells contained cells with media (Incubator Media), and 3 wells contained cells with the media removed (Incubator Dry). The removal of the media from the 3 wells was considered the start point of the 2 hours incubation of the incubator controls.

III.18 ROS Assay

Reactive oxygen species were assessed by measuring the oxidation of the redox sensitive dye 2',7'-dichlorodihydrofluorescein diacetate (H2DCF-DA, Sigma-Aldrich, UK). Following hydrolysis by intracellular esterases, the resultant H2DCF is unable to leave the cell and is then oxidised by ROS, to form the fluorescent DCF (dichlorofluorescein) molecule. The level of fluorescence is proportional to the degree of oxidative stress in the cells. Briefly, the cells were preloaded with H2DCF-DA dissolved in the growth medium (10 µM, final concentration) for 30 minutes. After the loading of the cells, both in the exposed samples and in the incubator controls a first fluorescence reading was taken and marked as pre-exposure. After the treatment (air/benzene exposure or 2 hours in the incubator), a second fluorescence reading was taken, marked as post-exposure.

DCF fluorescence was quantified before and after exposure using a Tecan Ifinite F200 pro plate reader (excitation wavelength: 485 nm, emission wavelength: 535 nm) using I-control V.1.9 software. To minimise the exposure of the cells to air, quantification of the fluorescence before the exposure was performed without removing the apical medium from the inserts. Three controls and three exposed samples were analysed. Cells that had not been loaded with H2DCF-DA were used as a negative control and to take into account background levels of fluorescence.

III.19 Comet Assay

DNA strand breaks were assessed by the alkaline comet assay (Singh et al., 1988). Briefly, after trypsinization, the cells were re-suspended in 150 µl PBS. 15 µl of the suspension were added to 150 µl of 0.5% (v/v) warm low melting point agarose (LMPA) and the cell suspension was added to a microscope slide that had been previously coated with 0.5% (v/v) normal melting point agarose (NMPA). After a coverslip was added, the slides were left on an ice-cold tray to allow the agarose to solidify. Once the agarose had solidified, the coverslip was removed and the slides were incubated for 1 hour at 4° C in lysis buffer (2.5 M NaCl, 0.1 M Na_2EDTA,

10 mM Tris base, 1% sodium N-lauryl sarcosinate, 10% DMSO and 1% Triton X-100, pH 10).

Next, the slides were then transferred to a horizontal electrophoresis tank containing electrophoresis buffer (NaOH 300 mM, EDTA 1 mM, pH 13.0) and DNA allowed to unwind for 20 minutes. DNA was then subjected to electrophoresis (32 V, 0.8 V/cm) for 20 minutes. The slides after the electrophoresis were transferred to the neutralization buffer (Tris HCl 0.4 M, pH 7.5) and washed 3 times for 5 minutes, followed by 1 wash in H_2O for 5 minutes. Finally, slides were stained with 50 µl of Sybr gold (Invitrogen, 10× solution in neutralisation buffer, UK). Slides were examined at 320× magnification using a fluorescence microscope (Zeiss axiovert 10, Zeiss, UK) fitted with a 515–560 nm excitation filter and a barrier filter of 590 nm. A USB digital camera (Merlin, Allied Vision Technologies, UK) received the images, which were analysed using a personal computer-based image analysis system Comet Assay IV (Perceptive instruments, UK). The software analysed each cell by assigning a proportion between the % of DNA in the tail and the % of DNA in the head. Other variables can also be analysed, such as the tail migration, which is the length of the tail and the tail moment, which is a product of both the tail migration and the % DNA in the tail. Due to the software limitations, it was not possible to include heavily damaged cells ("hedgehogs") in the count, as the nuclei and the whole cells would not be recognized by the software, leading to wrong results. However, they were taken in account by counting a standard number of cells and calculating the percentage of hedgehogs (therefore unquantifiable cells) over the total number of counted cells.
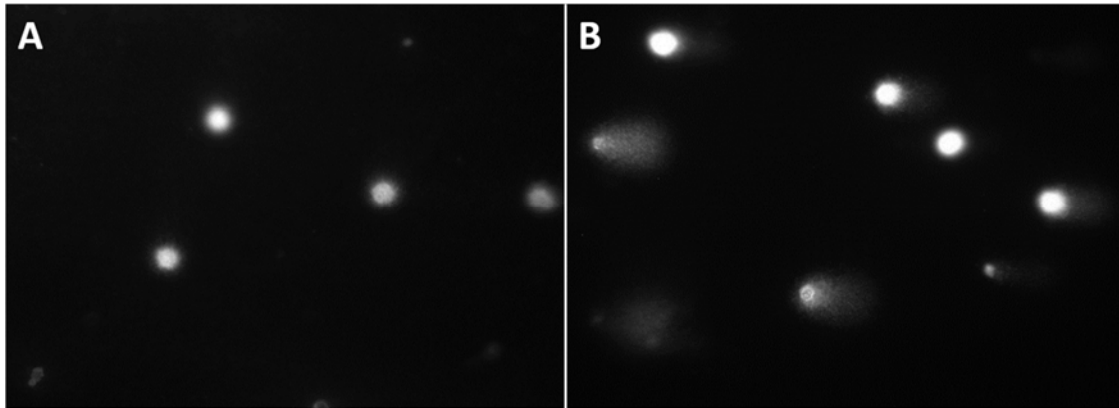
Figure 32: Comet examples.
A: control cells exposed to synthetic air for 2h;
B: Cells exposed to 0.1ppm benzene for 2h.
B shows higher tail intensities, which are indicative of DNA damage, a "hedgehog" is visible
at the bottom left corner of B.

A random sample of at least 300 nuclei on each slide was assessed and categorised into quantifiable or not quantifiable. Unquantifiable nuclei consisted largely of "hedgehogs", indicative of nuclei where most of the DNA was in the tail and probably represent nuclei from apoptotic and necrotic cells. A minimum of 60 nuclei were quantified per slide (N = 64 – 200). Percent tail DNA (TD %) was chosen to assess the extent of DNA damage in quantifiable cells, as this has been shown to suffer much less from inter-run variation than other Comet parameters because it is largely independent of electrophoresis voltage and run time (Olive and Durand, 2005).

IV. Assessment of VOC and PAH exposure in different population groups in Birmingham, UK

This chapter discusses urinary VOC and PAH concentrations across high- and low-exposure subjects, and associated oxidative stress drivers. The aim of the study was to assess the impact and contribution of various behaviours and sources of pollutants to the human exposure, assessing early biomarkers of effect.
Some of the data used in the analysis was generated as part of an HEI project (Harrison et al., 2009).

IV.1 Study population

The participating subjects were assigned to the different groups following the rules described in the Methodologies, section III.14 and III.1. Table 34 contains a summary of the demographic information gathered from the questionnaires. After analysing the data available from the questionnaires, it was observed that 38% of the MATCH volunteers had a garage attached to the house, the majority of which had the garage directly accessible from the house. 12.2% of MATCH volunteers declared to be exposed to ETS at home and 7.3% in the workplace. In the FIXAT project, 14.9% of the subjects declared to be exposed to ETS at home and 17.6% in the workplace. The samples from FIXAT and MATCH projects were analysed for urinary cotinine following the methodology described by Fustinoni et al. (2013) to estimate the effective ETS exposure and smoking habits of the subjects. We used different cut off concentration values from the ones reported in Fustinoni's reference paper to divide smokers from non-ETS exposed and ETS exposed subjects (urinary cotinine <1µg/L for non-ETS exposed, <50µg/L for ETS exposed, >50µg/L for active smokers). Analysing the results of the test, it was clear that some subjects, although declaring to be non-smokers, were actively smoking (2.8% of subjects in FIXAT project). Interestingly, there was a difference between the subjects that declared to be exposed to ETS, and the outcome of the cotinine test: 14.9% of the subjects declared to be exposed to ETS at home, and 17.6% to be exposed to ETS in the workplace, while only 11.1% of the total sample pool was found positive for ETS. Only 6.3%

of the control subjects were found positive for ETS exposure, and no subject from the living/working in a recently built/refurbished building were exposed to ETS according to the urinary cotinine analysis.

The main age group is between 26 and 35 years old for both FIXAT and MATCH subsets, however, the population is not evenly distributed along the subgroups as showed in Table 34. Genders are evenly represented in the total sample pool but the occupationally exposed subgroup was formed only by male subjects, which caused an uneven distribution among the subgroups. χ^2 test showed that the population of the subgroups was not evenly distributed for many of the variables such as average annual mileage and age range. The most important differences in distribution are the ones that are linked to higher exposure to VOCs, such as the use of a motor vehicle for work (85.7% of occupationally exposed) and average yearly mileage, which is generally higher on occupationally exposed subjects.

Table 34: *Summary of demographic information for FIXAT and MATCH project.*

		Project name					
		FIXAT (%)				MATCH (%)	χ² P value (FIXAT)
		Group				Group	
		Control (N=16)	Living/working in a new or refurbished building (N=15)	Occupationally exposed (N=14)	Total (N=45)	MATCH project (N=41)	
Gender	Male	33.3%	29.4%	100.0%	52.2%	34.1%	<0.001
	Female	66.7%	70.6%	0.0%	47.8%	65.9%	
Classified as a smoker (urinary cotinine measurement)	Non-ETS exposed (Cotinine < 1 µg/L)	93.8%	100.0%	67.9%	86.1%	12.2%	0.010
	ETS exposed (Cotinine < 50 µg/L)	6.3%	0.0%	25.0%	11.1%	34.1%	
	Smoker (Cotinine > 50 µg/L)	0.0%	0.0%	7.1%	2.8%	2.4%	
	Missing data	0.0%	0.0%	0.0%	0.0%	51.2%	
Classified as a smoker (urinary cotinine measurement) ETS threshold 10µg/L	Non-ETS exposed (Cotinine < 10 µg/L)	100%	100%	85.7%	94.6%	39%	0.136
	ETS exposed (Cotinine < 50 µg/L)	0%	0%	7.1%	2.7%	7.3%	
	Smoker (Cotinine < 50 µg/L)		0%	7.1%	2.7%	2.4%	
	Missing data	0%	0%	0%	0%	51.2%	
ETS at home	No	81.3%	94.1%	78.6%	85.1%	87.8%	0.417
	Yes	18.8%	5.9%	21.4%	14.9%	12.2%	
ETS at work	N/A	0.0%	43.3%	50.0%	36.5%	0.0%	<0.001
	No	87.5%	53.3%	14.3%	45.9%	92.7%	
	Yes	12.5%	3.3%	35.7%	17.6%	7.3%	
Age range	18-25	20.0%	17.6%	50.0%	28.3%	7.3%	0.003
	26-35	80.0%	76.5%	14.3%	58.7%	26.8%	
	36-45	0.0%	5.9%	0.0%	2.2%	19.5%	
	46-55	0.0%	0.0%	21.4%	6.5%	19.5%	
	56-65	0.0%	0.0%	14.3%	4.3%	19.5%	
	>65	0.0%	0.0%	0.0%	0.0%	7.3%	
Home-work commute	N/A	13.3%	11.8%	0.0%	8.7%	22.0%	<0.001
	<5 Miles	86.7%	58.8%	14.3%	54.3%	29.3%	
	5-10 Miles	0.0%	11.8%	71.4%	26.1%	34.1%	
	10-15 Miles	0.0%	0.0%	14.3%	4.3%	7.3%	
	15-20 Miles	0.0%	17.6%	0.0%	6.5%	2.4%	
	20-30 Miles	0.0%	0.0%	0.0%	0.0%	4.9%	

Commute method	N/A	13.3%	11.8%	0.0%	8.7%	24.4%	
	Car	6.7%	35.3%	78.6%	39.1%	43.9%	
	Train	6.7%	17.6%	0.0%	8.7%	4.9%	0.010
	Bus	26.7%	17.6%	7.1%	17.4%	7.3%	
	Cycle	46.7%	17.6%	7.1%	23.9%	9.8%	
	Walk	0.0%	0.0%	7.1%	2.2%	9.8%	
Travel time	N/A	13.3%	11.8%	0.0%	8.7%	22.0%	
	<5 minutes	0.0%	0.0%	7.1%	2.2%	2.4%	
	5-15 minutes	46.7%	23.5%	14.3%	28.3%	22.0%	
	15-30 minutes	33.3%	41.2%	64.3%	45.7%	36.6%	0.258
	30-45 minutes	6.7%	23.5%	14.3%	15.2%	9.8%	
	>60 minutes	0.0%	0.0%	0.0%	0.0%	7.3%	
Vehicle used for work	No	93.3%	82.4%	14.3%	67.4%	75.6%	<0.001
	Yes	6.7%	17.6%	85.7%	32.6%	24.4%	
Time spent in work vehicle	N/A	93.3%	82.4%	14.3%	65.2%	73.2%	
	<30 minutes	0.0%	17.6%	0.0%	6.5%	9.8%	
	30-60 minutes	6.7%	0.0%	21.4%	8.7%	9.8%	<0.001
	1-3 hours	0.0%	0.0%	50.0%	15.2%	0.0%	
	More than 3 hours	0.0%	0.0%	14.3%	4.3%	7.3%	
Average yearly mileage	N/A	53.8%	47.1%	0.0%	34.1%	14.6%	
	<5,000 miles	38.5%	35.3%	21.4%	31.8%	29.3%	
	5,000-15,000 miles	7.7%	11.8%	50.0%	22.7%	24.4%	0.003
	15,000-30,000 miles	0.0%	5.9%	28.6%	11.4%	24.4%	
	>30,000 miles	0.0%	0.0%	0.0%	0.0%	7.3%	
Garage attached to the house	No	93.8%	76.5%	64.3%	78.7%	61.0%	0.139
	Yes	6.3%	23.5%	35.7%	21.3%	39.0%	
Garage accessible from the house	No	93.8%	100.0%	64.3%	87.2%	68.3%	0.008
	Yes	6.3%	0.0%	35.7%	12.8%	31.7%	
Number of people living in the house	1	12.5%	5.9%	7.1%	8.5%	12.2%	
	2-3	62.5%	64.7%	64.3%	63.8%	51.2%	0.970
	4 or more	25.0%	29.4%	28.6%	27.7%	36.6%	

IV.2 Urinary BTEX, airborne VOCs and 8-oxo-dG concentration comparisons between FIXAT and MATCH populations

Unmetabolized BTEX, cotinine and 8-oxo-dG were measured in the urine samples using the methodologies described in sections III.7, III.8 and III.10 (Methodologies). Airborne VOCs from personal exposure were measured following the methodology described in III.5. A summary of the results is presented in Table 35. In order to avoid statistical problems due to the skewness of the data, the medians were used for the comparisons, which were performed with nonparametric tests. The median of urinary benzene did not show major differences between the various groups, except for the occupationally exposed subgroup. Although the MATCH samples were chosen within the higher exposure samples, the urinary benzene concentration appear to be the lowest of all the subgroups, even though the concentration range is the highest. It is important to remember that the MATCH samples were collected between 2005 and 2007, and they were not collected with the purpose of HS-SPMA-GC-MS analysis. This might have caused part of the VOCs to evaporate from the urine before the analyses. This loss affects mostly the compounds with the lowest relative concentrations, as they are closer to the LOD. When the same group is analysed for toluene, the expected higher concentration is reflected by the observed median.

Toluene is generally more abundant than benzene, therefore it is possible to measure it also in samples that have not been suitably stored for headspace analyses.

Table 35: Summary of urinary BTEX, cotinine and 8-oxo-dG with the available P.E. data.

	Group														
	Control			Living/working in a new or recently refurbished building						Occupationally exposed						MATCH project high exposed subjects		
	Sampling event			Sampling event						Sampling event						Sampling event		
	Single sampling			First sampling			Second sampling			First sampling			Second sampling			Single sampling		
	Median	Minimum	Maximum	Median	Minimum	Maximum	Median	Minimum	Maximum	Median	Minimum	Maximum	Median	Minimum	Maximum	Median	Minimum	Maximum
Cotinine (µg/L)	.14	<LOD	1.19	.13	<LOD	.45	.18	<LOD	.45	.27	<LOD	346.50	.27	<LOD	1125.29	3.46	<LOD	120.91
8-oxo-dG (nMol/M creatinine)	1.29	.52	2.34	1.25	.72	2.36	1.14	.90	2.16	1.15	.51	2.24	1.09	.80	2.04	1.28	.70	2.41
Urinary Benzene (ng/L)	75	58	121	69	51	98	69	53	134	89	60	271	95	61	282	55	15	306
Urinary Toluene (ng/L)	134	73	731	139	69	977	141	72	838	174	91	428	156	98	497	444	77	7003
Urinary Ethylbenzene (ng/L)	19	15	24	24	16	49	22	17	40	20	15	37	28	<LOD	67	62	18	465
½ Urinary m+p-xylene (ng/L)	34	30	42	36	30	52	36	30	41	35	32	52	44	31	87	92	15	228
Urinary o-xylene (ng/L)	27	22	118	35	21	84	33	23	82	36	23	50	36	26	83	35	<LOD	210
Urinary Naphthalene (ng/L)	46	29	402	44	27	98	45	30	125	43	26	56	42	26	57	28	<LOD	134
P.E. benzene (µg/m^3)	1.36	.52	2.70	1.54	.61	5.84	1.64	.65	1.99	5.95	.58	27.60	N/A	N/A	N/A	3.48	1.30	30.25
P.E. toluene (µg/m^3)	8.04	1.82	692.03	20.15	7.00	159.48	12.99	7.64	38.33	19.53	4.25	437.79	N/A	N/A	N/A	14.80	2.47	312.82
P.E. ethylbenzene (µg/m^3)	1.17	.31	3.71	1.61	.40	2.49	4.99	.28	6.35	7.03	1.39	247.54	N/A	N/A	N/A	2.24	.44	59.99
P.E. p-xylene (µg/m^3)	.92	.24	1.40	1.02	.30	2.25	4.29	.19	5.60	N/A	N/A	N/A	N/A	N/A	N/A	1.88	.05	74.70
P.E. mxylene (µg/m^3)	2.38	.63	4.36	2.68	.75	5.15	10.00	.44	14.82	N/A	N/A	N/A	N/A	N/A	N/A	5.14	.82	144.22
P.E. o-xylene (µg/m^3)	1.49	.37	3.22	1.70	.41	3.10	5.60	.25	9.55	N/A	N/A	N/A	N/A	N/A	N/A	2.65	.13	75.78
P.E. naphthalene (µg/m^3)	.76	.36	25.51	1.62	.08	47.58	1.31	.12	2.82	N/A	N/A	N/A	N/A	N/A	N/A	.72	.06	14.21
P.E. pyridine (µg/m^3)	.37	.16	3.55	.56	.13	5.42	.54	.38	2.11	.07	.06	.09	N/A	N/A	N/A	.18	.03	2.23
P.E. octaldehyde (µg/m^3)	6.93	2.59	10.81	5.58	1.71	27.86	9.12	1.82	16.38	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A

The measured level of benzene is generally low in all of the personal exposure measurements, well under the 8 hours tolerated concentration level for occupational exposure to benzene, which is 1 ppm (3.195 mg/m³) (HSE, 2011).

Toluene is the compound that is found in the higher concentrations, due to the various sources of exposure. In this case, although the median for both airborne and urinary toluene is coherent with the expected exposure (control < living/working in recently built or refurbished building < occupationally exposed), the higher concentrations (range) of airborne toluene are found in the control group, most likely due to certain activities of some subjects. Interestingly, urinary ethylbenzene appears to be much higher in the occupationally exposed subjects than in the other groups, which could indicate a source of ethylbenzene that is typical of some activities of the occupationally exposed group.

The urinary BTEX show a similar pattern, although the differences in the concentration between the different groups are not as high as the ones observed in the personal exposure. Benzene shows a remarkable difference between the occupationally exposed group and the others, however the most relevant difference between the groups is represented by the toluene again. Interestingly, urinary ethylbenzene does not show a pattern that is comparable to the personal exposure data in the occupationally exposed subjects.

When the first and the second sampling were compared (first sampling at the end of the working week and second sampling at the beginning of the working week), in some cases it was observed an increase of some of the urinary BTEX. This finding suggests that some of the subjects' activities of the weekend led the subjects to be exposed to some sources of the analysed compounds. Unfortunately, it was not possible to measure the personal exposure during the day off for the occupationally exposed subjects. In general, it was found that urinary BTEX concentrations are consistent with previously published data from Fustinoni's research group where the analyses were performed with the same methodology (Fustinoni et al., 2010).

Together with the urinary BTEX, urinary 8-oxo-dG was also analysed as a biomarker of DNA damage due to oxidative stress. Urinary 8-oxo-dG median and range were found to be very similar between all of the groups, no significant difference could be identified for any of the groups. In previous papers, it was reported that in general, the variation range of 8-oxo-dG is generally small, even for populations that are different, therefore it is plausible to expect a small variation in our sample subset which is formed mostly of general population (Evans et al., 2010).

The median urinary concentration of each compound in each subgroup was compared with the median concentrations of the other subgroups by nonparametric independent samples median test in SPSS, and the distributions were analysed with Kruskal-Wallis (KW) test and Kolmogorov-Smirnov (KS) test. Kruskal-Wallis test is a nonparametric equivalent of the ANOVA, however, it is used to compare the distributions of the cases, where the null hypothesis is that all the cases of the groups have the same type of distribution (Kruskal and Wallis, 1952). Kolmogorov-Smirnov (KS) test is used instead of KW when distributions of only two groups are compared (Kolmogorov–Smirnov Test, 2008).

No significant difference was observed in the distribution and in the median concentration of any urinary compound between the two sampling events in the living/working in a recently built/refurbished building group. Only in one case (urinary m+p-xylene) the null hypothesis (same distribution in all the groups) was rejected in the occupationally exposed group ($p=0.043$).

The same analysis was performed also comparing the medians and the distributions between the different subgroups, divided also by the sampling event when possible. The results evidenced a significant difference in the medians ($p=0.006$) and the distribution (KW $p<0.001$) of urinary cotinine within the groups. This finding could be explained by the presence of two active smokers in the occupationally exposed group, but also from the ETS exposure that is

typical in some groups (Table 34). As shown in Table 34, 100% of the subjects living/working

in a recently built/refurbished building, are classified as non-ETS exposed, and only a small

fraction of the controls is classified as ETS exposed. In MATCH project and in the

occupationally exposed group, the distribution of the subjects classified with the same

parameters, is much different, showing a significant change in the distribution when compared

to the control and living/working in new/recently refurbished buildings. Since these

classifications were based on the urinary cotinine levels, this difference in the distribution of

the subjects are reflected in the analyses when the groups are compared. The null hypothesis

for 8-oxo-dG median and distribution was retained ($p=0.473$ for the median), showing that the

changes in the urinary concentration of 8-oxo-dG between the subgroups are not statistically

significant. On the other hand, the medians of benzene ($p=0.002$), toluene ($p<0.001$) and

ethylbenzene ($p<0.001$) showed significant differences between the groups together with their

distributions (KW $p<0.001$ for all of the distributions). As mentioned before, these differences

could be arising from the presence of ETS exposed subjects and some active smokers, as

suggested from the similar results of the urinary cotinine. In order to investigate this possibility,

the groups were further divided in smokers, ETS exposed and non-ETS exposed, and the

analysis was repeated. For the non-ETS exposed subjects, the only compound that showed a

statistically significant difference in the median, was ethylbenzene ($p=0.016$), while the

distribution of benzene (KW $p=0.021$), toluene (KW $p=0.033$), ethylbenzene (KW $p<0.001$)

and m+p-xylene (KW $p=0.024$) showed a statistically significant difference. For the ETS

exposed subgroup, the medians of benzene ($p=0.028$), toluene ($p=0.028$) and ethylbenzene

($p=0.002$) showed a statistically significant difference. Similarly, the distribution of benzene

($p=0.022$), toluene (KW $p=0.009$) and ethylbenzene (KW $p=0.002$) were not the same in all

the groups. The smokers' subgroup did not show any significant difference, possibly due to the

high levels of urinary VOCs arising from smoking, but it could be also due to the small number

of subjects. In Table 36 are reported the median concentrations of the urinary VOCs, together with the observed statistically significant differences between the medians and the distributions of each category. Most of the highest values are recorded in the MATCH group, except for the urinary benzene for the ETS exposed subjects, which is found higher in the control group.

Table 36: Overview of the median urinary BTEX concentrations for each group, classified based on the ETS exposure.
(- indicates that none of the subjects were classified in that category, * indicates a statistically significant difference between the medians of the groups of the same category, + indicates a statistically significant difference between the distributions in the groups of the same category)

	Group											
	Control			Living/working in a new or recently refurbished building			Occupationally exposed			MATCH project high exposed subjects		
	Classified as a smoker			Classified as a smoker			Classified as a smoker			Classified as a smoker		
	Non-ETS exposed	ETS exposed	Smoker	Non-ETS exposed	ETS exposed	Smoker	Non-ETS exposed	ETS exposed	Smoker	Non-ETS exposed	ETS exposed	Smoker
	Median	Median	Median	Median	Median	Median	Median	Median	Median	Median	Median	Median
Urinary Benzene (ng/L)	73+	121+	-	69+	-	-	89+	103*+	179	58+	67*+	65
Urinary Toluene (ng/L)	144+	106*+	-	133+	-	-	158+	166*+	190	499+	530*+	583
Urinary Ethylbenzene (ng/L)	19*+	20*+	-	22*+	-	-	27*+	17*+	36	61*+	67*+	247
Urinary M+P-xylene (ng/L)	34+	33	-	36+	-	-	41+	33	47	83+	179	118
Urinary O-xylene (ng/L)	27	34	-	33	-	-	37	29	63	32	85	80
Urinary Naphthalene (ng/L)	46	54	-	45	-	-	42	39	50	93	24	28

These findings suggest that the smoking habits, together with the ETS exposure might represent a confounding factor for the analyses, as one of the main sources of the urinary BTEX could be represented by ETS exposure, although significant differences were observed between the groups even after being divided according to ETS/smoking classification. Another important factor to take in account is that each of the groups is formed by 14-15 subjects (except MATCH project with 41 subjects). Therefore when a further division of each group (control, living or working in a recently built/refurbished building, occupationally exposed and MATCH) in other subgroups, generates one or more subgroups with few cases, and it is

possible that the number of cases is too small to observe significant differences. Moreover, the

distribution of the ETS exposed samples consisted in two main groups of high ETS exposure

and low ETS exposure, causing the median concentration of both cotinine and urinary VOCs

to shift towards higher values, as shown in Figure 33. To counter for this problem, also taking

in account the differences observed in the distribution in Table 34, a different division in

subgroups was performed, using a higher value of urinary cotinine to define non-ETS exposed

subjects (10µg/L). Due to the generally low ETS exposure of the subjects, this passage allowed

to create two main subgroups based on higher and lower ETS exposure, maintaining a high

number of cases throughout the subgroups.
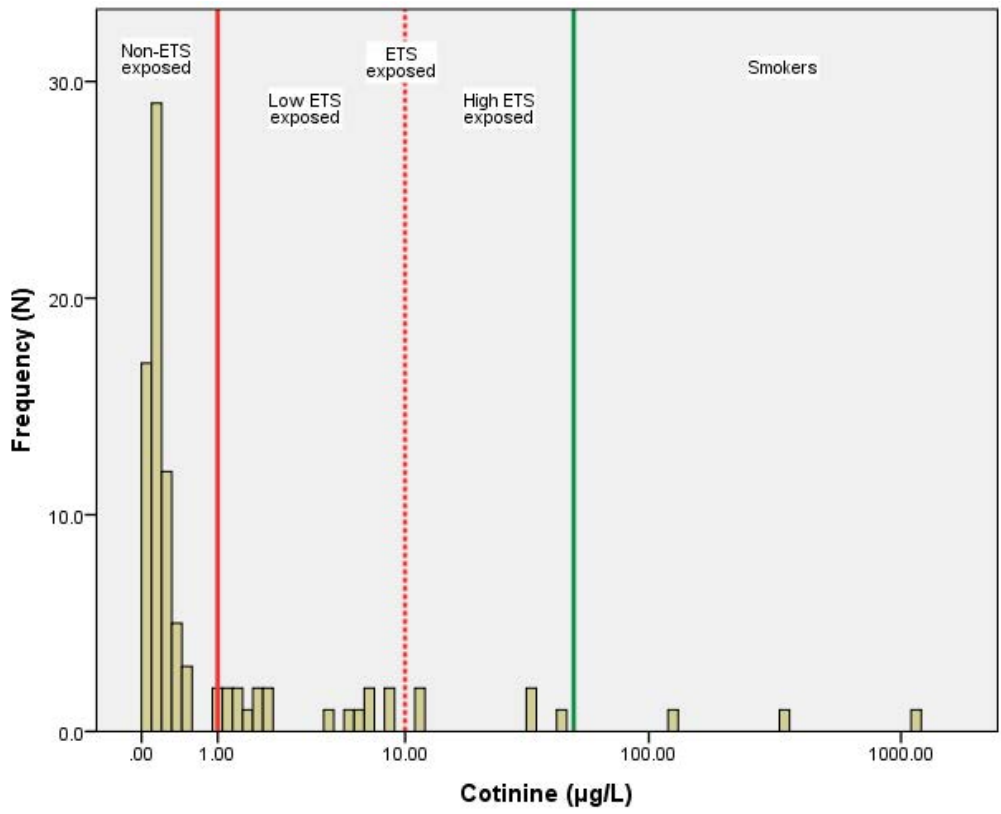
Figure 33: distribution of the FIXAT and MATCH samples based on the cotinine concentration. The red continuous line indicates the 1µg/L treshold of cotinine, the dashed red line indicates the 10µg/L threshold, and the green line represents the threshold for the smokers (50µg/L).

When the median concentrations of the urinary markers were compared after this

subgroup division, the median concentrations of cotinine of each subgroup were found to be

101

statistically different between the groups (FIXAT groups + MATCH) ($p=0.008$). Moreover, the distribution of the data for cotinine concentration was found to be different between the groups ($p<0.001$). In this case, the medians of urinary benzene ($p=0.032$), toluene ($p<0.001$) and ethylbenzene ($p<0.001$) showed a statistically significant difference between the groups. Also the distribution of benzene ($p=0.001$), toluene ($p=0.001$) and ethylbenzene ($p<0.001$) were found to be different between the groups.

In the other subgroup (high ETS exposure and smokers) the medians of urinary toluene ($p=0.007$) and ethylbenzene ($p=0.012$) showed a difference, together with the distributions ($p=0.014$ and $p=0.029$ respectively). No significant difference was observed for the urinary cotinine. Table 37 shows that the main difference for urinary cotinine is observed in MATCH project subjects. When the MATCH subjects were excluded from the statistical analyses, the median concentration of urinary cotinine did not show statistically significant differences between the groups, only the distribution showed a difference ($p=0.042$). After excluding the MATCH subjects, only the median concentration of urinary benzene showed a difference between the groups ($p=0.008$), while the distribution showed a difference for urinary benzene ($p=0.011$) and urinary ethylbenzene ($p=0.008$).

Table 37: Summary of the median concentrations of the measured urinary metabolites in all the groups. The sample pool was obtained including only the subjects with urinary cotinine concentration <10µg/L. (indicates a statistically significant difference in the median between the groups, + indicates a statistically significant difference in the distribution between the groups)*

	Group/sampling event					
	Control Single sampling	Living/working in a new or recently refurbished building First sampling	Living/working in a new or recently refurbished building Second sampling	Occupationally exposed, First sampling	Occupationally exposed Second sampling	MATCH single sampling
	Median	Median	Median	Median	Median	Median
Cotinine (µg/L)* +	0.14	0.12	0.18	0.24	0.23	1.80
8-oxo-dG (nMol/M creatinine)	1.29	1.37	1.15	1.15	1.06	1.25
Urinary Benzene (ng/L)* +	75	69	70	89	92	64
Urinary Toluene (ng/L)* +	134	126	134	174	152	459
Urinary Ethylbenzene (ng/L)* +	19	24	21	23	28	61
Urinary m+p-xylene (ng/L)	34	36	35	35	44	166
Urinary o-xylene (ng/L)	27	35	33	36	36	37
Urinary Naphthalene (ng/L)	46	44	46	41	42	28

Table 38: Summary of the concentrations of urinary metabolites for the group with urinary cotinine >10 µg/L. Control and living/working in new or recently refurbished building groups did not include any subject with such urinary concentrations of cotinine.

	Cotinine >10µg/L			
	Control	Living/working in a new or recently refurbished building	Occupationally exposed	MATCH project high exposed subjects
	Median	Median	Median	Median
Cotinine (µg/L)	.	.	196.66	22.73
8-oxo-dG (nMol/M creatinine)	.	.	1.48	1.46
Urinary Benzene (ng/L)	.	.	152	34
Urinary Toluene (ng/L)	.	.	198	840
Urinary Ethylbenzene (ng/L)	.	.	18	304
Urinary M+P-xylene (ng/L)	.	.	34	180
Urinary O-xylene (ng/L)	.	.	38	105
Urinary Naphthalene (ng/L)	.	.	48	28

These findings suggest that although smoking and ETS exposure are one of the main sources of urinary BTEX and exposure, a significant contribution to the exposure can also be attributable to different sources and activities. In Table 37 is shown a summary of the medians of each group after the elimination of smokers and subjects with high ETS exposure. The observed BTEX values are compatible with previously reported data for non smoking subjects (Fustinoni et al., 2010). It is still possible to identify a trend, especially with toluene, where higher levels of urinary cotinine tend to be associated with higher levels of urinary BTEX, and the results show that the difference is statistically significant.

As mentioned above, the main difference in urinary BTEX concentrations are represented by the MATCH subjects, where the median concentrations of most compounds (except benzene, o-xylene and naphthalene) are much higher than in any of the other groups. For the FIXAT project, toluene is found to be higher in the occupationally exposed subjects, together with benzene. Interestingly, most of the compounds did not show a significant change between the first and the second sampling, although a slight reduction of 8-oxo-dG median concentration is observed. Taking in account the work from Evans et al. (2010), where they reported changes in 8-oxo-dG around 20-30%, we could conclude that the change observed in both occupationally exposed and living/working in recently built/refurbished buildings between the first and the second sampling are relevant regarding the oxidative stress. There is a decrease of 17% between the median concentration of 8-oxo-dG of the first and the second sampling of the living/working in a recently built or refurbished building, and a decrease of 8% between the first and the second sampling of occupationally exposed subjects. These variations, however were found to be not statistically significant, due to the inter-personal variation of urinary 8-oxo-dG and the differences in the distribution of the data, and the relatively small sample pool. The difference in the concentration of urinary 8-oxo-dG between the two sampling events in two of the groups, although not being statistically significant, is highly

valuable, as it is obtained from two different samplings of the same subjects. This procedure, especially in the case of the occupationally exposed group that were sampled twice in a short period of time, minimizes the inter-sample variations.

A paired samples t-test was performed where two samplings were performed, in order to investigate any differences between the first and the second sampling. No statistically significant differences were observed for any of the urinary VOCs nor for the 8-oxo-dG, although for this last variable the significance was $p=0.072$. Marginally significant differences were observed for urinary ethylbenzene ($p=0.027$) and m+p-xylene ($p=0.026$) between the two samplings of the occupationally exposed subjects. These results could be due to the small number of samples in each subgroup, however the observed p values, except in the mentioned cases, were always found >0.200.

IV.3 Sub-categories comparisons

The collected data was analysed for skewness in order to determine the distribution. All of the biomarkers and the personal exposure data was found to be skewed towards the right (skewness value >0). All the following analyses were performed on the log transformed data. Even after the log transformation, urinary BTEX and naphthalene, showed a skewness coefficient ranging between 0.7 and 1.7, indicating that the data distribution is still not symmetrical after the transformation, this is probably due to the high variability between the different samples. A similar behaviour was observed also when the skewness was analysed for each group.

The mean concentrations of the compounds in each of the groups were compared using the ANOVA test followed by Bonferroni post hoc comparison, for each of the grouping variable obtained from the questionnaire data with multiple answers and T test for dichotomies. This procedure was performed in order to identify the groups that showed statistically significant differences in the urinary concentrations of the metabolites. Taking in account the results obtained in the previous section and due to the skewness of the data, the results obtained with the ANOVA test and the Bonferroni post hoc comparisons were then verified by nonparametric median comparison, and the distributions were compared by Kruskal-Wallis 1-way ANOVA and Kolmogorov-Smirnov tests.

Differences between genders:

Table 39: Representation of the analysed subgroups.

Control		Living/working in recently built or refurbished buildings		Occupationally exposed		MATCH	
Males	Females	Males	Females	Males	Females	Males	Females

No significant difference was observed in the variance of any of the urinary metabolites when the two genders were compared, while a significant difference was observed for the personal exposure data for benzene ($p=0.003$), toluene ($p=0.027$) and ethylbenzene ($p<0.001$). This result can be explained by the high difference on the personal exposure data of occupationally exposed subjects, which are only male volunteers, and the rest of the volunteers' personal exposure. This finding was confirmed by the nonparametric comparisons, which did not show any significant differences between the two genders. Only the personal exposure measurements of o-xylene ($p=0.042$) and m-xylene ($p=0.027$) showed a difference in the distribution between male and female subjects in the control subgroup.

FIXAT subgroups and MATCH comparison:

Table 40: Representation of the analysed subgroups.

Control	Living/working in recently built or refurbished buildings	Occupationally exposed	MATCH

Table 41: Summary of the statistically significant differences observed in the ANOVA test between the sampling subgroups.

	ANOVA test *p* value	Subgroup showing significant difference
PE benzene	<0.001	Occupationally exposed
PE ethylbenzene	<0.001	Occupationally exposed
PE toluene	0.044	Occupationally exposed
Urinary naphthalene	0.004	MATCH
Urinary VOCs	<0.001	MATCH
Urinary o-xylene	0.124	

ANOVA test was performed with Bonferroni post hoc multiple comparison, to evaluate the differences in the measured concentrations of airborne VOCs between the various groups. A significant difference was observed in the personal exposure data for benzene ($p<0.001$) and ethylbenzene ($p<0.001$), while a marginally significant difference was observed for toluene ($p=0.044$). These results are due to the major difference observed in the personal exposure data of the occupationally exposed subgroup, which is generally higher than all the other subgroups (FIXAT and MATCH). All of the urinary VOCs showed a statistically significant difference between the subgroups, with p values all under 0.001, except for o-xylene ($p=0.124$) and naphthalene ($p=0.004$). The findings were confirmed by the nonparametric comparisons, which showed similar results, except for all of the xylenes, which did not show significant differences.

Bonferroni post hoc comparison showed that only the MATCH project samples showed a statistically significant difference from the other groups.

Table 42: Summary of the statistically significant differences observed in the ANOVA test between the sampling subgroups, after elimination of MATCH subset.

	ANOVA test p value	Subgroup showing significant difference (FIXAT only)
Urinary Benzene	<0.001	Occupationally exposed
Urinary Cotinine	0.001	Occupationally exposed
Urinary Ethylbenzene	0.028	Occupationally exposed
Urinary m+p xylene	0.015	Occupationally exposed
Urinary Naphthalene	0.036	Occupationally exposed
PE Benzene	<0.001	Occupationally exposed
PE Ethylbenzene	<0.001	Occupationally exposed
PE Toluene	0.044	Occupationally exposed

To further evaluate the differences in the FIXAT subgroups only, the MATCH samples were removed from the calculations, and another ANOVA test was performed for the same

variables. In this case, statistically significant differences were observed only in the concentration urinary benzene ($p<0.001$) and cotinine ($p=0.001$). Marginally significant differences were observed for urinary ethylbenzene ($p=0.028$), m+p-xylene ($p=0.015$) and naphthalene ($p=0.036$). The personal exposure data showed statistically significant differences only for benzene ($p<0.001$) and ethylbenzene ($p<0.001$), and a marginally significant difference for toluene ($p=0.044$). Nonparametric comparisons confirmed the findings for benzene ($p=0.005$) and m+p-xylene ($p=0.036$) but not for urinary cotinine ($p=0.148$), however, the distribution of cotinine showed to be not the same between all the groups (KW $p=0.005$), suggesting non-homogeneous groups regarding the ETS exposure and the smoking habits. The nonparametric comparison of the personal exposure data showed significant differences between the subgroups for benzene ($p=0.001$), toluene ($p=0.029$), ethylbenzene ($p<0.001$) and pyridine ($p<0.001$).

Bonferroni post hoc comparison showed that the significant differences were mostly to be attributed to the occupationally exposed subjects. The cotinine levels were found to be the lowest in the living/working in a recently built or refurbished building, which however showed a statistically significant difference only with the occupationally exposed group. The control group, although having a higher mean concentration of urinary cotinine, showed a statistically significant difference with the occupationally exposed subjects. It is important to remember that the occupationally exposed group contained actively smoking and high ETS exposed subjects. Due to the strong correlation between smoking habits and urinary benzene, the results should be evaluated carefully. For urinary benzene, the difference was significant between the occupationally exposed and all the other subgroups, while for urinary ethylbenzene, m+p-xylene and naphthalene, the difference was significant only between the occupationally exposed and the control group. The personal exposure data showed a significant difference for benzene and ethylbenzene between the occupationally exposed subgroup and all the others,

while for toluene the significant difference was only between the occupationally exposed subjects and the control group.

Table 43:Summary of the statistically significant differences observed in the ANOVA test between the sampling subgroups after elimination of ETS exposed and smoking subjects.

	ANOVA test *p* value	Subgroup showing significant difference (FIXAT non-ETS exposed only)
Urinary Benzene	0.011	Occupationally exposed
Urinary Ethylbenzene	0.002	Occupationally exposed
Urinary m+p xylene	0.010	Occupationally exposed
PE Benzene	<0.001	Occupationally exposed
PE Ethylbenzene	<0.001	Occupationally exposed
PE pyridine	<0.001	Occupationally exposed
PE styrene	0.035	Occupationally exposed

Further comparison analyses were performed for the non-ETS exposed subjects (urinary cotinine <1µg/L), in order to eliminate the ETS exposure as a confounding factor. When the non-ETS exposed subjects' measurements (urinary cotinine <1µg/L) were compared between the different groups with the ANOVA test, no statistically significant difference was observed for urinary cotinine, between the different groups. Statistically significant differences were observed for urinary benzene ($p=0.011$), ethylbenzene ($p=0.002$), m+p-xylene ($p=0.010$). The personal exposure data showed a similar pattern, with statistically significant differences between the groups for benzene ($p<0.001$), ethylbenzene ($p<0.001$) pyridine ($p<0.001$) and styrene ($p=0.035$). These results were confirmed in the nonparametric tests, except for m+p-xylene. In addition, the median concentration of toluene in the personal exposure measurement showed a statistically significant difference between the groups.

The post hoc comparisons showed that only the difference between occupationally exposed and living/working in a recently built/refurbished building subgroups was statistically significant, with the occupationally exposed showing the higher concentration.

110

Urinary ethylbenzene concentration difference was found to be statistically significant between occupationally exposed and control subgroups. Urinary m+p-xylene difference was found to be statistically significant between the occupationally exposed group and all the others.

The personal exposure measurements all showed statistically significant differences between the occupationally exposed and all of the other subgroups. This could be due not only to the different airborne concentrations, but also to the different sorbent tubes used in the measurements of that subgroup. However, the fact that these differences are reflected in the urinary measurements, supports the hypothesis that the difference is due to the higher exposure of the subjects to those compounds.

FIXAT occupationally exposed and living/working in a recently built or refurbished building, first and second sampling comparison:

Table 44: Representation of the analysed subgroups.

Control	Living/working in recently built or refurbished buildings		Occupationally exposed		MATCH
Single sampling	1st sampling	2nd sampling	1st sampling	2nd sampling	Single sampling

Table 45: Summary of the ANOVA test results comparing the first sampling events of the various subgroups.

	ANOVA test p value	Subgroup showing significant difference (First sampling and single sampling)
Urinary benzene	0.006	Occupationally exposed
PE benzene	<0.001	Occupationally exposed
PE ethylbenzene	<0.001	Occupationally exposed

The sample pool, after being divided in groups, was then further separated, since both occupationally exposed subjects and living/working in a recently built/refurbished building had two sampling events. For the first sampling event, the significant differences were observed only for urinary benzene ($p=0.006$). Personal exposure data showed significant differences

between the subgroups for benzene ($p<0.001$) and ethylbenzene ($p<0.001$). The findings were confirmed by the nonparametric comparisons.

Post hoc comparisons in this case showed a statistically significant difference between occupationally exposed subjects and subjects living or working in a recently built or refurbished building, while the difference was not significant between any of the other groups and the control group, due to the high variance in the group. The personal exposure data showed a statistically significant difference for the benzene and ethylbenzene concentration between the occupationally exposed group and the others.

Table 46: Summary of the ANOVA test results comparing the second sampling event of the various subgroups.

