In vitro analysis of volatile organic compounds in search of potential biomarkers of lung cancer

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Declaration

This thesis is the result of approximately three and a half years of research conducted at the School of Environment and Life Sciences at the University of Salford. Throughout the period of the research, some of the contents of the literature review (Chapter 1) have been published in the two following review articles:

Schmidt, K. and Podmore, I. (2015) 'Current challenges in volatile organic compounds analysis as potential biomarkers of cancer', *Journal of Biomarkers*, 2015, Article ID 981458, (16 pp)

Schmidt, K. and Podmore, I. (2015) 'Solid Phase Microextraction (SPME) method development in analysis of Volatile Organic Compounds (VOCs) as potential biomarkers of cancer', *Journal of Molecular Biomarkers & Diagnosis*, 6(253), (10 pp)

Since the articles were published in the open access journals, I was free, as the main author, to use some parts of the published articles in my thesis.

Therefore, I declare that this thesis comprises only my original work towards the PhD and no portion of the work referred to in this thesis has been submitted in support of an application for another degree or professional qualification to the University of Salford or any other institution. Due acknowledgements have been made in the text to all other material used.

Abbreviations

ACN	acetonitrile
ADH	alcohol dehydrogenase
AKR	aldo-keto reductase
ALDH	aldehyde dehydrogenase
ATCC	American Type Culture Collection
ATP	adenosine triphosphate
AUC	area under the curve
2-B	2-bromobenzyl alcohol
BP	boiling point
BTEX	benzene, toluene, ethylbenzene, and xylenes
CHF	chloroform
CLCGP	Clinical Lung Cancer Genome Project
COPD	chronic obstructive pulmonary disease
CYP	cytochrome 450
DCC18	disk carbon active C18
DCM	dichloromethane
df	film thickness
DI	direct immersion
DME	dimethoxyethane
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethyl sulfoxide
DNTD	dynamic needle trap device
DSC18	disk silica C18
EBC	exhaled breath condensate
ECACC	European Collection of Authenticated Cell Cultures
EGFR	epidermal growth factor receptor
EI	electron ionisation
EML4-ALK	echinoderm microtubule-associated protein-like gene fused to the anaplastic lymphoma kinase gene
e-Nose	electronic nose
ER	estrogen receptor

ESI	electrospray ionisation
EtAc	ethyl acetate
EtOH	ethanol
EVA	ethylene vinyl acetate
2-F	2-fluorophenol
FBS	fetal bovine serum
FOM	foreskin melanocytes
FS	full scan
GC	gas chromatography
HBECs	human bronchial epithelial cells
HCl	hydrochloric acid
HCC-HMP	liver cancer cells with a high metastatic potential
HCC-LMP	liver cancer cells with a low metastatic potential
HDPE	high-density polyethylene
HDFs	human dermal fibroblasts
HEPES	(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)
HMLE	human mammary epithelial cells
HPLC	high performance liquid chromatography
HS	headspace
HUVEC	human umbilical vein endothelial cells
IC_{50}	the concentration of the drg inducing 50% cell growth inhibition
ID	internal diameter
I-ELCAP	The International Early Lung Cancer Action Program Investigators
IPA	2-propanol
IMS	ion mobility spectrometry
ISTD	internal standard
KEGG	Kyoto encyclopedia of genes and genomes
KRAS	Kirsten rat sarcoma viral oncogene homolog
LAS	laser absorption spectroscopy
LD	liquid desorption
LDPE	low-density polyethylene
LC-MS	liquid chromatography-mass spectrometry
LOD	limit of detection
LOQ	limit of quantification

MCC	multi-capillary column
MeOH	methanol
MDA	multivariate discriminant analysis
Mm	metastatic melanoma
MMSE	monolithic material sorptive extraction
MS	mass spectrometry
MT	MonoTrap
MW	molecular weight
NAD	nicotinamide adenine dinucleotide
NaOH	sodium hydroxide
NGM	Network Genomic Medicine
NHLFs	normal human lung fibroblasts
NIST	National Institute of Standards and Technology
NPV	negative predictive value
NR	natural rubber
NRC	National Research Council (United States)
NSCLC	non-small cell lung cancer
NTD	needle trap device
PR	progesterone receptor
OD	outside diameter
ODS	octadecyl functional group
P&T	purge and trap
PBS	phosphate buffered saline
PCA	principal component analysis
PFBHA	(O-(2,3,4,5,6-pentafluorophenyl) methylhdroxylamine hydrochloride)
PP	polypropylene
PPV	positive predictive value
PVC	polyvinyl chloride
PS	polystyrene
PTFE	polytetrafluoroethylene
PTR	proton transfer reaction
PUFA	polyunsaturated fatty aids
ROC	receiver operating characteristic

RGP	radial growth phase
RPMI	Roswell Park Memorial Institute
RSD	relative standard deviation
RT	retention time
SBSE	stir bar sorptive extraction
SCLC	small cell lung cancer
SD	standard deviation
SDME	single drop microextraction
SDR	short-chain reductase
SPME	solid phase microextraction
SIFT	selected ion flow tube
SIM	selected ion monitoring
SPI	single photon ionisation
SVI™	soil vapour intrusion
TBE	tracheobronchial epithelial cells
TD	thermal desorption
Tenax®	poly(2,6-diphenyl-p-phenylene oxide)
TFME	thin-film microextraction
TIC	total ion chromatogram
TOF	time-of-flight
TP53	tumour protein p53
VOC	volatile organic compound
VGP	vertical growth phase
WHO	World Health Organization

Notations

α	level of significance
A_a	peak area of the analyte
A_{ISTD}	peak area of the internal standard
β	headspace to sample phase ratio
C_0	initial concentration of the analyte in the sample phase
C_s	concentration of the analyte in the sample phase
C_g	concentration of the analyte in the headspace
K	partition coefficient; water:air distribution constant
$\lambda_{b:a}$	blood:air partition coefficient
$\lambda_{f:b}$	fat:blood partition coefficient
μ	mean of the population
m/z	mass-to-charge ratio
n	sample size
p-value	significance value (calculated probability)
ppb	parts-per-billion, 10^{-9}
ppm	parts-per-million, 10^{-6}
ppq	parts-per-quadrillion, 10^{-15}
ppt	parts-per-trillion, 10^{-12}
rpm	revolutions per minute
S/N	signal-to-noise ratio
V_g	volume of the headspace phase
V_s	volume of the sample phase
$\bar{x}$	mean of the sample

Abstract

Lung cancer is a leading cause of death from cancer worldwide. An early diagnosis and appropriate treatment are crucial in reducing mortality among people suffering from the disease. Therefore, one of the main focuses of lung cancer studies is on advances in its early detection. One of the most promising is the analysis of volatile organic compounds (VOCs). VOCs are a diverse group of carbon-based chemicals that are present in exhaled breath and biofluids, and may be collected from the headspace of these matrices. Different patterns of VOCs have been correlated with various diseases, cancer among them. Studies have shown that various cancer cells *in vitro* produce or consume specific VOCs that can serve as potential biomarkers to differentiate them from non-cancer cells.

The present study aimed at the detection, identification and semi-quantification of VOCs released or consumed by the adenocarcinoma human alveolar A549 cell line. For this purpose, gas chromatography with mass spectrometric detection was combined with two pre-concentration techniques: monolithic material sorptive extraction (MMSE) or extraction of thermal desorption (TD) sorbent tubes with an Easy-VOC^{TM} pump as a sample loading tool. MMSE is a new technique for the extraction of VOCs from various samples and it is used for the first time for the analysis of VOCs from cell culture medium. TD-GC-MS is a popular technique for VOCs analysis and it has been used for the first time here with Bio-VOC^{TM} pump in the studies of VOCs *in vitro.* The project also aimed at the comparison of the A549 VOC level trends to the trends of the normal human lung fibroblasts NHLF (MMSE experiment) and normal human bronchial BEAS-2B cells (TD experiment). In addition, the VOC patterns between the growing and confluent cells of the same cell line were compared for the first time.

In the MMSE experiment, seven VOCs were produced and 14 VOCs metabolised exclusively by the cancer cells. Among the released compounds were methylated hydrocarbons (2,4-dimethyl-1-heptene; 4-methylundecane; 2,3,6,7-tetramethyloctane 2,3,5-trimethylhexane and 2,3,5-trimethyldecane) and alcohols (cyclohexanol and 3-heptanol). The metabolised analytes were alcohols (4-decanol; 6-dodecanol; 2-ethylhexanol; 1-octanol), aldehydes (dodecanal; tetradecanal), ketones (acetophenone; cyclohexanone; 2-tetradecanone), phenols (phenol and 2-nitrophenol); an ether (2-