	ANOVA test p value	Subgroup showing significant difference (First sampling and single sampling)
Urinary benzene	0.035	Occupationally exposed
Urinary m+p xylene	0.014	Occupationally exposed
PE benzene	0.008	Post-hoc could not be performed
PE ethylbenzene	0.050	Post-hoc could not be performed

The second sampling event for the occupationally exposed subjects took place after the weekend, to allow the work related exposure build-up of the working week to be eliminated, but in this occasion the personal exposure was not measured, while for the subjects living or working in a recently built or refurbished building, the second sampling took place after one year. ANOVA test showed a marginally significant difference between the groups for urinary benzene ($p=0.035$) and for m+p-xylene ($p=0.014$) but not for any other urinary biomarker. However, in this case no significant difference was observed in the nonparametric comparisons. Post hoc comparison revealed that the difference was again between the occupationally exposed group and all the others for urinary benzene, and between occupationally exposed group and control group for urinary benzene and m+p-xylene. Taking in account the results of the nonparametric comparisons, it is possible that the difference

observed in the ANOVA test is due to the difference in the distribution of the occupationally exposed subgroup.

Airborne benzene ($p=0.008$) and ethylbenzene ($p=0.050$) showed a statistically significant difference for personal exposure, but due to the missing data for the occupationally exposed subjects, post hoc comparisons could not be performed.

8-oxo-dG comparison between FIXAT and MATCH subgroups:

Table 47: Representation of the analysed subgroups.

Control	Living/working in recently built or refurbished buildings	Occupationally exposed	MATCH

8-oxo-dG concentration did not show significant differences between any the groups in any of the tests performed.

ETS exposure classification based on urinary cotinine comparison:

Table 48: Representation of the analysed subgroups.

Control			Living/working in recently built or refurbished buildings			Occupationally exposed			MATCH
Non ETS exposed	ETS exposed	Smokers	Non ETS exposed	ETS exposed	Smokers	Non ETS exposed	ETS exposed	Smokers	Not included in the analyses

Another set of ANOVA tests were performed for the different ETS exposure groups. The ETS exposure classification, based on the urinary cotinine concentration (non-ETS exposed, ETS exposed, smoker), underlined a strong linkage between tobacco smoke and urinary benzene, as mentioned previously in section IV.2 and as reported in previous works (Manini et al., 2010; Lovreglio et al., 2011). Only the subjects from FIXAT project were selected, as the samples chosen from MATCH project were chosen on purpose along the highest exposures, including ETS.

Table 49: Summary of the results from ANOVA test between the three different ETS groups based on the urinary cotinine concentration.

	ANOVA test p value	Subgroup showing significant difference (First sampling and single sampling)
Urinary benzene	<0.001	Non-ETS exposed (lower conc.)
PE ethylbenzene	<0.001	Non-ETS exposed (lower conc.)

Only the urinary benzene showed a statistically significant difference between the subgroups ($p<0.001$), where the non-ETS exposed had the lowest urinary benzene concentration, with a statistically significant difference with the other two groups. The subjects classified as smokers and ETS exposed, showed a significant difference only with the non-ETS exposed, which is however coherent with previously published data (Manini et al., 2010). Furthermore, in the same work, the levels of 8-oxo-dG were found to be generally higher than the levels measured in our study, and showed a statistically significant difference between smokers and non-ETS exposed only in the higher concentrations. In our case, the smokers were a small portion of the total sample pool and, as mentioned in section IV.2, most of the ETS exposed subjects had very low ETS exposures.

Another significant difference was observed for personal exposure levels of ethylbenzene. In that case, although the majority of the smokers are occupationally exposed subjects, that have a generally higher exposure to ethylbenzene, the difference was found between the non-ETS exposed and the ETS exposed subgroups. The ETS exposed subjects, however, are more represented in the occupationally exposed group, which have a generally higher measured level of personal exposure to ethylbenzene.

Age range comparison:

Table 50: Representation of the analysed subgroups.

Control	Living/working in recently built or refurbished buildings	Occupationally exposed	MATCH
Age ranges	Age ranges	Age ranges	Age ranges

The age range subgroups were compared with the ANOVA test. Due to the distribution of the population, the results could not be taken in account, as the age range that showed statistically significant difference was the age range 36-45. This age range is represented mainly in MATCH project subjects and only a small proportion is represented in FIXAT project (Table 34), this would cause biased analyses due to the grouping of a single age range in high exposure group.

Commuting method comparison:

Table 51: Representation of the analysed subgroups.

Control	Living/working in recently built or refurbished buildings	Occupationally exposed	MATCH
Commuting methods	Commuting methods	Commuting methods	Commuting methods

Table 52: Summary of the results of ANOVA test between the various commuting methods for urinary and PE VOCs.

	ANOVA test p value	Subgroup showing significant difference (Commuting method)
Urinary toluene	0.006	Commuting on foot (higher conc.) vs. cycling (lower conc.)
Urinary naphthalene	0.009	Commuting on foot (higher conc.) vs. cycling (lower conc.)
Urinary o-xyene	0.024	Commuting on foot (higher conc.) vs. cycling (lower conc.)
PE ethylbenzene	0.023	Commuting on foot (higher conc.) vs. cycling (lower conc.)
PE pyridine	0.040	Commuting on foot (higher conc.) vs. cycling (lower conc.)

The different commuting methods (Table 34), when compared with the ANOVA test, showed statistically significant differences between the different commuting subgroups for urinary toluene ($p=0.006$) and naphthalene ($p=0.009$), and marginally significant differences for o-xylene ($p=0.024$). Post hoc test revealed that the higher levels of urinary toluene were measured in the subjects that commuted on foot, and the lowest for subjects who commuted by cycling. However, these findings were not confirmed by the nonparametric comparisons, where only o-xylene median concentration showed a statistically significant difference between the method of commute. The analysis of the personal exposure data showed a statistically significant difference of ethylbenzene ($p=0.023$) and pyridine ($p=0.040$) between the different commuting methods.

Table 53: Summary of the results of the nonparametric comparison tests between the different methods of commuting.

	Nonparametric test *p* value	Kind of difference observed in nonparametric test (Commuting method)
8-oxo-dG	0.040	Distribution
PE benzene	0.013	Median (Walking, highest concentration, cycling, lowest concentration)

Interestingly, MATCH project showed a statistically significant difference in the distribution of 8-oxo-dG between the different method of commute ($p=0.040$), together with a statistically significant difference of the median concentration of airborne benzene measured in the personal exposure ($p=0.013$). The statistically significant differences were observed between commuting by cycling, car and walking. Interestingly the commuting by car was not significantly different from commuting by walking, which showed the highest levels of urinary benzene. Also in this case, the distribution of the volunteers in the various commuting subgroups could play an important role on the interpretation of the data, but it is interesting to

notice that urinary toluene did not show any particular variation in the concentration for any of the other groupings. Interestingly, urinary naphthalene was found in the higher concentrations in the group that commute by cycling, and it showed a significant difference with the subjects that did not commute and that commuted by car.

Comparison between subjects using a vehicle for work and others:

Table 54: Representation of the analysed subgroups.

Control		Living/working in recently built or refurbished buildings		Occupationally exposed		MATCH	
Vehicle used for work	No vehicles used for work	Vehicle used for work	No vehicles used for work	Vehicle used for work	No vehicles used for work	Vehicle used for work	No vehicles used for work

Urinary benzene was found to be significantly different when grouped by the subjects that used a vehicle for work and the subjects who did not ($p=0.003$), even in this case the distribution of the subjects shows that most of the subjects that use a vehicle for work are in the occupationally exposed subgroup.

Comparison between groups based on average yearly mileage:

Table 55: Representation of the analysed subgroups.

Control	Living/working in recently built or refurbished buildings	Occupationally exposed	MATCH
Average yearly mileage	Average yearly mileage	Average yearly mileage	Average yearly mileage

Table 56: Summary of ANOVA results on the comparisons between the various categories based on the yearly mileage.

	ANOVA test *p* value	Subgroup showing significant difference (Average yearly mileage)
8-oxo-dG	0.003	<5,000 miles/year (vs. >30,000 miles/year) **lowest concentration observed for subjects between 5,000 and 15,000 miles/year**
Urinary toluene	0.004	>30,000 miles/year
Urinary benzene	0.016	<5,000 miles/year (vs. >30,000 miles/year)
Urinary ethylbenzene	0.014	>30,000 miles/year
Urinary m+p-xylene	0.033	<5,000 miles/year (vs. >30,000 miles/year)
Urinary o-xylene	0.032	<5,000 miles/year (vs. >30,000 miles/year)
PE benzene	0.003	<5,000 miles/year (vs. >30,000 miles/year)
PE ethylbenzene	0.016	<5,000 miles/year (vs. >30,000 miles/year)

When the sample pool is grouped by the average yearly mileage, it is interesting to notice that the 8-oxo-dG shows a statistically significant difference between the subgroups (p=0.003) together with urinary toluene (p=0.004), while a marginally significant difference is observed for urinary benzene (p=0.016), ethylbenzene (p=0.014), m+p-xylene (p=0.033) and o-xylene (p=0.032). These results are confirmed in the nonparametric comparisons for 8-oxo-dG, toluene and ethylbenzene, but not for the xylenes and ethylbenzene.

Interestingly, this pattern is not replicated for personal exposure, where a significant difference is observed for benzene (p=0.003) and a marginally significant difference is observed for ethylbenzene (p=0.016), while toluene does not show any significant difference between the subgroups (p=0.825). Similar results were observed also in the nonparametric comparisons.

Post hoc comparisons showed that 8-oxo-dG concentrations are lowest for the group that drives between 5,000 and 15,000 miles per year, and the difference is statistically significant between the groups of subjects that drive less than 5,000 miles per year and more than 30,000 miles per year. Also in this comparison, the distribution of the subjects in the subgroups can contribute to the observed differences, as most of the control subjects are driving 5,000 miles or less, while occupationally exposed subjects and MATCH project subjects have generally higher yearly mileages. In particular, MATCH project is the only group where the >30,000 miles/year subgroup is represented. However, the lowest observed concentrations for 8-oxo-dG were observed in the subgroup formed by subjects with an average yearly mileage between 5,000 and 15,000. This subgroup is largely represented by the occupationally exposed subjects, which, in general have slightly higher urinary concentrations of unmetabolized VOCs, but not higher levels of urinary 8-oxo-dG. This separation characterizes a group with higher levels of oxidative stress which can be correlated to the average yearly mileage that is compatible with a more intensive use of the car. Another interesting factor to observe, is the high level of 8-oxo-dG found in the groups with the least yearly mileage, or that do not commute using a car. This finding could suggest that the intensive use of a car (compatible with more time spent in traffic) contributes to the oxidative stress, but also a very minimal use of the car, with intensive use of alternative forms of transportation, could as well contribute to the oxidative stress. The results are summarised in Figure 34 where an increasing trend can be observed with the higher yearly mileages, but a high variability of the data is observed for the lower yearly mileages.

Urinary toluene and ethylbenzene were found to be higher in the subjects that drive more than 30,000 miles per year, while the other groups did not show statistically significant differences between them. That particular subgroup is represented only in the MATCH project, therefore the difference that is observed can be related to the higher urinary concentration of toluene in the MATCH subjects that might be arising from other sources.
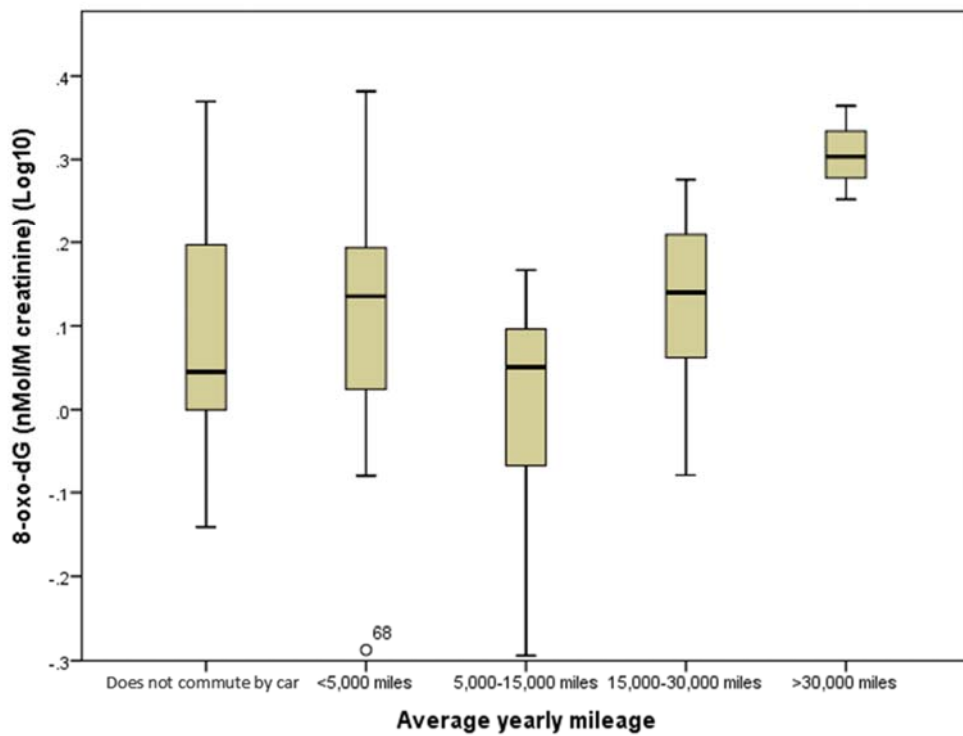
Figure 34: Boxplot showing the mean concentration of 8-oxo-dG in each category defined by the average yearly mileage (full data set).

Discussion of the data and conclusions on key comparisons and groups:

In conclusion, the data shows a linkage between ETS exposure and urinary VOCs. However, when the higher ETS exposure subjects are removed from the database, it is still possible to observe variations in the urinary VOC concentrations between the various groups. The most valuable differences were observed between the urinary VOCs concentration in the various subgroups when the higher ETS exposed subjects were removed from the database. Although the difference of median concentration of urinary cotinine between the groups was not found to be statistically significant, the urinary VOCs median concentrations still showed a statistically significant difference, suggesting that sources different from ETS are contributing to the exposure.

Another remarkable difference that was observed is the general reduction of the median concentration of 8-oxo-dG in the second samplings of occupationally exposed subjects (Table 37). Although this reduction is found to be not statistically relevant, it is still an important finding, as the observed variation is coherent with previously reported data for other activities (i.e. smoking) (Evans et al., 2010). Unfortunately, in our case due to the relatively small number of subjects and the high inter-personal variability of 8-oxo-dG, although a difference was observed, this was not found to be statistically significant between the two subgroups. A larger population would make this comparison more meaningful, because of the many factors that can influence oxidative stress, such as diet, health condition or inflammatory response to pollution-unrelated sources, which would be mitigated in a larger sample (Chen et al., 1999; Evans et al., 2010; Halliwell, 2007; Knaapen et al., 2006).

It is also important to underline the difference observed in the urinary concentration of 8-oxo-dG when the sample pool was divided by the average yearly mileage (Figure 34). Although this division is strictly connected with the distribution of the subjects between the subgroups (MATCH and occupationally exposed subjects generally had higher yearly

mileages), the outcome is still valuable, as it is possible to identify a significant difference in the oxidative stress biomarker between different categories. Moreover, the data suggests the existence of a threshold, which will be discussed in the next section, under which it is not possible to determine any differences, due to the contribution of other factors to the oxidative stress.

One of the most important factors to take in account in our dataset is the contribution of active smoking and ETS exposure to both oxidative stress and urinary VOCs. When the cases were pooled together, including non-ETS exposed subjects and low ETS exposed (urinary cotinine <10µg/L) in the same subgroup, it was possible to identify differences between the subgroups for all of the urinary VOCs, in some cases for the 8-oxo-dG, but the cotinine levels did not show significant differences. This finding underlines the importance of eliminating confounding factors such as ETS exposure, which can bias the data due to the high impact that it has on urinary VOCs even when a small number of samples is affected. Figure 35 shows also that the ETS exposure is not related to the 8-oxo-dG levels when the subjects are divided based on the average yearly mileage.
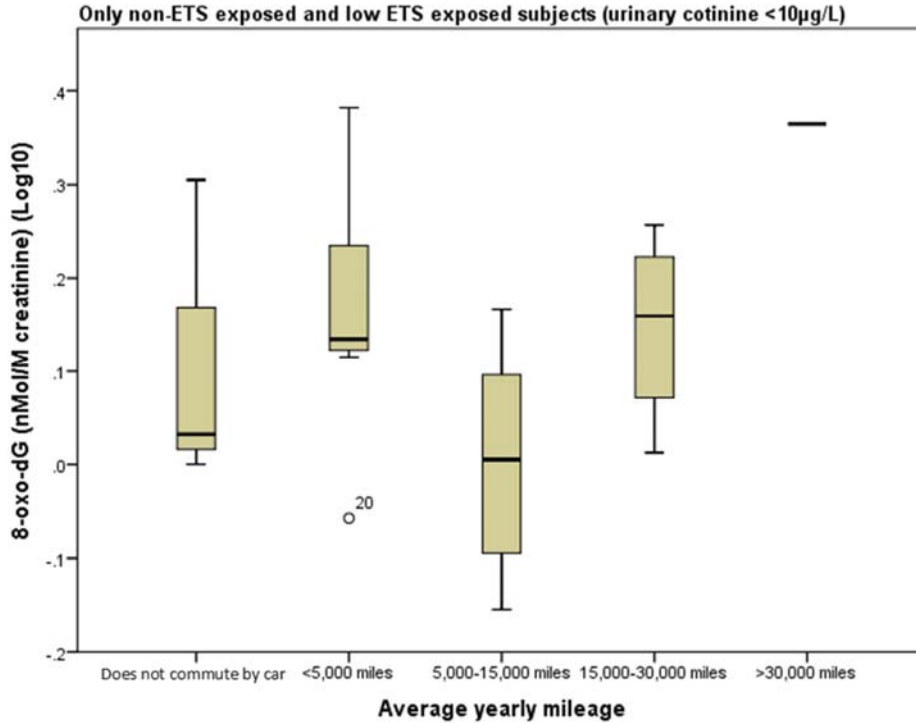
Figure 35: Boxplot showing the mean concentration of 8-oxo-dG in each category defined by the average yearly mileage, after the elimination of subject exposed to high levels of ETS and smokers.

Similar results were obtained when the urinary concentrations of 8-oxo-dG were compared between the different subgroups including and excluding the higher ETS exposed subjects and the smokers (Figure 36 and Figure 37). When the concentrations were compared including the smokers and higher ETS exposed subjects, no difference was observed between the groups (Figure 36). The distribution of ETS exposed subjects prevented the comparison of only non-ETS exposed subjects, due to the uneven distribution of ETS exposed subjects.
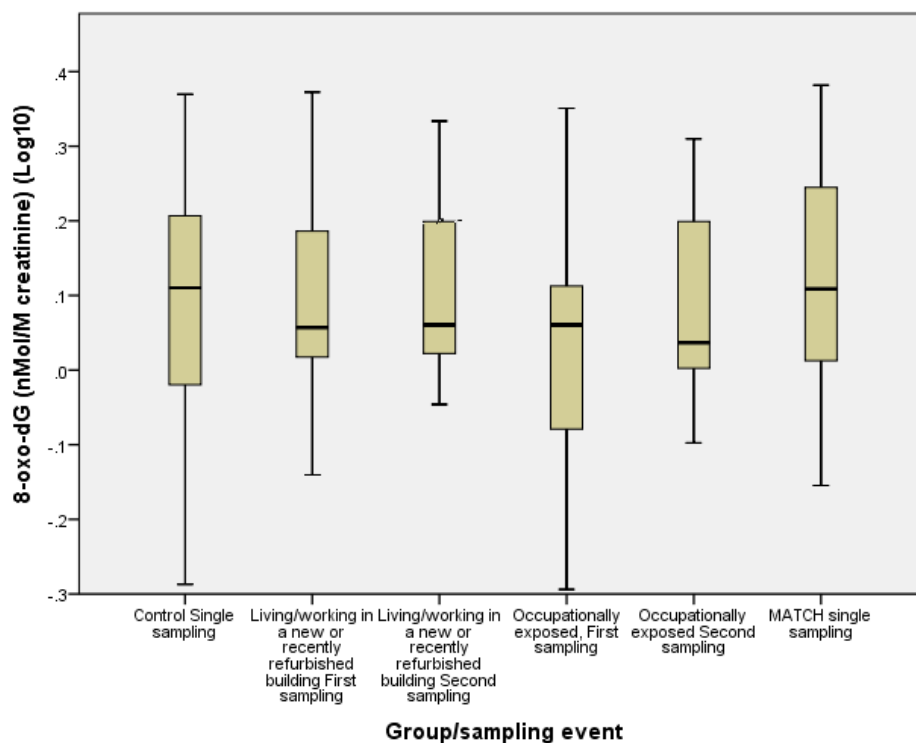
Figure 36:Boxplot showing the mean concentrations of 8-oxo-dG for each group (full data set).

Using a less stringent division, it was possible to eliminate the outliers (high ETS exposed and smoking subjects), without affecting the urinary cotinine median distribution, which, in fact, did not show statistically significant differences between the FIXAT and MATCH groups after the removal (as mentioned previously in this section). When only the subjects with higher ETS exposure and the smokers were removed from the database, it was possible to observe differences in the mean concentrations of urinary 8-oxo-dG (Figure 37). Although the observed differences were not statistically significant, it was possible to assess a decrease in the oxidative stress in the second sampling event in both living/working in a recently built or refurbished building, and occupationally exposed group.
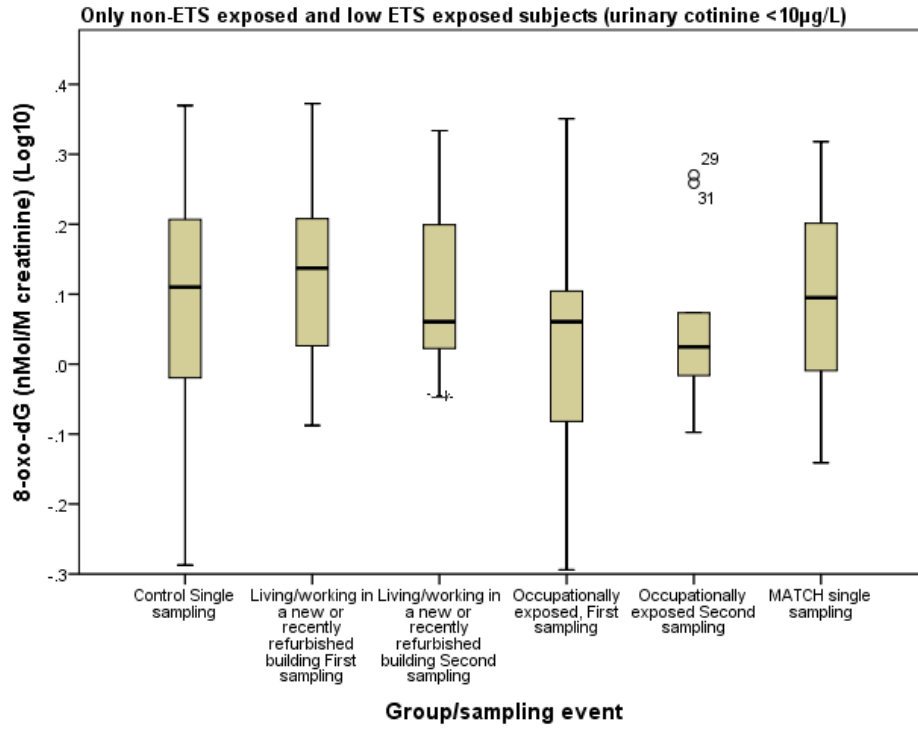
Figure 37: Boxplot showing the mean concentrations of 8-oxo-dG for each group and sampling event after the removal of high ETS exposed subjects and smokers.

IV.4 Correlation analyses

Urinary BTEX correlations in different subgroups and categories of ETS exposure

A bivariate Pearson correlation matrix was created with all the measured variables (Table 61). When the urinary metabolites were analysed, benzene was found to be negatively correlated with the rest of the urinary VOCs, while it was correlated with urinary naphthalene. This finding is in contrast with previously reported data, where benzene was found to be correlated to the other urinary VOCs (Fustinoni et al., 2010). Toluene instead, showed a significant correlation with the other VOCs, except for naphthalene. Due to these findings, three different variables were created, using the sum of different groups of compounds. The variable "Sum of BTEX + naphthalene" included the sum of all the urinary VOCs, "Sum of BTEX" included the sum of all the VOCs excluding naphthalene, and "Sum of TEX" included the sum of the urinary VOCs excluding benzene and naphthalene. The correlations were analysed again with the sample pool divided by subgroup, obtaining a correlation matrix for each subgroup of subjects. The obtained correlation coefficients maintained a pattern similar to the one observed for the complete pool only for the occupationally exposed group (p values all ≤0.010, with benzene showing no correlation and naphthalene showing a negative correlation), as shown in Table 58, and the MATCH project samples (p values all <0.001, except for benzene and naphthalene where, again, the correlation coefficients are negative), as shown in Table 57.

Table 57: Summary of correlations between urinary BTEX and naphthalene in the MATCH subgroup.

MATCH subgroup

		Urinary Benzene (ng/L) (Log10)	Urinary Toluene (ng/L) (Log10)	Urinary Ethylbenzene (ng/L) (Log10)	Urinary M+P-xylene (ng/L) (Log10)	Urinary O-xylene (ng/L) (Log10)
Urinary Toluene (ng/L) (Log10)	Pearson Correlation	-.117	1			
	Sig. (2-tailed)	.498				
Urinary Ethylbenzene (ng/L) (Log10)	Pearson Correlation	-.277	.686**	1		
	Sig. (2-tailed)	.102	.000			
Urinary M+P-xylene (ng/L) (Log10)	Pearson Correlation	-.491*	.763**	.907**	1	
	Sig. (2-tailed)	.024	.000	.000		
Urinary O-xylene (ng/L) (Log10)	Pearson Correlation	-.085	.740**	.697**	.781**	1
	Sig. (2-tailed)	.621	.000	.000	.000	
Urinary Naphthalene (ng/L) (Log10)	Pearson Correlation	.184	-.551**	-.209	-.288	-.431*
	Sig. (2-tailed)	.412	.006	.340	.340	.040

*. Correlation is significant at the 0.05 level (2-tailed).

**. Correlation is significant at the 0.01 level (2-tailed).

Table 58: Summary of the correlations between the urinary BTEX and naphthalene for the occupationally exposed subgroup.

Occupationally exposed subjects subgroup (FIXAT)

		Urinary Benzene (ng/L) (Log10)	Urinary Toluene (ng/L) (Log10)	Urinary Ethylbenzene (ng/L) (Log10)	Urinary m+p-xylene (ng/L) (Log10)	Urinary O-xylene (ng/L) (Log10)
Urinary Toluene (ng/L) (Log10)	Pearson Correlation	.373	1			
	Sig. (2-tailed)	.050				
Urinary Ethylbenzene (ng/L) (Log10)	Pearson Correlation	.316	.490**	1		
	Sig. (2-tailed)	.101	.008			
Urinary M+P-xylene (ng/L) (Log10)	Pearson Correlation	.261	.616**	.899**	1	
	Sig. (2-tailed)	.179	<0.001	<0.001		
Urinary O-xylene (ng/L) (Log10)	Pearson Correlation	.290	.478*	.592**	.629**	1
	Sig. (2-tailed)	.135	.010	.001	<0.001	
Urinary Naphthalene (ng/L) (Log10)	Pearson Correlation	-.123	-.443*	-.400*	-.492**	-.179
	Sig. (2-tailed)	.534	.018	.035	.008	.363

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

On the other hand, in the subjects of control group and those living/working in a recently built or refurbished building, not all the urinary VOCs were correlated with each other. The urinary VOCs from the group with volunteers living/working in recently build or refurbished buildings showed correlations between toluene and ethylbenzene ($p=0.048$), ethylbenzene and m+p-xylene ($p=0.004$) and m+p-xylene with o-xylene ($p<0.001$). The results are summarized in Table 59.

Table 59: Summary of the correlations between urinary BTEX and naphthalene for the FIXAT subgroup living/working in a new or recently refurbished building.

Living/working in a new or recently refurbished building subgroup (FIXAT)

		Urinary Benzene (ng/L) (Log10)	Urinary Toluene (ng/L) (Log10)	Urinary Ethylbenzene (ng/L) (Log10)	Urinary M+P-xylene (ng/L) (Log10)	Urinary O-xylene (ng/L) (Log10)
Urinary Toluene (ng/L) (Log10)	Pearson Correlation	-.389[*]	1			
	Sig. (2-tailed)	.034				
Urinary Ethylbenzene (ng/L) (Log10)	Pearson Correlation	-.219	.376[*]	1		
	Sig. (2-tailed)	.262	.048			
Urinary M+P-xylene (ng/L) (Log10)	Pearson Correlation	-.143	.115	.521[**]	1	
	Sig. (2-tailed)	.451	.544	.004		
Urinary O-xylene (ng/L) (Log10)	Pearson Correlation	-.280	.211	.263	.605[**]	1
	Sig. (2-tailed)	.134	.264	.176	.000	
Urinary Naphthalene (ng/L) (Log10)	Pearson Correlation	-.120	.081	-.215	-.153	.007
	Sig. (2-tailed)	.534	.677	.281	.428	.971

*. Correlation is significant at the 0.05 level (2-tailed).

**. Correlation is significant at the 0.01 level (2-tailed).

Urinary VOCs of the control group showed statistically significant correlations only between ethylbenzene and m+p-xylene ($p=0.003$) and between toluene and naphthalene ($p=0.019$), as shown in Table 60.

.

Table 60: Summary of the correlations between the urinary BTEX and naphthalene in the control subgroup.

		Urinary Benzene (ng/L) (Log10)	Urinary Toluene (ng/L) (Log10)	Urinary Ethylbenzene (ng/L) (Log10)	Urinary M+P-xylene (ng/L) (Log10)	Urinary O-xylene (ng/L) (Log10)
Urinary Toluene (ng/L) (Log10)	Pearson Correlation	.204	1			
	Sig. (2-tailed)	.449				
Urinary Ethylbenzene (ng/L) (Log10)	Pearson Correlation	-.011	-.293	1		
	Sig. (2-tailed)	.969	.289			
Urinary M+P-xylene (ng/L) (Log10)	Pearson Correlation	.064	.136	.705**	1	
	Sig. (2-tailed)	.814	.616	.003		
Urinary O-xylene (ng/L) (Log10)	Pearson Correlation	.076	.211	-.082	.349	1
	Sig. (2-tailed)	.779	.433	.772	.185	
Urinary Naphthalene (ng/L) (Log10)	Pearson Correlation	.147	.578*	-.487	-.255	.339
	Sig. (2-tailed)	.588	.019	.066	.340	.199

*. Correlation is significant at the 0.05 level (2-tailed).

**. Correlation is significant at the 0.01 level (2-tailed).

These changes of the correlation patterns, might suggest a different contribution of the single VOCs in the different groups, which could be caused by the different levels of exposure or by differences in the lifestyle of the subjects of each subgroup.

Table 61: Correlation table for urinary BTEX + naphthalene of FIXAT and MATCH projects.

FIXAT and MATCH projects urinary BTEX + naphthalene (N=121)

		Urinary Benzene (ng/L) (Log10)	Urinary Toluene (ng/L) (Log10)	Urinary Ethylbenzene (ng/L) (Log10)	Urinary m+p-xylene (ng/L) (Log10)	Urinary o-xylene (ng/L) (Log10)
Urinary Toluene (ng/L) (Log10)	Pearson Correlation	-.243[**]	1			
	Sig. (2-tailed)	.007				
Urinary Ethylbenzene (ng/L) (Log10)	Pearson Correlation	-.342[**]	.726[**]	1		
	Sig. (2-tailed)	<0.001	<0.001			
Urinary m+p-xylene (ng/L) (Log10)	Pearson Correlation	-.317[**]	.670[**]	.810[**]	1	
	Sig. (2-tailed)	.001	<0.001	<0.001		
Urinary o-xylene (ng/L) (Log10)	Pearson Correlation	-.123	.591[**]	.567[**]	.735[**]	1
	Sig. (2-tailed)	.181	<0.001	<0.001	<0.001	
Urinary Naphthalene (ng/L) (Log10)	Pearson Correlation	.260[**]	-.259[**]	-.362[**]	-.324[**]	-.207[*]
	Sig. (2-tailed)	.007	.007	<0.001	.001	.033

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

Correlations between urinary BTEX, cotinine and oxidative stress biomarker (8-oxo-dG)

According to previously reported data, exposure to benzene is correlated with DNA damage, and in particular to 8-oxo-dG (Manini et al., 2010).

A correlation matrix was created to investigate correlations between urinary VOCs and 8-oxo-dG (Table 62). The correlation between the variables created with the sum of the different urinary VOCs and each of the VOCs were analysed, and most were found to be statistically significant, with a high correlation coefficient. Table 62 shows also the correlation between the urinary VOCs and the urinary cotinine. In this case, 16 of the cotinine measurements (N=90) were under the detection limit (0.1 µg/L), and were left in the analysis substituting them with ½ LOD. Since the samples under the detection limit were a considerable fraction of the total (17%), the same analyses were performed again with the samples under the LOD for urinary cotinine removed from the database. This allowed us to evaluate the contribution of ETS exposure to the urinary VOC excretion.

The single VOCs showed relevant correlation with the DNA damage biomarker 8-oxo-dG for ethylbenzene ($p=0.045$) and xylenes ($p=0.019$ and $p=0.035$), which were generally measured at low concentrations in the samples. Urinary benzene was found to be not correlated to any of the other VOCs, as shown on Table 62, nor to any of the variables obtained by summing the concentration of each VOC, except for TEX, where it showed a negative correlation. Furthermore, no correlation between urinary benzene and 8-oxo-dG was observed, in contrast with previously reported data (Manini et al., 2010) which, however, focused on populations with higher exposure and active smokers. A marginally significant correlation was found between urinary benzene and urinary cotinine ($p=0.048$), which is consistent with previous work (Fustinoni et al., 2005). Interestingly, also all of the other measured VOCs except naphthalene were correlated to the urinary cotinine, suggesting a possible contribution of ETS to the VOC exposure. In Table 63 are shown the correlations between urinary cotinine

and VOCs after the removal of the points under the limit of detection. This passage was performed in order to investigate the contribution of tobacco smoke to the exposure in subjects that showed a measurable exposure. The correlations showed to be affected by the removal of the data points, becoming weaker, especially the correlation between urinary benzene and urinary cotinine became non-statistically significant. In general, all of the correlation coefficients decreased, together with the relative P values. Also, it is important to mention that 2 samples from the occupationally exposed were classified as smokers, and 22 other samples were classified as ETS exposed. The analyses were repeated separating the groups in ETS exposed and non-ETS exposed, no statistical analyses could be performed on the smokers due to the small sample pool (N=2).

In addition, the same analysis was performed with the independent measurements performed along with FIXAT project, which contained several samples from smokers and ETS exposed subjects, confirming a strong correlation between urinary cotinine and benzene (R=0.667, $p=0.050$) although the number of samples is limited (N=9). Another significant correlation in the independent measurements group was found between urinary benzene and 8-oxo-dG (R=0.732, $p=0.025$) and urinary naphthalene, again with cotinine (R=0.700, $p=0.036$). Also, urinary cotinine was found to be strongly correlated with 8-oxo-dG (R=0.706, $p=0.022$). These findings confirm that tobacco smoke is an important source of urinary benzene, and that there is a strong correlation between smoking habits and oxidative stress (Evans et al., 2010; Manini et al., 2006). Although the results obtained using the independent measurements were interesting, it is important to underline that the number of samples was very small (N=9) and therefore, the correlation values would be higher. Due to the small number of samples and the statistical issues that this might cause, the results obtained for this particular subgroup were not taken in account for further conclusions.

Table 62: Correlation table for the sums of the main urinary VOCs with the single VOCs, cotinine and 8-oxo-dG (full data set).

		Cotinine (µg/L) (Log10)	8-oxo-dG (nMol/M creatinine) (Log10)	Sum of urinary BTEX + naphthalene (ng/L) (Log10)	Sum of urinary BTEX (ng/L) (Log10)	Sum of urinary TEX (ng/L) (Log10)
8-oxo-dG (nMol/M creatinine) (Log10)	Pearson Correlation	.013	1			
	Sig. (2-tailed)	.901				
Sum of urinary BTEX+naphthalene (ng/L) (Log10)	Pearson Correlation	.449**	.194*	1		
	Sig. (2-tailed)	<0.001	.041			
Sum of urinary BTEX (ng/L) (Log10)	Pearson Correlation	.453**	.206*	.995**	1	
	Sig. (2-tailed)	<0.001	.030	<0.001		
Sum of urinary TEX (ng/L) (Log10)	Pearson Correlation	.417**	.194*	.987**	.992**	1
	Sig. (2-tailed)	<0.001	.041	<0.001	<0.001	
Urinary Benzene (ng/L) (Log10)	Pearson Correlation	.209*	-.052	-.161	-.143	-.245*
	Sig. (2-tailed)	.048	.592	.092	.137	.010
Urinary Toluene (ng/L) (Log10)	Pearson Correlation	.399**	.165	.967**	.975**	.987**
	Sig. (2-tailed)	<0.001	.083	<0.001	<0.001	<0.001
Urinary Ethylbenzene (ng/L) (Log10)	Pearson Correlation	.434**	.193*	.778**	.782**	.794**
	Sig. (2-tailed)	<0.001	.045	<0.001	<0.001	<0.001
Urinary m+p-xylene (ng/L) (Log10)	Pearson Correlation	.424**	.240*	.748**	.751**	.745**
	Sig. (2-tailed)	<0.001	.019	<0.001	<0.001	<0.001
Urinary o-xylene (ng/L) (Log10)	Pearson Correlation	.378**	.200*	.687**	.681**	.673**
	Sig. (2-tailed)	<0.001	.035	<0.001	<0.001	<0.001
Urinary Naphthalene (ng/L) (Log10)	Pearson Correlation	-.102	-.178	-.170	-.279**	-.281**
	Sig. (2-tailed)	.359	.083	.099	.006	.006

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

Table 63: Correlation table between urinary VOCs and cotinine after the removal of the cotinine measurements under the limit of detection.

		Cotinine (µg/L) (Log10)
8-oxo-dG (nMol/M creatinine) (Log10)	Pearson Correlation	.043
	Sig. (2-tailed)	.714
Sum of urinary BTEX+naphthalene (ng/L) (Log10)	Pearson Correlation	.403**
	Sig. (2-tailed)	<0.001
Sum of urinary BTEX (ng/L) (Log10)	Pearson Correlation	.409**
	Sig. (2-tailed)	<0.001
Sum of urinary TEX (ng/L) (Log10)	Pearson Correlation	.371**
	Sig. (2-tailed)	.001
Urinary Benzene (ng/L) (Log10)	Pearson Correlation	.200
	Sig. (2-tailed)	.089
Urinary Toluene (ng/L) (Log10)	Pearson Correlation	.335**
	Sig. (2-tailed)	.003
Urinary Ethylbenzene (ng/L) (Log10)	Pearson Correlation	.419**
	Sig. (2-tailed)	<0.001
Urinary m+p-xylene (ng/L) (Log10)	Pearson Correlation	.415**
	Sig. (2-tailed)	.001
Urinary o-xylene (ng/L) (Log10)	Pearson Correlation	.443**
	Sig. (2-tailed)	<0.001
Urinary Naphthalene (ng/L) (Log10)	Pearson Correlation	-.132
	Sig. (2-tailed)	.286

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

The two analysed groups were non-ETS exposed and ETS exposed. The non-ETS exposed group was analysed also with the full data set, without the removal of the samples with cotinine concentration <LOD. All of the variables were analysed for correlation with cotinine and 8-oxo-dG (Table 64). Interestingly, in this case, the 8-oxo-dG was not correlated to any of the BTEX nor to the urinary cotinine in both the non-ETS exposed subgroups (full data set and dataset without samples with cotinine <LOD). Urinary cotinine was correlated to all of the

combinations of BTEX sums, toluene and o-xylene, with the highest significance with the sum of BTEX + naphthalene ($p=0.010$). In the ETS exposed subgroup a very strong correlation between urinary cotinine and 8-oxo-dG was observed, suggesting a possible contribution of ETS (univocally identified by the cotinine) to the oxidative stress. Moreover, 8-oxo-dG was found to have statistically significant correlation with all of the urinary combinations of BTEX sums and all of the single urinary VOCs except benzene, while for naphthalene the observed correlation coefficient was negative. Although the sample size for the ETS exposed subgroup is not very large, the group is composed of subjects that belong to all of the FIXAT subgroups and MATCH. These findings were confirmed by the analysis of the same correlations using Spearman nonparametric correlations, which provided similar results.

It is interesting to observe that the in the ETS exposed subjects no significant correlation between urinary cotinine and urinary BTEX was observed. Although our sample pool for ETS exposed subjects is relatively small, this observation is in contrast with previous literature (Manini et al., 2006). One of the possible explanations to the lack of correlation, could be related to the groups where the ETS exposed subject come from, which are mainly MATCH and FIXAT occupationally exposed (Table 34). Occupationally exposed subjects are exposed to higher VOCs concentrations, which in this particular case act as confounding factor for ETS exposure. Another important information to take in account is that urinary BTEX and cotinine have different half-lives. Although they have a similar route of excretion which involves glomerular reabsorption (Jatlow, 2003), VOCs generally have an half-life that is shorter than cotinine. VOCs blood concentration drops to 20-30% of the initial concentration in 2-4 hours (Janasik et al., 2008; Bois et al., 1996; Fustinoni et al., 2009), while cotinine half-life is longer. Cotinine has been reported to reach $T_{1/2}$ in approximately 17 hours (Benowitz, 1996). This difference between the half-lives of the compounds could affect the results, depending on the ETS exposure time, as cotinine would not be at $T_{1/2}$ while the VOCs would be past that point.

Since the urine sampling was performed in the morning, it is possible that for some of the subjects the ETS exposure or smoking event happened several hours before. This, together with one or more bladder voiding, would cause variability in the measured VOCs, although it is still possible to measure the trace of the exposure, this would be affected by the time gap, while urinary cotinine would not. 8-oxo-dG, being a marker of oxidative stress and inflammation, is produced also after the exposure event. The correlation found between urinary cotinine and 8-oxo-dG would suggest that these two compounds have similar half-lives, and are good biomarkers of ETS exposure, even after several hours from the exposure event.

The correlation in the non-ETS exposed subjects between urinary cotinine and urinary VOCs would both place the exposure events later in the sampling day or, more likely, suggest a low ETS contamination that was not reported, as it was not detected. One possible route of exposure would be a contamination coming from outdoor air.

Another route of exposure to ETS would be third hand smoke. Third hand smoke exposure is defined as the exposure arising from residual tobacco smoke contamination that remains after a cigarette has been extinguished (Winickoff et al., 2009). Third hand smoke is proven to be a source of most of the compounds associated with second hand smoke and cigarette smoke, however, many of the compounds last for a short time in the gas phase, and are found on surfaces, due to adsorption. One of the compounds that is absorbed on the surfaces is nicotine, together with particulate matter, which can be resuspended by mechanic action (i.e. dusting, vacuum cleaning) (Sleiman et al., 2014; Matt et al., 2008). Third hand ETS exposure can also be caused by the off gassing of smoke residuals from clothing of smokers that the subjects might have been in contact with (Chien et al., 2011; Fleming et al., 2012).

Table 64: Correlation table for urinary cotinine, 8-oxo-dG and urinary VOCs divided by ETS exposure (FIXAT + MATCH).