methoxydiphenylmethane), an ester (pentanoic acid, 2,4-dimethyl-3-oxo-, methyl ester) and a hydrocarbon (tetradecane). 2,4-Dimethylheptane; 2,6-di-*tert*-butyl-1,4-benzoquinone; 1-phenylethanol and 2-pentadecanone were released by both A549 and NHLF cell lines. The cancer cells were observed, however, to emit the VOCs at a higher level than the fibroblasts. Benzaldehyde; 2-ethylhexanol; hexanal and 1-nonanol were found to be consumed by both the cancer and NHLF cells, however, at a greater rate by the former. In the TD experiment, 2,3,5-trimethylhexane and *tert*-butanol were produced exclusively by the A549 cells, while ethyl acetate solely by the BEAS-2B cells. Acetophenone, benzaldehyde and 2-methylbutanal were metabolised and acetone was produced at higher levels by the cancer cells than by the BEAS-2B cells.

The possible use of the analysed VOCs as potential biomarkers of lung cancer is discussed, along with the suggestion and discussion of the possible metabolic pathways leading to the uptake and release of these VOCs by the analysed cells. Also the discussion of poor correlation between different *in vitro* studies, as well as between *in vivo* and *in vitro* studies of VOCs as potential biomarkers of cancer, is undertaken.

Chapter 1

Introduction*

1.1 Lung cancer

1.1.1 Lungs

The lungs, (the primary organ of respiration in humans) are the lower part of the respiratory tract which starts with the trachea divided into primary bronchi. These structures are lined with pseudostratified ciliated columnar epithelium, interspersed with goblet cells which produce mucous. The epithelium is shown in Figure 1.1.

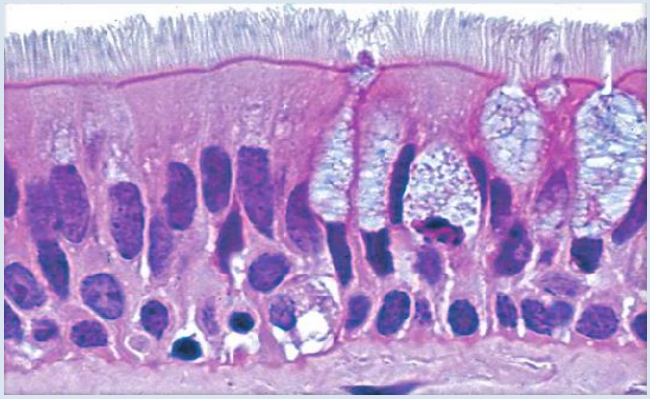

Figure 1.1 Ciliated pseudostratified columnar epithelium with goblet cells (Flickr, 2011).

Bronchi undergo branching into bronchioles, which are also lined with epithelial cells, and are encircled with smooth muscle cells. Bronchioles divide into alveolar ducts and then sacks which turn into alveoli where gas exchange takes place. There are two types of alveoli cells: type I pneumocytes and type II pneumocytes. Type I are squamous epithelial cells with thin cell membrane to enable gas exchange while type two are larger, line the alveoli and produce epithelial lining fluid (to reduce the surface tension of the alveoli).

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Alveoli also contain alveolar macrophages (Eroschenko, 2008). Figure 1.2 is a diagram depicting the main parts of the lungs.