		NON-ETS EXPOSED (Full data set N=66)		NON-ETS EXPOSED (Cotinine <LOD removed N=50)		ETS EXPOSED (N=22)	
		Cotinine (µg/L) (Log10)	8-oxo-dG (nMol/M creatinine) (Log10	Cotinine (µg/L) (Log10)	8-oxo-dG (nMol/M creatinine) (Log10)	Cotinine (µg/L) (Log10)	8-oxo-dG (nMol/M creatinine) (Log10)
8-oxo-dG (nMol/M creatinine) (Log10)	Pearson Corr.	-.025	1	.003	1	.556[**]	1
	Sig. (2-tailed)	.840		.982		.007	
Sum of urinary BTEX + naphthalene (ng/L) (Log10)	Pearson Corr.	.347[**]	-.028	.365[**]	.074	.152	.449[*]
	Sig. (2-tailed)	.004	.826	.010	.611	.500	.036
Sum of urinary BTEX (ng/L) (Log10)	Pearson Corr.	.334[**]	.013	.354[*]	.112	.139	.470[*]
	Sig. (2-tailed)	.006	.920	.013	.445	.536	.027
Sum of urinary TEX (ng/L) (Log10)	Pearson Corr.	.314[*]	-.015	.329[*]	.094	.118	.475[*]
	Sig. (2-tailed)	.010	.907	.021	.520	.600	.026
Urinary Benzene (ng/L) (Log10)	Pearson Corr.	.215	.097	.281	.049	-.014	-.208
	Sig. (2-tailed)	.083	.439	.050	.739	.952	.353
Urinary Toluene (ng/L) (Log10)	Pearson Corr.	.345[**]	-.031	.321[*]	.068	.072	.458[*]
	Sig. (2-tailed)	.005	.802	.025	.643	.749	.032
Urinary Ethylbenzene (ng/L) (Log10)	Pearson Corr.	-.026	-.169	-.129	-.120	.184	.500[*]
	Sig. (2-tailed)	.842	.186	.388	.421	.413	.018
Urinary m+p-xylene (ng/L) (Log10)	Pearson Corr.	.059	.015	.085	.016	.132	.579[*]
	Sig. (2-tailed)	.643	.906	.570	.917	.627	.019
Urinary o-xylene (ng/L) (Log10)	Pearson Corr.	.082	.075	.415[**]	.156	.245	.474[*]
	Sig. (2-tailed)	.514	.551	.003	.285	.273	.026
Urinary Naphthalene (ng/L) (Log10)	Pearson Corr.	.115	-.231	.079	-.179	.243	-.492[*]
	Sig. (2-tailed)	.369	.069	.599	.229	.347	.045

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

Urinary BTEX and metabolites correlations with personal exposure data

Personal exposure data was analysed for correlations with the urinary data, the relevant correlations are reported in Table 65. Urinary cotinine was found to be correlated with benzene (p=0.039), ethylbenzene (p=0.015) and m-xylene (p=0.044) from the personal exposure measurement. Surprisingly none of the personal exposure BTEX were correlated to the 8-oxo-dG. Interestingly 8-oxo-dG was correlated to pyridine and octaldehyde. Pyridine is an ETS marker that is measured in the personal exposure (Brunnemann et al., 1996; Bi et al., 2005), while octaldehyde is compound that is commonly used in perfumes and it showed the capability of triggering inflammatory response in lung cells (Song et al., 2013). Octaldehyde was measured only in FIXAT project, while pyridine was available also from the MATCH analyses (Aquilina et al., 2010).

Pyridine was analysed for correlation with urinary cotinine, as both are markers for ETS. Interestingly, when the full data set was analysed (MATCH + FIXAT), no significant correlation was found between the two, which is in contrast to what has been reported before (Aquilina et al., 2010). When the data set was separated and each database (FIXAT and MATCH) was analysed alone, we observed that in MATCH project urinary cotinine and pyridine were significantly correlated (p<0.001) with a high Pearson coefficient (0.774). FIXAT database did not show significant correlation between pyridine and urinary cotinine. This finding can be explained from the fact that MATCH project involved the study of the effects of ETS exposure before and after the smoking ban, therefore higher levels of ETS were measured. In addition, the selected samples from MATCH were the samples with higher VOC exposure, which involved also higher ETS exposure, as demonstrated in the urinary cotinine concentrations. On the other hand, the selection of the volunteers in FIXAT project was aimed to maintain ETS exposure as low as possible, due to the known role of ETS exposure as a confounder and source of VOCs. Furthermore, the higher levels of ETS exposure, measured

from the urinary cotinine, were recorded in the occupationally exposed subjects, which were monitored for P.E. only during the work shift, missing out the rest of the day, and therefore not allowing us to measure the extent of ETS exposure through pyridine.

The different combinations of the sums of urinary BTEX, did not show any significant correlation with any of the VOCs measured in the personal exposure. On the other hand, the sum of the BTEX measured in the personal exposure, was correlated with the urinary benzene (p=0.021). Many of the VOCs from the personal exposure data were found to be correlated with the urinary benzene: toluene (p=0.023), ethylbenzene (p<0.001), p-xylene (p=0.027), m-xylene (p=0.029) and o-xylene (p=0.010), as reported in Table 65. The only positive significant correlation between the same airborne and urinary VOC was found for naphthalene (p<0.001). Interestingly, pyridine was found to be negatively correlated with urinary benzene (p=0.032), considering that pyridine is a marker of ETS and the concentration of urinary benzene is affected by ETS exposure, this finding would be in contrast with previously reported evidence. Moreover, pyridine did not show any correlation with urinary cotinine (p=0.844). The measured pyridine levels were low in FIXAT project, and in several of the cases under the LOD (0.1µg/m³). Also in this case, the readings under the LOD were substituted with ½LOD. This passage, due to the amount of samples <LOD, could create an artifactual correlation. To counter that possibility, the data points under the LOD for pyridine were removed and the correlations were analysed again (Table 66). After this correction, all the urinary VOCs except benzene showed an increased statistical significance and correlation coefficient with urinary cotinine, suggesting a possible contribution of ETS exposure to the urinary VOC concentration, despite the lack of correlation between pyridine and urinary cotinine. Interestingly, when the samples from MATCH project only were analysed for the same correlations, urinary cotinine was found to be strongly correlated with pyridine (R= 0.774, p<0.001). This could be due to the higher levels of ETS to which the subjects were exposed in MATCH project.

Table 65: Correlation table for P.E. VOCs and urinary cotinine, 8-oxo-dG and VOCs (full data set, N=83).

		Cotinine (µg/L) (Log10)	8-oxo-dG (nMol/M creatinine) (Log10)	Sum of urinary BTEX + naphthalene (ng/L) (Log10)	Sum of urinary BTEX (ng/L) (Log10)	Sum of urinary TEX (ng/L) (Log10)	Urinary Benzene (ng/L) (Log10)	Urinary Toluene (ng/L) (Log10)	Urinary Ethylbenzene (ng/L) (Log10)	Urinary m+p-xylene (ng/L) (Log10)	Urinary o-xylene (ng/L) (Log10)	Urinary Naphthalene (ng/L) (Log10)
Sum of personal exposure BTEX (Log10)	Pearson Correlation	.220	-.029	-.069	-.041	-.060	.278[*]	-.051	.045	-.164	-.089	-.125
	Sig. (2-tailed)	.117	.806	.572	.738	.626	.021	.675	.715	.237	.469	.363
P.E. benzene (µg/m^3) (Log10)	Pearson Correlation	.255[*]	-.045	.047	.067	.059	.116	.074	.173	-.059	-.103	-.137
	Sig. (2-tailed)	.039	.682	.676	.550	.596	.297	.504	.121	.635	.354	.261
P.E. toluene (µg/m^3) (Log10)	Pearson Correlation	.239	-.141	-.076	-.057	-.083	.250[*]	-.074	-.043	-.151	-.052	-.024
	Sig. (2-tailed)	.054	.192	.495	.610	.454	.023	.508	.702	.219	.640	.842
P.E. ethylbenzene (µg/m^3) (Log10)	Pearson Correlation	.298[*]	-.093	-.042	-.016	-.058	.392[**]	-.038	-.043	-.151	-.119	-.077
	Sig. (2-tailed)	.015	.392	.709	.884	.601	<0.001	.732	.700	.220	.284	.528
P.E. p-xylene (µg/m^3) (Log10)	Pearson Correlation	.243	.101	.058	.095	.081	.267[*]	.099	.188	-.102	-.087	-.290[*]
	Sig. (2-tailed)	.082	.394	.637	.436	.511	.027	.416	.128	.463	.477	.032
P.E. m-xylene (µg/m^3) (Log10)	Pearson Correlation	.281[*]	.102	.092	.128	.113	.263[*]	.132	.200	-.073	-.055	-.281[*]
	Sig. (2-tailed)	.044	.389	.454	.293	.355	.029	.280	.104	.602	.651	.037
P.E. pyridine (µg/m^3) (Log10)	Pearson Correlation	-.025	.242[*]	.044	.023	.027	-.258[*]	-.034	.058	.231	.220[*]	.049
	Sig. (2-tailed)	.844	.024	.693	.839	.809	.019	.763	.607	.058	.046	.692
P.E. o-xylene (µg/m^3) (Log10)	Pearson Correlation	.173	.134	.013	.049	.030	.310[**]	.047	.109	-.149	-.093	-.241
	Sig. (2-tailed)	.221	.257	.912	.689	.806	.010	.700	.381	.281	.448	.076
P.E. naphthalene (µg/m^3) (Log10)	Pearson Correlation	-.242	-.161	-.209	-.250[*]	-.261[*]	.165	-.255[*]	-.236	-.229	-.156	.494[**]
	Sig. (2-tailed)	.084	.174	.085	.038	.030	.177	.034	.054	.095	.201	<0.001
P.E. octaldehyde (µg/m^3) (Log10)	Pearson Correlation	-.016	.411[*]	-.017	-.027	-.029	.086	-.053	-.088	-.060	.127	.033
	Sig. (2-tailed)	.933	.019	.928	.885	.875	.638	.772	.645	.745	.487	.859

**. Correlation is significant at the 0.01 level (2-tailed). ; *. Correlation is significant at the 0.05 level (2-tailed).

Table 66: Urinary cotinine and 8-oxo-dG correlations with urinary VOCs and airborne pyridine after removal of points under the pyridine LOD (N=49) for the whole sample pool.

		Cotinine (µg/L) (Log10)	8-oxo-dG (nMol/M creatinine) (Log10)
Cotinine (µg/L) (Log10)	Pearson Correlation	1	
	Sig. (2-tailed)		
8-oxo-dG (nMol/M creatinine) (Log10)	Pearson Correlation	.075	1
	Sig. (2-tailed)	.609	
Sum of urinary BTEX+naphthalene (ng/L) (Log10)	Pearson Correlation	.683[**]	.154
	Sig. (2-tailed)	<0.001	.243
Sum of urinary BTEX (ng/L) (Log10)	Pearson Correlation	.687[**]	.183
	Sig. (2-tailed)	<0.001	.166
Sum of urinary TEX (ng/L) (Log10)	Pearson Correlation	.677[**]	.170
	Sig. (2-tailed)	<0.001	.199
Urinary Benzene (ng/L) (Log10)	Pearson Correlation	-.099	-.087
	Sig. (2-tailed)	.505	.514
Urinary Toluene (ng/L) (Log10)	Pearson Correlation	.667[**]	.132
	Sig. (2-tailed)	<0.001	.319
Urinary Ethylbenzene (ng/L) (Log10)	Pearson Correlation	.701[**]	.192
	Sig. (2-tailed)	<0.001	.153
Urinary m+p-xylene (ng/L) (Log10)	Pearson Correlation	.690[**]	.293[*]
	Sig. (2-tailed)	<0.001	.037
Urinary o-xylene (ng/L) (Log10)	Pearson Correlation	.527[**]	.204
	Sig. (2-tailed)	<0.001	.121
Urinary Naphthalene (ng/L) (Log10)	Pearson Correlation	-.167	-.356[*]
	Sig. (2-tailed)	.291	.011
P.E. pyridine (µg/m^3) (Log10)	Pearson Correlation	.155	.161
	Sig. (2-tailed)	.287	.207

[**]. Correlation is significant at the 0.01 level (2-tailed).

[*]. Correlation is significant at the 0.05 level (2-tailed).

The observed results suggest that ETS exposure plays a role in VOC exposure, especially when the whole dataset is analysed (Table 62 and Table 63). The findings are consistent with previously reported data (Fustinoni et al., 2010), where ETS exposure and active smoking are reported to be one of the main sources of benzene intake, although when the data points under the LOD of cotinine were removed, the correlation with urinary benzene

became not statistically significant. This behaviour could be related to the selection of the samples, which included mostly subjects with low ETS exposure, with only a small portion of subjects with higher ETS exposures. When the dataset is divided in different subgroups based on the measured urinary cotinine (Table 64), the correlation between ETS and benzene becomes weak and it loses significance. On the other hand, a strong correlation between urinary cotinine and 8-oxo-dG was observed for the ETS exposed subgroup. In the same subgroup, many urinary VOCs were found to be strongly correlated to the 8-oxo-dG (toluene, ethylbenzene and xylenes) but surprisingly, not urinary benzene. In the same group, urinary cotinine was found to be not correlated to any of the urinary VOCs or to any combination of sum of urinary BTEX. Interestingly the non-ETS exposed subgroup, showed a strong correlation between urinary VOCs and cotinine, but no correlation was found between the urinary VOCs and the 8-oxo-dG.

Due to the distribution of the ETS exposed subjects in each subgroup, it is possible that the outcome is affected by the levels of exposure of each group. Although several ETS exposed subjects were found in the control group, in the occupationally exposed and in the MATCH project, no ETS exposure was recorded in the subjects living/working in a recently built/refurbished building. Since each group is characterized by different behaviour and exposure to different sources, the ETS contribution not being evenly distributed along the groups could cause a bias in the measurements. To assess the effect of the lack of ETS exposed subjects in one of the groups, an ANOVA test was performed to assess the differences of the urinary cotinine and VOCs between the various subgroups. The ANOVA test did not show significant difference between the subgroups when the urinary cotinine levels were compared for the ETS exposed subjects ($p=0.517$), indicating similar levels of ETS exposure throughout the subgroups. However, a significant difference between the subgroups was observed when the sums of the urinary BTEX were compared (BTEX + naphthalene $p=0.039$; BTEX $p=0.038$,

143

TEX p=0.016). For the single VOCs the only relevant differences were observed for toluene (p=0.013), ethylbenzene (p=0.004), m+p-xylene (p=0.010) and o-xylene (p=0.037). As described in section IV.3 under the ETS exposure classification comparison paragraph, the only significant difference between the different levels of ETS exposure was found in the levels of urinary benzene. On the other hand, in this case, only the ETS exposed subjects were analysed, and the different groups were compared identifying statistically significant differences between them. The main components of the ETS exposed subgroups were occupationally exposed and MATCH subjects. Referring to Table 37, it is possible to observe that occupationally exposed subjects have the highest levels of urinary benzene and naphthalene, while the MATCH subjects have generally higher urinary concentrations of toluene, ethylbenzene and m+p-xylene. These findings might suggests that although the mean ETS exposure is homogeneous throughout the different subgroups, the different behaviour of the subjects of each subgroup affects the VOCs exposure. However, although the two groups are significantly different in terms of activities of the subjects and time of sampling (MATCH samples were collected before and immediately after the smoking ban), analysing the results reported in Table 64, it is possible to identify a threshold of exposure. This conclusion is based on the finding that for subjects exposed to low levels of VOCs, such as the control group and the living/working in recently built/refurbished buildings, one of the main sources of VOC exposure can be identified as ETS exposure, even though the urinary cotinine levels are very low and they are categorized as non-ETS exposed. Only few subjects reported ETS exposure (N=11), however, only 2 were classified as ETS exposed based on the urinary cotinine. Most of the subjects did not report any ETS exposure, therefore we could assume that the subjects were not aware of the exposure. This conclusion, taking in account also the possibility that some subjects did not report an ETS exposure event even being aware of the exposure, draws the attention towards the sources of

144

urinary cotinine and VOCs. As mentioned previously, third hand smoke is a possible explanation, together with contamination from outdoor air.

VOCs and ETS exposure do not seem to have an effect on 8-oxo-dG in these subjects, therefore the variability of the oxidative stress biomarker in these subjects is related to other sources.

On the other hand, subjects that are exposed to higher levels of ETS, such as occupationally exposed subjects and MATCH project subjects, are most probably exposed also to other sources of VOCs (especially the occupationally exposed subjects) that, however, are confounded by the exposure generated by ETS, as shown in Table 34.

IV.5 **Threshold of effect**

The threshold of effect becomes very evident when the data is represented graphically: in Figure 38 is reported a scatter plot representing the relationship between urinary cotinine and 8-oxo-dG, where a reference line was placed at the corresponding cotinine concentration threshold between non-ETS exposed and ETS exposed subjects (1µg/L). There is an evident separation between the non-ETS exposed subjects (left side of the reference line) and the ETS exposed subjects (right side of the reference line). The variability of the 8-oxo-dG in the non-ETS exposed subjects does not show any correlation with the urinary concentration of cotinine. The ETS exposed subjects and the smokers showed a correlation between the level of ETS exposure, measured by the urinary cotinine, and the amount of 8-oxo-dG measured.
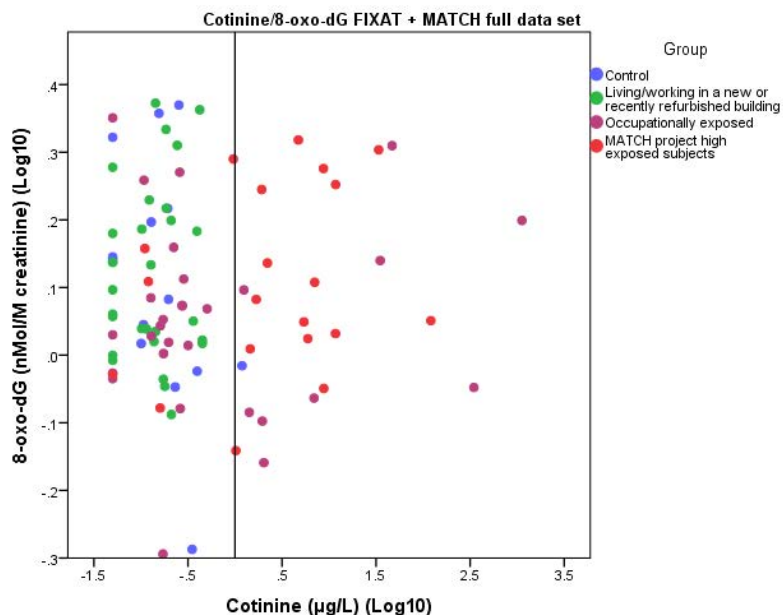
Figure 38: Scatter plot representing the full data set for MATCH and FIXAT project showing the relationship between urinary cotinine and 8-oxo-dG. The vertical line represents the threshold of discrimination between ETS exposed and non ETS exposed subjects (urinary cotinine=1µg/L).

Figure 39 shows the relationship between ETS exposure and the sum of the measured urinary BTEX + naphthalene (which showed the higher correlation between the various sums combinations with urinary cotinine in Table 66). The reference line is placed also in this case at the concentration threshold between non-ETS exposed and ETS exposed subjects. Interestingly, a correlation pattern is observed on the two sides of the reference line. A linear regression line was calculated, with an R^2 value of 0.202. It is interesting to notice that even if the subjects are considered non-ETS exposed, the relationship of urinary VOCs with cotinine is still very strong, suggesting that in our project the main sources of BTEX exposure could be attributed to ETS exposure, even at very low levels. From the equation of the regression line, we calculated the intersection point with the reference line, in order to identify the threshold of effect for 8-oxo-dG, which was identified as 2.74 (550 ng/L of total BTEX + naphthalene). This value ("threshold of effect") was then reported on a third graphic (Figure 40).

146
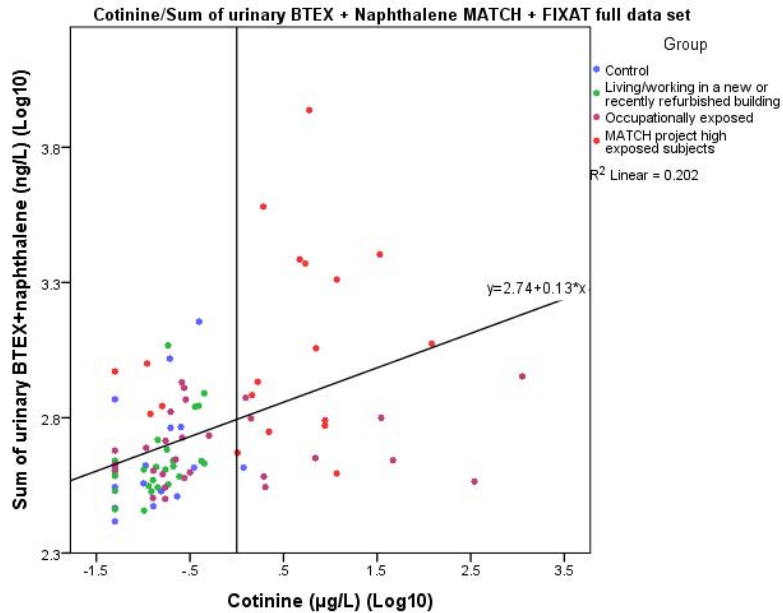
Figure 39: Scatter plot representing the full data set for MATCH and FIXAT project showing the relationship between the sum of urinary BTEX + naphthalene and cotinine. The vertical line represents the threshold of discrimination between ETS exposed and non ETS exposed subjects (urinary cotinine=1µg/L).

In Figure 40 is possible to observe an effect threshold, where the urinary BTEX + naphthalene with a urinary concentration higher than the found threshold value show a correlation with 8-oxo-dG. Under the threshold of effect, the variability of 8-oxo-dG is not dependent on the urinary BTEX + naphthalene concentration.

This findings suggest that VOC or ETS exposure are not responsible for oxidative stress under a certain level which is consistent with non-ETS exposed subjects, and the causes of the variation in the 8-oxo-dG for the non-ETS exposed subjects has to be searched elsewhere. However, the same findings suggest that in our samples, one of the causes of oxidative stress in ETS exposed subjects and smokers can be considered the VOCs, of which the ETS has proven to be one of the main sources.
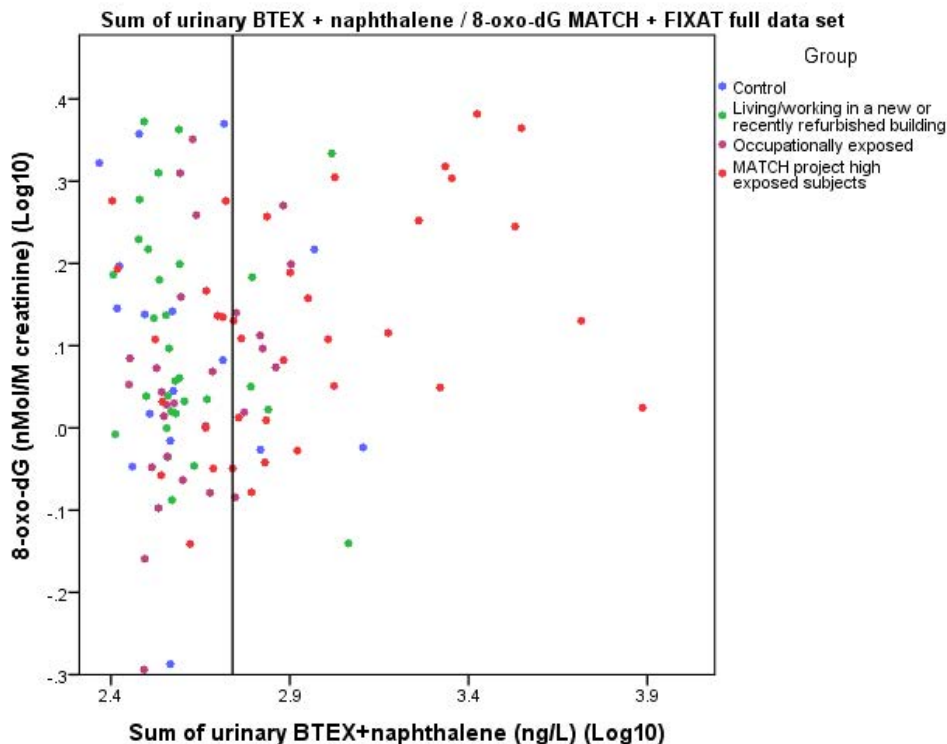
Figure 40: Scatter plot representing the full data set for MATCH and FIXAT project showing the relationship between the sum of urinary BTEX + naphthalene and 8-oxo-dG. The vertical reference line identifies the intersection between the regression line of Figure 39 and the threshold of discrimination between ETS exposed and non ETS exposed subjects.

In Table 65 and Table 66, is reported the correlation between personal exposure measurements and urinary VOCs and metabolites. As mentioned, airborne benzene is surprisingly not correlated with any of the urinary VOCs, however it showed a correlation with urinary cotinine. Airborne toluene, on the other hand, showed a significant correlation with urinary benzene, but not with urinary toluene. Similar results were obtained by using the sum of the airborne VOCs, which showed a correlation only with urinary benzene. Urinary benzene showed significant correlations with all of the airborne VOCs. This behaviour has been observed previously by Fustinoni et al. (2010) although in their study the airborne concentrations were found to be higher than in both FIXAT and MATCH. Moreover, in our study, subjects were selected among non-ETS exposed and a division was made between ETS

148

exposed and non-ETS exposed subjects. Our findings are coherent with the hypothesis that is made in Fustinoni's work, where benzene is suggested as a potential marker of environmental exposure to BTEX (Fustinoni et al., 2010). However, in our study we observed a correlation between all of the urinary BTEX and cotinine, and between urinary cotinine and airborne measured pollutants, suggesting that for our population, one of the main sources of exposure to BTEX could be ETS, even at very low concentrations. This is confirmed for some of the airborne BTEX in personal exposure, as shown in Table 67, where the only compounds that showed a significant correlation with 8-oxo-dG were cotinine in the ETS exposed subgroup (p=0.007), and pyridine in the full data set (p=0.024). Moreover, benzene, ethylbenzene and m-xylene showed a significant correlation with urinary cotinine in the full data set, only toluene showed a significant correlation with urinary cotinine in the non-ETS exposed subgroup, and in the ETS exposed subgroup significant correlations were observed between urinary cotinine and airborne benzene, toluene and pyridine. These observations are in line with previously reported data that links cigarette smoke to BTEX, where the most abundant compounds are toluene and benzene (Darrall et al., 1998; Bi et al., 2005).

Another important finding is that no combination of the sums of urinary BTEX + naphthalene, BTEX or TEX was found correlated with any of the airborne pollutants, except for pyridine (Table 65). Only urinary naphthalene showed a significant correlation (p<0.001), this could suggest a different source of exposure or metabolic pathway for naphthalene, which relates better than airborne BTEX to its urinary correspondent. Moreover, airborne and urinary naphthalene did not show any correlation with urinary cotinine, suggesting an alternative source of exposure other than ETS exposure, as previously observed by Aquilina et al. (2010). This would explain the high correlation observed between the airborne and the urinary compound, since it would not be affected by the confounding effect of ETS. This hypothesis is compatible with previously reported data on the BTEX and naphthalene content of cigarette

149

smoke, where naphthalene was found to have a significantly lower concentration in cigarette smoke, compared to the concentrations of the BTEX mixture (Bi et al., 2005; Ding et al., 2005; Darrall et al., 1998). Based on the same works, the ratios with the total VOCs of the single compounds of both personal exposure and urinary BTEX and naphthalene median concentrations were calculated. This procedure was performed for each of the FIXAT groups and MATCH, analysing the full data set, the smokers, the subjects with detectable levels of urinary cotinine and for subjects with urinary cotinine concentration <LOD. The subgroups obtained were then compared with the reported ratios of BTEX and naphthalene contained in mainstream cigarette smoke.

Table 67: overview of correlations between P.E. measurements, urinary cotinine and 8-oxo-dG based on ETS exposure classification.

		TOTAL (N=87)		NON ETS (N=47)		ETS (N=18)	
		8-oxo-dG (nMol/M creatinine) (Log10)	Cotinine (µg/L) (Log10)	8-oxo-dG (nMol/M creatinine) (Log10)	Cotinine (µg/L) (Log10)	8-oxo-dG (nMol/M creatinine) (Log10)	Cotinine (µg/L) (Log10)
8-oxo-dG (nMol/M creatinine) (Log10)	Pearson Correlation	1		1		1	
	Sig. (2-tailed)						
Cotinine (µg/L) (Log10)	Pearson Correlation	.013	1	-.025	1	.556**	1
	Sig. (2-tailed)	.901		.840		.007	
P.E. benzene (µg/m^3) (Log10)	Pearson Correlation	-.045	.255*	-.181	.182	.355	.510*
	Sig. (2-tailed)	.682	.039	.224	.221	.149	.031
P.E. toluene (µg/m^3) (Log10)	Pearson Correlation	-.141	.239	-.212	.303*	.020	.575*
	Sig. (2-tailed)	.192	.054	.152	.038	.939	.013
P.E. ethylbenzene (µg/m^3) (Log10)	Pearson Correlation	-.093	.298*	-.147	.193	.086	.415
	Sig. (2-tailed)	.392	.015	.324	.194	.734	.087
P.E. p-xylene (µg/m^3) (Log10)	Pearson Correlation	.101	.243	.248	-.004	.359	.285
	Sig. (2-tailed)	.394	.082	.133	.980	.207	.323
P.E. m-xylene (µg/m^3) (Log10)	Pearson Correlation	.102	.281*	.251	-.005	.364	.321
	Sig. (2-tailed)	.389	.044	.129	.976	.201	.263
P.E. pyridine (µg/m^3) (Log10)	Pearson Correlation	.242*	-.025	.250	-.243	.391	.492*
	Sig. (2-tailed)	.024	.844	.090	.099	.109	.038
P.E. o-xylene (µg/m^3) (Log10)	Pearson Correlation	.134	.173	.278	-.015	.377	.269
	Sig. (2-tailed)	.257	.221	.091	.929	.184	.353
P.E. naphthalene (µg/m^3) (Log10)	Pearson Correlation	-.161	-.242	-.248	.199	.067	.166
	Sig. (2-tailed)	.174	.084	.133	.230	.821	.571
**. Correlation is significant at the 0.01 level (2-tailed). ; *. Correlation is significant at the 0.05 level (2-tailed).							

151

IV.6 Airborne and urinary BTEX ratios analysis

Table 68: Comparison between the relative contributions of each compound to the total BTEX + naphthalene amount.

	Benzene	Toluene	Ethylbenzene	m+p-xylene	o-xylene	naphthalene	
Cigarette smoke	**35.7%**	**49.5%**	**4.9%**	**8.1%**	**1.6%**	**0.2%**	
Indoor air	**21.6%**	**59.5%**	**3.6%**	**11.2%**	**2.9%**	**1.2%**	**Reference values (airborne) (Bi et al., 2005)**
Outdoor air	**23.7%**	**49.0%**	**5.2%**	**16.9%**	**4.5%**	**0.6%**	
Smoking home	**29.6%**	**53.1%**	**3.5%**	**10.4%**	**2.5%**	**0.9%**	
Non-smoking home	**15.5%**	**64.1%**	**3.6%**	**11.7%**	**3.4%**	**1.7%**	
FIXAT smokers (occupationally exposed)	31.7%	33.7%	6.4%	8.3%	11.1%	8.8%	Urinary measured values

IV.7 In Airborne and urinary BTEX ratios analysis

Table 68 are reported the ratios of each BTEX compound and naphthalene in mainstream tobacco smoke (Bi et al., 2005), in other main microenvironments (Kim et al., 2001), and in the urine samples of the smokers found in FIXAT project. The smokers were present only in the occupationally exposed subjects, which carried the personal exposure sampling kit only during the work shift. Since the subjects did not smoke during the shift, the personal exposure data is not reported. A similarity of the ratios can be observed between the urinary BTEX of the smokers and the cigarette smoke, although toluene, naphthalene and o-xylene show a difference with the reference cigarette smoke ratios. As mentioned in section IV.2, occupationally exposed subjects showed higher levels of xylenes and ethylbenzene when compared to the other groups, therefore it is possible that the contribution of the occupational exposure is higher than the confounding effect of tobacco smoke. However, the differences in the proportions are relatively small, indicating a contribution of tobacco smoke towards the

overall exposure. However, the changes that can be observed between mainstream cigarette smoke and the different microenvironments, are mainly affecting the ratios of benzene and toluene, while the other measured compounds have either small variations or are present in such small concentrations that any small variation in the measurement would affect the calculated ratios.

A similar comparison was performed with the full data set of personal exposure and urinary BTEX + naphthalene (Table 69, Figure 41). The personal exposure measurements showed a generalized different pattern of the BTEX and naphthalene, except for the MATCH project (sampling performed before and after the smoking ban). However, the apportionments of the VOCs in MATCH and FIXAT control, were very similar, while a higher contribution of toluene was observed for the subjects living/working in a recently built or refurbished building. These ratios however, are discordant with most of the reference values. This might be due to the lower amounts of benzene sources in outdoor air, or to a higher contribution of indoor sources of other VOCs, such as xylenes. The observed apportionments, however, show only one part of the data, since the total concentrations are not included. The median total BTEX + naphthalene measured in the various groups were 16.22 µg/m^3 for FIXAT control, 30.32 µg/m^3 for FIXAT living/working in a new or recently refurbished building, and 30.91 µg/m^3 for MATCH samples. FIXAT occupationally exposed subjects had a total of 32.51 µg/m^3, only with benzene, toluene and ethylbenzene. Analysing these concentration differences, since the apportionments are similar for FIXAT control and MATCH, it is possible to conclude that the subjects could have been exposed to similar sources. Especially between FIXAT control group and MATCH, the airborne BTEX apportionment is extremely similar, however, the concentration of the total VOCs is significantly higher in MATCH subjects. Subjects living in a new or recently refurbished building showed higher total airborne VOCs concentrations in

153

the personal exposure, with a major contribution of toluene towards the total exposure (Figure 41).

Interestingly, the urinary concentrations of the compounds show a similar pattern to the reference values in most of the groups, except for MATCH (Table 69, Figure 42). The apportionments for each group do not change significantly, and the main affected compound appears to be the benzene. This finding would suggest common sources of VOCs. In the urinary VOCs from FIXAT living/working in a new or recently refurbished building, it was not observed the same increase of toluene relative concentration compared with the other groups. MATCH subjects showed a significantly lower relative concentration of urinary benzene. This could be due to the samples being stored for a long period with some cycles of freezing/thawing, causing the loss of different amounts of each compound, changing the relative proportions of some of the compounds.

Apportionment of airborne and personal exposure BTEX + naphthalene
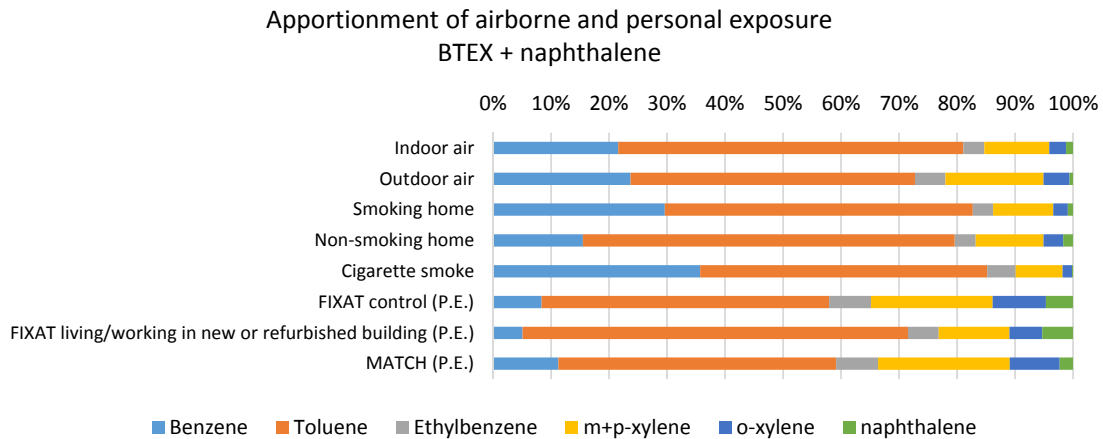
Figure 41: Graphical representation of BTEX + naphthalene apportionment in FIXAT and MATCH personal exposure and in the main microenvironments.

Apportionment of urinary BTEX + naphthalene in FIXAT groups and MATCH
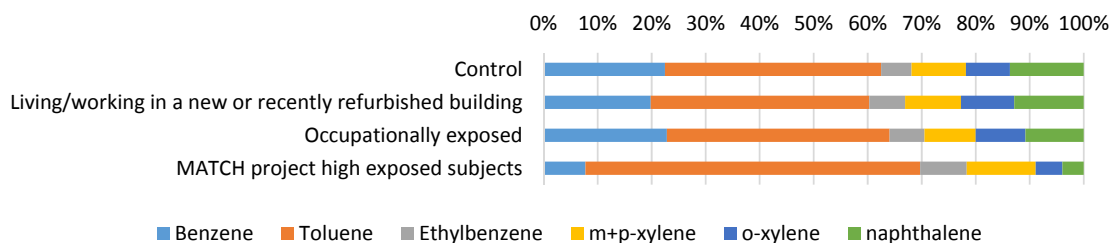
Figure 42: Graphical representation of urinary BTEX + naphthalene apportionment in FIXAT and MATCH.

Table 69: Comparison between the relative contributions of each compound to the total BTEX + naphthalene amount. Personal exposure and urinary concentrations are analysed for each group.

	Benzene	Toluene	Ethylbenzene	m+p-xylene	o-xylene	naphthalene	
FIXAT control (P.E.)	8.4%	49.6%	7.2%	21.0%	9.2%	4.7%	
FIXAT living/working in new or refurbished building (P.E.)	5.1%	66.5%	5.3%	12.2%	5.6%	5.3%	Full data set personal exposure
FIXAT occupationally exposed (P.E.)	18.3%	60.1%	21.6%	N/A	N/A	N/A	
MATCH (P.E.)	11.3%	47.9%	7.2%	22.7%	8.6%	2.3%	
FIXAT control (Urinary)	22.4%	40.0%	5.7%	10.1%	8.1%	13.7%	
FIXAT living/working in new or refurbished building (Urinary)	19.9%	40.1%	6.9%	10.4%	10.1%	12.7%	Full data set urinary BTEX + naphthalene
FIXAT occupationally exposed (Urinary)	22.4%	43.8%	5.0%	8.8%	9.1%	10.8%	
MATCH (Urinary)	7.7%	62.0%	8.7%	12.8%	4.9%	3.9%	

In order to investigate the contribution of ETS to the urinary VOC concentrations, the dataset was also divided according to the cotinine concentration. Taking into account the results in

155

Figure 39, it was decided to also separate the subjects based on the detectability of urinary cotinine. This last group was created as it was shown that even under the threshold of discrimination between ETS exposed and non-ETS exposed subjects, a relationship between urinary cotinine and VOC concentration was still present. The obtained subgroups, based on the ETS exposure classification groups were compared in Figure 43. The graphical representation underlines that ETS exposure and active smoking affect mostly urinary benzene, while the other urinary VOCs tend to maintain the original proportions.

Table 70: Comparison between the relative contributions of each compound to the total BTEX + naphthalene amount for samples with detectable and non-detectable levels of urinary cotinine. Personal exposure and urinary concentrations are analysed for each group.

	Benzene	Toluene	Ethylbenzene	m+p-xylene	o-xylene	naphthalene	
Cigarette smoke	**35.7%**	**49.5%**	**4.9%**	**8.1%**	**1.6%**	**0.2%**	**Reference values**
Non ETS-exposed FIXAT (urinary)	20.7%	40.9%	6.1%	10.1%	9.5%	12.6%	Urinary measures
ETS exposed FIXAT (urinary)	26.8%	41.0%	4.8%	8.6%	8.0%	10.7%	
Smoker FIXAT (urinary)	31.7%	33.7%	6.4%	8.3%	11.1%	8.8%	

Apportionment of urinary BTEX + naphthalene
separated by urinary cotinine concentration
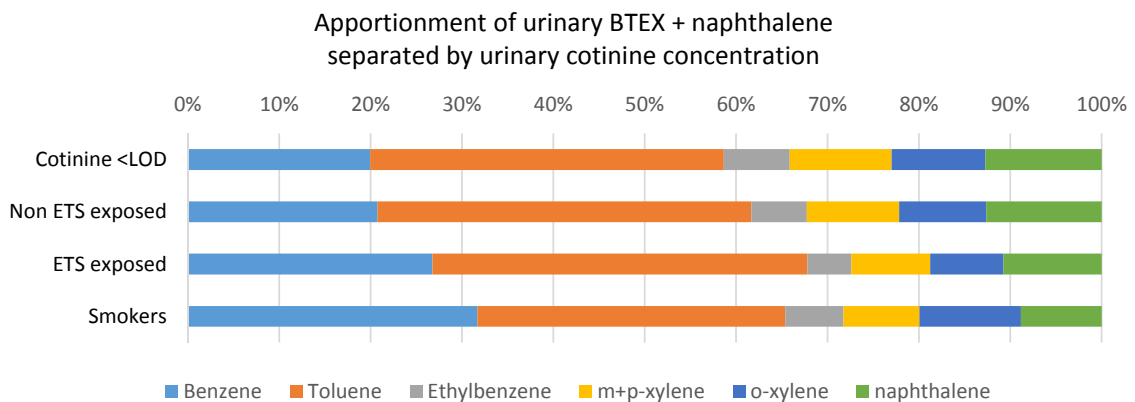
Figure 43: Graphical representation of urinary BTEX + naphthalene apportionments for each level of ETS exposure (FIXAT project only).

Table 70 shows the comparison of the proportions of each urinary VOC versus the mainstream cigarette smoke VOCs proportions. As mentioned previously, the apportionment that shows the most similarity is the urinary VOC profile of the smokers. However, the differences observed between the other groups are minimal, being mostly limited to benzene and toluene. Within the FIXAT project, also the sums of the total urinary VOCs were found to be consistent, with a median of 354 ng/L for the non-ETS exposed, 386 ng/L for the ETS exposed subjects. The smokers, unsurprisingly, had a total urinary VOCs concentration of 563 ng/L. Considering the changes in the proportions observed in Figure 43, it is possible to conclude that smoking mainly affects urinary benzene, however it is also a source of toluene, ethylbenzene and xylenes, which are less affected in terms of proportions but showed higher urinary concentrations.

In general, it was observed that the urinary naphthalene was found to be always in higher proportions, this would indicate a source of exposure other than ETS, however, when compared to the various microenvironments, also the other urinary VOCs showed higher proportions. As mentioned previously, naphthalene was also the only urinary VOC that showed a significant correlation with the airborne correspondent. This discrepancy of both urinary and airborne apportionments with the reference values could be explained by the different microenvironments visited by each subject. The overlapping contributions of each microenvironment or activity would make impossible to trace back the contribution of each microenvironment using only the apportionment. In these cases the availability of tracers for typical confounding factors or activities like urinary cotinine for ETS, becomes of crucial importance.

The lack of correlation between personal exposure and urinary biomarkers has been observed previously for general population and described by Sørensen et al. (2003b). This could be explained referring to the half-life of the VOCs in the human body (see introduction,

section I.5). Since most of the VOCs have a rapid excretion curve in the first part of their half-lives, it is possible that part of the VOCs absorbed during the exposure events of the first part of the sampling day were excreted or metabolized before the urine sampling. This dynamic absorption, metabolization and excretion, does not happen with the sorbent tubes, where all of the compounds that the subjects are exposed to throughout the sampling event, are adsorbed in the tube and are not released until the analysis.

This hypothesis could explain the lack of correlation for most of the low concentration urinary VOCs and their personal exposure correspondents, which appear to be more affected by this effect. This effect is particularly evident when the personal exposure and urinary measurement apportionments of benzene and toluene in the living/working in a recently built or refurbished building are compared. In this case the observed apportionment of benzene in airborne benzene is less than 10%, while in the urine the relative amount is around 20%. This change can be due to the different time of sampling of the urine compared to the airborne sampling and the difference between sorption tubes and excretion. However, the analyses on the threshold of effect and on the pattern of the relative concentrations of the VOCs suggest that one of the sources for most of the samples could be ETS. Even when the samples were classified as non-ETS exposed, the urinary VOCs showed a tendency to increase with the increase of urinary cotinine, suggesting that any amount of ETS would contribute towards the exposure. FIXAT sample pool was composed by general population, with very low VOC exposures, therefore any source, even with a small contribution, could have affected the measurements. This effect is observable in Figure 43 for the different levels of urinary cotinine, where the urinary benzene shows an increasing trend with the increase of ETS exposure. Although no correlation was found between personal exposure and urinary VOC concentration, the obtained results show the importance of markers and tracers for specific activities that are known to generate exposure. The apportionment of both urinary and airborne VOCs can

158

provide information on different microenvironments, activities or sources. However, since both measurements only give a cumulative information on the exposure during the sampling event, it is not possible to separate the various contributions of each activity or source only analysing the VOC data. Furthermore, in our case the population had very low levels of exposure, which proved to be sensitive to confounding factors, such as ETS, at levels that are generally reported to have a negligible effect.

IV.8 Considerations on 8-oxo-dG correlations

As mentioned previously regarding the threshold of effect, the lack of correlation between urinary metabolites and 8-oxo-dG for the low level exposures (non-ETS exposed subgroup) could depend on a similar reason. It is likely that for low exposures such as the ones observed in the non-ETS exposed subgroup, other sources of oxidative stress could interfere with the observed levels of 8-oxo-dG, or that the measured exposure levels were not high enough to cause an observable effect. Furthermore, it is important to remember that the subjects that were analysed in this study were volunteers from the general population, which would have other sources of oxidative stress that were not included in this study. Another important aspect to consider is the level of 8-oxo-dG measured. In this study, the observed 8-oxo-dG levels were generally lower than other previously reported data for similar studies for occupationally exposed subjects, as shown in Table 71:

Table 71: Comparison between FIXAT + MATCH projects and previously reported data.

Study sample subjects	8-oxo-dG Geometric mean (μMol/M creatinine)	8-oxo-dG Geometric Std Deviation
FIXAT control	1.26	1.49
FIXAT living /working in a new or recently refurbished building	1.29	1.37
FIXAT occupationally exposed	1.13	1.39
MATCH high exposed subjects	1.31	1.38
Manini et al. 2010 Gasoline pump attendants	1.71	0.60
Bagryantseva et al., 2010 Bus drivers	5.67 (mean)	2.50 (std deviation)

Within the measured airborne VOCs, another interesting correlation that was observed is the one between 8-oxo-dG and the airborne octaldehyde (p=0.019) measured in the personal exposure (Table 65). When translated in a graphic (Figure 44), the correlation appears evident. Unfortunately, only the measurements from control and living/working in a recently built/refurbished building groups were available for octaldehyde. However, the two groups are homogeneous, and the trend is present in both groups.

Octaldehyde (also known as octanal) is a compound used in perfumes that provides a citrus smell. It can be found in many household products and in perfumes. It is classified as an irritant (Clayton, 1995), and *in-vitro tests* showed that exposure of A549 cells to octaldehyde results in production of interleukin-6 and interleukin-8 (IL-6 and IL8) which are known triggers of the inflammatory process. It is possible that exposure to octaldehyde might trigger an inflammatory response, that can result in an increase of oxidative stress due to the immune response to the inflammation (Knaapen et al., 2006; Klebanoff, 2005). Another possibility is that octaldehyde is an ingredient of some (or several) consumer product(s), which might contain a compound that has not been measured in our project, which causes an increase of oxidative stress. In the

last case, it would be possible to use octaldehyde to trace and identify the product or the group of products or behaviours that led to the increased oxidative stress.
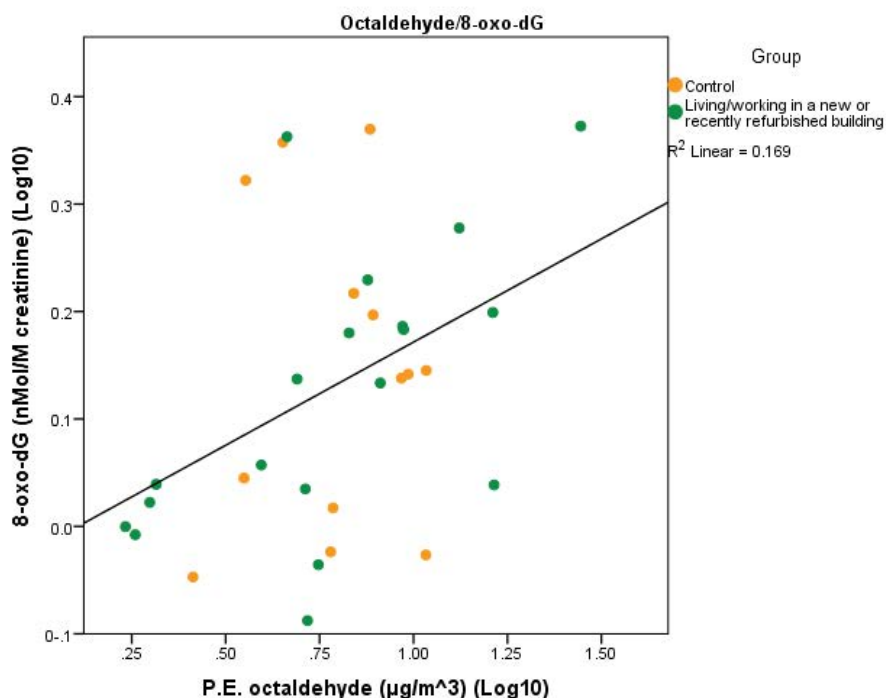
Figure 44: Scatter plot representing the relationship between octaldehyde and 8-oxo-dG.

IV.9 Correlation among urinary biomarkers and personal exposure to PAHs and quinones

In addition to airborne VOCs, airborne PAHs and quinones were also analysed for personal exposure, following the procedure described by Delgado-Saborit et al. (2013). Further data was obtained from MATCH project database for personal exposure measurements of PAHs and for urinary PAHs metabolites (Harrison et al., 2009; Delgado Saborit et al., 2009). All of the available measurements were analysed for correlations with urinary VOCs, cotinine and 8-oxo-dG.

The data showed a positive skewness, therefore, the measurements were log10 transformed.

When the full dataset (FIXAT + MATCH) was analysed, many of the analysed PAHs and quinones were found to be correlated mainly with cotinine and toluene, however, other

statistically significant correlations were observed with other markers. The statistically

significant correlations are presented in Table 72:

Table 72: Statistically significant correlations of airborne PAHs and quinones and urinary markers.

Airborne PAH	Correlation with urinary marker (full data set) (N=60)	Correlation in non ETS exposed subjects (N=45)
Acenaphthylene	Cotinine (R=0.375, *p*=0.007)	Not significant
	Toluene (R=0.343, *p*=0.010)	Not significant
Acenaphthene	Cotinine (R=0.289, *p*=0.040)	Not significant
	Ethylbenzene (R=0.450, *p*=0.001)	Not significant
Fluorene	Ethylbenzene (R=0.401, *p*=0.006)	Not significant
	m+p-xylene (R=0.298, *p*=0.040)	Not significant
Phenanthrene	8-oxo-dG (R=0.328, *p*=0.011)	Not significant
	m+p-xylene (R= 0.457, *p*=0.001)	Not significant
Fluoranthene	Cotinine (R=0.434, *p*=0.001)	Not Significant
	Toluene (R=0.450, *p*<0.001)	(R=0.405, *p*=0.006)
	Ethylbenzene (R=0.539, *p*<0.001)	Not significant
Pyrene	Cotinine (R=0.331, *p*=0.014)	Not significant
Chrysene	Cotinine (R=0.331, *p*=0.017)	Not Significant
Benzo(b)fluoranthene	Cotinine (R=0.278, *p*=0.042)	Not significant
	Toluene (R=0.324, *p*=0.012)	(R=0.379, *p*=0.010)
	Ethylbenzene (R=0.360, *p*=0.006)	Not significant
Benzo(k)fluoranthene	Cotinine (R=0.297, *p*=0.029)	Not significant
	Toluene (Not significant)	(0.338, *p*=0.023)
Indeno(1,2,3-cd)pyrene	Toluene (R=0.290, *p*=0.024)	Not significant
Benzo[ghi]perylene	Ethylbenzene (R=0.326, *p*=0.013)	Not significant
Coronene	Toluene (R=0.332, *p*=0.026)	Not significant
9,10-anthraquinone	Toluene (R=0.308, *p*=0.039)	(R=0.341, *p*=0.027)

As mentioned above, correlations were observed mainly with urinary cotinine and toluene,

which is the most abundant compound between the measured urinary VOCs. This pattern of

correlation might indicate a common source for both VOCs and PAHs. However, the

correlations between urinary cotinine and most of the airborne PAHs suggest that one of the

162

sources of these compounds could be also ETS. As previously reported in other works, not only VOCs but also PAHs are present in high concentration in cigarette smoke (Bi et al., 2005). Another suggested source of ETS related PAHs could be third hand smoke (Fleming et al., 2012). It has been previously reported that dust in smokers houses contains higher levels of PAHs and a correlation between cotinine in the dust and PAHs has been observed (Hoh et al., 2012). When the sample pool is divided based on ETS exposure, the correlations of airborne PAHs with cotinine lose statistical significance in both the ETS exposed and the non ETS exposed subgroup. However, the correlation between some of the airborne PAHs and toluene is conserved, suggesting a different source for the PAHs, together with ETS. 9,10-anthraquinone has been reported to be formed both as a derivate of oxidation of anthracene via UV light and ozone reaction, and directly emitted by diesel engines (Albinet et al., 2007). The traffic source would be in accordance with the observed correlation of anthraquinone with toluene. The correlation of PAHs with toluene strongly suggests a traffic related source, together with the ETS, since in the non ETS exposed subjects, although the correlation with cotinine is not significant, the correlation with toluene does not lose significance. Moreover fluoranthene, benzo(b)fluoranthene and benzo(k)fluoranthene have been linked with traffic and industrial emissions and are routinely monitored (Lodovici et al., 2003; Villar-Vidal et al., 2014).

In addition, benzo(k)fluoranthene, in this subgroup shows a statistically significant correlation with urinary toluene (R=0.338, $p=0.023$) that was not observed in the full data set.

The same analyses were performed on the ETS exposed subjects, however, the amount of samples with available measurements was very limited (N=8). In this case, we observed a similar behaviour to the one described in section IV.4: the urinary cotinine was not found to be correlated with any of the airborne PAHs while several PAHs showed a correlation with 8-oxo-dG, suggesting the presence of a threshold of effect also in this case. Unfortunately, the

available number of data was too low to perform similar analyses and determine a threshold of effect for the oxidative stress.

Table 73: Summary of the main correlations between airborne PAHs and urinary biomarkers for the ETS exposed subgroup.

Airborne PAH	Correlation with urinary marker (ETS-exposed)
Acenaphthene	8-oxo-dG (R=0.941, *p=0.017*) (N=5)
Phenanthrene	8-oxo-dG (R=0.849, *p*=0.008) Urinary ethylbenzene (R=0.782, *p*=0.022) (N=8)
Pyrene	8-oxo-dG (R=0.735, *p*=0.038) (N=8)
Benzo[ghi]perylene	8-oxo-dG (R=0.717, *p*=0.035) (N=8)
Benzo(a)anthracene	o-xylene (R=0.710, *p*=0.048) (N=8)
Chrysene	o-xylene (R=0.739, *p*=0.036) (N=8)
Dibenz(a,h)anthracene	o-xylene (R=0.790, *p*=0.020) (N=8)
Fluoranthene	Urinary toluene (R=0.763, *p*=0.028) (N=8)

In the ETS exposed group, significant correlations were observed between 8-oxo-dG and acenaphthene, phenanthrene, pyrene, benzo[ghi]perylene. Airborne PAHs were found to be correlated also with urinary VOCs, mainly with urinary o-xylene, which was correlated with benzo(a)anthracene, chrysene, dibenz(a,h)anthracene. The other correlations were observed between urinary ethylbenzene and phenanthrene, and between fluoranthene and urinary toluene. The results are summarized in Table 73. Negative correlations were observed between urinary naphthalene and most of the airborne PAHs, but unfortunately the number of available data was too small ($N \leq 5$). Due to the very low number of cases, the results obtained for these

correlations were not considered in further analyses, although the results are interestingly similar to the ones observed with the urinary VOCs and the threshold of effect. The observed behaviour would suggest a similar effect, where ETS plays a role as a source of PAHs, but the effects (i.e. oxidative stress) are observed only for the higher levels.

For the MATCH project samples, together with airborne measurements of PAHs, also some urinary PAH metabolites measurements were available. Despite the limited amount of available measurements (N=20), a Pearson correlation matrix was calculated. In general, the urinary PAH metabolites were found to be correlated within themselves, with R values ranging from 0.589 up to 0.892, consistent with the findings of previous analyses of the full data set (Aquilina et al., 2010). Unfortunately, no significant correlations were identified between the urinary PAH metabolites and the urinary VOCs, cotinine and 8-oxo-dG. However, both 1- and 2-hydroxyphenanthrene showed a marginally significant correlation with airborne acenaphthylene (R=0.672, p=0.047, N=9) and 1-hydroxypyrene showed a marginally significant correlation with fluoranthene (R=0.613, p=0.045, N=11).

It has been suggested that urinary metabolites of PAHs should be normalized versus urinary creatinine due to the excretion as glucuronide conjugates of the hydroxy-PAHs (Jongeneelen et al., 1987; Jongeneelen, 2001), therefore the urinary PAH metabolite data was used after normalization against creatinine.

Interestingly, although MATCH subjects were exposed to higher levels of ETS, no correlation was found between any of the urinary PAHs and urinary cotinine. It is important to mention that the sample size was relatively small, and this might have affected negatively some of the analyses. However, in this case, the single hydroxy PAHs did not show correlations with any of the airborne VOCs. The only correlations were found between 1- and 2-hydroxyphenanthrene and hydroxypyrene with two airborne PAHs that are not their parent compounds (acenaphthylene and fuoranthene). This finding is consistent with previously

reported analyses on the full MATCH data set (Aquilina et al., 2010). This finding would also suggest a different source for both hydroxypyrene and hydroxyphenanthrene (e.g. dietary intake). The analysis of MATCH project data proposed dietary intake as a primary source of urinary PAH metabolites (Harrison et al., 2009; Aquilina et al., 2010). PAH exposure is known to cause DNA adducts and DNA breaks (Jongeneelen, 2001), and 1-hydroxypyrene, together with 2-naphthol, showed a significant correlation with 8-oxo-dG in leukocytes, although this relationship is affected by genetic polymorphism (Kim et al., 2003). However, we did not observe such correlations in our data.

These findings suggest that most of the airborne PAHs are correlated with ETS, due to the relationship that has been observed with urinary cotinine. Moreover, the measured PAH species appear to have a common source with toluene, which is likely to be due to other sources such as traffic, in fact a loss of significance of the correlation with cotinine was observed when the non-ETS exposed group was analysed. This loss of significance was not observed for the unmetabolized VOCs, as showed in the results presented in section IV.4, suggesting that unmetabolized VOCs could be better biomarkers of low level ETS exposure. However, this same finding suggests that even low levels of ETS, which are generally classified as negligible, can be a confounding factor in general population.

Another interesting finding was the correlation of phenanthrene with 8-oxo-dG, and the lack of correlation of the same PAH with urinary cotinine. These finding shows a weak but significant link between airborne phenanthrene with oxidative stress, suggesting an action (either direct or indirect) of this compound on the DNA, and at the same time, no evidence of ETS as a potential source. Although it has been observed that without metabolic activation *in vitro*, phenanthrene is inactive on DNA (Platt et al., 2008), a disruption on redox balance in neutrophils has been reported, where exposure led to reduction of neutrophils function. Phenanthrene is known to be subjected to metabolic activation and cause oxidative stress via

redox cycling (Asahi et al., 2014). Moreover, in a previous work from Singh et al. (2008), a correlation between PAH exposure and oxidative stress levels has been observed. Although in that case no specific DNA damage biomarkers were analysed, our findings are in line with the conclusion of that work, as we identified phenanthrene as a possible contributor to DNA damage via oxidative stress, even in general population.

IV.10 Principal component analysis

The correlations between the variables of the dataset were analysed with principal component analysis (PCA) in order to determine the main variables that influence the variance of the dataset. Due to the high number of variables and their different provenance and availability, the analysis was performed in successive steps, adding a group of variables at a time.

Urinary cotinine is a biomarker of ETS exposure and smoking habits, while 8-oxo-dG is a biomarker of oxidative stress, which is one of the effects of exposure. Therefore, the two compounds were always kept in the analyses due to their relationship with all of the data.

The first batch of analyses was performed introducing all the urinary VOC measurements (BTEX and naphthalene). Missing data was substituted with the mean for each variable as a standard procedure of the SPSS software in order to maximise the number of cases included in the analyses, that in case of missing values for a variable, would not be included. However, when variables containing substituted values, the analyses were repeated without the substitution. In the first batch, with the urinary BTEX and naphthalene, no data was missing, and only in a small amount of cases, the readings were under the LOD. The readings <LOD were substituted with ½ LOD.

The full dataset was analysed, including all of the FIXAT and MATCH samples, the smokers were not excluded. Prior to analysis, the data was checked for adequacy with a Kaiser-Meyer-

Olkin (KMO) test and a Bartlett's test of sphericity. For the full data set, KMO score was 0.719 (suggested minimum is 0.6) and Bartlett's test was statistically significant ($p<0.001$).

The communalities table showed a generally high coefficient of the extraction result for all of the variables, as shown in Table 74.

Table 74: Communalities table for the full data set.

Communalities

	Initial	Extraction
Cotinine (µg/L) (Log10)	1.000	.752
8-oxo-dG (nMol/M creatinine) (Log10)	1.000	.645
Urinary Benzene (ng/L) (Log10)	1.000	.829
Urinary Toluene (ng/L) (Log10)	1.000	.663
Urinary Ethylbenzene (ng/L) (Log10)	1.000	.870
Urinary M+P-xylene (ng/L) (Log10)	1.000	.922
Urinary O-xylene (ng/L) (Log10)	1.000	.677
Urinary Naphthalene (ng/L) (Log10)	1.000	.595

The components were extracted until an Eigenvalue<1 was obtained. Component 1 explained 44.9% of the variance, component 2 16.3%, and component 3 13.2%. Coefficients under 0.1 were suppressed, due to their low relevance. The results of this extraction are reported in Table 75.

Table 75: Component matrix for the full data set (urinary metabolites only).

Component Matrix

	Component (% of variance)		
	1 (44.9%)	2 (16.3%)	3 (13.2%)
Cotinine (µg/L) (Log10)	.418	.759	
8-oxo-dG (nMol/M creatinine) (Log10)	.292	-.227	.713
Urinary Benzene (ng/L) (Log10)	-.207	.775	.430
Urinary Toluene (ng/L) (Log10)	.780	.122	-.199
Urinary Ethylbenzene (ng/L) (Log10)	.927		-.106
Urinary M+P-xylene (ng/L) (Log10)	.958		
Urinary O-xylene (ng/L) (Log10)	.811		-.136
Urinary Naphthalene (ng/L) (Log10)	-.498	.237	-.540

The components obtained reflect the results observed in the correlation analyses. The loadings of first component, which represents the majority of the total variance, indicate that the variance is mainly represented by the urinary VOCs and the urinary cotinine, while a minor contribution is given by the urinary 8-oxo-dG. Interestingly, in this component, urinary benzene and naphthalene have a negative coefficient. This component is likely to be related to the overall exposure to VOCs of the subjects, with a minor contribution from ETS (represented by the urinary cotinine), with a small significance of the oxidative stress biomarker towards the variance.

The second component could be related to the ETS, as the main loadings are on urinary cotinine and urinary benzene, which are both linked to ETS exposure. Interestingly, in this case 8-oxo-dG has a negative coefficient, in contrast to the coefficient found in component 1.

The third component represents mainly urinary benzene and 8-oxo-dG, and could represent the linkage between benzene, which is the main compound responsible for DNA damage, with the relative DNA damage biomarker.
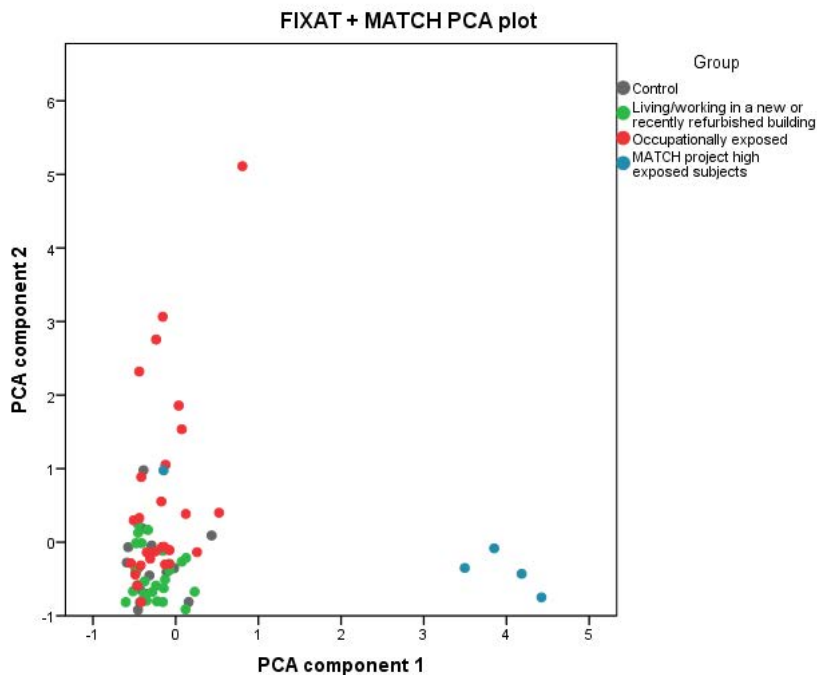
Figure 45: Scatter plot of component 1 and 2 obtained by PCA. Two main clustering are observed for occupationally exposed and MATCH subjects.

Figure 45 shows a scatter plot obtained with the component 1 and 2 (61.2% of variance explained), where two clusters are defined, one with MATCH subjects and one with occupationally exposed subjects. No clustering or separation is observed between control and living/working in a recently built or refurbished groups.

8-oxo-dG in the first two components does not show a high coefficient, and in addition, in the second component it shows a negative coefficient with a similar value observed in component 1. Only in component 3 the coefficient value becomes significantly higher, taking in account the percent contribution towards the total variance of the third component (13.2%), this result suggests that oxidative stress does not contribute much towards the variance of this set of variables. A higher contribution is provided by urinary cotinine, which has generally high coefficients, especially in component 2, suggesting that ETS exposure accounts for a good portion of the variability of this set of variables in the full data set. Urinary TEX show very high coefficients in component 1, but the loadings become smaller in component 2 and some

170

of them have a negative coefficient in component 3. However, component 1 alone represents over 44% of the total variance, suggesting that urinary TEX are the main contributors towards the variance in our data pool. Considering the loading of urinary cotinine in component 1 and 2, it is possible to include also cotinine as a main contributor towards the total variance. Interestingly, benzene shows a negative coefficient in component 1, but it shows a high coefficient in component 2, together with urinary cotinine, underlining the linkage between ETS exposure or smoking habits and urinary benzene concentration. Benzene is not the highest concentration compound in the observed samples, and the measurement showed a low amount of outliers, except in the cases of high ETS exposure and active smoking. This might explain the lower relevance of benzene in the first component and a higher loading in the second together with urinary cotinine. However, we did not observe a correlation between urinary benzene and cotinine in the ETS exposed subjects. This could be explained by an overall lower concentration of benzene in the exposure, that is associated with a higher metabolism, which has been suggested although there is an open debate on the topic (Rappaport et al., 2013; Price et al., 2013).

Furthermore, all of the BTEX have similar half-lives, and the excretion rate is similar across the BTEX mixture, as mentioned in section I.5. All of the BTEX share a similar metabolic pathway, which involves CYP 2E1. Although different effects have been observed for exposures to mixtures such as metabolic suppressions and interactions, the particular cases consisted in high exposures (above 25ppm), which are significantly higher than the exoposures levels of our study (Bois et al., 1996; Heinrich-Ramm et al., 2000; Miller and Edwards, 1999). When in component 3 8-oxo-dG shows a high coefficient, urinary benzene is the only compound suggesting a linkage between the two, which would be compatible to the known benzene genotoxicity (Hartwig, 2010; Barreto et al., 2009; Snyder and Hedli, 1996). Taking in account the coefficients for benzene and 8-oxo-dG observed in component 2, this linkage

appears to be weak in this population. However, within all the compounds analysed, benzene is the only known carcinogen and a correlation between urinary benzene and oxidative stress have been previously observed (Andreoli et al., 2012; Snyder and Hedli, 1996; Manini et al., 2010).

Naphthalene does not appear to contribute much towards the variance of the dataset, and in components 1 and 3, it shows a negative coefficient.

As a second step, personal exposure measurements were added to the variable set, and the same procedure was repeated. Within the personal exposure measurements, together with BTEX and naphthalene, also octaldehyde, pyridine, cumene, dipentene and styrene were included in the analysis. KMO test for this subset, returned a value of 0.769, and the sphericity test was significant (p<0.001). The components were extracted until an Eigenvalue<1 was reached. It is important to mention that the missing data was substituted with the mean in each variable. This operation was performed in order to avoid excluding variables that contained missing data. Since occupationally exposed and MATCH subjects' samples were not analysed for all the compounds, the substitution with the mean allowed to use all of the variables. Occupationally exposed subjects' personal exposure was sampled only in the first sampling day, and in that event, only benzene, toluene, ethylbenzene and pyridine were analysed. Substituting the mean in missing values can affect the test, therefore, the same procedure was performed again excluding the missing values.

When the full data set with the substituted values were analysed, 7 components were extracted, which explained a total of 76.9% of the variance. However, the first 5 components explained a cumulative 68% of the variance. Also in this case, coefficients <0.1 were suppressed. The results of the extraction are reported in Table 76.

.

Table 76: Component matrix for the full data set (urinary metabolites and personal exposure measurements).

Component Matrix

	Component (% of variance)						
	1 (28.6%)	2 (14.7%)	3 (12.8%)	4 (6.1%)	5 (5.7%)	6 (4.6%)	7 (4.3%)
Cotinine (µg/L) (Log10)	.116	.477	-.127	.245	.352	.346	.243
8-oxo-dG (nMol/M creatinine) (Log10)		.312	.163	-.115	-.545	.202	.315
Urinary Benzene (ng/L) (Log10)	.195	-.281	-.379	.229		.387	.482
Urinary Toluene (ng/L) (Log10)		.819		.191			-.186
Urinary Ethylbenzene (ng/L) (Log10)		.879	.116				-.218
Urinary M+P-xylene (ng/L) (Log10)		.838	.297	.139			
Urinary O-xylene (ng/L) (Log10)		.698	.284	.287	.125		.239
Urinary Naphthalene (ng/L) (Log10)	-.149	-.424		.501		.130	-.248
P.E. octaldehyde (µg/m^3) (Log10)	.322		.371	.145	-.386	.527	
P.E. benzene (µg/m^3) (Log10)	.588	.137	-.441		.193	.126	-.233
P.E. toluene (µg/m^3) (Log10)	.461		-.311	.196	.445	-.197	.308
P.E. ethylbenzene (µg/m^3) (Log10)	.718		-.444	.103	.207	.197	
P.E. p-xylene (µg/m^3) (Log10)	.861	.135	-.314		-.264	-.140	
P.E. m-xylene (µg/m^3) (Log10)	.873	.164	-.321		-.219		
P.E. pyridine (µg/m^3) (Log10)			.567	.111	-.230	-.265	.392
P.E. o-xylene (µg/m^3) (Log10)	.885		-.257		-.284	-.106	
P.E. cumene (µg/m^3) (Log10)	.676	-.108	.533	-.248	.293		
P.E. dipentene (µg/m^3) (Log10)	.414	-.135	.497	.284		.253	-.251
P.E. n-propylbenzene (µg/m^3) (Log10)	.657		.493	-.390	.302		
P.E. styrene (µg/m^3) (Log10)	.513	-.246	.361	.151	.183	-.415	.189

In this case, a major contribution of the personal exposure was observed in the explanation of the variance. Coefficients in component 1 were higher for P.E. ethylbenzene and xylenes. P.E. benzene, toluene, cumene, n-propylbenzene and styrene showed moderate contribution towards the variance, while a minor contribution was observed for dipentene, octaldehyde and urinary benzene. This pattern reflects the main differences between the groups observed in section IV.3. Component 2 showed higher coefficients for urinary TEX and cotinine, similarly to the previous analysis (Table 75), suggesting that the main contribution

towards the variance of the data pool is provided by P.E. benzene, ethylbenzene, xylenes and urinary toluene, ethylbenzene and xylenes (cumulative coefficient loading for component 1 and $2 = 43.3\%$). Component 3 shows a dominance of P.E. octaldehyde, pyridine, cumene, dipentene and styrene, with high coefficient, while a minor contribution towards the variance, is attributable to benzaldehyde and urinary benzene, this last one with a negative coefficient. From component 4 to component 7, we can observe a redundancy of the coefficients that, together with the low component relevance in explaining the variance (only 6.1% for component 4), suggest that those components are not significant towards the analysis.

When the analysis was repeated eliminating the cases with missing values, the KMO score was not sufficient to validate the analysis (0.439). Taking in account the number of components extracted and the coefficients obtained in Table 76, this analysis could be considered inconclusive, due to the low percentage of variance explained by each component.

The analysis was then repeated excluding the variables with missing data. For the personal exposure measurements, only benzene, toluene, ethylbenzene were used, obtaining a KMO score of 0.716. Bartlett's test of sphericity was found to be significant ($p<0.001$). Interestingly, in the communalities table, the extraction coefficient of urinary benzene was found to be low (0.254). Three components were extracted, the results of the analyses are reported in Table 77.

In this case, component 1 showed a prevalence of high coefficients in the urinary VOCs, specifically toluene, ethylbenzene, and xylenes and a moderately high coefficient was observed for urinary cotinine and 8-oxo-dG. Component 2 showed high coefficients for all of the P.E. measurements, together with urinary cotinine, suggesting that the two variables are linked. Component 3 showed a high coefficient for urinary naphthalene and 8-oxo-dG, which showed a negative coefficient.

174

A possible interpretation of the components is similar to the one proposed for Table 75, where the first component represents the differences in the exposure to VOCs of the subjects, which are mostly represented by the urinary TEX and cotinine, and are not reflected by the P.E. measurements. The second component could represent the contribution of ETS towards the exposure, as urinary cotinine and urinary benzene have slightly higher coefficients, and a higher VOC exposure could be also the reason for the higher coefficients of the P.E. VOCs.

While the third component in Table 75 clearly represents the linkage between exposure and effect, in this case (Table 77) it is not possible to determine any relationship with 8-oxo-dG, which has a low coefficient in the first two components and a negative coefficient in the third.

The variance is mainly related to different levels of exposures, and in particular the personal exposure measurements of benzene, toluene and ethylbenzene are to be considered the main contributors to the variance. Within the urinary metabolites, cotinine plays an important role in contributing towards the variance, both with personal exposure and urinary BTEX. Within the urinary BTEX and naphthalene, the main compounds responsible of the variance are generally toluene, ethylbenzene and xylenes. Urinary benzene contributes to the variance, but never in a measure that is comparable to the other urinary VOCs, moreover, benzene coefficient in component 1 and 2 has similar values, but in the first component it shows a negative contribution, while in the second component the contribution is positive. This suggests that the contribution of urinary benzene towards the variance is very limited, especially when also personal exposures are included in the analyses. These analyses also suggest that naphthalene is not responsible for the variance of the data, reflecting the previous observations in section IV.4.

Table 77: Component matrix for urinary metabolites and main personal exposure measurements (full data set).

Component Matrix

	Component		
	1	2	3
	(35.8%)	(22.5%)	(10.5%)
Cotinine (µg/L) (Log10)	.425	.590	.342
8-oxo-dG (nMol/M creatinine) (Log10)	.341	-.367	-.595
Urinary Benzene (ng/L) (Log10)	-.379	.332	
Urinary Toluene (ng/L) (Log10)	.790	.109	.343
Urinary Ethylbenzene (ng/L) (Log10)	.927	.144	
Urinary M+P-xylene (ng/L) (Log10)	.962		
Urinary O-xylene (ng/L) (Log10)	.816		
Urinary Naphthalene (ng/L) (Log10)	-.546		.666
P.E. benzene (µg/m^3) (Log10)		.815	-.274
P.E. toluene (µg/m^3) (Log10)	-.255	.642	
P.E. ethylbenzene (µg/m^3) (Log10)	-.238	.873	-.196

A third PCA analysis was attempted, including the measured PAHs and quinones in the variables. However, any combination did not return a KMO score that was acceptable for the analyses. Moreover, in the occupationally exposed subjects, airborne PAHs and quinones were not measured, significantly reducing the size of the data set.

In conclusion, PCA analysis showed a dominance of urinary toluene, ethylbenzene and xylenes as responsible factors explaining the variance of urinary measurements, followed by cotinine and, lastly, by 8-oxo-dG (Table 75). When the variables were analysed together with personal exposure measurements, the P.E. showed to provide a higher contribution to the variance of the samples, compared to the urinary markers (Table 76). However, it must be considered that the lack of the measurements of some compounds in the occupationally exposed subjects might have affected the results. In fact, when only the measured airborne VOCs were added to the variable pool, the main contribution to the variance was provided by the urinary VOCs (Table 77). Cotinine, in both cases, showed to contribute towards the

variance, confirming the linkage between ETS and both urinary and airborne VOCs. 8-oxo-dG did not appear to be one of the main contributors towards the data variance, although it did show a moderate contribution.

PCA analysis results suggest that urinary BTEX are a valuable tool for evaluating exposure, the findings also confirm that one of the sources of VOCs in our samples can be related to ETS exposure. Unfortunately, due to the lack of some measurements, airborne PAHs could not be included in the analyses. This last step could have provided further insights on the characterization of the groups, taking in account the results obtained in section IV.9.

IV.11 Linear regressions

Due to the gaps in available data for some of the variables, a single regression model was found to be unsuitable to model all the variables together. The model could be calculated only using the cases that did not have any missing data in any of the variables, unless a correction policy is applied. All of the models were calculated on 75% of the sample, and validated against the remaining 25%. A first linear regression was calculated including all of the measured urinary VOCs and cotinine, P.E. VOCs, airborne PAHs and the information gathered through the questionnaires. This was possible only for the control group and for both samplings of the living/working in a new or recently refurbished building group (N=44). The included variables, together with all of the measured airborne and urinary VOCs and airborne PAHs, were: group/sampling event, gender, age range. These variables for the groups included in the analysis were found homogeneous, only the age range showed a statistically significant difference in the occupationally exposed subjects (section IV.1). The dependent variable was set to be 8-oxo-dG, in order to investigate if it was possible to create a model that would estimate the oxidative stress based on the exposure.

177

The model was developed on SPSS, using the stepwise option, which allowed to add the optimum number of variables to obtain the best fit on the data. The obtained models only included airborne PAHs and the age range. This finding is compatible with the results shown in section IV.9 and IV.10. The results are summarized in Table 78.

Table 78: Summary of the linear regression model for 8-oxo-dG based on FIXAT project samples. Occupationally exposed subjects were excluded due to the lack of airborne PAH data.

Variables Entered/Removed[a]

Model	Variables Entered	Variables Removed	Method
1	Benzo(a)pyrene	.	Stepwise (Criteria: Probability-of-F-to-enter <= .050, Probability-of-F-to-remove >= .100).
2	Age range	.	Stepwise (Criteria: Probability-of-F-to-enter <= .050, Probability-of-F-to-remove >= .100).
3	9,10 anthraquinone	.	Stepwise (Criteria: Probability-of-F-to-enter <= .050, Probability-of-F-to-remove >= .100).
4	Anthracene	.	Stepwise (Criteria: Probability-of-F-to-enter <= .050, Probability-of-F-to-remove >= .100).

a. Dependent Variable: 8-oxo-dG (nMol/M creatinine)

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
4	.908[d]	.825	.775	.20828

d. Predictors: (Constant), Benzo(a)pyrene, Age range, 9,10 anthraquinone, Anthracene

The obtained coefficients (Table 79) were then validated by calculating the 8-oxo-dG values for the 25% of the data that was not included in the model, and the normalized mean bias (NMB) was calculated using the following equation (Harrison et al., 2009):

Equation 3

$$NMB(\%) = \frac{\sum_{i=1}^{n}(y_{predicted} - y_{measured})}{\sum_{i=1}^{n}(y_{measured})} \times 100$$

Table 79: Summary of coefficients, standard error and significance (FIXAT project without occupationally exposed subjects).

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta		
4	(Constant)	.748	.265		2.819	.014
	Benzo(a)pyrene	.140	.026	.604	5.305	.000
	Age range	.361	.110	.377	3.281	.005
	9,10 anthraquinone	-.651	.182	-.416	-3.579	.003
	Anthracene	1.324	.519	.300	2.552	.023

a. Dependent Variable: 8-oxo-dG (nMol/M creatinine)

The obtained equation is reported below.

Equation 4:

$$Y = 0.140\,a + 0.361\,b - 0.651\,c + 1.324\,d + 0.748$$

Where a was benzo(a)pyrene (μg/m³), b was the age range, c was the 9,10 anthraquinone (μg/m³), and d was anthracene (μg/m³).

Unfortunately, due to the missing data for some of the measurements, the sample number had to be drastically reduced, and this excluded the occupationally exposed and the MATCH study subjects. The results suggested that the main contribution to the oxidative stress, for this subset of samples, is coming from airborne PAHs exposure, although 9, 10 anthraquinone has a negative coefficient, suggesting a negative contribution of that compound. The validation of the model was performed by calculating the 8-oxo-dG concentration on the 25% of the data that was randomly selected and excluded from the model calculation, using the obtained equation. The calculated values accuracy was then evaluated by NMB (Equation 3). The obtained value for NMB was -33%, which suggests that our model, for this data set, tends to underpredict values. Although this value does not seem to be very far from the actual values, it is important to remember that 8-oxo-dG variations are often minimal. Therefore, although the model does provide an estimation of the oxidative stress generated by exposure

to certain compounds, the main conclusion that we can draw from the result, is that benzo(a)pyrene, anthracene, 9,10-anthraquinone, together with the age range, are the main contributors to oxidative stress in an heterogeneous general population. However, this data alone is not sufficient to build a reliable model for prediction of oxidative stress with the amount of data that we have available.

In order to include all of the variables and to analyse the full data set, the missing values were substituted with the mean. The obtained model (Table 80) did not have a significant R^2 value (R^2=0.101).

Table 80: Overview of model obtained with the full data set (FIXAT + MATCH) and missing values substituted with the mean.

Coefficients^a

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta		
2	(Constant)	1.361	.051		14.631	.000
	P.E. naphthalene (µg/m^3)	-.019	.008	-.238	-2.327	.022
	Urinary O-xylene (ng/L)	.003	.002	.224	2.185	.032

a. Dependent Variable: 8-oxo-dG (nMol/M creatinine)

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
2	.318	.101	.080	.41979

Another model was calculated, using the full data set, this time without substituting the missing data with the mean, the cases were excluded listwise, including in the analyses only the cases with a complete set of data. In order to maximise the amount of cases included in the analysis, we included only the variables that were measured in all of the groups. Therefore, the model was calculated using urinary VOCs and cotinine, and airborne benzene, toluene, and ethylbenzene, which were measured for all the subjects. Airborne PAHs were not included, as

they were not measured for the occupationally exposed subjects and that would cause the exclusion of a whole subgroup. The dependent value was again 8-oxo-dG.

When the model was calculated, no variables were entered in the equation. The same result was obtained also using only the FIXAT samples. The outcome of these calculations shows that the variables that are available for all the subjects are not suitable to calculate a regression model for 8-oxo-dG. This finding is in accordance with the results presented in Table 78, where none of the urinary biomarkers was included in the model. The result is also in accordance to the weak relationship observed between 8-oxo-dG and most of the measured compounds, suggesting that although we can observe a relationship, between several compounds and 8-oxo-dG, we do not have the statistical power to build a model for this population.

The outcome of these calculations suggests that the main contributors to the oxidative stress, in this particular data set, are not VOCs. However, due to the relatively small sample set, it is also possible that a reliable model cannot be calculated. When the sample subset was integrated with MATCH samples, the data pool became very heterogeneous, due to the different levels of exposure and to the different sources. This inhomogeneity might have affected negatively the calculations. Moreover, the amount of missing data, in this sample pool, affects the results and the possibility of using all of the variables, since in some cases certain compounds were not measured. All of these factors did not allow to calculate a model with the complete variable set, however, when only the variables that had no missing data were included, the obtained model was not satisfactory, although we were able to obtain a model (Equation 4:). The obtained model suggested that benzo(a)pyrene, which is a known human carcinogen (IARC, 2012), age range, 9,10-anthraquinone, which is a possible human carcinogen (IARC, 2010), and anthracene, which is the most abundant compound of the PAH mixture, are predictors of the 8-oxo-dG concentration. The predictions obtained with that model, however, due to the normalized mean bias (-33%), could not predict the 8-oxo-dG

concentration accurately enough to be valuable, due to the typically low variations (20% in smokers) of the DNA damage biomarker (Evans et al., 2010).

V. High PAH exposure and oxidative stress: a case study of Jeddah, Saudi Arabia

This chapter details results from a sampling campaign at three locations in Jeddah. The objective was to assess the impact of high level exposure to PAHs on early biomarkers of effect in a population of school children living near an oil refinery and a heavy traffic ring road.
The data discussed in this chapter was collected by Dr Magdy Shamy, King Abdulaziz University, Jeddah, Saudi Arabia.

V.1 Introduction and study population

Airborne PAH data was collected at three locations in Jeddah, Saudi Arabia. The locations were three schools, placed at different distances from an oil refinery.

Location A (Pitrumin) is a school in the immediate neighbourhood (700m) of an oil refinery, location B (Qweiza) is a school located next to a heavy traffic ring road at a distance of 5 Km from the refinery. Location C (Obhur) is a school located in the north part of Jeddah, next to the red sea, far from the refinery and traffic, which is used as a rural/background site. The air sampling was performed using polyurethane foam (PUF) filters for the vapour phase and quartz filters for the particle phase (Trasande et al., 2015).

Along with the air measurements of the PAHs, urine samples were collected the morning after the air measurement from the children attending each school. The urine samples were analysed for hydroxylated metabolites of phenanthrene (OH-phenanthrene) and pyrene (OH-pyrene) (1-, 2-/9-, 3-, 4-hydroxyphenanthrene, 1-hydroxypyrene) and creatinine. The creatinine concentration was used as a normalization factor. Along with the urine samples, dietary information, age, body mass index (BMI), physical information, pulmonary function and information on any disease were also collected through questionnaires.

The air and urine sampling were performed on the same day for locations A and B, while for location C the sampling was performed four weeks later.

Several studies have been performed previously on the concentration of PAHs metabolites in urine, which were synthesized in the PhD dissertation from Li (2009) (Table 81), where the readings show a decreasing trend during the years. The most influent works in terms of number and exposure levels are Heudorf & Angerer, (2001) and Li et al., (2008). Heudorf & Angerer studied high exposed subjects, for whom exposure arose from parquet glue containing high levels of PAHs, while Li et al. treated US general population. The concentrations of urinary OH-pyrene from Jeddah school children (Table 82) are generally much higher than the previously reported values, indicating a very high exposure of the population to PAHs.

Table 81: Overview of urinary PAH metabolite 1-OH-pyrene in different populations over the years (Li 2009).

Population - Country	Age range	N	Median concentration (range) (ng/g creatinine)	Reference
Adults – Atlanta USA	31-62	8 subjects (439 samples)	67 (17-5839)	Li 2009
Adults – Atlanta USA	25-58	8 subjects (427 samples)	58 (7-1963)	Li et al. 2010
US population - USA	≥20	1625 subjects	41 (15-233)	Li et al. 2008
Adults - Germany	≥20	495 subjects	88 (LOD-1172)	Heudorf & Angerer 2001
Non-smoking adults - Italy	22-81	327 subjects	149 (52-654)	Roggi et al. 1997
General population and ETS exposed - UK	18->66	100 subjects (500 samples)	Geom. Mean 96 (19-656)	Aquilina et al., 2010
Present study	10-14	167 subjects (501 samples)	264 (15-2476)	

Table 82: Summary of concentrations of 1-OH-pyrene and sum of OH-phenanthrene species in Jeddah school children.

Species	N	Median concentration (ng/g creatinine)	Range (ng/g creatinine)
1-OH-Pyrene [Day 1]	167	267	15-1632
1-OH-Pyrene [Day 2]	167	259	25-2476
1-OH-Pyrene [Day 3]	167	267	30-1904
Sum of OH-Phenanthrene [Day 1]	167	512	84-3499
Sum of OH-Phenanthrene [Day 2]	167	492	112-2349
Sum of OH-Phenanthrene [Day 3]	167	519	122-9591

Li (2009) reported that the main source of urinary PAH metabolites is dietary ingestion of PAHs as for example burned or chargrilled food. However, in that study the levels of exposure were much lower than the ones from Jeddah school children. In our case, having only three days of urinary biomarkers for each site to compare with the air measurements makes it difficult to find a correlation between air concentration and urinary metabolites. Although there are major differences between the air measurements in the three sites (Figure 46 and Figure 47), the differences between the urinary concentrations are not so remarkable, as shown in Figure 46 and Figure 49. Interestingly, the airborne concentrations measured in school site A and B (next to the oil refinery and to the ring road, respectively) are significantly higher than the concentrations measured in the MATCH study, which took place in UK before the smoking ban (Harrison et al., 2009). The reported airborne concentrations for pyrene and phenanthrene from MATCH study are in the order of 1 ng/m^3, while in this case, site A and B show concentrations up to 10 times higher for pyrene and up to 30 times higher for phenanthrene.
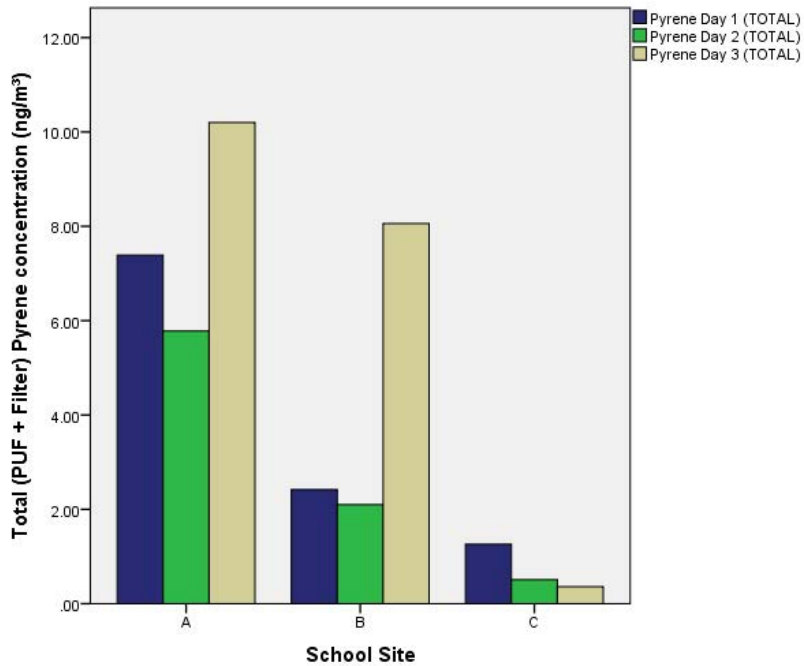
Figure 46: Concentrations of airborne pyrene on the three days before each urine sampling, pyrene concentrations are sensibly different between the three sites.
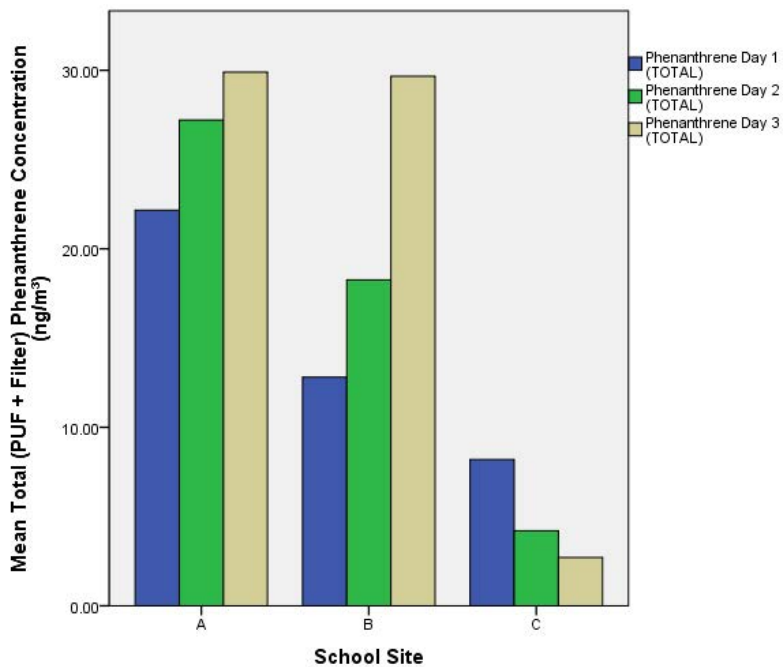
Figure 47: Concentrations of airborne phenanthrene on the three days before each urine sampling, phenanthrene concentrations are sensibly different between the three sites.

Air concentration of phenanthrene showed a different pattern when compared to airborne pyrene, showing higher concentrations on the second day of sampling for site A and B. However, the urinary metabolite did not show a similar pattern as showed in Figure 49, and the measured levels did not show significant differences across the three locations in terms of day to day variation in each site. Similarly, urinary OH-pyrene did not show any significant changes nor any similarity with the pattern of the airborne measurements on a daily basis, as shown in Figure 48. However, for both urinary metabolites, it is possible to observe a lower concentration in the rural background site.
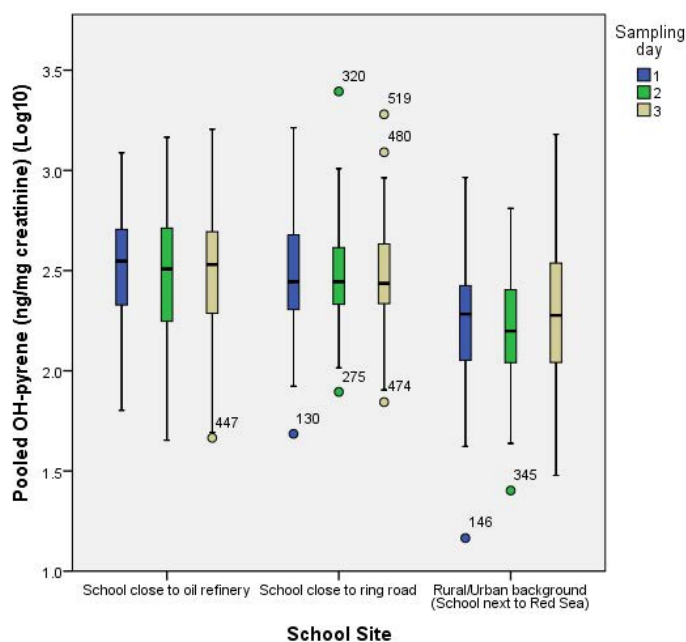
Figure 48: Box plot of the urinary concentration of OH-pyrene, no remarkable difference between the three days can be identified, and the difference between the three sites is less evident.
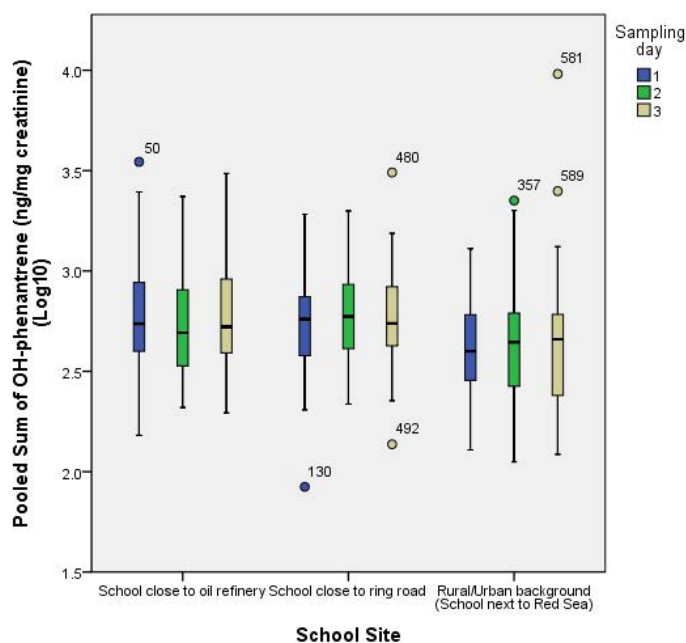
Figure 49: Box plot of the urinary concentration of the sum of OH-phenanthrene, no remarkable difference between the three days can be identified.

In this study, OH-pyrene was analysed together with the different species of OH-phenanthrene. Due to the high level of correlation between the various OH-phenanrene species, the sum of the total OH-phenanthrene concentrations was used for the analyses. Samples were log transformed prior analysis, and then ANOVA test was performed with Bonferroni post hoc comparison within the subgroups created based on the school site. The ANOVA test showed a statistically significant difference between the site subgroups for the urinary OH-phenanthrene measured in all of the samples on each day (p=0.009 for day 1; p=0.06 for day 2; p=0.009 for day 3). OH-pyrene levels also compared with ANOVA test, and it showed statistically significant differences in all of the sampling days (p=0.003 for day 1; p=0.001 for day 2; p=0.002 for day 3). Further information was obtained with the Bonferroni post hoc comparison, which showed a statistically significant difference between the samples from the rural background (location C) and the location next to the ring road (location B) for all the three days for both metabolites. However, there was no statistically significant difference

187

between the samples taken from the school next to the refinery (location A) and location B on any day. Interestingly, no statistically significant difference was observed between location A and location C on any day. The whole three-day data was then pooled together and the three groups were again compared with the ANOVA test with Bonferroni post hoc comparison. In this case, the differences were more relevant. The three groups showed statistically significant difference for both metabolites ($p<0.001$ for OH-phenanthrenes and $p<0.001$ for OH-pyrene). The Bonferroni post hoc comparison showed a difference between the location C and the others for OH-phenanthrene ($p<0.001$ C-B; $p<0.001$ C-A) and for OH-pyrene ($p<0.001$ C-A and C-B). The data was then analysed on a boxplot for both metabolites (Figure 50 and Figure 51) and although the difference is not evident, it is possible to observe a lower concentration for both metabolites in the samples from site C.
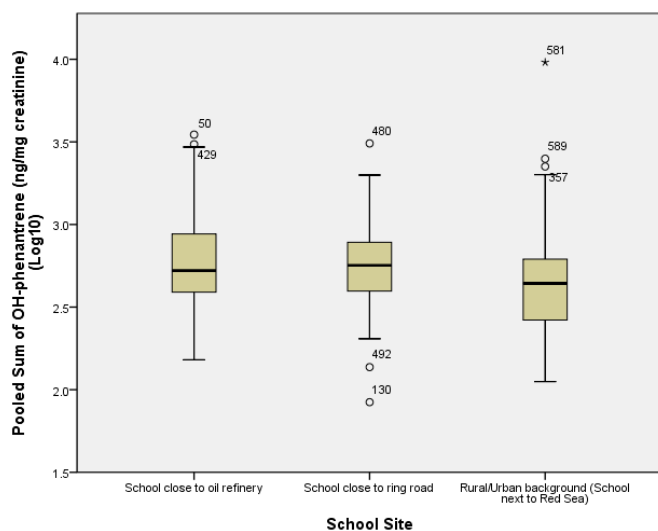
Figure 50: Boxplot showing the pooled data for urinary OH-phenanthrenes divided by the school site.
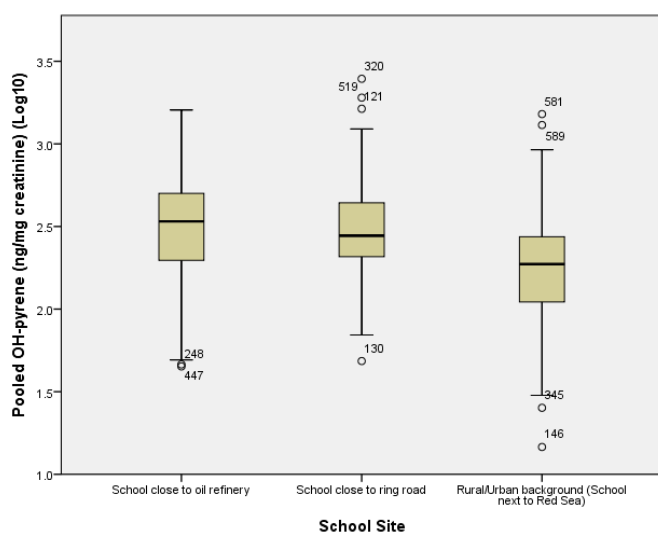
Figure 51: Boxplot showing the pooled data for urinary OH-pyrene divided by the school site.

In light of the findings for urinary 1-hydroxypyrene and airborne pyrene we could conclude that although the dietary exposure plays a primary role in the contribution to the urinary metabolites, the chronic exposure of the subjects of this study is so high that the contribution from the diet, although present, does not invalidate the evaluation of airborne exposure to PAHs of this particular set of subjects.

189

V.2 8-oxo-dG analysis

Urinary 8-oxo-dG was analysed as a DNA damage biomarker in order to study a possible correlation between the exposure level and the level of DNA damage. No correlation was found between 8-oxo-dG and the OH-pyrene ($p=0.237$) or the sum of OH-phenanthrenes ($p=0.255$).

The population was divided in different subgroups based on the school site, and the level of 8-oxo-dG were compared. Unfortunately, the available samples only included one sampling event (day 2) of subjects living next to the oil refinery and next to the ring road. The ANOVA test showed a statistically significant difference between the two available groups ($p=0.001$), that were then plotted on a boxplot graphic (Figure 52). As a control reference, FIXAT + MATCH project's geometric mean (red) ± geometric standard deviation (black) reference lines were added to the graphic, showing significantly higher values for the Saudi samples. The median concentrations and the ranges are reported in Table 83. However, due to the large differences in the populations, the FIXAT + MATCH samples could not be included in the statistical analyses.

Table 83: Comparison of the median concentrations and ranges of urinary 8-oxo-dG between the UK and Saudi subjects.

Group name	Median 8-oxo-dG concentration (ng/g creatinine)	Range (ng/g creatinine)
Jeddah schoolchildren School next to ring road	1.70	0.87-4.12
Jeddah schoolchildren School next to oil refinery	1.48	0.67-3.40
FIXAT subjects	1.14	0.51-2.36
MATCH high level exposed subjects	1.28	0.72-2.41

Interestingly, the subjects at the school next to the ring road showed the higher concentration between the two groups. Another important factor to take in account is the health status of the subjects, therefore the population was divided in groups based on the diseases that they declared in the questionnaires as showed in Table 84, and the 8-oxo-dG levels of each group were compared. The ANOVA test showed a statistically significant difference between the groups, but the Bonferroni post hoc comparison could not be performed due to the small number of cases in some of the groups. The data was then plotted on a boxplot for visualization (Figure 53). It is clear from the figure that certain health conditions are likely to affect the amount of 8-oxo-dG excreted in the urine. Digestive tract issues appear to be an important factor, together with urinary tract problems and interestingly blood diseases. The digestive tract subgroup contains only one subject that is affected of favism, while the others all declared to suffer from diarrhoea. For the blood diseases, only one subject suffered from rheumatic hearth condition, which could be a source of inflammation, while the other subjects all suffered of anaemia-related problems. Interestingly, subjects suffering of pulmonary diseases did not show a significantly higher level of 8-oxo-dG, while the subjects with urinary tract problems, predictably showed a significantly higher level.

Table 84: Groups based on the declared diseases.

No disease declared (N=154)	N/A
Neurological (N=8)	Nervous
	Dizziness, adenoids and headache
	Low visual activity
	Headache
	Hydrocephalus
	Nocturnal enuresis
Digestive tract (N=3)	Diarrhoea
	Favism
Blood (N=5)	Rheumatic heart
	Sickle cell anaemia
	Anaemia
	Sickle cell anaemia carrier
Allergy and bone pain (N=6)	Allergy
	Bone pains
Pulmonary (N=21)	Bronchial asthma
	Cough
Urinary tract (N=2)	Burning micturition
	Urinary tract stone
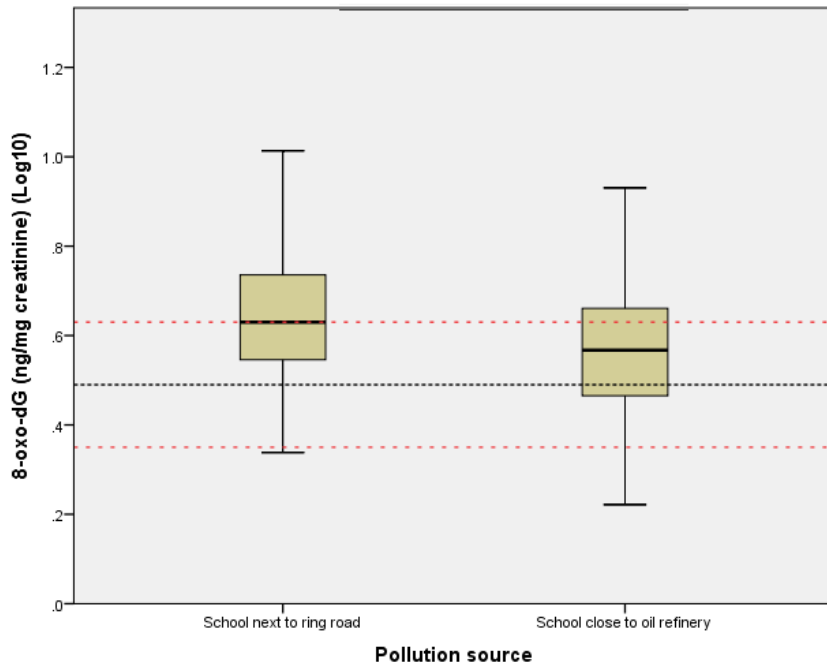
Figure 52: Boxplot showing the urinary concentration of 8-oxo-dG of the subjects from sites A and B. Dotted line represents the geometric mean of FIXAT+MATCH project ± standard deviation (red).
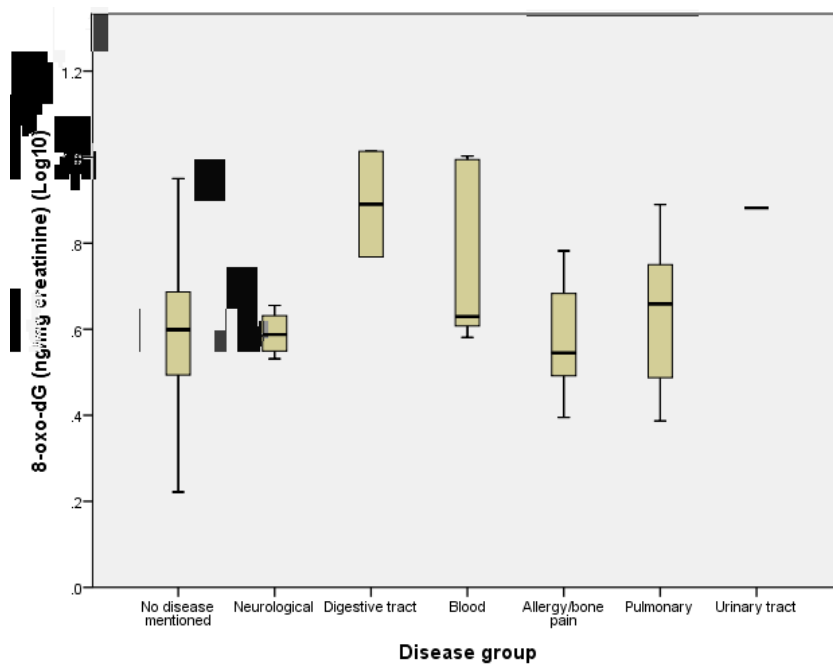
Figure 53: Boxplot showing the urinary concentration of 8-oxo-dG of the subjects divided by the declared disease group.

193

Another relevant factor to analyse was the relationship between the district where each subject lives and the urinary 8-oxo-dG, which would give further information on the contribution of the exposure at home versus the exposure at the school. In this case, the subjects living next to the oil refinery (Pitrumin, 700m away from the refinery) showed a higher concentration of 8-oxo-dG compared to the subjects living in the other districts. Unfortunately, the subjects that indicated "other" in the questionnaires, did not provide further information on the residence location. The subjects living in Qweiza area, were the subjects living next to the ring road, 6Km away from the oil refinery, while the urine samples from Obhur area were not made available.

The same analyses with the same groups were carried out also for the PAHs urinary metabolites to investigate if the pattern seen with the 8-oxo-dG was consistent with the other data. If compared with the urinary concentrations of PAH metabolites, the available data for 8-oxo-dG seems to have a similar pattern, especially for OH-pyrene (Figure 55, Figure 56).
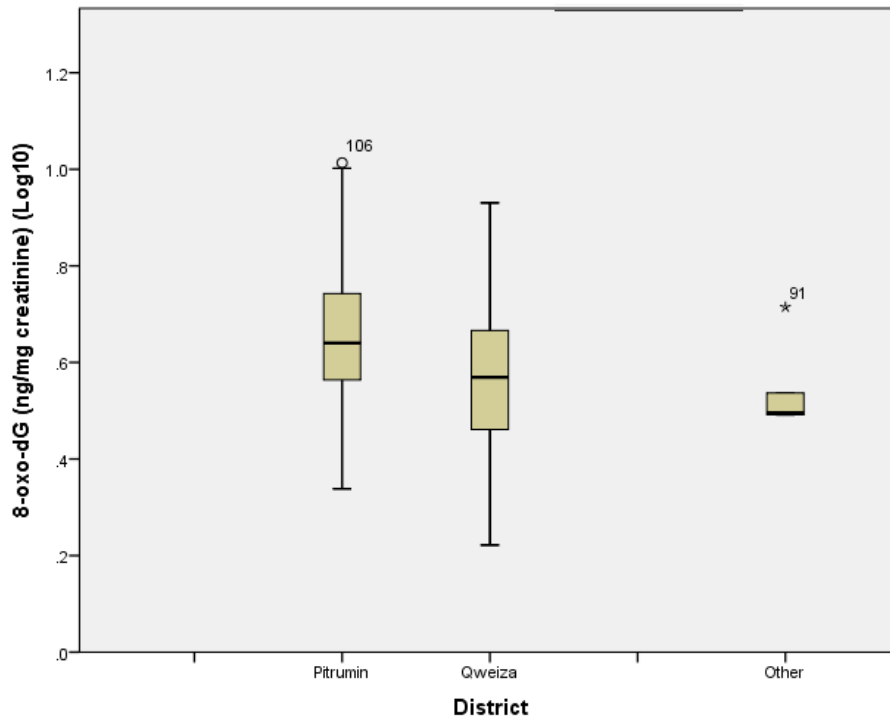
Figure 54: 8-oxo-dG concentrations divided by the living district.
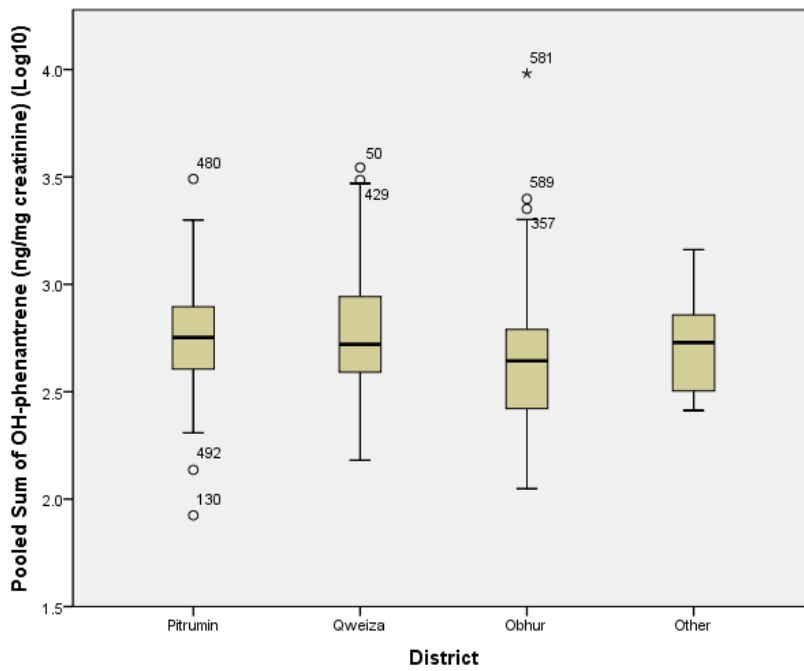
Figure 55: OH-phenanthrene concentrations divided by the living district.
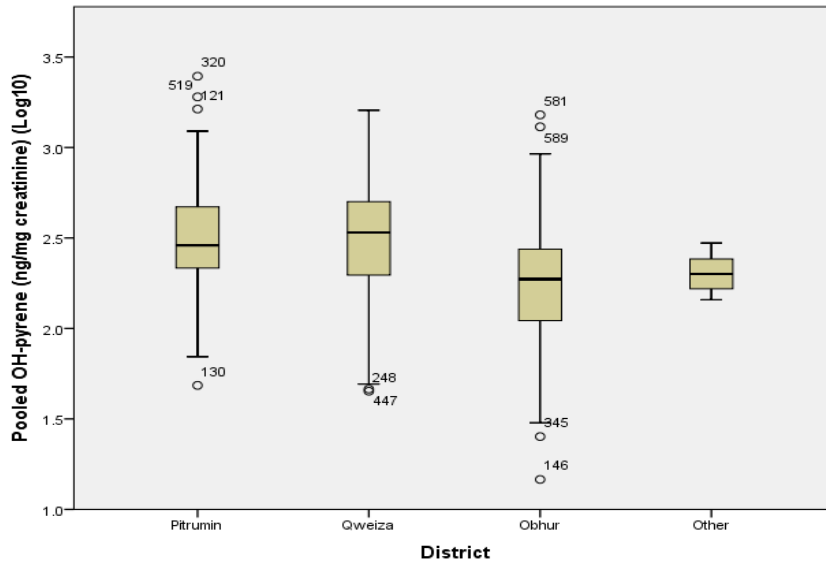
Figure 56: OH-pyrene concentrations divided by the living district.

When analysed for OH-pyrene and OH-phenanthrene, the health condition subgroups did not show significant differences (Figure 57 and Figure 58), although higher concentrations of urinary OH-pyrene were observed in the digestive tract disease subgroup.
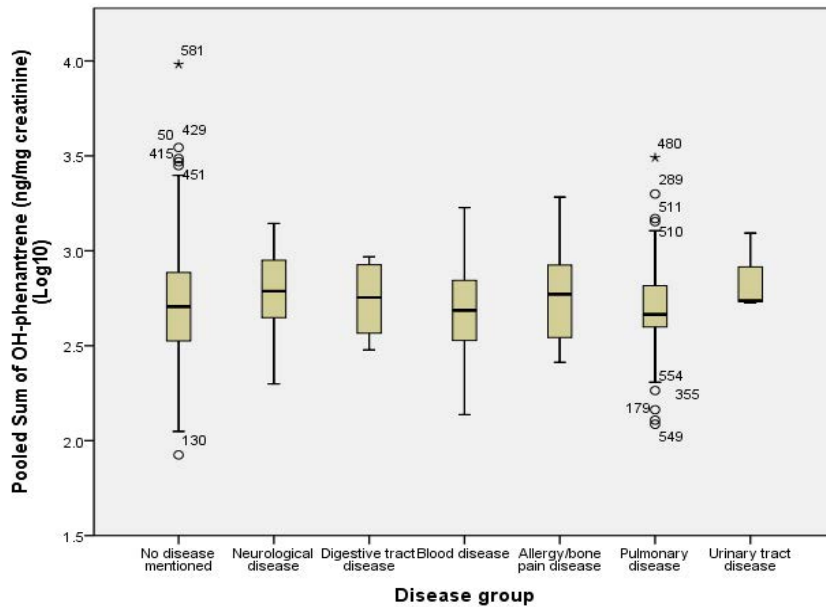
Figure 57: Boxplot of urinary concentration of OH-phenanthrene divided by the declared disease.

196
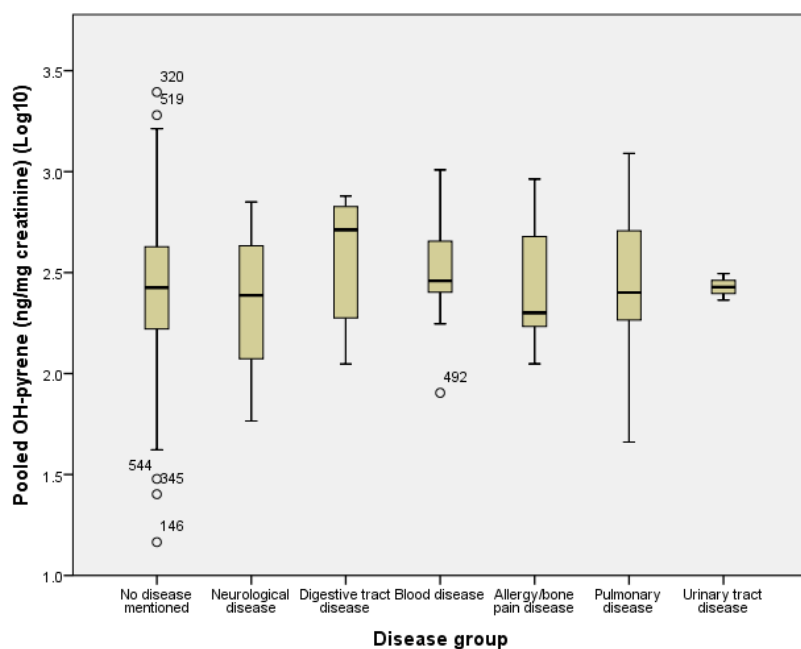
Figure 58: Boxplot of urinary concentration of OH-pyrene divided by the declared disease.

These findings underline the importance of choosing healthy subjects for DNA damage biomonitoring, as any illness or disease can affect the results. It is impossible to determine if the exposure can cause any of the diseases, although it would be possible to hypothesize that lung-related diseases and even allergies could be correlated to the chronic exposure to pollutants, as it has been previously suggested for both PAHs and VOCs (Sørensen et al., 2003a; Rossner et al., 2008; Röder-Stolinski et al., 2008). No significant differences were observed in the urinary concentration of the metabolites for each disease group, except for the urinary tract diseases (Figure 57), and digestive tract diseases (Figure 58). In the case of urinary tract diseases, the number of subjects with that problem is very small (N=2), as well as for the digestive tract diseases (N=3), and the difference in the concentration is not big enough to be related to the disease. Although the main contribution to 8-oxo-dG is likely to be generated from the health condition of the subjects, a difference was observed when the 8-oxo-dG levels were compared based on the living district (Figure 54). This could indicate that the location of the house is also a factor that can influence the levels of oxidative stress of the subjects.

197

V.3 Correlation and statistical analysis

The way the experiment was performed is limiting in terms of possible analyses of the database that go beyond the correlation between the variables and comparison between different subgroups, since the urine samples were collected only on three days in two schools and on three different days in the school in the background site. Although the air sampling campaign lasted for a month, the airborne concentration data that can be used for the correlations with the urinary metabolites is limited to the days where the urine was sampled. The sampling of the urine has been performed only on three days instead of spreading it throughout the month.

Pearson correlation analysis was conducted for all of the variables to highlight the correlations between the variables. To select possible relevant grouping variables, each group was associated with a score, corresponding to the expected level of exposure (e.g. 1 for the background site, 2 for the ring road site, and 3 for the site near the oil refinery). Pearson correlation with the data was then analysed to investigate if there was an increasing pattern for any of the biomarkers that was coherent with the score assigned.

The first correlation that is found is between the income, the school location and the airborne concentration. The same high correlation level is found also between the cooking appliance and the airborne concentration. This can be explained by the fact that the volunteers are living in the various districts based on their income and therefore the different districts have different cooking appliances (some areas might have more gas cookers than others) as well as different levels of exposure. As shown in Table 85, a trend between the geographical distribution is evident, with the subjects living next to the oil refinery belonging to the lowest income interval, in opposition to the majority of the subjects in the highest income interval living in the rural background site. This observation would suggest an environmental inequality, where the cheaper houses are located closer to pollution sources. This inequality is

reflected also in the air quality of the premises of each group, and the consequent health effects, especially as this trend was found also in the urinary PAHs metabolites and 8-oxo-dG, as shown in Figure 59, Figure 60 and Figure 54. Unfortunately, it is not possible to determine if the levels of the PAHs metabolites are influenced by the airborne exposure or from the different cooking appliance effect on food cooking (different temperatures could cause the food to burn more easily).

Table 85: Overview of subjects' distribution based on cooking appliance, district and income.

| | | District | | | |
| | | Pitrumin | Qweiza | Obhur | Other |
		Count	Count	Count	Count
Cooking appliance type	Not answered	2	2	0	0
	Gas	55	60	34	5
	Electric	6	5	16	0
	Gas and Electric	3	1	10	0

| | | Income | | | |
| | | Not answered | <3000 SR | 3000-9000 SR | >9000 SR |
		Count	Count	Count	Count
District	Pitrumin	2	**36**	24	4
	Qweiza	3	19	**36**	10
	Obhur	2	1	21	**36**
	Other	0	2	3	0

Table 85 shows also the distribution of the population in the different districts. Pitrumin and Qweiza district showed a similar distribution of cooking appliances, mainly being gas cookers, a slight different distribution was observed for Obhur district, which had a higher number of electric cooking appliances. As mentioned, a significant difference in the distribution was also observed for the residence district of the population divided by the income intervals. A chi-square test was performed to evaluate the differences between the groups, and in both cases the difference in the distribution was statistically significant ($p<0.001$ in both cases). This difference in the distribution among the cooking appliance type could be partly

responsible for the variations observed in the urinary metabolites (Figure 59 and Figure 60). However, the subjects not living in any of those districts, showed the lowest concentrations of urinary OH-PAHs, suggesting that the geographical placement might have a role in the exposure to PAHs (Figure 55 and Figure 56). However, this pattern is not followed by the 8-oxo-dG concentrations, which were found to be higher in the subjects with gas + electric appliances (Figure 61).
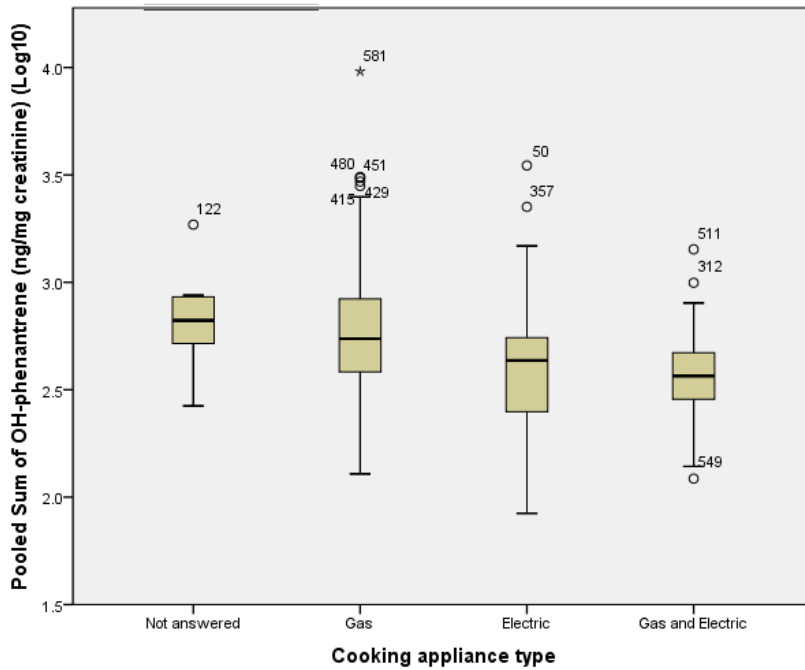
Figure 59: Levels of OH-phenanthrene grouped by cooking appliances type.
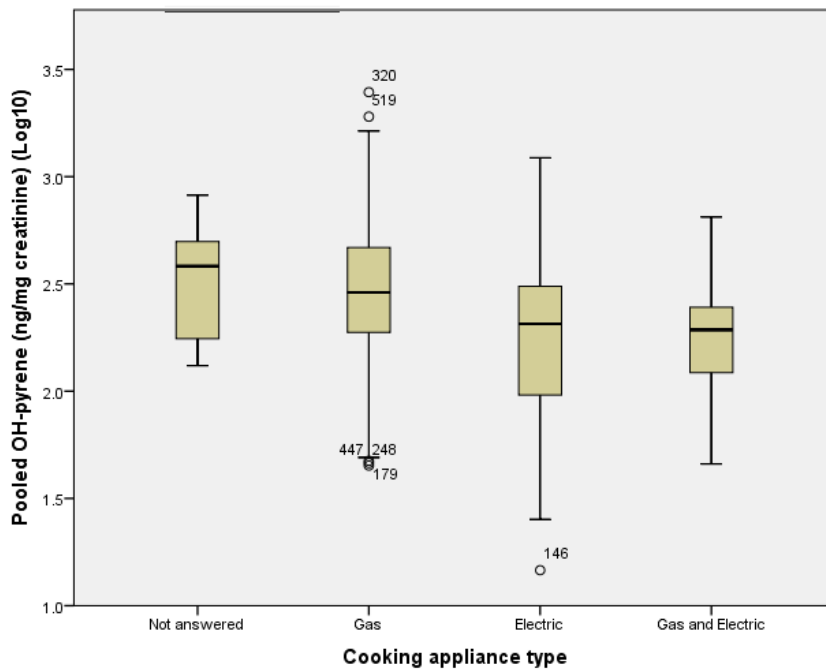
Figure 60: Levels of OH-pyrene grouped by cooking appliances type.
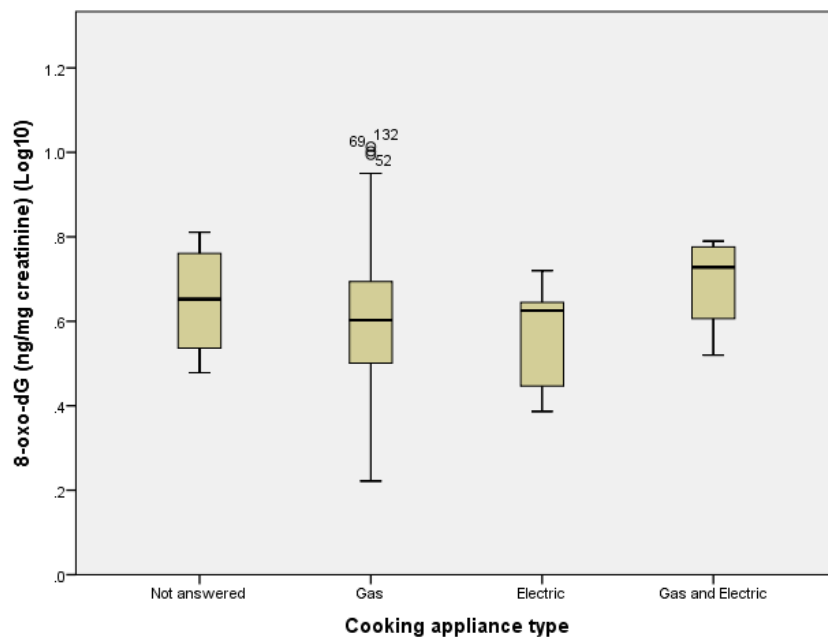
Figure 61: Levels of 8-oxo-dG for each cooking appliance group.

A similar strong correlation was found between the airborne PAH concentration and the distance between the school and the house, confirming the dominance of geographical positioning for the correlation with airborne PAHs levels. All of these correlations highlight a population that is divided mostly by the geographical position and the three groups are not homogeneous between them. Even if this grouping is not helpful to identify characterizing variables from the database, it can be used to identify the three groups by level of exposure since all the variables that are correlated to the geographical distribution can be included in the single variable of the volunteer's house location.

Although marginally significant correlations were found between the cooking appliances and various PAH metabolites, the observed differences between the subgroups are more likely to be related to the district of provenience of the subjects (Table 85). In fact, the correlations were more consistent and significant between the urinary PAH metabolites and the airborne PAH concentrations. Therefore, although a different cooking appliance can affect the way food is cooked, the variable could be represented from the district, due to the strong

correlation with airborne PAHs and the distribution of the cooking appliances in the different districts.

Surprisingly no significant correlations were found between the urinary PAH metabolites and the use of incense, smokers in the house and the habit of kitchen fan use.

The main source of urinary PAH metabolites reported in the literature is suggested to be the diet, specifically from burnt or charbroiled food (Strickland et al., 1996; Harrison et al., 2009; Aquilina et al., 2010; Roggi et al., 1997b). However, when analysed in the current study with an ANOVA test, the PAH metabolites, did not show statistically significant differences ($p=0.475$ for OH-phenanthrenes and $p=0.082$ for OH-pyrenes) between the different subgroups (Figure 62 and Figure 63).
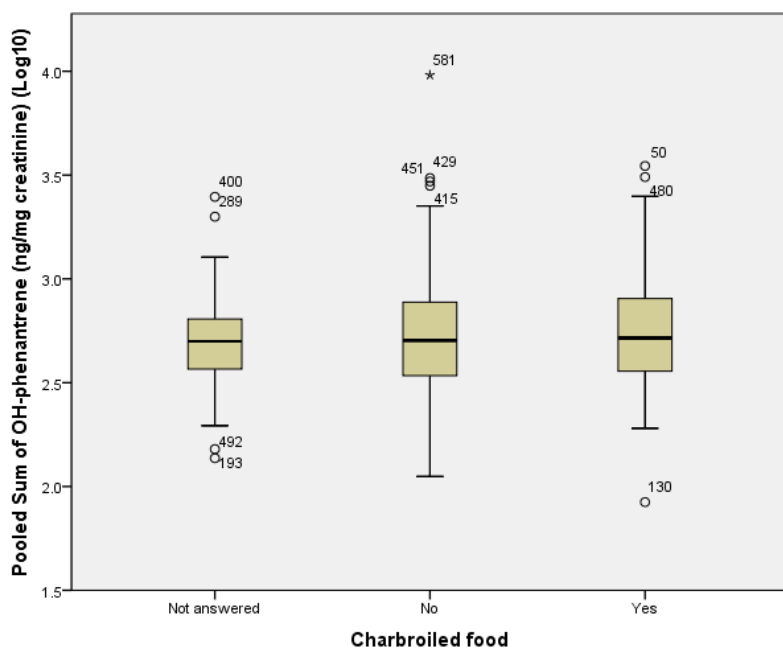
Figure 62: Boxplot showing the sum of OH-phenanthrenes urinary concentrations, divided by charbroiled food consumption.
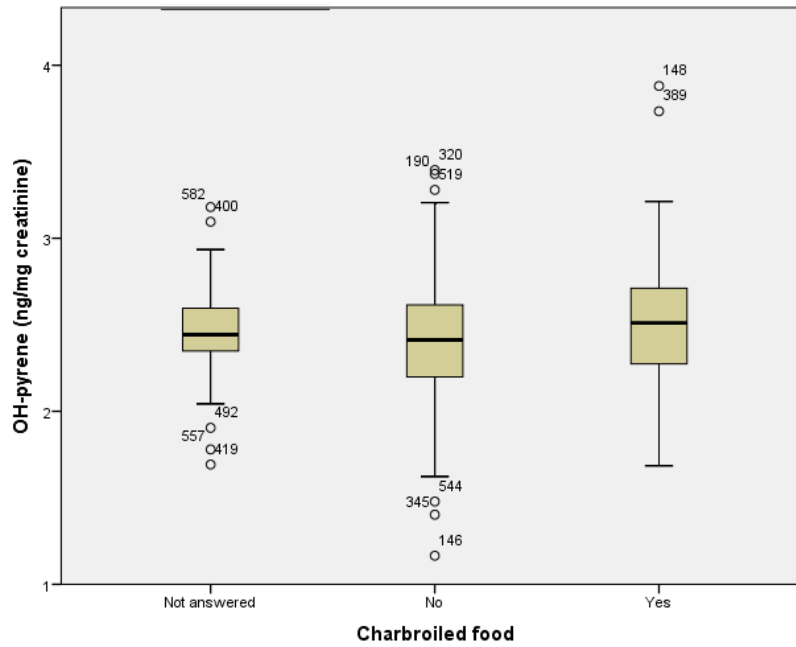
Figure 63: Boxplot showing the OH-pyrene urinary concentrations, divided by charbroiled food consumption.

In Table 86 are summarized the frequencies of each disease group divided by the school site. Since the number of declared disease cases is small, it is not possible to determine a correlation between the geographical area (and therefore the level of exposure) and a predominance of a certain kind of disease. Furthermore, the diseases seem to be evenly distributed between the three groups. The only uneven groups are the allergy/bone pain and blood diseases, which are more frequent in site B (school next to ring road).

Table 86: Frequency table of disease groups divided by school site.

			Count
Disease group	No disease mentioned	Rural/Urban background (School next to Red Sea)	45
		School next to ring road	47
		School close to oil refinery	62
	Neurological	Rural/Urban background (School next to Red Sea)	4
		School next to ring road	2
		School close to oil refinery	2
	Digestive tract	Rural/Urban background (School next to Red Sea)	1
		School next to ring road	1
		School close to oil refinery	1
	Blood	Rural/Urban background (School next to Red Sea)	0
		School next to ring road	5
		School close to oil refinery	0
	Allergy/bone pain	Rural/Urban background (School next to Red Sea)	0
		School next to ring road	5
		School close to oil refinery	1
	Pulmonary	Rural/Urban background (School next to Red Sea)	9
		School next to ring road	9
		School close to oil refinery	3
	Urinary tract	Rural/Urban background (School next to Red Sea)	1
		School next to ring road	1
		School close to oil refinery	0

Interestingly the lung capacity (measured by spirometry) does not seem to be affected by the PAH exposure. In fact no relevant correlation between lung function and airborne or urinary PAH concentration and no significant difference in the subgroups was observed with other tests.

No significant correlation was observed between the urinary PAH metabolites and the corresponding airborne PAH between the three sites in the single day exposure for day 1, while

for day 2 a marginally significant correlation ($p=0.05$) was observed between airborne phenanthrene (filter + PUF) and OH-phenanthrene. Interestingly the airborne phenanthrene was correlated with the OH-pyrene ($p=0.09$), which was not correlated with airborne pyrene.

For day 3, airborne phenanthrene was found to be highly correlated with the urinary OH-phenanthrenes ($p=0.003$) and OH-pyrene ($p=0.001$). Airborne pyrene was found to be highly correlated with the urinary OH-pyrene ($p=0.002$) and marginally correlated with OH-phenanthrenes ($p=0.011$). However, due to the correlations being calculated only over one day, it is not possible to draw conclusions on this set of analyses.

When the three day data was analysed for correlations, a statistically significant correlation was found between OH-phenanthrenes and the airborne phenanthrene ($p<0.001$) and between OH-pyrene and airborne pyrene ($p<0.001$).

However, when the whole 3-day data was analysed for correlations, the urinary OH-pyrene and OH-phenanthrenes showed statistically significant correlation ($p\leq0.001$) with all of the airborne PAHs, with R values ranging from a minimum of 0.142 (benzo(a)pyrene / OH-phenanthrenes) to a maximum of 0.269 (phenanthrene / OH-pyrene).

It is important to underline that these correlations have been calculated with only three time points for the airborne pollutants, as the urine sampling was performed only on three days for each school, so the results might be debatable. The correlation observed, reflects mainly the difference evidenced when the population was divided by school site where, on average, the urinary concentrations showed a pattern that was similar to the airborne concentration.

As mentioned previously, PAH metabolites do not enter the urine by passive diffusion like the unmetabolized VOCs, as they are excreted as glucuronide conjugates (Jongeneelen et al., 1987; Jongeneelen, 2001), in this case we obtained better results using the measurement normalized against creatinine.

These results suggest that in this particular population, there is a correlation between the single event exposure (although the sampling period is very limited) and the urinary metabolites. Although it has been previously reported that the main source of urinary PAH metabolites is the dietary intake of the compounds (Strickland and Kang, 1999; Harrison et al., 2009; Aquilina et al., 2010), in this particular case, the exposure levels are so high that the exposure is influencing the amount of urinary metabolites.

When the subgroups were compared with the ANOVA test, both urinary metabolites concentrations showed statistically significant difference between the site C (rural background) and the sites A and B, but it was not possible to determine any significant difference between the site A and B (next to the oil refinery and next to the ring road). It is important to consider that the two sites are only 5 Km away from each other and the ring road is downwind to the refinery, even though in Figure 46 and Figure 47 is showed that the airborne concentrations are significantly different. Perhaps this result further underlines the influence of food preparation and cooking appliances as PAH sources more than airborne concentration of the pollutants. Another interesting observation is the difference between the geographical location of the residence, which was strongly correlated with the income. This suggests that the population is divided in different areas based on the income, likely because of the housing prices. The population with lower income was concentrated mainly in the vicinity of the oil refinery, while the population in the median range of income was split between the vicinity of the oil refinery and the ring road, with a majority of subjects in the latter. There was a distinct separation in the population with highest income, which were prevalently located in the rural background site. Possibly the vicinity to the oil refinery affects the housing prices, or the area prevalently contains cheaper accommodation, however, this separation generates also a difference in the exposure, as showed in Figure 55 and Figure 56. These differences suggest a social difference that affects the exposure, and potentially the health condition of the subjects. The 8-oxo-dG

data did not provide meaningful data regarding the correlation with the exposure, the urinary 8-oxo-dG concentration was not correlated with any of the PAH metabolites, and it did not show significant differences between the sites, although a difference could be observed (Figure 52). A major problem with the 8-oxo-dG analysis was that no control samples (from site C) were provided, making it difficult to understand if there is a difference between subjects with different levels of exposure at school. When the 8-oxo-dG concentration was compared between the groups created on the base of the district of residence, the subjects living in the "other" districts (not the refinery or ring road district) showed a significantly lower level of 8-oxo-dG, but the sample pool was too small to draw any solid conclusion (N=5). The result, however, is promising and suggests a significant difference based on the location of the house and therefore the vicinity to the pollution sources. Another important finding was the level of 8-oxo-dG in the various disease group, showing a significant difference in people suffering of digestive tract and blood disease, which underlines the importance of recruiting only healthy subjects to avoid misinterpretation of the data. In this specific case, it is likely that the digestive tract disease could have caused higher readings of 8-oxo-dG, in particular diarrhoea. Diarrhoea, as a symptom, could be linked to the inflammatory bowel disease, which is known to be related to oxidative stress and inflammation (Goyal et al., 2014).

A proposal for future studies would be to analyse the urine for other metabolites that are less prone to diet contaminations like unmetabolized VOCs or VOC metabolites together with the analysis of 8-oxo-dG of the whole sample set.

It has not been possible to draw further conclusions or try a regression model, because although the metabolites show a correlation with the exposure, the usable airborne data is limited to only 3 days. Furthermore, 8-oxo-dG in our case did not show any correlation with the PAH

metabolites, while it showed a correlation with 1-OH-pyrene in previous studies (Al-Saleh et al., 2013). Our results could be affected by the lack of data for the control site, which does not allow us to have a low exposed control group.

VI.1 Introduction

People spend much of their time indoors where they are continuously exposed to low concentrations of a wide variety of chemicals including volatile organic compounds (VOCs). It has been demonstrated that building materials, furniture and consumer products are a source of low level VOC exposure contributing together with the outdoor air to the exposure of general population (Delgado-Saborit et al., 2011; Wolkoff, 2012). Indoor exposure to VOCs has been reported to cause a number of symptoms ranging from irritation of the respiratory system to sick building syndrome (Brinke et al., 2004; Wang et al., 2013). In addition, VOCs are also reported to contribute to oxidative stress induction via production of reactive metabolites and hence have the potential to damage DNA (Knaapen et al., 2006; Lu et al., 2007; Saint-Georges et al., 2008; Snyder and Hedli, 1996; Sørensen et al., 2003a; Wang et al., 2013). Exposure to VOCs such as styrene can also lead to an inflammatory response in the lungs that can then result in oxidative stress (Bönisch et al., 2012; Röder-Stolinski et al., 2008; Fubini and Hubbard, 2003; van Berlo et al., 2010). It has been suggested that other compounds such as benzene and toluene can trigger an inflammatory response, but DNA damage caused by such response has not been supported by solid evidence (Pariselli et al., 2009; Wang et al., 2013). The relationship between exposure to benzene and DNA damage has been extensively studied,

especially for occupational exposures, demonstrating that benzene is involved in DNA damage through reactive metabolic intermediates (Snyder and Hedli, 1996; Hartwig, 2010) and generation of reactive oxygen species (ROS) (Barreto et al., 2009). Because of the detailed knowledge of its metabolism, benzene serves as an ideal positive control test compound for the development of new toxicological assays and tests. Furthermore, in several studies of human exposure to benzene at medium and low concentrations (0.001 ppm – 0.03 ppm), a correlation was found between levels of urinary benzene, benzene metabolites and urinary 8-oxo-2'-deoxyguanosine (8-oxo-dG) a biomarker that is widely considered to be associated with ROS related DNA damage and repair (Andreoli et al., 2012; Manini et al., 2010; Bagryantseva et al., 2010).

A recent review (Hartwig, 2010) summarised the possible mechanisms through which benzene and its metabolites can cause DNA damage. *In vivo*, after absorption, benzene is metabolized by cytochrome P450 2E1 (CYP2E1) in the liver to produce metabolites including: phenol, catechol, hydroquinone and 1,2,4-trihydroxybenzene. These closed ring metabolites then undergo further metabolism in the bone marrow to form *o-* and *p*-benzoquinone. *p*-Benzoquinone is thought to be the toxic metabolite responsible for myelotoxicity due to its high reactivity and is known to form adducts with proteins and DNA (Rappaport et al., 2005; Linhart et al., 2011). Bone marrow, due to the high rate of cell mitosis and lower DNA repair capacity (Buschfort-Papewalis et al., 2002) is a sensitive tissue for DNA instability, resulting in possible health effects sooner than other tissues.

All of the phenolic benzene metabolites are chemically active in cells, increasing the oxidative DNA damage, directly or through further metabolism. Therefore, although the main target tissues and the metabolism of benzene have been studied and understood, it is important to develop models to assess the toxicity caused by VOC exposure of other metabolically

competent tissues including the lung epithelium, which is a principal route of exposure to benzene and other VOCs. Such models could also be useful to study other VOCs, as currently there is little evidence of any linkage between exposure and DNA damage for other VOC species, even if it has been suggested from studies in animal models and *in vitro* studies (Sarma et al., 2011; Singh et al., 2009).

Currently there are only few detailed studies on the development of *in vitro* models to examine the effects of such pollutants on the lung. Pariselli et al. (2009) developed a method to expose lung cells to various concentrations of benzene and toluene, evaluating some of the effects caused by the pollutants on lung cells growing on permeable supports that allow exposure of cells to gas mixtures, modelling a real life situation of exposure.

Although Pariselli et al. (2009) reported interesting results on inflammatory response triggered by VOC exposure and cell viability, no further studies on DNA damage or ROS production were reported deriving from lung epithelium models. We therefore aimed to progress by exploring the feasibility of an air-liquid interface (ALI) *in vitro* model using lung epithelial cells grown on a permeable membrane. The main focus of this study was on the short-term effects of exposure of lung cells to VOCs with two main endpoints: oxidative stress and DNA damage.

VI.2 Methodologies

The experiments and analyses were conducted following the methodologies described in sections III.15 - III.19 (Methodologies)

VI.3 RESULTS

Visual analysis of the cells before and after the exposure demonstrated little mechanical stress and slight drying sustained by the cells due to the airflow, but this did not result in overt cell toxicity as assessed by light microscopy (data not shown).

VI.4 Induction of Oxidative Stress

Cells were analysed for levels of ROS before and after the exposure together with a control that was exposed only to synthetic air and a set of incubator controls both with and without apical medium. The results presented in Figure 64 clearly indicate that cells exposed to 0.3 ppm benzene had elevated levels of intracellular ROS compared to controls as assessed by the fluorescein oxidation assay. The presented results are based on two independent replicates (each including three technical replicates) for 0.03 ppm and 0.3 ppm benzene, and a single measurement of three technical replicates for 0.1 ppm benzene. Incubator control results are based on 5 independent replicates. The means were compared using ANOVA with Bonferroni post-hoc comparisons. The only statistically significant difference between the pre and post exposure was observed for the 0.3 ppm benzene exposed cells ($p < 0.001$).

Pre/Post Exposure Fluorescence
(Fluorescence Units)
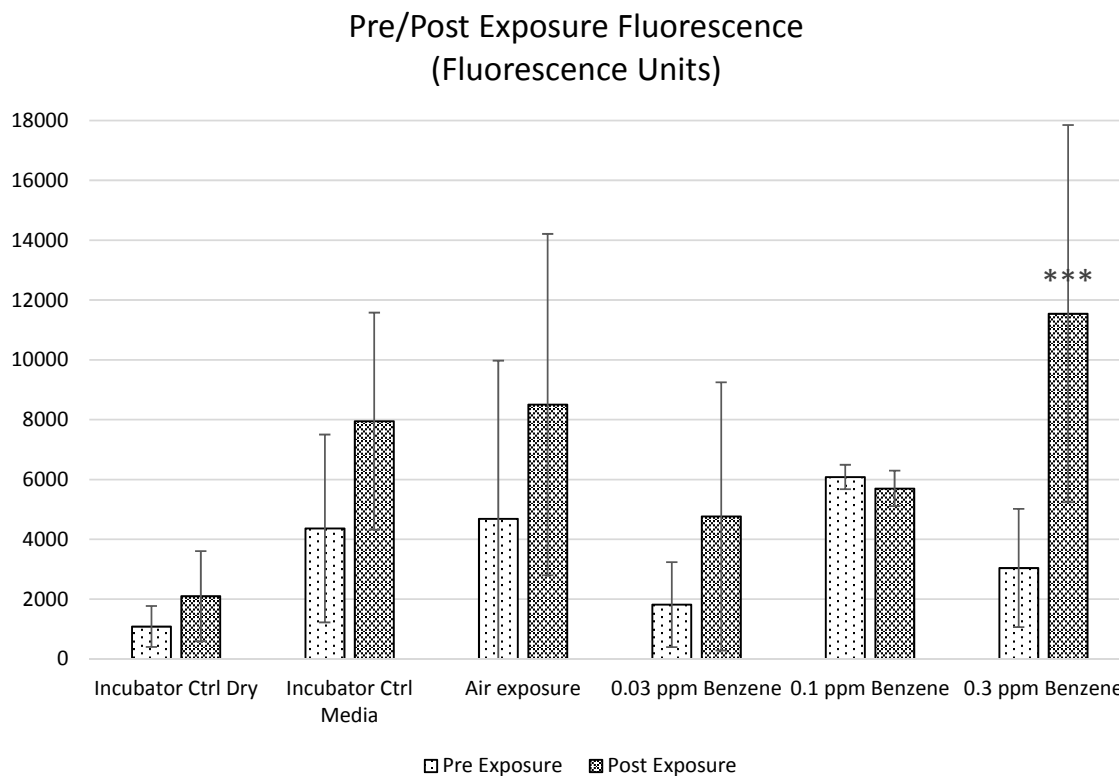
Figure 64: Summary of the DCF fluorescence measurements before and after exposure. Error bars represent standard deviation. (***) indicates a statistically significant difference between the pre and the post exposure.

A generalized increase of fluorescence was observed in all the treatments, including the incubator controls, suggesting a baseline production of ROS in normal cell metabolism. Unfortunately, in the case of 0.1ppm benzene exposure, the pre-exposure condition showed a high reading, most likely due to media background fluorescence. These findings are comparable with previously published data (Pariselli et al., 2009). Although further tests need to be performed, treatment with benzene shows an oxidative effect in the cells, which could be related to either an inflammatory response to benzene itself, or more probably to oxidising metabolites such as benzoquinones (Snyder and Hedli, 1996).

VI.5 Induction of DNA Strand Breaks

Following exposure of cells to air (Controls), low (0.03 ppm) and medium (0.1 ppm) benzene concentrations, the incidence of highly damaged "hedgehog" cells was below 15% of the total number of cells counted. In contrast the number of not quantifiable nuclei was increased at higher benzene concentrations (23–60% of unquantifiable nuclei, N=300). The high frequency of "hedgehogs" at the highest concentration of benzene exposure tested (0.3 ppm) made DNA damage quantification difficult and is probably indicative of direct benzene cytotoxicity to cells at this concentration of benzene exposure.

Table 87: Descriptive statistics of the Comet assay data (% tail intensity).

	N (Cells)	Mean	Median	Standard Deviation	Variance	*H* (Variance/ Mean)
Incubator Controls (Media)	1550	4.52	0.74	9.40	70.98	20.61
Incubator Controls (Dry)	1376	6.73	3.40	11.80	135.26	18.98
Controls	1028	7.40	3.15	11.60	147.82	20.77
0.03 ppm benzene	314	11.49	3.21	15.58	243.83	21.26
0.1 ppm benzene	318	16.02*	5.53*	21.28	456.49	28.37
0.3 ppm benzene	375	12.27*	5.13*	16.90	303.75	23.29

The % tail intensity was used as a metric to quantify DNA damage in the cells. Experimental data were pooled from 5 controls and one of the independent replicates of each exposure, which consisted of 3 technical replicates for each exposure, and the findings are summarised in Table 87 and Figure 65 and Figure 66. Each exposure event was compared with the relative control. The reported data refers to a single exposure experiment and the relative controls. Two other independent replicates were performed for the 0.03 ppm and 0.3 ppm exposure, obtaining similar results (Table 87, appendix X.9). Differences were observed between the absolute values of the tail intensities, however the ratios between the exposed and the control cells were found to be similar between the replicates. The ratios were compared by a Kruskal-Wallis

215

independent samples test, which retained the null hypothesis of the samples having the same distribution (p=0.102). An independent samples median test also retained the null hypothesis, suggesting that the medians of the ratios are similar (p=0.354). The observed differences of the absolute values between the independent replicates are likely due to different starting conditions of the cells. Tail intensities of each exposed sample were compared with its relative air exposed control using Friedman's 2-way ANOVA by ranks. The only statistically significant differences were observed between the samples exposed to 0.1 ppm and 0.3 ppm benzene and their relative controls. No statistically significant difference was observed between air exposed controls and incubator controls under any condition.

A clear concentration-dependent induction of DNA strand breaks following the exposure to benzene (0.03–0.3 ppm) was observed as assessed by the exposed/control ratio of mean tail intensity (Figure 67). In general, due to the large numbers of "hedgehogs", the cells exposed to 0.3 ppm benzene, showed a decreased amount of DNA damage compared to the cells exposed to 0.1 ppm benzene. Linear regression analysis showed a significant correlation between the level of benzene exposure and the extent of DNA damage when the value was normalized against the relative controls (R^2=0.718). An increase of DNA damage was observed in all the exposures, however a statistically significant difference with the control was observed only for 0.1 ppm and 0.3 ppm benzene concentrations (Figure 65). Figure 66 represents the pooled data of each exposure experiment in a scatter plot. Data was plotted to evaluate the increase of dispersion of the readings with higher benzene exposure, indicating a generalized increase in DNA damage related to the concentration of airborne benzene. Importantly, we observed a statistically significant increase in levels of DNA-strand breaks in cells exposed to 0.1 ppm benzene were there was no evidence of direct cytotoxicity to the cells.

Mean Tail Intensities (%)
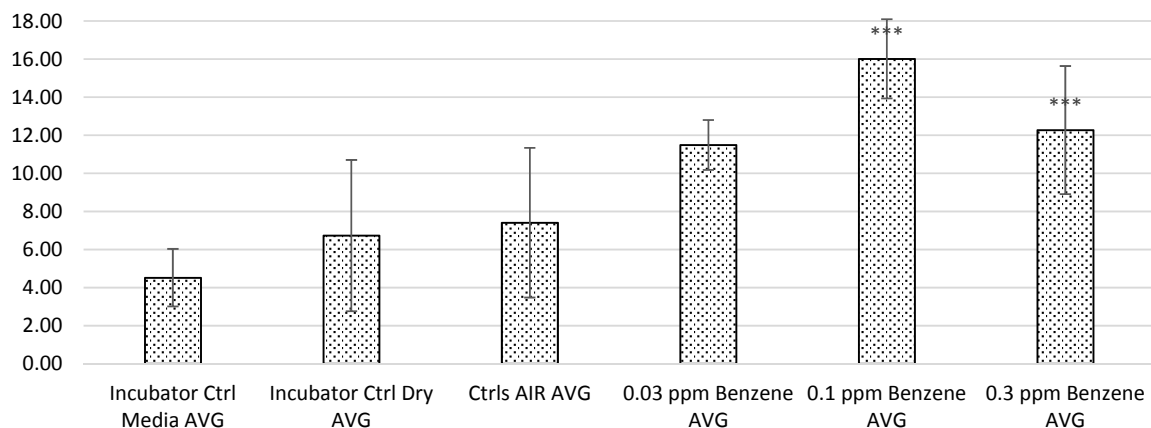
Figure 65: Mean tail intensity (%) of the control and exposed cells. Error bars represent the standard deviation of the means. (***) indicates a statistically significant difference between the exposed sample and their relative controls. The figure includes 3 independent replicates for the controls, and a single independent replicate for the exposed cells. Each independent replicate consisted of 3 technical replicates.

The coefficient of dispersion (*H*) (Tice et al., 2000), expressed as the ratio of variance/mean, was also calculated to further evaluate the data (Table 87). *H* is an accepted parameter used to express the dispersion of the Comet data and is sensitive to a large DNA damage response in a small proportion of cells and is also less affected by inter-experimental variability. All of these parameters were consistent with a concentration-dependent increase in DNA strand breaks following exposure of cells to benzene.
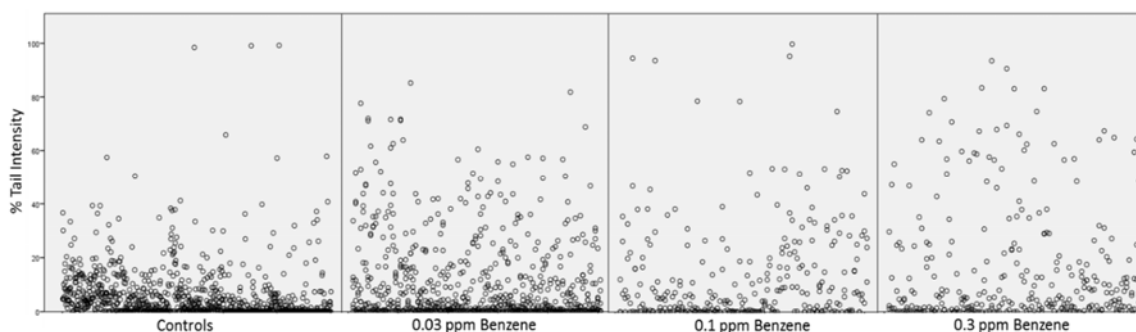
Figure 66: Graphical representation of the % tail intensities measured in the Comet assay as a function of benzene concentration. The figure includes 5 independent replicates for the controls, 2 independent replicates for 0.03 and 0.3ppm benzene, and a single independent replicate for 0.1ppm benzene.

To minimise the effects of inter-experiment variability, the ratio of mean % tail intensity between the treated cells and the controls of the same exposure experiment were compared. This enhanced the difference between the low level exposure (2h 0.03 ppm) and the higher levels. The reported ratios (Figure 67) are obtained by averaging the ratio of exposed/control mean tail intensity (%) of each independent replicate. Interestingly, the difference between the ratios of the two higher conditions was smaller than the difference in the ratios observed between the 0.03 ppm and 0.1 ppm benzene, as shown in Figure 67. Moreover, the higher concentration showed a lower ratio than 0.1 ppm exposed cells, with a higher variability. This is likely due to the higher amount of unquantifiable cells in the highest benzene concentration, which made the quantification difficult.

218
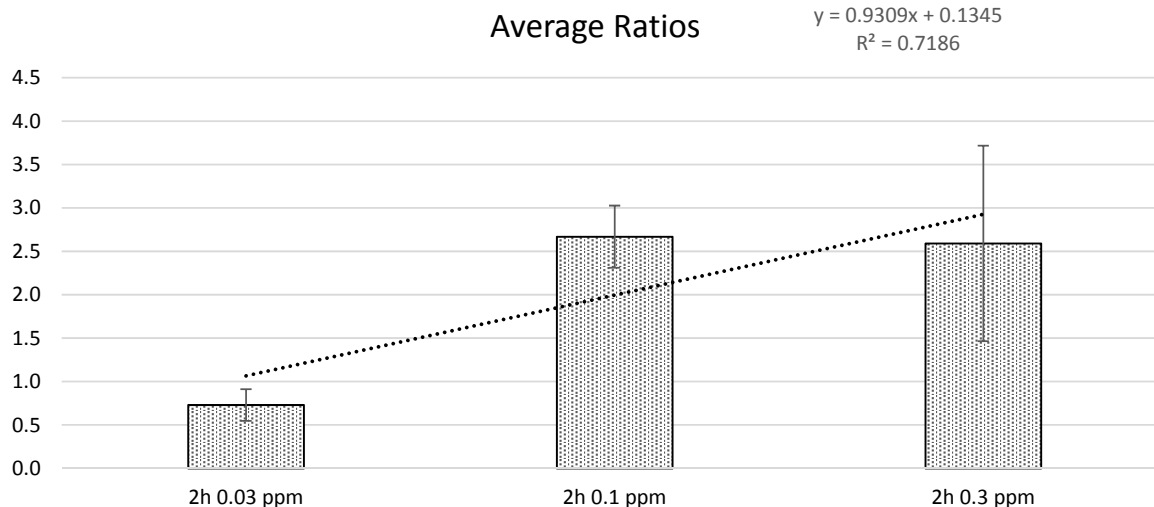
Figure 67: Average ratio between mean % tail intensities of treated cells and each of the relative controls of 3 independent replicates for 0.03 ppm and 0.3ppm and of 2 independent replicates for 0.1 ppm. Error bars represent standard deviation between the ratios of each replicate.

VI.6 DISCUSSION

The aim of this pilot study was to develop an *in vitro* system to study the short-term effects of airborne exposure of lung cells to toxic chemicals in a physiologically relevant model. Although carcinoma cells were used instead of primary cells, A549 cells are commonly used as a toxicological model for lung tissue. Moreover, they represent a relevant model as they express CYP2E1, which is required for the metabolic activation of benzene which, *in-vivo* has a multi-organ toxicity (Snyder and Hedli, 1996; Hartwig, 2010; Bois et al., 1996). Benzene was chosen as a test pollutant due to its known genotoxic effects. The benzene concentrations used in this work lie between typical ambient air and occupational exposure levels. Delgado-Saborit et al. (2009; 2011) report personal exposure concentrations measured in the UK of 0.15-30.2 $\mu g\ m^{-3}$ (0.05-9.3 ppb) while in Saudi Arabia, ambient air benzene concentrations ranged from 0.09-1.1 ppb (Alghamdi et al., 2014) and in 43 Chinese cities varied between 0.7-10.4 ppb (Barletta et al., 2005). These compare with concentrations in this study of 0.03-0.3 ppm (30-300 ppb), and the European Union ambient air quality standard of 5 $\mu g\ m^{-3}$ (1.5 ppb).

On the other hand, the occupational exposure limit for benzene recommended by both NIOSH (US) and HSE (UK) is 1 ppm time-weighted average over an 8-hour shift.

As previously reported, a limitation of many *in vitro* cellular models of lung toxicity is that cells are exposed to test chemicals dissolved in cell culture media (Pariselli et al., 2009). This is physiologically unrealistic, and furthermore the media itself can have a matrix effect, and the uptake and the interaction of the pollutants with the cells does not model a real life situation (Ritter et al., 2001). The exposure vessel custom designed and used in this study proved to be a useful and reliable tool to evaluate the effect of benzene on cultured lung A549 cells. It also represents a more physiologically relevant model than exposure of cells to benzene dissolved in cell culture media. Development of permeable cell culture inserts has allowed the development of models whereby lung epithelial cells can be grown in a monolayer and the apical medium can be removed during the exposure to airborne chemicals, thus better modelling a lung epithelium and allowing exposure of cells to controlled atmospheres. This method of exposure is much closer to the *in vivo* situation, providing a more realistic model for exposure studies. For logistical reasons, the gas flow rate was higher than previously reported in the literature (10 ml/min vs. 2 ml/min). However, this variable did not affect the performance of the exposure vessel or the cells negatively, as shown by the comparisons between incubator controls and synthetic air exposed controls (Figure 64 and Figure 65). However, some mechanical stress was observed in the cells at the centre of the airflow by optical microscopy analyses, which can be solved in future experiments by reducing the flow rate as suggested by Pariselli et al. (2006). A longer exposure (4h) of cells to synthetic air was also tested, but the majority of the cells did not survive during the experiment. Overall, our findings confirmed the feasibility of the experiments and the reliability of the model.

Measurement of ROS levels using the DCF assay showed a significant increase of oxidised fluorescein in cells exposed to 0.3 ppm benzene for 2 hours. This finding is in accordance to previously reported data that has linked benzene exposure with inflammation and ROS production (Pariselli et al., 2009; Wang et al., 2013; Barreto et al., 2009; Mögel et al., 2011; Sørensen et al., 2003a). Furthermore, this data is consistent with a previous report by Mögel et al. (2011) that A459 cells express CYP2E1, that is involved in the benzene metabolism and ROS production. Intracellular ROS production could be related to the production of oxidising metabolites of benzene such as benzoquinone as suggested by Hartwig (2010).

Metabolism of benzene has also been linked to DNA damage due to reactive benzene metabolites and ROS generation, which was confirmed in the current study by the results of the Comet assay. Figure 66 shows the direct correlation between the benzene concentration and DNA damage, and importantly there is evidence of DNA strand breaks at concentrations that were not directly toxic to the cells. One of the possible mechanisms of action could be through the known reactive benzene metabolite p-benzoquinone. One of its cellular targets is topoisomerase-II, which is inhibited, generating DNA double strand breaks (DSB) which are then repaired by homologous recombination (HR) or non-homologous end-joining (NHEJ). NHEJ is thought to be more error prone than HR, however DSB damage can lead to DNA aberrations by base deletions or translocations. Furthermore, the metabolism of benzene through redox cycling and generation of ROS is proposed as another main source of DNA damage. When hydroquinone or catechol are oxidised in the presence of oxygen, superoxide (O_2^-) is formed, which is then transformed into H_2O_2 by superoxide dismutase. H_2O_2 is not responsible for DNA damage directly, but it increases the oxidative potential of the cell, and in the presence of transition metals ions, hydroxyl radical ($\bullet$OH) is formed, which reacts with lipids, proteins and DNA. $\bullet$OH is responsible for the formation of 8-oxo-dG and other

nucleobase alterations due to oxidation (Halliwell and Aruoma, 1991). H_2O_2 production is also

linked to an inflammatory response of which, as stated above, VOC exposure has been reported

to be a trigger.

In addition to non-toxic concentrations, relatively high concentrations of benzene (compared

to the concentrations that can be found in everyday life), which have been reported to have

cytotoxic effects as well as genotoxic effects, were also studied. In the current study, we saw

evidence of benzene cytotoxicity following exposure of cells for 2h at a concentration of 0.3

ppm as indicated by the fact that a large number of cells nuclei were not quantifiable by the

Comet assay, but appeared instead as "hedgehogs". Further evidence of that effect is

represented by the tail intensity data normalised against the controls (Figure 67) and the *H*

value decreasing at the higher concentration. In the 0.3 ppm exposure a significant fraction of

nuclei in the Comet assay appeared as "hedgehogs" and were classified as unquantifiable (23-

60% of unquantifiable nuclei, N=300 per slide), while at lower levels of benzene, the number

of "hedgehogs" was negligible. Although the significance of hedgehog nuclei in the Comet

assay is not entirely clear, they may represent nuclei derived from either apoptotic or necrotic

cells, although other studies have shown that cells sustaining extensive DNA damage can

survive and repair the damage taken (Collins, 2004). These results could provide a further

insight on the interpretation of the data reported by Pariselli et al. (2009). In that study, the tests

were performed after incubating the cells in complete medium for 24h after the exposure,

allowing the cells to respond to the pollutants and their effects. It is known that large amount

of DNA damage and bulky adducts can trigger apoptotic response (Roos and Kaina, 2006), and

furthermore benzene has been shown to be capable of inducing apoptosis by production of

ROS and DNA damage in leukaemia cells (Sarma et al., 2011). In our study, cells were

analysed immediately after 2h exposure, not allowing sufficient time for the apoptotic response

to happen, therefore there is the possibility that most of the "hedgehogs" we observed in the higher benzene exposure could eventually undergo apoptosis.

The Comet assay data confirmed the genotoxic activity of benzene not only in its widely acknowledged target organs (bone marrow), but also in metabolically competent cells derived from lung epithelial cells. Although the mechanism of action was not fully elucidated, generation of ROS arising from benzene exposure was observed, as well as an association between exposure to benzene and DNA damage.

In conclusion, with this pilot study we have confirmed that A549 cell line represents a valid model to test exposure and DNA damage arising from airborne exposure to benzene. Although the metabolism of benzene *in vivo* is more complex and involves different tissues, A549 cells appear to produce a response that is comparable to the one found in the target organs of those compounds *in vivo*. Further metabolomic and proteomic studies would be needed to confirm the theory, and further studies with different endpoints will be needed in order to have a better understanding of benzene metabolism and its correlation with DNA damage. Furthermore, the results obtained by studying a well known carcinogen confirmed the validity of this model for analysing not only the effects of exposure of lung cells to benzene, but also opens the way for analysing other common VOC with the same procedure, to assess the potential DNA damage caused by exposure.

VII. Conclusions

VII.1 Assessment of VOC and PAH exposure in different population groups in Birmingham, UK

The main outcome of this study was that although a correlation between exposure to VOCs and variations in the level of oxidative stress can be observed for general population, a prediction model could not be calculated due to the limited amount of samples that affected the precision of the model. The precision of the prediction model was of crucial importance to be a valuable tool, since the changes in 8-oxo-dG concentration are generally very small. Different categories of general population, although being exposed to similar concentrations of pollutants, have proven to be very variable in terms of lifestyle. These heterogeneous categories, together with the limited amount of subjects proved that there are also differences in lifestyle that can affect oxidative stress, which in some cases are not directly related to exposure. General population is often referred at as a control group for occupational exposure studies, however, we observed a variance which should be taken in consideration especially when the group is used as a comparison factor.

We observed significant correlations between urinary VOCs and urinary cotinine even in subjects that were categorized as "non-ETS exposed". These findings would suggest a role of ETS as a contributor to VOC exposure even at concentrations that are not categorized as second hand smoking but can still be detected by urinary cotinine measurement. This finding would also suggest the contribution of third hand smoke towards the exposure, however, although aged smoke has been reported as a source of PAHs and nicotine (Winickoff et al., 2009; Matt et al., 2008; Hoh et al., 2012), long term persistency of VOCs due to aged smoke has not been proved (Sleiman et al., 2014). Another possible route of exposure to third hand

tobacco smoke could be from visited places. Interestingly, the ETS exposure levels (although urinary cotinine was still detected) in the subjects living/working in new or recently refurbished buildings were generally lower. Taking in account that by definition, the control group should not have any new furniture or carpets, we could speculate that in some cases the house or furniture were exposed to tobacco smoke and are still releasing persistent pollutants such as PAHs and nicotine (Matt et al., 2008). The hypothesis of third hand tobacco smoke exposure would be in accordance with the observed correlation between airborne PAHs and cotinine (Fleming et al., 2012). Third hand tobacco smoke, however, does not fully explain the correlation observed between urinary cotinine and VOCs in the non-ETS exposed subjects. Although it has been proved that certain cloths can off gas VOCs after being exposed to tobacco smoke (Chien et al., 2011), this explanation would be applicable only to relatively fresh exposures (i.e. presence in a room of a person who smoked outside). VOCs, due to their characteristics of volatility, would not be likely to remain in an ambient or off gas from clothing after long periods.

A slight difference was observed for 8-oxo-dG between the different subgroups of FIXAT project, although this difference was not found to be statistically significant. This underlines a shortcoming of this study, which lies mostly in the number of participants. The variations in the levels of oxidative stress are generally reported to be minimal between groups with significantly different levels of exposure (e.g. non-smokers vs smokers which differ on average by ~20% (Evans et al., 2010)), while the sample pool of this study was mostly general population. Even the occupationally exposed subjects were not exposed to significantly higher levels of VOCs compared to the control subjects.

Significant differences in urinary VOCs, however, were observed between the FIXAT and MATCH project subjects. MATCH subjects were exposed mainly to high levels of ETS, and the samples included in this study were chosen within the higher exposed subjects.

Although differences were observed within the markers of exposure, no significant difference was observed in 8-oxo-dG within the groups. This lack of observable results could be sourced back to the general homogeneity of the exposure levels, as within our sampling groups we did not have levels of exposure that stood out, except for MATCH. Moreover, the sample number was too small to observe statistically significant differences on the levels of a metabolite that is known to have small variations. In addition, the spot sampling of different subjects, accounted for more variability in the oxidative stress measurements. Different subjects with different lifestyle, had different levels of oxidative stress. In a relatively small sample pool, it is not an easy task to determine the causes and the effects of the oxidative stress.

Although we observed that exposure to low levels of benzene does have an effect on cells that are metabolically competent towards the compound (chapter VI), we failed to observe those effect on the FIXAT or MATCH population. This is likely due to the different activities and other substances that the subjects are exposed to, that generate a baseline level of oxidative stress that cannot be related only to VOC exposure. Statistically significant correlations between urinary VOCs and 8-oxo-dG were observed in the ETS exposed subjects (Table 64). Interestingly, in this case, the correlations were observed between the various combinations of sums of urinary BTEX + naphthalene and 8-oxo-dG and between toluene, ethylbenzene, xylenes and 8-oxo-dG. Another significant correlation was observed between urinary naphthalene and 8-oxo-dG but in this case the coefficient was negative. Still in Table 64, the non-ETS exposed subjects showed statistically significant correlations between the combinations of sums of urinary BTEX + naphthalene and urinary cotinine. The same subgroups of samples showed correlations between urinary cotinine and urinary toluene and o-xylene. These findings suggest that even at extremely low ETS exposures, in our sample pool, the main contributors to the urinary VOCs are probably ETS and third hand tobacco smoke. It is important to remember that the subjects that participated in this study belong to the general

226

population, and they spent most of their time indoors. Moreover, more than 47% of the non-ETS exposed subjects had an indoors job (either office work or not), which would reduce the chances of ETS exposure. The measured levels of cotinine for this subset are extremely low, however, a statistically significant correlation is observed. The coefficients of correlation are low (R=0.347, urinary BTEX + naphthalene and cotinine), suggesting that the correlation between urinary BTEX and cotinine is weak but, however, statistically significant (p=0.004). The observed variability suggests that ETS is not the only contributor towards the urinary VOCs, but the observations suggest that it is one of the main ones.

Furthermore, a threshold of effect was observed for VOC exposure upon oxidative stress (Figure 38, Figure 39 and Figure 40). We observed that above the ETS exposed threshold (urinary cotinine >1µg/L) a relationship between urinary BTEX + naphthalene and 8-oxo-dG could be found. Under that threshold, no kind of relationship between urinary VOCs and 8-oxo-dG was detected. However, when the relationship between urinary cotinine and urinary BTEX + naphthalene was considered (Figure 39) a correlation between urinary cotinine and urinary BTEX + naphthalene was observed for non-ETS exposed subjects, while in the ETS exposed subjects, the correlation was observed between urinary cotinine and 8-oxo-dG, and more importantly, between urinary BTEX + naphthalene and 8-oxo-dG (Table 64). From these results, it is possible to conclude that for our sample pool, the point of discrimination between non-ETS exposed subjects and ETS exposed subjects, also represents a threshold of effect for VOC exposure. Under that threshold, 8-oxo-dG is not correlated to the urinary concentration of VOCs, while above the threshold, the correlation becomes more evident. The observed threshold coincides with the point of intersection between the regression line of the cotinine/urinary BTEX + naphthalene plot, and the discriminant urinary cotinine concentration between ETS exposed and non-ETS exposed subjects. In terms of urinary concentration of total VOCs the threshold was identified at 550 ng/L of BTEX + naphthalene. The proportions of the

single compounds are variable in each sample, however, subjects with higher total urinary VOCs concentrations showed higher 8-oxo-dG concentrations (Figure 40).

In conclusion, the definition "threshold of effect" is due to the difference observed above and under the reference. Above the threshold of effect, it is possible to see a relationship between the urinary BTEX + naphthalene and 8-oxo-dG, under the threshold, the variance of the 8-oxo-dG is not related to the urinary BTEX and it might be caused from other sources or activities. The threshold of effect represents the data reported in Table 64.

In addition, in section IV.9 we observed a statistically significant correlation between airborne phenanthrene and 8-oxo-dG. This finding suggests that for the concentrations observed in FIXAT and MATCH project, phenanthrene exposure has an effect on oxidative stress or it represents the effect of the PAH mixture, as it is one of the most abundant of the compounds (Aquilina et al., 2010). Furthermore, the lack of correlation observed between airborne phenanthrene and urinary cotinine, suggests that tobacco smoke is not the main source of phenanthrene in our study. Unfortunately, we were not able to measure the urinary metabolites of the PAHs with our instruments in the FIXAT samples. Although the urinary PAH metabolites measurements were available for MATCH project, no correlation was found between any of the urinary PAH metabolites and 8-oxo-dG. Similar results were obtained with the samples from Jeddah schoolchildren, where no correlation between urinary PAH metabolites and 8-oxo-dG was observed (V.3).

No significant differences in the urinary levels of VOCs or 8-oxo-dG were observed in subjects living or working in new or recently refurbished buildings after 1 year (IV.2). An important factor to consider is that the number of samples was relatively small, and we could only perform a spot sampling, so it is possible that the measured 8-oxo-dG values could be affected by other factors. However, the significance of the t-test was not far from being statistically significant ($p=0.087$). This result could suggest that with a bigger sample size, it

might be possible to find a statistically significant difference, by reducing the inter-sample variation. Marginally significant differences were observed in the urinary VOC concentrations of the occupationally exposed subjects at the end of the working week and after the weekend, for ethylbenzene ($p=0.023$) and m+p-xylene ($p=0.017$), suggesting a work-related source of these two compounds. However, also in this case, the lifestyle of some volunteers (smoking and ETS exposure) could be a confounding factor. In particular, in this case the commitment factor played a key role, as together with positively committed subjects, there were subjects that declared to be non-smokers, which was in contrast with the measurement of urinary cotinine. Also in this case, the sample number was relatively small, and the sampling was performed only in two events for each subject. Although the two urine sampling events were close to each other (Saturday morning at the end of the working week and Monday morning at the beginning of the working week), and the variations in the subjects' lifestyle was minimal, we could not identify statistically significant differences in 8-oxo-dG concentration between the two days.

PCA analyses (IV.10) evidenced a difference between urinary and airborne VOC data, where generally the urinary VOC measurements had a higher significance in explaining the variance of the data set. However, the differences in some of the airborne samplings (occupationally exposed group) could possibly have influenced the results due to the limited amount of compound measured. Moreover, when all the variables were included in the analyses for the full data set, too many factors were extracted, underlining the need of a higher number of samples for successful extractions. Within the urinary biomarkers, xylenes, ethylbenzene and toluene were found to be the main contributors to the variance. 8-oxo-dG although present within the principal components, was not one of the main contributors towards the variance. Another variable that was always present within the principal components, was the urinary cotinine, which proved to be another important marker to take in account even for subjects that

are classified as non-ETS exposed. PCA also confirmed the utility and feasibility of sampling campaigns based on general population and low exposed individuals. Moreover, when only the urinary biomarkers were analysed we were able to identify three principal components which allowed to interpret as lifestyle related VOC exposure, ETS and oxidative stress caused by exposure (Table 75).

The results obtained by the calculation of a linear model for 8-oxo-dG underlined some of the shortcomings of this study. Although we obtained significant predictors for 8-oxo-dG (benzo(a)pyrene, age range, 9,10-anthraquinone, anthracene), we were not able to obtain a good prediction for 8-oxo-dG on the validation subset. This is likely due to the limited amount of samples available to build the model, and to the high inter-individual variance of 8-oxo-dG, which, together with the generally small span of the variation in the concentration, make the calculation of an accurate model difficult.

Another important factor that must be taken in account and that can affect the results dramatically is the volunteers' commitment. Although in this study all the subjects voluntarily applied for the monitoring, not everyone was committed and interested in the study. Many subjects volunteered within the university, and had a scientific background, or however had a basic understanding of a personal sampling experiment. This subset was generally very open in providing information about the sampling day. This allowed us to collect good data and information about the sampling events. However, in some cases the subjects tended to provide extremely detailed information, or even overestimate some of the questions. A fitting example is given by some of the subjects that answered positively to the question regarding ETS (both at home and in the workplace), however, according to the cotinine analyses, the exposure they were subjected to was not relevant and the subjects were not categorized as ETS exposed. Upon investigations, the subjects explained that they considered to be exposed to ETS because of occasional exposures happening outside of the workplace (ETS in the workplace) or because

230

their smoker partners smoked outside of the house (ETS at home). This information alone, without urinary cotinine measurement, would have been misleading in the analyses.

On the other hand, subjects that did not have a scientific background, tended to provide the more honest and "correct" answers to the questionnaires. However, in some cases they were reluctant to provide details about their day. This was evident in some of the occupationally exposed subjects: some subjects were interested in the study and very collaborative, mainly driven by curiosity. Other subjects were reluctant to provide information and in some cases, did not provide true information to some of the questions (some subjects declared to be non-smokers, which was in contrast with the measured amount of urinary cotinine).

The "commitment" factor is often underestimated or not mentioned in the studies, and especially if a significant amount of data is gathered from questionnaires, it should be taken in great consideration. It is important to have a committed population, and in some cases it is preferable that the volunteers are not overcommitted, in order to avoid false positives or incongruent data. In conclusion, the commitment factor should be taken in account especially for information that cannot be double assessed with a measurement (i.e. ETS exposure), in order to avoid bias in the collected information.

In conclusion, with this project, we demonstrated the possible application of headspace microextraction for biomonitoring of low exposed subjects, together with personal exposure sampling. Furthermore, the measurement of oxidative stress biomarkers showed the influence of exposure to different pollutants (ETS, VOCs, PAHs) towards the oxidative stress. We were able to link exposure to some of the pollutants to early effects biomarkers (8-oxo-dG), which suggests that indoor and outdoor exposures to low levels of pollutants can have an effect on the oxidative stress. We managed to identify a threshold of effect for urinary VOCs, as a pivotal point between exposure and early effect (measured as oxidative stress). This threshold of effect defines the point where the exposure level leads to an observable effect on the oxidative stress.

Under that threshold, although it is possible to find a correlation between urinary cotinine and urinary VOCs, it is not possible to observe an effect.

This study can be considered a pilot for further projects based on general population, and the difficulties encountered in this project can be used to enhance future studies. The crucial aspects to be considered would be:

-Sample number

-Subjects commitment

-Number of measurements per subject

-Timing of the biological samplings

VII.2 PAH and oxidative stress biomonitoring in Jeddah school children

The analyses of the data obtained from Saudi Arabia, showed the contribution of outdoor pollution sources towards the urinary PAHs metabolites. Although in previous works it has been suggested that the main contribution towards urinary OH-PAHs arises from dietary intake (Harrison et al., 2009), we observed differences in the urinary PAH metabolites concentration between populations living in different districts and being exposed to different levels of pollution.

Similar differences in the urinary concentration of the PAH metabolites were observed between subjects using different cooking appliances, which however were also related to different areas, which might have a stronger effect on the PAH exposure. Moreover, no difference was observed between subjects eating charbroiled food and subjects eating non-charbroiled food. Charbroiled food is a known source of urinary PAHs, as dietary intake is generally suggested to be one of the main sources of exposure (Strickland and Kang, 1999; Strickland et al., 1996). Our findings suggest that in this case dietary exposure could be not the only source of exposure, as airborne PAHs showed to have an effect on the urinary

concentration of their metabolites when the subjects were divided based on the residence area. This difference suggests an inequality based on the wealth of the population, since the subjects with lower income were mostly residing in the vicinity of the oil refinery, while the majority of subjects in the higher income were residing in the rural background site. The residence area is likely to be affected by the income and this results in a difference in the air quality and higher exposure to pollutants of the subjects with lower income.

Differences were observed in the urinary 8-oxo-dG levels between subjects living in different areas. Unfortunately, due to the limited availability of samples and sampling events, it was not possible to investigate in depth the correlations between 8-oxo-dG and the measured PAHs. However, the lack of correlation between urinary PAH metabolites and the oxidative stress biomarker observed in this case, would suggest other sources of oxidative stress, which have not yet been analysed, although significantly higher concentrations of 8-oxo-dG were observed in relation to some disease conditions. Due to the sampling sites location (next to an oil refinery and a heavy-traffic ring road) it is possible that VOCs play a major role in the contribution to the oxidative stress, instead of PAHs.

The results suggest possible further developments with a set of analyses similar to the ones performed for FIXAT project. This would allow to explore a broader spectrum of metabolites and biomarkers, in order to identify possible contributors to the oxidative stress in this particular population.

VII.3 In-vitro test of exposure to controlled benzene concentration on metabolically competent cells

With this pilot experiment, we tested the feasibility of an *in-vitro* test to study the early effects of exposure to pollutants. We used benzene as it is a known carcinogen and its metabolic pathway has been extensively studied. We designed a custom exposure vessel in order to

233

expose cells to controlled atmospheres. With this project we intended to test the feasibility of the methodology, in order to open the way to more detailed future studies. Moreover, we aimed to create a system to expose the cells in a physiologically relevant way, creating a toxicological model that is closer to the real life situation than the classical model. We managed to have successful exposures of cells to air and to benzene at three key concentrations. We were able to observe early effects of exposure on DNA, with a dose-effect relationship.

These findings suggested that both the design of the exposure vessel and the A549 cells, are a valid toxicological model to study the exposure to VOCs. The designed model could be used for future projects, in order to evaluate the effects of exposure to various pollutants, or to study the metabolic pathways of different compounds.

VII.4 Shortcomings of the studies

Each project showed a number of weaknesses and shortcomings that, however, provided useful information for future projects and development.

FIXAT project

With FIXAT project, we encountered difficulties in developing the method for measurement of quinones in urine (benzoquinone, hydroquinone and PAH quinones), due to the low concentrations of the metabolites we were analysing. The main problem is represented by confounding factors, as it is possible that some biomarkers can be also related to normal metabolism, such as hippuric acid, which is a metabolite of toluene, but can also be a result of catabolism of proteins or metabolism of naturally occurring benzoic acid (Fustinoni et al., 2000). Confounding factors and specificity are generally not a major issue, since most of the studies are performed either on occupationally exposed subjects or on populations that have a high baseline exposure (e.g. highly polluted cities/areas, smokers). The importance of the specificity of the biomarkers is inversely proportional to the exposure level. When analysing general population, the confounding factors can provide a significant contribution towards certain biomarkers, affecting the significance of the analyses. In our study, we noticed an important contribution of ETS, even in cases that were classified as non ETS exposed. This suggests that the exposure that does not arise from ETS is so small that cigarette smoke, even at extremely low concentration, can affect the biomarkers. A possible solution for this issue, would be the search for new biomarkers. However, this task is not easy, as urine is a complex matrix and there are significant variations induced by variables such as diet, genetic polymorphisms, and many more. This variability makes the typical biomarker discovery approaches (e.g. metabolomics) difficult, as it is hard to isolate metabolites and biomarkers that are only related to exposure to pollutants.

We could not see statistically significant differences between our groups when urinary 8-oxo-dG concentrations were compared, which might be caused by the relatively small number of subjects in each group and the small variability of 8-oxo-dG concentrations. Probably, with larger study populations, it would be possible to detect statistically significant differences within different groups of general population.

PAH and oxidative stress biomonitoring in Jeddah schoolchildren

The analyses performed on the samples obtained from Saudi Arabia did not show the same correlations observed between PAHs (airborne and urinary) and oxidative stress. Although the 8-oxo-dG levels were higher than the ones measured in FIXAT and MATCH projects (Figure 52, section V.2), the comparison would not be significant, as the two subsets are coming from different populations with different age ranges, lifestyles and genetic subsets. The main shortcomings of this study were the sampling campaign, which only provided samples from three days, with the control samples not collected at the same time as the exposed subjects. Furthermore, the control urine samples, and the urine samples of day 1 and 3 of the campaign were not made available for 8-oxo-dG measurement. This made it difficult to draw conclusions based on the oxidative stress only. In this study, no personal exposure data was collected, and all the correlations were calculated using fixed site data, which provided only three data points, and only for urinary metabolites. Although the sample size was large, the sampling time span was limited and it was not possible to assess correlations between airborne concentrations of PAHs and their urinary metabolites or 8-oxo-dG. Further measurements of urinary VOCs were not included in this campaign, making it difficult to make comparisons with FIXAT and MATCH results, since the only overlapping measurements were the ones of 8-oxo-dG for FIXAT and 1-OH-pyrene and 8-oxo-dG for part of the samples from MATCH.

Benzene effects on DNA damage using a novel *in-vitro* exposure device

The study on DNA damage caused by exposure to low concentrations of benzene using A549 cells provided good results. However, since it was a pilot study in quest of observable effects in order to proceed with further studies, few replicates were performed. DCF assay was performed only on one of the concentrations, in order to evaluate if there was production of ROS arising from exposure to benzene. The study was aimed to assess if it was possible to observe an effect that was caused by the exposure, and the result was positive.

However, this *in-vitro* system lacks one of the other main contributors towards oxidative stress, which is the immune system. Often, exposure is related to oxidative stress damage that is caused from the immune system reaction to the exposure (Klebanoff, 2005; van Berlo et al., 2010; Knaapen et al., 2006; Schottenfeld and Beebe-Dimmer, 2006; Wang et al., 2013). In our case, it would not be possible to observe such secondary effects. However, it would be possible to measure inflammation biomarkers (e.g. interleukin-8) which are strongly correlated to inflammatory response and neutrophils chemotaxis.

VIII.   Future directions

The results obtained from these studies underlined a need of new biomarkers of exposure for general population.

It was not possible to determine a "fingerprint of exposure" due to the high variability of the samples, although the population could be considered homogeneous. One of the main directions that would be worth following is to identify metabolomic profiles of different groups of subjects, using a large sample base. Similar studies are undergoing (Smolders et al., 2014), however they mainly focus on biomonitoring. A metabolomic approach on a large cohort of subjects could add useful information on exposure and allow to identify a fingerprint of

exposure on a complex matrix such as urine. This would not be possible in small scale experiments, due to the high variability of the samples and the analysed matrix.

Another interesting field of study would be to develop further our cell exposure vessel, creating a hybrid, more complex system, with different tissues that would serve as a more complete model for exposure and toxicological studies. Recent developments on 3D cell cultures and organ-on-a-chip systems could be implemented and improved for specific exposure experiments (Huh et al., 2012; Bhatia and Ingber, 2014).

IX. Bibliography

Albinet, A., Leoz-Garziandia, E., Budzinski, H., et al. (2007) Polycyclic aromatic hydrocarbons (PAHs), nitrated PAHs and oxygenated PAHs in ambient air of the Marseilles area (South of France): Concentrations and sources. **Science of the Total Environment**, 384: 280–292

Al-Saleh, I., Alsabbahen, A., Shinwari, N., et al. (2013) Polycyclic aromatic hydrocarbons (PAHs) as determinants of various anthropometric measures of birth outcome. **The Science of the total environment** [online], 444: 565–78. Available from: http://www.ncbi.nlm.nih.gov/pubmed/23314068 [Accessed 2 May 2014]

De Alwis, G.K.H., Needham, L.L. and Barr, D.B. (2009) Automated solid phase extraction, on-support derivatization and isotope dilution-GC/MS method for the detection of urinary dialkyl phosphates in humans. **Talanta** [online], 77 (3): 1063–1067. Available from: <Go to ISI>://WOS:000262761300021 [Accessed 22 May 2012]

Andreoli, R., Protano, C., Manini, P., et al. (2012) Association between environmental exposure to benzene and oxidative damage to nucleic acids in children. **Med Lav**, 103 (5): 324–337

Aquilina, N.J., Delgado-Saborit, J.M., Meddings, C., et al. (2010) Environmental and biological monitoring of exposures to PAHs and ETS in the general population. **Environment International** [online], 36 (7): 763–771. Available from: <Go to ISI>://WOS:000280886700015

Asahi, M., Kawai, M., Toyama, T., et al. (2014) Identification and quantification of in vivo metabolites of 9,10-phenanthrenequinone in human urine associated with producing reactive oxygen species. **Chemical research in toxicology** [online], 27 (1): 76–85. Available from: http://www.ncbi.nlm.nih.gov/pubmed/24443938 [Accessed 26 November 2014]

Bagryantseva, Y., Novotna, B., Rossner, P., et al. (2010) Oxidative damage to biological macromolecules in Prague bus drivers and garagemen: Impact of air pollution and genetic polymorphisms. **Toxicology Letters** [online], 199 (1): 60–68. Available from: <Go to ISI>://000283910500009

Barreto, G., Madureira, D., Capani, F., et al. (2009) The role of catechols and free radicals in benzene toxicity: an oxidative DNA damage pathway. **Environmental and molecular mutagenesis** [online], 50 (9): 771–80. Available from: http://www.ncbi.nlm.nih.gov/pubmed/19449395 [Accessed 28 July 2013]

Benowitz, N.L. (1996) Cotinine as a biomarker of environmental tobacco smoke exposure. **Epidemiologic reviews**, 18 (2): 188–204

Van Berlo, D., Wessels, A., Boots, A.W., et al. (2010) Neutrophil-derived ROS contribute to oxidative DNA damage induction by quartz particles. **Free radical biology & medicine** [online], 49 (11): 1685–93. Available from: http://www.ncbi.nlm.nih.gov/pubmed/20828610 [Accessed 4 August 2014]

Bhatia, S.N. and Ingber, D.E. (2014) Microfluidic organs-on-chips. **Nature biotechnology** [online], 32 (8): 760–772. Available from: http://www.ncbi.nlm.nih.gov/pubmed/25093883 [Accessed 7 August 2014]

Bi, X., Sheng, G., Feng, Y., et al. (2005) Gas- and particulate-phase specific tracer and toxic organic compounds in environmental tobacco smoke. **Chemosphere** [online], 61 (10): 1512–22. Available from: http://www.ncbi.nlm.nih.gov/pubmed/15975627 [Accessed 21 November 2014]

De Blas, M., Navazo, M., Alonso, L., et al. (2012) Simultaneous indoor and outdoor on-line hourly monitoring of atmospheric volatile organic compounds in an urban building. The role of inside and outside sources. **The Science of the total environment** [online], 426: 327–35. Available from: http://www.ncbi.nlm.nih.gov/pubmed/22542255 [Accessed 9 December 2014]

Boersma, M.G., Balvers, W.G., Boeren, S., et al. (1994) NADPH-cytochrome reductase catalysed redox cycling of 1,4-benzoquinone; hampered at physiological conditions, initiated at increased pH values. **Biochemical Pharmacology** [online], 47 (11): 1949–1955. Available from: http://www.sciencedirect.com/science/article/pii/000629529490068X

Bois, F.Y., Jackson, E.T., Pekari, K., et al. (1996) Population toxicokinetics of benzene. **Environmental health perspectives** [online], 104 Suppl (December): 1405–11. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1469729&tool=pmcentrez &rendertype=abstract [Accessed 26 March 2014]

Bolden, A.L., Kwiatkowski, C.F. and Colborn, T. (2015) New Look at BTEX: Are Ambient Levels a Problem? **Environmental Science & Technology** [online], p. 150415000233002. Available from: http://pubs.acs.org/doi/abs/10.1021/es505316f

Bönisch, U., Böhme, A., Kohajda, T., et al. (2012) Volatile organic compounds enhance allergic airway inflammation in an experimental mouse model. **PloS one** [online], 7 (7): e39817. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3389035&tool=pmcentrez &rendertype=abstract [Accessed 4 March 2013]

Brinke, J., Selvin, S. and Hodgson, A. (2004) Development of new volatile organic compound (VOC) exposure metrics and their relationship to "sick building syndrome" symptoms. **Indoor …** [online], (510): 140–152. Available from: http://onlinelibrary.wiley.com/doi/10.1111/j.1600-0668.1998.t01-1-00002.x/abstract [Accessed 14 March 2013]

Brunnemann, K.D., Prokopczyk, B., Djordjevic, M. V, et al. (1996) Formation and analysis of tobacco-specific N-nitrosamines. **Critical reviews in toxicology** [online], 26 (2): 121–37. Available from: http://www.ncbi.nlm.nih.gov/pubmed/8688156 [Accessed 2 November 2014]

Buschfort-Papewalis, C., Moritz, T., Liedert, B., et al. (2002) Down-regulation of DNA repair in human CD34(+) progenitor cells corresponds to increased drug sensitivity

and apoptotic response. **Blood** [online], 100 (3): 845–53. Available from: http://www.ncbi.nlm.nih.gov/pubmed/12130494 [Accessed 23 June 2014]

Campisi, J., Morreo, G. and Pardee, A.B. (1984) Kinetics of G1 transit following brief starvation for serum factors. **Experimental Cell Research** [online], 152 (2): 459–466. Available from: http://linkinghub.elsevier.com/retrieve/pii/0014482784906475 [Accessed 19 July 2014]

Campo, L., Cattaneo, A., Consonni, D., et al. (2011) Urinary methyl tert-butyl ether and benzene as biomarkers of exposure to urban traffic. **Environment International** [online], 37 (2): 404–411. Available from: <Go to ISI>://000287620100013

Chen, L., Bowen, P.E., Berzy, D., et al. (1999) Diet modification affects DNA oxidative damage in healthy humans. **Free Radical Biology and Medicine**, 26 (98): 695–703

Cheng, J., Sulpizi, M. and Sprik, M. (2009) Redox potentials and pKa for benzoquinone from density functional theory based molecular dynamics. **The Journal of chemical physics** [online], 131 (15): 154504. Available from: http://www.ncbi.nlm.nih.gov/pubmed/20568869 [Accessed 19 March 2012]

Chien, Y.C., Chang, C.P. and Liu, Z.Z. (2011) Volatile organics off-gassed among tobacco-exposed clothing fabrics. **Journal of Hazardous Materials** [online], 193: 139–148. Available from: http://dx.doi.org/10.1016/j.jhazmat.2011.07.042

Clayton, G.D. and F.E.C. (1995) G. D. Clayton and F. E. Clayton (eds). Patty's industrial hygiene and toxicology. Toxicology, 4th edn, vol. ii, parts A - f. John Wiley & Sons, New York, Part A, 1993; Parts B -; F, 1994; 5045 pp., total price £944/$1350. **Journal of Applied Toxicology** [online], 15 (5): 421–421. Available from: http://doi.wiley.com/10.1002/jat.2550150514 [Accessed 4 November 2014]

Collins, A.R. (2004) The comet assay for DNA damage and repair: principles, applications, and limitations. **Molecular biotechnology** [online], 26 (3): 249–61. Available from: http://www.ncbi.nlm.nih.gov/pubmed/15004294 [Accessed 10 June 2014]

Collins, R.H., Feldman, M. and Fordtran, J.S. (1987) Colon cancer, dysplasia, and surveillance in patients with ulcerative colitis. A critical review. **The New England journal of medicine** [online], 316 (26): 1654–8. Available from: http://www.ncbi.nlm.nih.gov/pubmed/3295551 [Accessed 18 September 2014]

Darrall, K.G., Figgins, J.A., Brown, R.D., et al. (1998) Determination of benzene and associated volatile compounds in mainstream cigarette smoke. **The Analyst** [online], 123 (5): 1095–1101. Available from: http://pubs.rsc.org/en/content/articlehtml/1998/an/a708664d [Accessed 22 November 2014]

Delgado Saborit, J.M., Aquilina, N.J., Meddings, C., et al. (2009) Measurement of personal exposure to volatile organic compounds and particle associated PAH in three UK regions. **Environmental science & technology** [online], 43 (12): 4582–8. Available from: http://www.ncbi.nlm.nih.gov/pubmed/19603680 [Accessed 24 January 2015]

Delgado-Saborit, J.M., Alam, M.S., Godri Pollitt, K.J., et al. (2013) Analysis of atmospheric concentrations of quinones and polycyclic aromatic hydrocarbons in vapour and

particulate phases. **Atmospheric Environment** [online], 77: 974–982. Available from: http://linkinghub.elsevier.com/retrieve/pii/S1352231013004536 [Accessed 25 November 2014]

Delgado-saborit, J.M., Aquilina, N.J., Meddings, C., et al. (2011) Relationship of personal exposure to volatile organic compounds to home, work and fixed site outdoor concentrations. **Sci Total Environ** [online], 409 (3): 478–488. Available from: <Go to ISI>://MEDLINE:21112612

Delgado-Saborit, J.M., Aquilina, N.J., Meddings, C., et al. (2011) Relationship of personal exposure to volatile organic compounds to home, work and fixed site outdoor concentrations. **The Science of the total environment** [online], 409 (3): 478–88. Available from: http://www.ncbi.nlm.nih.gov/pubmed/21112612 [Accessed 18 March 2012]

Ding, Y.S., Trommel, J.S., Yan, X.J., et al. (2005) Determination of 14 Polycyclic Aromatic Hydrocarbons in Mainstream Smoke from Domestic Cigarettes. **Environmental Science & Technology** [online], 39 (2): 471–478. Available from: http://pubs.acs.org/doi/abs/10.1021/es048690k [Accessed 22 November 2014]

Edwards, R.D., Jurvelin, J., Koistinen, K., et al. (2001) VOC source identification from personal and residential indoor, outdoor and workplace microenvironment samples in EXPOLIS-Helsinki, Finland. **Atmospheric Environment** [online], 35 (28): 4829–4841. Available from: http://linkinghub.elsevier.com/retrieve/pii/S1352231001002308 [Accessed 13 December 2014]

Evans, M.D., Olinski, R., Loft, S., et al. (2010) Toward consensus in the analysis of urinary 8-oxo-7,8-dihydro-2'-deoxyguanosine as a noninvasive biomarker of oxidative stress. **FASEB journal : official publication of the Federation of American Societies for Experimental Biology** [online], 24 (4): 1249–60. Available from: http://www.ncbi.nlm.nih.gov/pubmed/19966135 [Accessed 7 December 2012]

Fleming, T., Anderson, C., Amin, S., et al. (2012) Third-hand tobacco smoke: Significant vector for PAH exposure or non-issue? **Integrated Environmental Assessment and Management**. 8 (4) pp. 763–764

Foster, K. a, Oster, C.G., Mayer, M.M., et al. (1998) Characterization of the A549 cell line as a type II pulmonary epithelial cell model for drug metabolism. **Experimental cell research** [online], 243 (2): 359–66. Available from: http://www.ncbi.nlm.nih.gov/pubmed/9743595 [Accessed 13 June 2014]

Fubini, B. and Hubbard, A. (2003) Reactive oxygen species (ROS) and reactive nitrogen species (RNS) generation by silica in inflammation and fibrosis. **Free Radical Biology and Medicine** [online], 34 (12): 1507–1516. Available from: http://linkinghub.elsevier.com/retrieve/pii/S0891584903001497 [Accessed 27 July 2014]

Fustinoni, S., Buratti, M., Giampiccolo, R., et al. (2000) Comparison between blood and urinary toluene as biomarkers of exposure to toluene. **International archives of**

occupational and environmental health [online], 73 (6): 389–96. Available from: http://www.ncbi.nlm.nih.gov/pubmed/11007342 [Accessed 16 March 2014]

Fustinoni, S., Campo, L., Polledri, E., et al. (2013) A Validated Method for Urinary Cotinine Quantification Used to Classify Active and Environmental Tobacco Smoke Exposure. **Current Analytical Chemistry** [online], 9 (3): 447–456. Available from: http://www.ingentaconnect.com/content/ben/cac/2013/00000009/00000003/art00014

Fustinoni, S., Consonni, D., Campo, L., et al. (2005) Monitoring low benzene exposure: comparative evaluation of urinary biomarkers, influence of cigarette smoking, and genetic polymorphisms. **Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology** [online], 14 (9): 2237–44. Available from: http://www.ncbi.nlm.nih.gov/pubmed/16172237 [Accessed 25 March 2014]

Fustinoni, S., Giampiccolo, R., Pulvirenti, S., et al. (1999) Headspace solid-phase microextraction for the determination of benzene, toluene, ethylbenzene and xylenes in urine. **Journal of Chromatography B: Biomedical Sciences and Applications** [online], 723 (1-2): 105–115. Available from: <Go to ISI>://000078700800012 [Accessed 4 April 2013]

Fustinoni, S., Mercadante, R., Campo, L., et al. (2007) Comparison Between Urinary o - Cresol and Toluene as Biomarkers of Toluene Exposure. **Journal of Occupational and Environmental Hygiene** [online], 4 (1): 1–9. Available from: http://www.tandfonline.com/doi/abs/10.1080/15459620601044844 [Accessed 18 March 2014]

Fustinoni, S., Mercadante, R. and Campo, L. (2009) Self-collected urine sampling to study the kinetics of urinary toluene (and o-cresol) and define the best sampling time for biomonitoring. **International archives of occupational and environmental health** [online], 82 (6): 703–13. Available from: http://www.ncbi.nlm.nih.gov/pubmed/19125274 [Accessed 25 March 2014]

Fustinoni, S., Rossella, F., Campo, L., et al. (2010) Urinary BTEX, MTBE and naphthalene as biomarkers to gain environmental exposure profiles of the general population. **The Science of the total environment** [online], 408 (14): 2840–9. Available from: <Go to ISI>://WOS:000278579600007 [Accessed 4 April 2013]

Gaikwad, N.W., Yang, L., Weisenburger, D.D., et al. (2009) Urinary biomarkers suggest that estrogen-DNA adducts may play a role in the aetiology of non-Hodgkin lymphoma. **Biomarkers** [online], 14 (7): 502–512. Available from: <Go to ISI>://000272715700008

Goyal, N., Rana, A., Ahlawat, A., et al. (2014) Animal models of inflammatory bowel disease: A review. **Inflammopharmacology**. 22 pp. 219–233

Gromiec, J.P. and Piotrowski, J.K. (1984) Urinary mandelic acid as an exposure test for ethylbenzene. **International archives of occupational and environmental health** [online], 55 (1): 61–72. Available from: http://link.springer.com/10.1007/BF00378068 [Accessed 25 March 2014]

Halliwell, B. (2007) Biochemistry of oxidative stress. **Biochemical Society transactions**, 35: 1147–1150

Halliwell, B. and Aruoma, O.I. (1991) DNA damage by oxygen-derived species Its mechanism and measurement in mammalian systems. **FEBS Letters** [online], 281 (1-2): 9–19. Available from: http://linkinghub.elsevier.com/retrieve/pii/0014579391803476 [Accessed 23 June 2014]

Harrison, R.M., Delgado-Saborit, J.M., Baker, S.J., et al. (2009) Measurement and modeling of exposure to selected air toxics for health effects studies and verification by biomarkers. **Res Rep Health Eff Inst** [online], (143): 3–100. Available from: <Go to ISI>://MEDLINE:19999825

Harrison, R.M., Thornton, C. a, Lawrence, R.G., et al. (2002) Personal exposure monitoring of particulate matter, nitrogen dioxide, and carbon monoxide, including susceptible groups. **Occupational and environmental medicine** [online], 59 (10): 671–9. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1740216&tool=pmcentrez&rendertype=abstract [Accessed 29 September 2014]

Hartwig, A. (2010) The role of DNA repair in benzene-induced carcinogenesis. **Chemico-biological interactions** [online], 184 (1-2): 269–72. Available from: http://www.ncbi.nlm.nih.gov/pubmed/20056111 [Accessed 23 June 2014]

Heinrich-Ramm, R., Jakubowski, M., Heinzow, B., et al. (2000) Biological monitoring for exposure to volatile organic compounds (VOCs) (IUPAC Recommendations 2000). **Pure and Applied Chemistry**. 72 (3) pp. 385–436

Hemat, H., Wittsiepe, J., Wilhelm, M., et al. (2012) High levels of 1-hydroxypyrene and hydroxyphenanthrenes in urine of children and adults from Afghanistan. **Journal of Exposure Science and Environmental Epidemiology**, 22: 46–51

Heudorf, U. and Angerer, J. (2001) Internal exposure to PAHs of children and adults living in homes with parquet flooring containing high levels of PAHs in the parquet glue. **International archives of occupational and environmental health** [online], 74 (2): 91–101. Available from: http://www.ncbi.nlm.nih.gov/pubmed/11317712

Hinwood, A., Farrar, D., Berko, H., et al. (2003) **Technical Report No. 6: BTEX personal exposure monitoring in four Australian cities.** Canberra

Hoh, E., Hunt, R.N., Quintana, P.J.E., et al. (2012) Environmental tobacco smoke as a source of polycyclic aromatic hydrocarbons in settled household dust. **Environmental Science and Technology**, 46: 4174–4183

HSE (2011) EH40 / 2005 Workplace exposure limits (second edition). **HSE books** [online], ISBN 978 0. Available from: http://www.hse.gov.uk/pubns/priced/eh40.pdf

Hu, Y., Zhou, Z., Xue, X., et al. (2006) Sensitive biomarker of polycyclic aromatic hydrocarbons (PAHs): urinary 1-hydroxyprene glucuronide in relation to smoking and low ambient levels of exposure. **Biomarkers** [online], 11 (4): 306–318. Available from: <Go to ISI>://000240494300002

Huh, D., Torisawa, Y., Hamilton, G. a, et al. (2012) Microengineered physiological biomimicry: organs-on-chips. **Lab on a chip** [online], 12 (12): 2156–64. Available from: http://www.ncbi.nlm.nih.gov/pubmed/22555377 [Accessed 10 July 2014]

IARC (2010) IARC Monographs on the Evaluation of Carcinogenic Risks to Humans. Some Chemicals Present in Industrial and Consumer Products, Food and Drinking-water. **IARC monographs** [online], 101. Available from: http://monographs.iarc.fr/ENG/Monographs/vol101/

IARC (2012) IARC monographs on the evaluation of carcinogenic risks to humans. Chemical Agents and Related Occupations. **IARC monographs** [online], 100F: 111–144. Available from: http://monographs.iarc.fr/ENG/Monographs/vol100F/index.php

Ikeda, M. (1999) Solvents in urine as exposure markers. **Toxicology letters** [online], 108 (2-3): 99–106. Available from: http://www.ncbi.nlm.nih.gov/pubmed/12592578

Jacob, P., Wilson, M. and Benowitz, N.L. (2007) Determination of phenolic metabolites of polycyclic aromatic hydrocarbons in human urine as their pentafluorobenzyl ether derivatives using liquid chromatography-tandem mass spectrometry. **Analytical Chemistry** [online], 79 (2): 587–598. Available from: http://www.ncbi.nlm.nih.gov/pubmed/17222024

Janasik, B., Jakubowski, M. and Jalowiecki, P. (2008) Excretion of unchanged volatile organic compounds (toluene, ethylbenzene, xylene and mesitylene) in urine as result of experimental human volunteer exposure. **International Archives of Occupational and Environmental Health** [online], 81 (4): 443–449. Available from: <Go to ISI>://WOS:000252160400008

Jatlow, P. (2003) Correction of Urine Cotinine Concentrations for Creatinine Excretion: Is It Useful? **Clinical Chemistry** [online], 49 (11): 1932–1934. Available from: http://www.clinchem.org/cgi/doi/10.1373/clinchem.2003.023374 [Accessed 1 November 2014]

Jenkins, P.L., Phillips, T.J., Mulberg, E.J., et al. (1992) Activity patterns of Californians: Use of and proximity to indoor pollutant sources. **Atmospheric Environment. Part A. General Topics** [online], 26 (12): 2141–2148. Available from: http://linkinghub.elsevier.com/retrieve/pii/0960168692904027 [Accessed 29 September 2014]

Jongeneelen, F. (2001) Benchmark guideline for urinary 1-hydroxypyrene as biomarker of occupational exposure to polycyclic aromatic hydrocarbons. **The Annals of Occupational Hygiene** [online], 45 (1): 3–13. Available from: http://linkinghub.elsevier.com/retrieve/pii/S0003487800000090 [Accessed 12 October 2014]

Jongeneelen, F., Anzion, R. and Henderson, P. (1987) Determination of hydroxylated metabolites of polycyclic aromatic hydrocarbons in urine. **Journal of Chromatography …** [online], 413: 227–32. Available from: http://www.ncbi.nlm.nih.gov/pubmed/3558672 [Accessed 12 October 2014]

Kerzic, P.J., Liu, W.S., Pan, M.T., et al. (2010) Analysis of hydroquinone and catechol in peripheral blood of benzene-exposed workers. **Chemico-Biological Interactions** [online], 184 (1-2): 182–188. Available from: http://www.ncbi.nlm.nih.gov/pubmed/20026093 [Accessed 22 May 2012]

Kim, S., Vermeulen, R., Johnson, B.A., et al. (2006) Using urinary biomarkers to elucidate dose-related patterns of human benzene metabolism. **Carcinogenesis** [online], 27 (4): 772–781. Available from: <Go to ISI>://WOS:000236252600011

Kim, Y.D., Lee, C.H., Nan, H.M., et al. (2003) Effects of genetic polymorphisms in metabolic enzymes on the relationships between 8-hydroxydeoxyguanosine levels in human leukocytes and urinary 1-hydroxypyrene and 2-naphthol concentrations. **Journal of Occupational Health**, 45: 160–167

Kim, Y.M., Harrad, S. and Harrison, R.M. (2001) Concentrations and Sources of VOCs in Urban Domestic and Public Microenvironments. **Environmental Science & Technology** [online], 35 (6): 997–1004. Available from: http://pubs.acs.org/doi/abs/10.1021/es000192y [Accessed 13 December 2014]

Klebanoff, S.J. (2005) Myeloperoxidase: friend and foe. **Journal of leukocyte biology** [online], 77 (5): 598–625. Available from: http://www.ncbi.nlm.nih.gov/pubmed/15689384 [Accessed 18 September 2014]

Knaapen, A.M., Güngör, N., Schins, R.P.F., et al. (2006) Neutrophils and respiratory tract DNA damage and mutagenesis: a review. **Mutagenesis** [online], 21 (4): 225–36. Available from: http://www.ncbi.nlm.nih.gov/pubmed/16870698 [Accessed 9 December 2014]

Kolmogorov–Smirnov Test (2008). In **The Concise Encyclopedia of Statistics SE - 214** [online]. Springer New York. pp. 283–287. Available from: http://dx.doi.org/10.1007/978-0-387-32833-1_214

Kostrzewski, P. (1993) [Micromethods for determining organic solvents in blood by headspace analysis]. **Medycyna pracy** [online], 44 (2): 131–9. Available from: http://www.ncbi.nlm.nih.gov/pubmed/8377638 [Accessed 25 March 2014]

Kroll, M., Chesler, R., Hagengruber, C., et al. (1986) Automated determination of urinary creatinine without sample dilution: theory and practice. **Clin Chem**, 32: 446–452

Kruskal, W.H. and Wallis, W.A. (1952) Use of Ranks in One-Criterion Variance Analysis. **Journal of the American Statistical Association** [online], 47: 583–621. Available from: http://www.ncbi.nlm.nih.gov/pubmed/21527941\nhttp://www.ncbi.nlm.nih.gov/pubmed/21900426\nhttp://www.tandfonline.com/doi/abs/10.1080/01621459.1952.10483441

Lam, P.M.W., Mistry, V., Marczylo, T.H., et al. (2012) Rapid measurement of 8-oxo-7,8-dihydro-2'-deoxyguanosine in human biological matrices using ultra-high-performance liquid chromatography-tandem mass spectrometry. **Free radical biology & medicine** [online], 52 (10): 2057–63. Available from:

http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3404459&tool=pmcentrez
&rendertype=abstract [Accessed 6 April 2013]

Leinonen, A., Vuorensola, K., Lepola, L.M., et al. (2006) Liquid-phase microextraction for sample preparation in analysis of unconjugated anabolic steroids in urine. **Analytica Chimica Acta** [online], 559 (2): 166–172. Available from: <Go to ISI>://000235572600004

Li, Z. (2009) **CHARACTERIZATION OF POLYCYCLIC AROMATIC HYDROCARBONS (PAH) IN AIRBORNE PARTICLES AND ASSESSMENT OF HUMAN EXPOSURE TO PAHS**. Georgia Institute of Technology

Li, Z., Romanoff, L.C., Lewin, M.D., et al. (2010) Variability of urinary concentrations of polycyclic aromatic hydrocarbon metabolite in general population and comparison of spot, first-morning, and 24-h void sampling. **Journal of exposure science & environmental epidemiology** [online], 20 (6): 526–35. Available from: http://www.ncbi.nlm.nih.gov/pubmed/19707251 [Accessed 8 October 2014]

Li, Z., Sandau, C.D., Romanoff, L.C., et al. (2008) Concentration and profile of 22 urinary polycyclic aromatic hydrocarbon metabolites in the US population. **Environmental research** [online], 107 (3): 320–31. Available from: http://www.ncbi.nlm.nih.gov/pubmed/18313659 [Accessed 8 October 2014]

Lim, D., Ikeda, A., Vu, K.K.-T., et al. (2011) Method development for the measurement of quinone levels in urine. **Journal of chromatography. B, Analytical technologies in the biomedical and life sciences** [online], 879 (30): 3592–8. Available from: http://www.ncbi.nlm.nih.gov/pubmed/22024390 [Accessed 14 March 2013]

Lin, H.-S., Jenner, A.M., Ong, C.N., et al. (2004) A high-throughput and sensitive methodology for the quantification of urinary 8-hydroxy-2'-deoxyguanosine: measurement with gas chromatography-mass spectrometry after single solid-phase extraction. **The Biochemical journal** [online], 380 (Pt 2): 541–8. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1224185&tool=pmcentrez
&rendertype=abstract

Lin, Y.S., Vermeulen, R., Tsai, C.H., et al. (2007) Albumin adducts of electrophilic benzene metabolites in benzene-exposed and control workers. **Environmental Health Perspectives** [online], 115 (1): 28–34. Available from: <Go to ISI>://000243299200028

Linhart, I., Mikes, P., Kralik, A., et al. (2011) Metabolism of N(2)-(4-hydroxyphenyl)guanine, a DNA adduct formed from p-benzoquinone, in rat. **Toxicology Letters** [online], 205 (3): 273–278. Available from: <Go to ISI>://MEDLINE:21718765

Lodovici, M., Venturini, M., Marini, E., et al. (2003) Polycyclic aromatic hydrocarbons air levels in Florence, Italy, and their correlation with other air pollutants. **Chemosphere**, 50: 377–382

Lovreglio, P., D'Errico, M.N., Fustinoni, S., et al. (2011) Biomarkers of internal dose for the assessment of environmental exposure to benzene. **Journal of Environmental**

Monitoring [online], 13 (10): 2921–2928. Available from: <Go to ISI>://WOS:000295579000031

Lu, C.-Y., Ma, Y.-C., Lin, J.-M., et al. (2007) Oxidative DNA damage estimated by urinary 8-hydroxydeoxyguanosine and indoor air pollution among non-smoking office employees. **Environmental research** [online], 103 (3): 331–7. Available from: http://www.ncbi.nlm.nih.gov/pubmed/17034784 [Accessed 13 March 2013]

Manini, P., De Palma, G., Andreoli, R., et al. (2006) Environmental and biological monitoring of benzene exposure in a cohort of Italian taxi drivers. **Toxicology Letters** [online], 167 (2): 142–151. Available from: <Go to ISI>://000242948400007

Manini, P., De Palma, G., Andreoli, R., et al. (2010) Occupational exposure to low levels of benzene: Biomarkers of exposure and nucleic acid oxidation and their modulation by polymorphic xenobiotic metabolizing enzymes. **Toxicology Letters** [online], 193 (3): 229–235. Available from: http://www.sciencedirect.com/science/article/B6TCR-4Y7P6VH-1/2/f66b480499bd8795b4cf186a0d930e42

Matt, G.E., Quintana, P.J.E., Hovell, M.F., et al. (2008) Residual tobacco smoke pollution in used cars for sale: air, dust, and surfaces. **Nicotine & tobacco research : official journal of the Society for Research on Nicotine and Tobacco**, 10 (9): 1467–1475

Medinsky, M.A., Schlosser, P.M. and Bond, J.A. (1994) Critical issues in benzene toxicity and metabolism: the effect of interactions with other organic chemicals on risk assessment. **Environmental health perspectives** [online], 102 Suppl: 119–24. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1566790&tool=pmcentrez&rendertype=abstract [Accessed 20 November 2013]

Melikian, A.A., O'Connor, R., Prahalad, A.K., et al. (1999) Determination of the urinary benzene metabolites S-phenylmercapturic acid and trans,trans-muconic acid by liquid chromatography-tandem mass spectrometry. **Carcinogenesis** [online], 20 (4): 719–726. Available from: <Go to ISI>://WOS:000079714300029

Miller, M.J. and Edwards, J.W. (1999) Possible preferential metabolism of xylene isomers following occupational exposure to mixed xylenes. **International Archives of Occupational and Environmental Health**, 72: 89–97

Missia, D. a., Demetriou, E., Michael, N., et al. (2010) Indoor exposure from building materials: A field study. **Atmospheric Environment** [online], 44 (35): 4388–4395. Available from: http://dx.doi.org/10.1016/j.atmosenv.2010.07.049

Mögel, I., Baumann, S., Böhme, A., et al. (2011) The aromatic volatile organic compounds toluene, benzene and styrene induce COX-2 and prostaglandins in human lung epithelial cells via oxidative stress and p38 MAPK activation. **Toxicology** [online], 289 (1): 28–37. Available from: http://www.ncbi.nlm.nih.gov/pubmed/21801798 [Accessed 4 March 2013]

Olive, P.L. and Durand, R.E. (2005) Heterogeneity in DNA damage using the comet assay. **Cytometry. Part A : the journal of the International Society for Analytical**

Cytology [online], 66 (1): 1–8. Available from:
http://www.ncbi.nlm.nih.gov/pubmed/15924303 [Accessed 10 July 2014]

Paci, E., Pigini, D., Cialdella, A.M., et al. (2007) Determination of free and total S-
phenylmercapturic acid by HPLC/MS/MS in the biological monitoring of benzene
exposure. **Biomarkers** [online], 12 (2): 111–122. Available from: <Go to
ISI>://000244350500001

Pariselli, F., Sacco, M.G. and Rembges, D. (2006) Dynamic in Vitro Exposure of Human
Derived Lung Cells to Indoor Priority Pollutants. **Epidemiology** [online], 17 (Suppl):
S228. Available from:
http://content.wkhealth.com/linkback/openurl?sid=WKPTLP:landingpage&an=00001
648-200611001-00585 [Accessed 5 November 2013]

Pariselli, F., Sacco, M.G. and Rembges, D. (2009) An optimized method for in vitro exposure
of human derived lung cells to volatile chemicals. **Experimental and toxicologic
pathology : official journal of the Gesellschaft für Toxikologische Pathologie**
[online], 61 (1): 33–9. Available from:
http://www.ncbi.nlm.nih.gov/pubmed/18650076 [Accessed 11 June 2014]

Platt, K.L., Aderhold, S., Kulpe, K., et al. (2008) Unexpected DNA damage caused by
polycyclic aromatic hydrocarbons under standard laboratory conditions. **Mutation
research** [online], 650 (2): 96–103. Available from:
http://www.ncbi.nlm.nih.gov/pubmed/18160334 [Accessed 26 November 2014]

Price, P.S., Rey, T.D., Fontaine, D.D., et al. (2013) Letter to the editor in response to low-
dose metabolism of benzene in humans: Science and obfuscation rappaport et al.
(2013). **Carcinogenesis**. 34 (7) pp. 1692–1696

Pryor, W.A. (1988) Why is the hydroxyl radical the only radical that commonly adds to
DNA? Hypothesis: it has a rare combination of high electrophilicity, high
thermochemical reactivity, and a mode of production that can occur near DNA. **Free
radical biology & medicine** [online], 4 (4): 219–23. Available from:
http://www.ncbi.nlm.nih.gov/pubmed/2834274 [Accessed 18 September 2014]

Putnam, D.F. (1971) **Composition and Concentrative Properties of Human Urine**., NASA
CR (July)

Qu, Q., Shore, R., Li, G., et al. (2003) Validation and evaluation of biomarkers in workers
exposed to benzene in China. **Res Rep Health Eff Inst** [online], (115): 1–87.
Available from: <Go to ISI>://MEDLINE:12931845

Rappaport, S.M., Kim, S., Lan, Q., et al. (2010) Human benzene metabolism following
occupational and environmental exposures. **Chemico-Biological Interactions**
[online], 184 (1-2): 189–195. Available from:
http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3072712&tool=pmcentrez
&rendertype=abstract [Accessed 22 May 2012]

Rappaport, S.M., Kim, S., Thomas, R., et al. (2013) Low-dose metabolism of benzene in
humans: science and obfuscation. **Carcinogenesis** [online], 34 (1): 2–9. Available
from:

http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3584950&tool=pmcentrez&rendertype=abstract [Accessed 17 March 2014]

Rappaport, S.M., Waidyanatha, S., Yeowell-O'Connell, K., et al. (2005) Protein adducts as biomarkers of human benzene metabolism. **Chemico-Biological Interactions** [online], 153: 103–109. Available from: <Go to ISI>://000230065500012

Ritter, D., Knebel, J.W. and Aufderheide, M. (2001) In vitro exposure of isolated cells to native gaseous compounds--development and validation of an optimized system for human lung cells. **Experimental and toxicologic pathology : official journal of the Gesellschaft für Toxikologische Pathologie** [online], 53 (5): 373–86. Available from: http://www.ncbi.nlm.nih.gov/pubmed/11817107

Röder-Stolinski, C., Fischäder, G., Oostingh, G.J., et al. (2008) Styrene induces an inflammatory response in human lung epithelial cells via oxidative stress and NF-kappaB activation. **Toxicology and applied pharmacology** [online], 231 (2): 241–7. Available from: http://www.ncbi.nlm.nih.gov/pubmed/18554678 [Accessed 4 March 2013]

Rodila, R.C., Kim, J.C., Ji, Q.C., et al. (2006) A high-throughput, fully automated liquid/liquid extraction liquid chromatography/mass spectrometry method for the quantitation of a new investigational drug ABT-869 and its metabolite A-849529 in human plasma samples. **Rapid Communications in Mass Spectrometry** [online], 20 (20): 3067–3075. Available from: <Go to ISI>://000241332500009

Roggi, C., Minoia, C., Sciarra, G.F., et al. (1997a) Urinary 1-hydroxypyrene as a marker of exposure to pyrene: an epidemiological survey on a general population group. **Science of The Total Environment** [online], 199 (3): 247–254. Available from: http://linkinghub.elsevier.com/retrieve/pii/S0048969797054582 [Accessed 8 October 2014]

Roggi, C., Minoia, C., Sciarra, G.F., et al. (1997b) Urinary 1-hydroxypyrene as a marker of exposure to pyrene: an epidemiological survey on a general population group. **Science of The Total Environment**, 199 (3): 247–254

Roginsky, V.A., Pisarenko, L.M., Bors, W., et al. (1999) The kinetics and thermodynamics of quinone-semiquinone-hydroquinone systems under physiological conditions. **Journal of the Chemical Society-Perkin Transactions 2** [online], (4): 871–876. Available from: <Go to ISI>://WOS:000079747400030

Roos, W.P. and Kaina, B. (2006) DNA damage-induced cell death by apoptosis. **Trends in molecular medicine** [online], 12 (9): 440–50. Available from: http://www.ncbi.nlm.nih.gov/pubmed/16899408 [Accessed 11 July 2014]

Rossner, P., Svecova, V., Milcova, A., et al. (2008) Seasonal variability of oxidative stress markers in city bus drivers. Part II. Oxidative damage to lipids and proteins. **Mutation research** [online], 642 (1-2): 21–7. Available from: http://www.ncbi.nlm.nih.gov/pubmed/18436262 [Accessed 4 March 2013]

Sabatini, L., Barbieri, A., Indiveri, P., et al. (2008) Validation of an HPLC-MS/MS method for the simultaneous determination of phenylmercapturic acid, benzy1mercapturic

acid and o-methylbenzyl mercapturic acid in urine as biomarkers of exposure to benzene, toluene and xylenes. **Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences** [online], 863 (1): 115–122. Available from: http://www.ncbi.nlm.nih.gov/pubmed/18255357

Saint-Georges, F., Abbas, I., Billet, S., et al. (2008) Gene expression induction of volatile organic compound and/or polycyclic aromatic hydrocarbon-metabolizing enzymes in isolated human alveolar macrophages in response to airborne particulate matter (PM2.5). **Toxicology** [online], 244 (2-3): 220–30. Available from: http://www.ncbi.nlm.nih.gov/pubmed/18178302 [Accessed 4 March 2013]

Sarma, S.N., Kim, Y.-J., Song, M., et al. (2011) Induction of apoptosis in human leukemia cells through the production of reactive oxygen species and activation of HMOX1 and Noxa by benzene, toluene, and o-xylene. **Toxicology** [online], 280 (3): 109–17. Available from: http://www.ncbi.nlm.nih.gov/pubmed/21144877 [Accessed 29 July 2013]

Scheepers, P.T.J., Konings, J., Demirel, G., et al. (2010) Determination of exposure to benzene, toluene and xylenes in Turkish primary school children by analysis of breath and by environmental passive sampling. **Science of the Total Environment** [online], 408 (20): 4863–4870. Available from: <Go to ISI>://MEDLINE:20619876

Schottenfeld, D. and Beebe-Dimmer, J. (2006) Chronic inflammation: a common and important factor in the pathogenesis of neoplasia. **CA: a cancer journal for clinicians** [online], 56 (2): 69–83. Available from: http://www.ncbi.nlm.nih.gov/pubmed/16514135 [Accessed 18 September 2014]

Shang, Y., Fan, L., Feng, J., et al. (2013) Genotoxic and inflammatory effects of organic extracts from traffic-related particulate matter in human lung epithelial A549 cells: The role of quinones. **Toxicology in Vitro** [online], (January). Available from: http://linkinghub.elsevier.com/retrieve/pii/S088723331300009X [Accessed 21 January 2013]

Shin, S.-H. and Jo, W.-K. (2014) Volatile organic compound concentrations in newly built apartment buildings during pre- and post-occupancy stages. **International Journal of Environmental Analytical Chemistry** [online], 94 (4): 356–369. Available from: http://dx.doi.org/10.1080/03067319.2013.814125

Singh, M.P., Reddy, M.M.K., Mathur, N., et al. (2009) Induction of hsp70, hsp60, hsp83 and hsp26 and oxidative stress markers in benzene, toluene and xylene exposed Drosophila melanogaster: role of ROS generation. **Toxicology and applied pharmacology** [online], 235 (2): 226–43. Available from: http://www.ncbi.nlm.nih.gov/pubmed/19118569 [Accessed 1 July 2013]

Singh, N.P., McCoy, M.T., Tice, R.R., et al. (1988) A simple technique for quantitation of low levels of DNA damage in individual cells. **Experimental cell research** [online], 175 (1): 184–91. Available from: http://www.ncbi.nlm.nih.gov/pubmed/3345800 [Accessed 13 June 2014]

Singh, V.K., Patel, D.K., Jyoti, et al. (2008) Blood levels of polycyclic aromatic hydrocarbons in children and their association with oxidative stress indices: an Indian

perspective. **Clinical biochemistry** [online], 41 (3): 152–61. Available from: http://www.ncbi.nlm.nih.gov/pubmed/18154730 [Accessed 26 November 2014]

Sleiman, M., Logue, J.M., Luo, W., et al. (2014) Inhalable constituents of thirdhand tobacco smoke: chemical characterization and health impact considerations. **Environmental science & technology** [online], 48: 13093–101. Available from: http://www.ncbi.nlm.nih.gov/pubmed/25317906

Smolders, R., Den Hond, E., Koppen, G., et al. (2014) Interpreting biomarker data from the COPHES/DEMOCOPHES twin projects: Using external exposure data to understand biomarker differences among countries. **Environmental Research** [online], pp. 1–10. Available from: http://linkinghub.elsevier.com/retrieve/pii/S001393511400276X [Accessed 20 October 2014]

Snyder, R. and Hedli, C.C. (1996) An overview of benzene metabolism. **Environmental Health Perspectives** [online], 104: 1165–1171. Available from: <Go to ISI>://A1996WC46600009

Song, M.-K., Lee, H.-S., Choi, H.-S., et al. (2013) Octanal-induced inflammatory responses in cells relevant for lung toxicity: Expression and release of cytokines in A549 human alveolar cells. **Human & experimental toxicology** [online], 33 (7): 710–721. Available from: http://www.ncbi.nlm.nih.gov/pubmed/24130214 [Accessed 29 October 2014]

Sørensen, M., Autrup, H., Møller, P., et al. (2003a) Linking exposure to environmental pollutants with biological effects. **Mutation Research/Reviews in Mutation Research** [online], 544 (2-3): 255–271. Available from: http://europepmc.org/abstract/MED/14644327 [Accessed 14 March 2013]

Sørensen, M., Autrup, H., Møller, P., et al. (2003b) Linking exposure to environmental pollutants with biological effects. **Mutation Research/Reviews in Mutation Research** [online], 544 (2-3): 255–271. Available from: http://linkinghub.elsevier.com/retrieve/pii/S1383574203000899 [Accessed 14 March 2013]

Sørensen, M., Skov, H., Autrup, H., et al. (2003c) Urban benzene exposure and oxidative DNA damage: influence of genetic polymorphisms in metabolism genes. **The Science of the total environment** [online], 309 (1-3): 69–80. Available from: http://www.ncbi.nlm.nih.gov/pubmed/12798093 [Accessed 26 May 2012]

Strickland, P. and Kang, D. (1999) Urinary 1-hydroxypyrene and other PAH metabolites as biomarkers of exposure to environmental PAH in air particulate matter. **Toxicology letters** [online], 108 (2-3): 191–9. Available from: http://www.ncbi.nlm.nih.gov/pubmed/10511262 [Accessed 11 March 2014]

Strickland, P., Kang, D. and Sithisarankul, P. (1996) Polycyclic aromatic hydrocarbon metabolites in urine as biomarkers of exposure and effect. **Environmental health perspectives** [online], 104 Suppl (October): 927–32. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1469694&tool=pmcentrez &rendertype=abstract [Accessed 11 March 2014]

Thatcher, T. and Layton, D. (1995) Deposition, resuspension, and penetration of particles within a residence. **Atmospheric Environment** [online], 29 (13): 1487–1497. Available from: http://www.sciencedirect.com/science/article/pii/135223109500016R [Accessed 9 September 2014]

Tice, R.R., Agurell, E., Anderson, D., et al. (2000) Single cell gel/comet assay: guidelines for in vitro and in vivo genetic toxicology testing. **Environmental and molecular mutagenesis** [online], 35 (3): 206–21. Available from: http://www.ncbi.nlm.nih.gov/pubmed/10737956 [Accessed 13 July 2014]

Trasande, L., Urbina, E.M., Khoder, M., et al. (2015) Polycyclic aromatic hydrocarbons, brachial artery distensibility and blood pressure among children residing near an oil refinery. **Environmental Research** [online], 136: 133–140. Available from: http://linkinghub.elsevier.com/retrieve/pii/S0013935114003521 [Accessed 24 November 2014]

Verdina, A., Galati, R., Falasca, G., et al. (2001) Metabolic polymorphisms and urinary biomarkers in subjects with low benzene exposure. **Journal of Toxicology and Environmental Health-Part A** [online], 64 (8): 607–618. Available from: <Go to ISI>://000172692300002

Villar-Vidal, M., Lertxundi, A., Martinez López de Dicastillo, M.D., et al. (2014) Air polycyclic aromatic hydrocarbons (PAHs) associated with PM2.5 in a north cantabric coast urban environment. **Chemosphere** [online], 99: 233–238. Available from: http://dx.doi.org/10.1016/j.chemosphere.2013.11.006

Waidyanatha, S., Rothman, N., Fustinoni, S., et al. (2001) Urinary benzene as a biomarker of exposure among occupationally exposed and unexposed subjects. **Carcinogenesis** [online], 22 (2): 279–286. Available from: http://carcin.oxfordjournals.org/content/22/2/279.abstract

Waidyanatha, S., Rothman, N., Li, G.L., et al. (2004) Rapid determination of six urinary benzene metabolites in occupationally exposed and unexposed subjects. **Analytical Biochemistry** [online], 327 (2): 184–199. Available from: <Go to ISI>://000220791200006

Wang, F., Li, C., Liu, W., et al. (2013) Effects of subchronic exposure to low-dose volatile organic compounds on lung inflammation in mice. **Environmental toxicology** [online], pp. 1–9. Available from: http://onlinelibrary.wiley.com/doi/10.1002/tox.21844/full [Accessed 24 August 2013]

Weisel, C.P. (2010) Benzene exposure: an overview of monitoring methods and their findings. **Chemico-biological interactions** [online], 184 (1-2): 58–66. Available from: http://www.ncbi.nlm.nih.gov/pubmed/20056112 [Accessed 22 September 2014]

Winickoff, J.P., Friebely, J., Tanski, S.E., et al. (2009) Beliefs about the health effects of "thirdhand" smoke and home smoking bans. **Pediatrics**, 123: e74–e79

Wolkoff, P. (1995) Volatile organic compounds - Sources, measurements, emissions, and the impact on indoor air quality. **Indoor Air** [online], pp. 1–73. Available from: <Go to ISI>://A1995TN98500001

Wolkoff, P. (2012) Indoor air pollutants in office environments: Assessment of comfort, health, and performance. **International journal of hygiene and environmental health** [online], 216 (4): 371–394. Available from: http://www.ncbi.nlm.nih.gov/pubmed/22954455 [Accessed 11 March 2013]

List of research papers and presentations

Peer-reviewed articles

[1] **Mascelloni M.**, Cooke M. S., Fustinoni S., Mercadante R., Polledri E., Olgiati L., Campo L., Hodges, N., Harrison R. M., Delgado-Saborit J. M. Relevance of Oxidative Stress Related to Exposure to Volatile Organic Compounds. **Manuscript in preparation.**

[2] **Mascelloni M.**, Hodges, N., Harrison R. M., Delgado-Saborit J. M. V. Benzene effects on DNA damage and oxidative stress upon A549 lung epithelium cells using a novel exposure system. **Toxicol Lett. 2015 Aug 19;237(1):38-45. doi: 10.1016/j.toxlet.2015.05.015. Epub 2015 May 26.**

Conference papers

[1] Mascelloni, M., Harrison, R.M., Viant, M. and Delgado-Saborit, J.M. 2011. Development of analytical methods to characterise biomarkers of exposure and effect to low-level VOCs. In: *From Exposure to Health Effects: Novel Approaches to find the Linkage. Birmingham, United Kingdom, 17 May, 2011.* Birmingham, United Kingdom, 17 May, 2011 [Poster Presentation]

[2] Mascelloni M., Cooke M. S., Fustinoni S., Mercadante R., Polledri E., Olgiati L., Campo L., Harrison R. M., Delgado-Saborit J. M., 2013. Relationship between Relationship between exposure to low level VOCs and oxidative stress. In: *ISBM (International Symposium on Biological Monitoring in Occupational and Environmental Health). Manchester, United Kingdom*, 9th-11th September 2013. [Poster Presentation]

X. Annex

X.1 Synthetic urine

Method development for extractions and testing was performed using synthetic urine, prepared according to Putnam (1971). The synthetic urine composition was:

Albumin 0.1 g/L

Ammonium sulphate 1 g/L

Ascorbic acid 0.02 g/L

Bilirubin 0.005 g/L

Citric acid 0.5 g/L

Creatine 0.5 g/L

Creatinine 1.5 g/L

L-cystine 0.1 g/L

D-glucuronic acid 0.5 g/L

Hippuric acid 1 g/L

L-hystidine 0.5 g/L

Magnesium sulphate 1 g/L

Phenol 0.5 g/L (only added in first batch as it generated interference)

Urea 15 g/L

Uric acid 0.5 g/L

Glycine 0.5 g/L

Synthetic urine was used as a matrix for method development to ensure reliability within different methodologies of extraction and analysis.

FIXAT- Project
PARTICIPANTS BASELINE QUESTIONNAIRE

Volunteers ID: [　　　　　　]   Date: [　　　　　　]

PART A: GENERAL INFORMATION ABOUT YOUR HOUSE

A.1   DESCRIBE THE LOCATION OF THE HOME.   <u>Please tick one box</u>

Rural area	☐
Suburb	☐
City centre	☐

A.2   DESCRIBE THE TYPE OF DWELLING.   <u>Please tick one box</u>

Flat	☐
Centre terrace house	☐
End terrace house	☐
Semi-detached house	☐
Detached house	☐

A) IF YOU DO NOT LIVE IN A FLAT, PLEASE GO TO QUESTION A.6

A.3   ON WHICH FLOOR IS THE FLAT LOCATED?

(Please specify, e.g. 1, 2, 3 or Basement =B, Ground Floor = F)   ☐

A.4   WHAT IS IMMEDIATELY BELOW THE FLOOR OF YOUR FLAT?
<u>Please tick one box</u>

The ground	☐
Another flat	☐
Garage	☐
Other (please describe below)	☐

A.5   WHAT IS IMMEDIATELY ABOVE YOUR FLAT?
<u>Please tick one box</u>

The roof – is it a top floor flat	☐
Another flat	☐
Other (please describe below)	☐

A.6   APPROXIMATELY WHEN WAS YOUR HOUSE BUILT?   Please tick one box

Less 3 months ago	☐
more than 3 months - 1 year	☐
1- 5 years	☐
More than 5 year	☐

A.7   HAVE YOU DONE EXTENSIONS OR REDECORATIONS TO YOUR HOME?

Less 3 months ago	☐
3 months - 1 year	☐
More than 1 year	☐
N/A	☐

A.8   FOR EACH ROOM IN THE TABLE BELOW, PLEASE WRITE HOW MANY MONTHS AGO   YOU DID EACH OF THE FOLLOWING
If more than 12 months write X

	Wallpapering		Carpeting / Lino	Sanding / Stripping	Painting	New furniture
Kitchen						
Living rooms						
Bedrooms						
Other rooms						

IF NO NEW CARPET/LINO HAS BEEN LAID PLEASE GO TO A.12

A.9   WAS RUBBER-BACKED NYLON CARPET LAID? Please tick one box

Yes	☐
No	☐
Don't know	☐

A.10   WAS THE CARPET/LINO GLUE TO THE FLOOR? Please tick one box

Yes	☐
No	☐
Don't know	☐

A.11   HAS ANY SEALANT BEEN USED IN THE LAST 12 MONTHS?
    This question is about sealants used for waterproofing around baths, pipes etc. Please tick one box

Yes	☐
No	☐
Don't know	☐

A.12   DO YOU HAVE A GARAGE?

Yes	☐
No	☐

257

A.13   IS THE GARAGE PART OF YOUR HOME (OR DIRECTLY ATTACHED TO
        THE UNDERNEATH OR SIDE OF YOUR HOME) ?

Yes	☐
No	☐

A.14   (a)   DO YOU KEEP A CAR IN THE GARAGE?

Usually	☐
Sometimes	☐
Never	☐

A.15   (b)   WHAT TYPE OF FUEL DOES THE CAR RUN ON?

Unleaded petrol	☐
Diesel	☐
Don't know	☐

A.16   WHICH ROOM HAS A DOOR TO THE GARAGE? Please tick one box

Hall	☐
Kitchen	☐
Utility room	☐
Living room	☐
None	☐
Other (Please describe below)	☐

A.17   IS ANY OF THE FOLLOWING ITEMS STORED/USED IN:

	GARAGE	IN THE HOUSE
PETROL VEHICLE	☐	☐
DIESEL VEHICLE	☐	☐
PETROL LAWNMOWER	☐	☐
OPEN PAINTS	☐	☐
CLOSED PAINTS	☐	☐
SOLVENTS	☐	☐
INKS/TONER	☐	☐
PETROL	☐	☐
DIESEL	☐	☐
FERTILISER	☐	☐
GLUES	☐	☐
SPARE BUILDING MATERIAL	☐	☐
SPARE CARPETS	☐	☐
TUMBLE DRY	☐	☐

PART B: HEATING & COOKING

B.1   HOW MANY ROOMS DO YOU USUALLY HEAT AT THIS TIME OF YEAR?
<u>Write a number in each box</u>

Living rooms   *(include studies, dining rooms etc. but not kitchen diners or living rooms with kitchen included)* ☐

Living room or dining room which includes kitchen ☐

Kitchen ☐

Bedrooms ☐

Bathrooms ☐

Other rooms ☐

B.2   REGARDING THE MAIN METHOD OF HEATING AT THIS TIME OF YEAR?
WHAT FUEL DO YOU USE FOR YOUR MAIN HEATING? <u>Please tick one box</u>

Natural gas	☐
Electricity	☐
Bottled gas	☐
Other (Please describe below)	☐
Not applicable	☐

WHAT TYPE OF HEATING SYSTEM DO YOU USE FOR YOUR MAIN HEATING? <u>Please tick one box</u>

Electric storage heaters	☐
Central heating with radiators	☐
Warm air central heating	☐
Individual heaters or fires in each heated room	☐
Other (Please describe below)	☐
No applicable	☐

B.3   WHERE IS YOUR BOILER LOCATED?

Kitchen	☐
Hallway	☐
Under stairs	☐
Other (Please describe below)	☐

B.4   HAVE YOU USED ANY ADDITIONAL TYPE OF HEATING DURING THE SAMPLING DAY?

Yes	☐
No	☐

If no, please go to B.6

B.5   WHAT TYPE OF ADDITIONAL HEATING DO YOU USE MOST?
        WHAT FUEL DOES IT USE? Please tick one box

	Sampling day	Sampling week
Natural gas	☐	☐
Electricity	☐	☐
Bottled gas	☐	☐
Coal /coke	☐	☐
Wood	☐	☐
Paraffin	☐	☐
Other (Please describe below)	☐	☐

```
┌──────────────────────────────────────────────────────┐
│                                                      │
└──────────────────────────────────────────────────────┘
```

   WHAT TYPE OF HEATING SYSTEM DO YOU USE FOR YOUR ADDITIONAL
HEATING?   Please tick one box

	Sampling day	Sampling week
Electric storage heaters	☐	☐
Central heating with radiators	☐	☐
Warm air central heating	☐	☐
Individual heaters or fires in each heated room	☐	☐
	☐	☐
	☐	☐
Other (Please describe below)	☐	☐

```
┌──────────────────────────────────────────────────────┐
│                                                      │
└──────────────────────────────────────────────────────┘
```

B.6   DO YOU USE ANY FURTHER GAS OR SOLID FUEL WHICH YOU HAVE NOT
INCLUDED IN YOUR MAIN OR ADDITIONAL HEATING (NOT INCLUDING COOKING
FUEL)?

	Sampling day	Sampling week
Yes (please describe below)	☐	☐
No	☐	☐

If yes, please describe in box below

```
┌──────────────────────────────────────────────────────┐
│                                                      │
└──────────────────────────────────────────────────────┘
```

B.7   AT WHAT HOURS WAS YOUR HOUSE HEATED?
        Please mark off the boxes to show when you have heating on

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24

Midnight   Midday

260

B.8   WHAT MAIN COOKING FUEL DO YOU USE? <u>Please tick one box</u>

Natural gas   □
Electricity   □
Bottled gas   □
Other (Please   □
describe below)

IF YOU DO NOT USE GAS FOR COOKING PLEASE GO TO PART B.11

B.9   HOW MANY HOURS WAS YOUR GAS COOKER USED ON THE SAMPLING DAY?

Hours

B.10   DO YOU EVER USE THE GAS COOKER, WHEN YOU ARE NOT COOKING, TO
HEAT THE KITCHEN (OR ANY OTHER PART OF THE HOME)? <u>Please tick one box</u>

Yes, regularly   □
Yes, sometimes   □
Yes, only   □
occasionally
No, never   □

B.11   DO YOU HAVE A COOKER HOOD?
*Include cooker hoods which extract air to the outside, but NOT hoods which only
filter air and return it to the kitchen.*

Yes   □
No   □
If no, please go to PART C

B.12   HOW OFTEN IS THE COOKER HOOD USED?   <u>Please tick one box</u>

Fan not used   □
Fan sometimes used   □
Fan normally used   □
when room in use

PART C: WINDOWS & VENTILATION

C.1 PLEASE INDICATES WHICH DIRECTION THE WINDOWS IN YOUR HOME FACES. Please tick more than one box if applicable

	Street Side	Back Garden	Side Street/Side Alley	Other (please specify)
Kitchen				
Bathroom				
Living room				
Your bedroom				
Other*				
Other*				

*Please specify (e.g. dining room, second bedroom, corridor, etc.)

C.2 HOW OFTEN WAS THE WINDOWS OPEN DURING THE SAMPLING DAY?
Please tick one box for each room type

	All or most of the time	Part of the day	Only when needed	Rarely or never	No window	Don't know
Kitchen						
Bathroom						
Living rooms						
Bedrooms						
Other rooms						

C.3 DID YOU LEAVE WINDOWS OPEN AT NIGHT DURING THE SAMPLING DAY?
Please tick one box for each room type

	Yes	How long?	No	Don't know
Kitchen				
Bathroom				
Living rooms				
Bedrooms				
Other rooms				

C.4 DO YOU HAVE DOUBLE GLAZING?

Yes ☐
No ☐

C.5   DO YOU HAVE ANY ELECTRIC EXTRACTOR FANS?

This is a question about electric fans which extract air from the home to the outside. These fans are fitted in a window or wall, you may have one in a ceiling which blows air up a pipe and through the roof. Do __not__ include cooker hoods. Please tick one box.

Yes   ☐
No   ☐
If no, please go to PART C.7

C.6   PLEASE INDICATE WHETHER THERE IS A FAN IN THE ROOMS LISTED IN THE TABLE BELOW, AND WHETHER IT IS USED, BY TICKING THE APPROPRIATE BOXES

	No fan	Fan present, but not used	Fan sometimes used	Fan normally used when room in use
Kitchen				
Bathroom				
Other rooms				

C.7   WOULD YOU SAY THAT WINDOWS AND/OR VENTILATORS IN YOUR HOME PROVIDE ADEQUATE FRESH AIR? Please tick one box

Usually   ☐
Sometimes ☐
Never   ☐

PART D: THINGS THAT AFFECT THE AIR IN YOUR HOME

D.1   DO YOU SMOKE INDOORS AT HOME?

Yes   ☐
No   ☐

D.2   DOES ANYONE ELSE IN YOUR HOUSEHOLD SMOKE INDOORS AT HOME?

Yes   ☐
No   ☐

D.3   DOES ANYONE ELSE REGULARLY SMOKE INDOORS AT HOME?

Yes   ☐
No   ☐

IF NOBODY SMOKES PLEASE GO TO D.5

D.4 FOR EACH PERSON WHO SMOKES INSIDE YOUR HOME PLEASE ESTIMATE THE AMOUNT SMOKED PER WEEK INSIDE YOUR HOME.

PERSON	1	2	3	4	5	6
Cigarettes (number)						
Pipe tobacco (oz)						
Small cigars (number)						
Large cigars (number)						

D.5 HAVE YOU NOTICED ANY PATCHES OF MOULD ON THE WALLS OR CEILINGS OF YOUR HOME AT ANY TIME IN THE LAST 12 MONTHS?

Yes ☐
No ☐

If yes, please indicate in which room(s) in the box below

D.6 DO YOU OWN ANY PETS (WHICH ENTER THE HOUSE) ?

Yes ☐
No ☐
If no, please go to D.8

If yes, please indicate in which room(s) in the box below

D.7 WHICH ROOMS ARE YOUR PET ALLOWED IN?

Kitchen ☐
Living rooms ☐
Bedrooms ☐
Other rooms ☐

D.8 HAVE YOU USED ANY GERMICIDE, PESTICIDE, OR PARASITE KILLER ON:

	Sampling day	Sampling week
Yes (please describe below)	☐	☐
No	☐	☐

D.9 DID YOU DO ANY DIY?

Sampling day	Sampling week	Sampling month
☐	☐	☐
☐	☐	☐

If yes, please specify any recent DIY in the box below, including approximately when it was done.

D.10 PLEASE TICK TO INDICATE HOW OFTEN DO YOU DO THE FOLLOWING

	Most days or every day	About once a week	Less often	Rarely / never
Hoover				
Dust				

D.11 PLEASE TICK TO INDICATE HOW OFTEN YOU USE THE FOLLOWING

	Most days or every day	About once a week	Less often	Rarely / never
Aerosol insect killer				
Aerosol air freshener				
Other aerosol				

D.12 Can you provide us with your approximated weight?
in kg. or lbs.

X.3 Activity diary sample

FIXAT Project- ACTIVITY DIARY

Volunteer ID [   ]   **Date:** [   ]

Time	Where are you?	What are you doing?	Are there any windows or doors open? Y/N	Anyone smoking? Y/N	Location number for		Level of exercise			
					Places visited	Travelling	Rest	Low	Med	High
0000-0030										
0030-0100										
0100-0130										
...	...	...	...	...	...	...	...	...	...	...
2230-2300										
2300-2330										
2330-2400										

NOW PLEASE COMPLETE A LOCATION SHEET FOR EACH PLACE VISITED AND EACH TIME YOU TRAVELLED

X.4 High VOC/PAH exposure activity questionnaire sample

FIXAT Project- Sampling Questionnaire												

		Volunteer ID			Measurement Date							

Activities	Please Tick used		Specify the activity you are doing	For how long?	What products are you using?	What is your location?	Is there any ventilation?		Level of exercise			
	day	week					Yes	No	Rest	Low	Med	High
Cleaning	☐	☐										
Dusting *(e.g. furniture polish)*	☐	☐										
Vacuuming	☐	☐										
Aerosol and perfume use (*including plug-in air fresheners)*	☐	☐										
Cosmetics (e.g. Nail polish, nail remover or glue)	☐	☐										
Solvent use *(e.g. fertiliser, de-icer, insect spray)*	☐	☐										
Dry cleaning	☐	☐										
Candle burning	☐	☐										
Use of a photocopier	☐	☐										
Use of fireplace	☐	☐										
Use of any other fossil fuels *(e.g. petrol lawn mower)*	☐	☐										
Visit to petrol station/refuelling car	☐	☐										
DIY - Painting	☐	☐										
DIY - Wallpapering	☐	☐										
DIY – Gluing	☐	☐										
DIY – Other *(please specify)*	☐	☐										
Gardening *(e.g. lawn mowing)*	☐	☐										
Not Applicable - non of above activities done.	☐	☐										

X.5 Food preparation, self-medication or prescribed drugs and caffeinated drinks questionnaire sample

Sampling Date: [   ]   Sampling ID: [   ]

Please list here where your meals were prepared:								
	Where?				Plastic container?			Other, please specify:
	Restaurant	Home (prepared from scratch)	Fast food or Take away	Ready made food	Re-heated in	Kept in	N/A	
Breakfast								
Snack								
Lunch								
Dinner								
Other snack								

IN THE LAST 3 DAYS DID YOU CONSUMED ANY:		
	Average amount per day (average cup of 250-300ml)	
Tea	_____ Cups □ N/A	
Coffee	_____ Cups □ N/A	
Prescribed drugs or self-medication products: _____	Amount per day: _____	Amount per dose: _____
Vitamines or supplements (if any, please list): _____ _____	Amount per day: _____ _____	Amount per dose: _____ _____
Soft drinks (1 can = 330ml): Cola	_____ ml □ N/A	
Sport drinks (e.g. Gatorade)	_____ ml □ N/A	
Energy drinks (e.g. Red bull)	_____ ml □ N/A	
Other drinks please list: _____	_____ ml □ N/A	
_____	_____ ml □ N/A	
_____	_____ ml □ N/A	
_____	_____ ml □ N/A	

X.6 Dietary questionnaire sample

DRINKS

	MEALS					QUANTITIES (PER DAY)					SPECIFICATIONS / COMMENTS
DRINKS	Breakfast	Snack	Lunch	Dinner	Other Snack	Glass 1 125mL	Glass 2 250mL	Cup 30mL	Mug/Can 300mL	Pint/Bottle 550mL	Glass 1= 125mL; Glass 2 = 250mL; Cup=30mL; Mug = 300mL; Pint = 550mL
Water ☐ Tap water ☐ Glass bottled water ☐ Plastic bottled water											
Coffee and Tea ☐ Coffee ☐ Tea											
Juice Specify:											
Milk ☐ Whole ☐ Semi-skimmed ☐ Skimmed ☐ Other, specify:											
Soft drinks ☐ Cola ☐ Energy drink ☐ Sport drink ☐ other, specify:											
Alcohol ☐ Beer, Ale, Lager ☐ Spirits ☐ Wine ☐ other, specify:											
Other drinks Specify:											

CEREALS, SWEETS, SNACKS

	MEALS					QUANTITIES (PER DAY)					SPECIFICATIONS / COMMENTS
CEREALS, SWEETS, SNACKS	Breakfast	Snack	Lunch	Dinner	Other Snack	Small portion	Normal portion	Large portion	Packed or canned food	Other, Specify	
Cereals ☐ Muesli ☐ Corn flakes ☐ Oats ☐ other, specify:											Normal individual portion = 30 grams
Digestive Biscuits ☐											Specify quantity:
Cookies ☐ Homemade ☐ Packed											Specify quantity:
Cake ☐ Homemade ☐ Packed											Normal individual portion = 1 of 8 cake slices
Chocolate ☐ Dark ☐ Milk ☐ White ☐ other, specify:											Normal individual portion = 2 chunks of a chocolate bar
Sweets Specify:											Specify quantity:
Snacks ☐ Crisps ☐ Nuts ☐ Chocolate snacks ☐ other, specify:											

268

FRUIT AND VEGETABLES

	MEALS					QUANTITIES (PER DAY)					SPECIFICATIONS / COMMENTS
	Breakfast	Snack	Lunch	Dinner	Other Snack	Small portion	Normal portion	Large portion	Tick if ORGANIC	Other, Specify	
Fruit											
☐ Apple ☐ Orange											Specify quantity: _____
☐ Pear ☐ Banana ☐ Dried fruit											
☐ other, specify:											
Salad											
☐ Lettuce ☐ Tomato ☐ Cucumber											
☐ Other Ingredients:											
Dressing											
☐ Oil (specify kind) ☐ Vinegar											
☐ other, specify:											
Vegetables (Meal 1)											☐ raw ☐ boiled ☐ roasted
☐ Spinach ☐ Beans ☐ Carrots											☐ steamed ☐ fried ☐ baked
☐ Peas ☐ Cauliflower ☐ Potatoes											
☐ other, specify:											
Vegetables (Meal 2)											☐ raw ☐ boiled ☐ roasted
☐ Spinach ☐ Beans ☐ Carrots											☐ steamed ☐ fried ☐ baked
☐ Peas ☐ Cauliflower ☐ Potatoes											
☐ other, specify:											

PULSES AND SOUPS

	MEALS					QUANTITIES (PER DAY)					SPECIFICATIONS / COMMENTS
	Breakfast	Snack	Lunch	Dinner	Other Snack	Small portion	Normal portion	Large portion	Tick if ORGANIC	Packed, canned or ready made food	Other, Specify
Pulses											
☐ Beans ☐ Peas											Normal individual portion = 250-300mL
☐ other, specify:											
Soups											
☐ Meat broth ☐ Fish broth ☐ Vegetables broth											Normal individual portion = 250-300mL
☐ other, specify:											

PASTA AND RICE

	MEALS					QUANTITIES (PER DAY)					SPECIFICATIONS / COMMENTS
	Breakfast	Snack	Lunch	Dinner	Other Snack	Small portion	Normal portion	Large portion	Packed, canned or ready made food	Other, Specify	
Rice											☐ Boiled ☐ Risotto ☐ Steamed
☐ Basmati ☐ Normal ☐ Brown											☐ Paella
☐ other, specify:											Normal individual portion = 75g
Dry Pasta											Normal individual portion = 75g
☐ Long pasta (e.g. spaghetti) ☐ Short pasta (e.g. penne)											
☐ other, specify:											
Fresh pasta											Normal individual portion = 120g
☐ Noodles ☐ Cannelloni ☐ Lasagne											
☐ other, specify:											

SANDWICHES, WRAPS, PIZZA

	MEALS					QUANTITIES (PER DAY)			SPECIFICATIONS / COMMENTS
	Breakfast	Snack	Lunch	Dinner	Other Snack	Please specify quantity	Packed or ready made food	Other, Specify	
Sandwich, please specify ingredients									
Wrap, please specify ingredients									
Pizza, please specify ingredients									
Burger, please specify ingredients									
other, specify:									

MEAT, FISH, VEGETARIAN SUBSTITUTES, EGGS

	MEALS					QUANTITIES (PER DAY)				SPECIFICATIONS / COMMENTS
	Breakfast	Snack	Lunch	Dinner	Other Snack	Small portion	Normal portion	Large portion	Packed, canned or ready made food	Other, Specify
										Normal individual portion for fish = 140g / Normal individual portion for meat = 80g
Meat										
□ Poultry □ Pork □ Beef										□ raw □ boiled □ roasted
□ Lamb										□ steamed □ fried □ barbecue
□ other, Specify:										□ Cured meat □ Smoked
										□ other, Specify:
Fish										
□ Oilyfish □ White fish □ Shellfish										□ raw □ boiled □ roasted
□ Seafood										□ steamed □ fried □ baked
□ other, Specify:										□ smoked
										□ other, Specify:
Vegetarian substitutes										
□ Tofu □ Soyburger □ Soy-meat substitute										□ raw □ boiled □ roasted
□ other, Specify:										□ steamed □ fried □ baked
										□ other, Specify:
Eggs										□ raw □ boiled □ roasted
□ Specify quantity:										□ steamed □ fried □ baked

JAMS, PRESERVES AND SUGARS

	MEALS					QUANTITIES (PER DAY)			SPECIFICATIONS / COMMENTS
	Breakfast	Snack	Lunch	Dinner	Other Snack	Please specify how many tea spoons	Tick if ORGANIC	Packed, canned or ready made food	
									1 tea spoon = 4g
Jam									
□ Home made □ Ready-made									
□ other, Specify:									
Marmelade									
□ Home made □ Ready-made									
□ other, Specify:									
Sugars									
□ White sugar □ Brown sugar □ Sweetener									
□ Honey									
□ Specify quantity:									

DAIRY PRODUCTS

	MEALS					QUANTITIES (PER DAY) Please specify quantity			SPECIFICATIONS / COMMENTS
	Breakfast	Snack	Lunch	Dinner	Other Snack		Packed, canned or ready made food	Other, Specify	
Yoghurt									1 regular portion = 125g
□ Low-fat □ Full-fat									
□ other, Specify:									
Cheese									1 regular portion = 9g (size of a small match box)
□ Please specify:									
Butter									
□ Specify quantity:									
Spreads									
□ Specify quantity:									

BREADS

	MEALS					QUANTITIES (PER DAY)					SPECIFICATIONS / COMMENTS
	Breakfast	Snack	Lunch	Dinner	Other Snack	Slices (quantity)	Bread rolls (quantity)	Baguette (quantity)	Packed or ready made food	Other, Specify	
Bread											
□ White bread □ Brown bread □ Handmade bread											
□ other, Specify:											

SALT AND SPICES

	MEALS					QUANTITIES (PER DAY)				SPECIFICATIONS / COMMENTS
	Breakfast	Snack	Lunch	Dinner	Other Snack	1 pinch	1 teaspoon	more	Other, Specify	
Salt (specify)										
Spices (specify)										

OILS, SAUCES AND DRESSINGS

	MEALS					QUANTITIES (PER DAY)				SPECIFICATIONS / COMMENTS
	Breakfast	Snack	Lunch	Dinner	Other Snack	1 teaspoon	up to 2 soup spoons	more (e.g.frying)	Other, Specify	
Oil										1 teaspoon = 5mL 1 soup spoon = 10mL
□ Olive oil □ Peanut oil □ Sunflower oil										
□ other, Specify:										
□ Specify quantity:										
Sauces										
□ Tomato □ Bolognese □ Carbonara										
□ Thai □ Curry □ Mayonnaise										
□ Ketchup □ Sour cream □ Caesar cream										
□ other, specify:										

X.7 ETS exposure questionnaire sample

VOLUNTEERS ID:		**MEASUREMENT DATE**	

TIME OF EXPOSURE: e.g. 3pm – 4pm _____

      WHEN YOU ARE EXPOSED TO PEOPLE SMOKING PLEASE COMPLETE ONE OF THESE FORMS

1)   **PLEASE COMPLETE THE FOLLOWING TABLE:**

How far was the smoker from you?	How many people were smoking?	Approx how many cigarettes were smoked?	How long were you exposed to the smoke for?
Less than 2 metres			
More than 2 metres			

2)   **WHO WAS THE SMOKER?**
- ☐ A FRIEND OR RELATIVE IN MY COMPANY
- ☐ A PERSON WHO WAS NOT IN MY COMPANY
- ☐ A PASSER BY

3)   **WHERE WERE YOU EXPOSED TO THE SMOKE?**
- ☐ OUTSIDE, IN AN OPEN SPACE
- ☐ INSIDE, IN AN ENCLOSED SPACE
- ☐ OTHER, PLEASE DESCRIBE

_____

FOR OPEN SPACES:

4)   **IF YOU WERE IN AN OPEN SPACE PLEASE DESCRIBE IT:**
- ☐ PRIVATE GARDEN
- ☐ PARK
- ☐ PLAYGROUND
- ☐ STREET
- ☐ BUS STOP
- ☐ OTHER, PLEASE DESCRIBE

_____

5)   **HOW LONG WERE YOU IN THE OPEN SPACE?**
      (e.g. 3pm – 4pm)

FOR ENCLOSED SPACES:

6)   **IF YOU WERE IN AN ENCLOSED SPACE PLEASE SAY WHERE YOU WERE:**

7)   **HOW SMOKY IS THE ROOM?**
- ☐ NOT SMOKY AT ALL
- ☐ SLIGHTLY SMOKY, PEOPLE ARE SMOKING OCCASSIONALLY
- ☐ SMOKY, PEOPLE ARE FREQUENTLY SMOKING
- ☐ VERY SMOKY, THERE ARE PEOPLE CONSTANTLY SMOKING

8)   **HOW VENTILATED IS THE ROOM?**
- ☐ IT IS WELL VENTILATED
- ☐ THERE IS SOME VENTILATION
- ☐ IT IS NOT VENTILATED
- ☐ I DON'T KNOW

9)   **ARE THERE ANY SOURCES OF VENTILATION IN THE ROOM?**
- ☐ OPEN WINDOWS
- ☐ OPEN DOORS
- ☐ FAN
- ☐ CEILING FAN
- ☐ AIR EXTRACTORS
- ☐ AIR CONDITIONING
- ☐ PASSIVE VENTILATION
- ☐ DON'T KNOW

10)   **HEN YOU WERE EXPOSED TO THE SMOKE WERE ANY HEATING SOURCES ON?**
- ☐ YES
- ☐ NO
- ☐ DON'T KNOW

X.8 Travelling questionnaire sample

LOCATION SHEET FOR TRAVELLING - Information About your Journey					
	Volunteers ID			**Measurement Date**	
Location number	**1**	**2**	**3**	**4**	**5**
Length of time travelling? (e.g. 30mins)					
Start time of travelling?					
If return journey along same route, what time is return?					
Where are you travelling from?					
Where are you travelling to?					
How are you travelling?					
Car/Taxi	☐	☐	☐	☐	☐
Motorbike	☐	☐	☐	☐	☐
Bus	☐	☐	☐	☐	☐
Electric Train	☐	☐	☐	☐	☐
Diesel Train	☐	☐	☐	☐	☐
Metro/Underground	☐	☐	☐	☐	☐
Cycling	☐	☐	☐	☐	☐
Walking	☐	☐	☐	☐	☐
How busy are the roads?					
Not busy *(very few cars around)*	☐	☐	☐	☐	☐
Busy at times *(busy on some roads)*	☐	☐	☐	☐	☐
Busy *(constant moving traffic)*	☐	☐	☐	☐	☐
Very busy *(congested/stationary traffic)*	☐	☐	☐	☐	☐
Not Applicable (travelling by train/metro)	☐	☐	☐	☐	☐
Is anyone smoking?					
No *(not smoky at all)*	☐	☐	☐	☐	☐
Occasionally *(slightly smoky)*	☐	☐	☐	☐	☐
Frequently *(smoky)*	☐	☐	☐	☐	☐
Constantly *(very smoky)*	☐	☐	☐	☐	☐
Please name the areas travelled through or the bus or train route taken: (e.g. Harborne-Edgbaston-City Centre, Bus Number 22, Train Route - Cross City Line - New Street to Erdington, e.t.c.)					
Location number (continued from previous page)	**1**	**2**	**3**	**4**	**5**
Please name the roads travelled along: (e.g. Hagley Road-Broad Street-Queensway-A38M-A38 Tyburn Road e.t.c.)					
If you are travelling by car, taxi or motobike please complete the following questions:					
Are you:					
Driving	☐	☐	☐	☐	☐
Passenger	☐	☐	☐	☐	☐
Do you own the car/motorbike?					
Yes	☐	☐	☐	☐	☐
No	☐	☐	☐	☐	☐
Make of car/motorbike?					
Model of car/motorbike?					
Fuel type:					
Petrol	☐	☐	☐	☐	☐
Diesel	☐	☐	☐	☐	☐
Engine size					
Year of manufacture					
Is the air conditioning used?					
Yes	☐	☐	☐	☐	☐
No	☐	☐	☐	☐	☐
Is the fan/heater used?					
Yes	☐	☐	☐	☐	☐
No	☐	☐	☐	☐	☐
Where was the briefcase kept?					
In the seating area of the car	☐	☐	☐	☐	☐
In the boot of the car	☐	☐	☐	☐	☐
In the travel case of the bike	☐	☐	☐	☐	☐
Other	☐	☐	☐	☐	☐
Any other information you would like to tell us about:					

X.9 Supplementary information on Comet assay

	Mean Tail Intensity (%)	Median Tail Intensity (%)	Exposed/Control Ratios
Replicate 1			
Ctrl 0.03	11.85	5.35	0.97
Benzene 0.03	11.49	3.21	
Ctrl 0.1	5.29	1.04	3.03
Benzene 0.1	16.02	5.53	
Ctrl 0.3	2.94	0.27	4.18
Benzene 0.3	12.27	5.13	
Replicate 2			
Ctrl 0.03	16.61	5.38	0.63
Benzene 0.03	10.52	1.96	
Ctrl 0.1	N/A	N/A	
Benzene 0.1	N/A	N/A	
Ctrl 0.3	9.54	5.93	1.70
Benzene 0.3	16.19	2.63	
Replicate 3			
Ctrl 0.03	11.39	3.76	0.69
Benzene 0.03	7.84	3.64	
Ctrl 0.1	3.62	0.16	2.31
Benzene 0.1	8.36	1.40	
Ctrl 0.3	7.01	4.01	1.59
Benzene 0.3	11.15	4.08	

Supplementary table*: Overview of the mean and median tail intensities of each of the independent replicates, including their relative air exposed controls. The reported data consists of the average of 3 technical replicates for each concentration.*
In replicate 1 it was possible to perform a better measurement of the tail intensities for the 0.3 ppm benzene concentration, however, on average the exposed/control ratio was lower in the cells exposed to 0.3 ppm than in the cells exposed to 0.1 ppm.