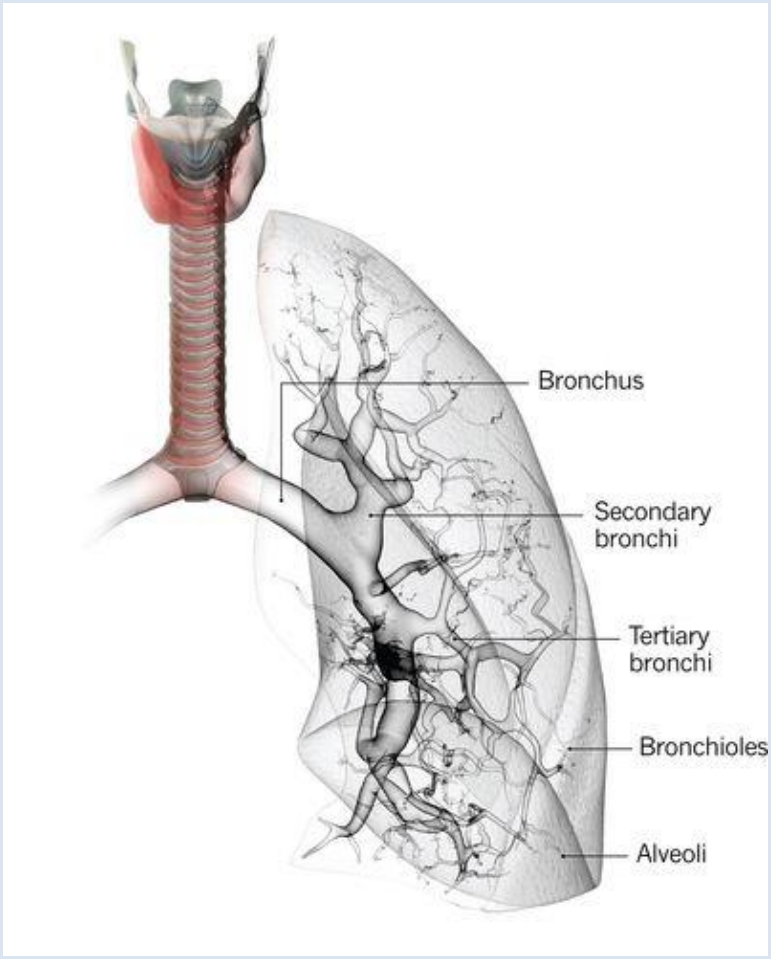

Figure 1.2 Lung diagram [reprinted by permission from Macmillan Publishers Ltd: Nature, 512, S2-S3, Bender, (2014), copyright 2014].

1.1.2 Classification of lung cancer

Lung cancer (lung carcinoma, pulmonary carcinoma) is a disease that is characterised by uncontrolled cell growth in epithelia of the trachea, bronchi or lungs. If this growth starts in the lung tissue, it is primary lung cancer rather than secondary lung cancer, where the tumour has its origin in a different part of the body and then spreads into the lung tissues. There are two main types of lung cancer depending on the cell morphology: small-cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). NSCLC includes three

subtypes: squamous cell carcinoma, adenocarcinoma, and large cell carcinoma. The classification and diagnosis is based on histological features determined by light microscopy. However, dividing lung cancer into SCLC and NSCLC for diagnosis is not sufficient. The diagnosis of advanced NSCLC is based on the precise classification of the histological subtype, in combination with molecular diagnostic methods to detect mutations of epithelial growth factor receptors or alternations in anaplastic lymphoma kinase, so that an appropriate treatment can be chosen (Petersen, 2011; Dong Y. *et al*., 2016).

The approximate incidence of the different lung cancer subtypes is shown in Figure 1.3. The most common lung cancer subtype is adenocarcinoma, accounting for 40% of all lung cancer cases. Squamous cell carcinoma is the second most common lung cancer (30% occurrence). Less common are SCLC and large cell carcinoma with 15% incidence each (Bender, 2014). Figure 1.3 shows also pictorial examples of each of the major types of lung cancer.

SCLC typically has its origin in the central parts of the lungs. It is usually poorly differentiated, aggressive with metastasis and is almost always caused by smoking (WHO, 2008). The cells of SCLC may have a varied morphology: comma-shaped oat cells, small homogenous round cells, spindle-shaped cells or larger intermediate cells (NRC, 1991). Figure 1.3 (A) presents SCLC showing relatively large cells.

Large cell carcinoma is less associated with smoking, it usually has its origin in the distal bronchi but it can begin in any part of the lung. It is generally undifferentiated and tends to grow and spread quickly (Khuder, 2001; WHO, 2003). The main morphological characteristic of this type of cancer are relatively large cells (NRC, 1991). Large cell carcinoma is shown in Figure 1.3 (B).

Adenocarcinoma is also less associated with smoking; in fact it is the most common type of lung cancer in never-smokers (Sun *et al.*, 2007). Over the last few decades adenocarcinoma has became the most common type of lung cancer, overtaking squamous cell carcinoma (WHO, 2008). This cancer usually arises in the outer section of the lung, in the cells that line the alveoli, and forms glands. Early metastasis to the brain, adrenal glands and pleura is characteristic of this cancer. Adenocarcinoma is defined by the formation of mucus (intercellular or discrete), or by characteristic growth patterns such as papillary differentiation, glandular or acinar growth or a single-layer wall of cells along

the alveolar septum and bronchioles (Petersen, 2011). The mucinous type of adenocarcinoma is shown in Figure 1.3 (C).

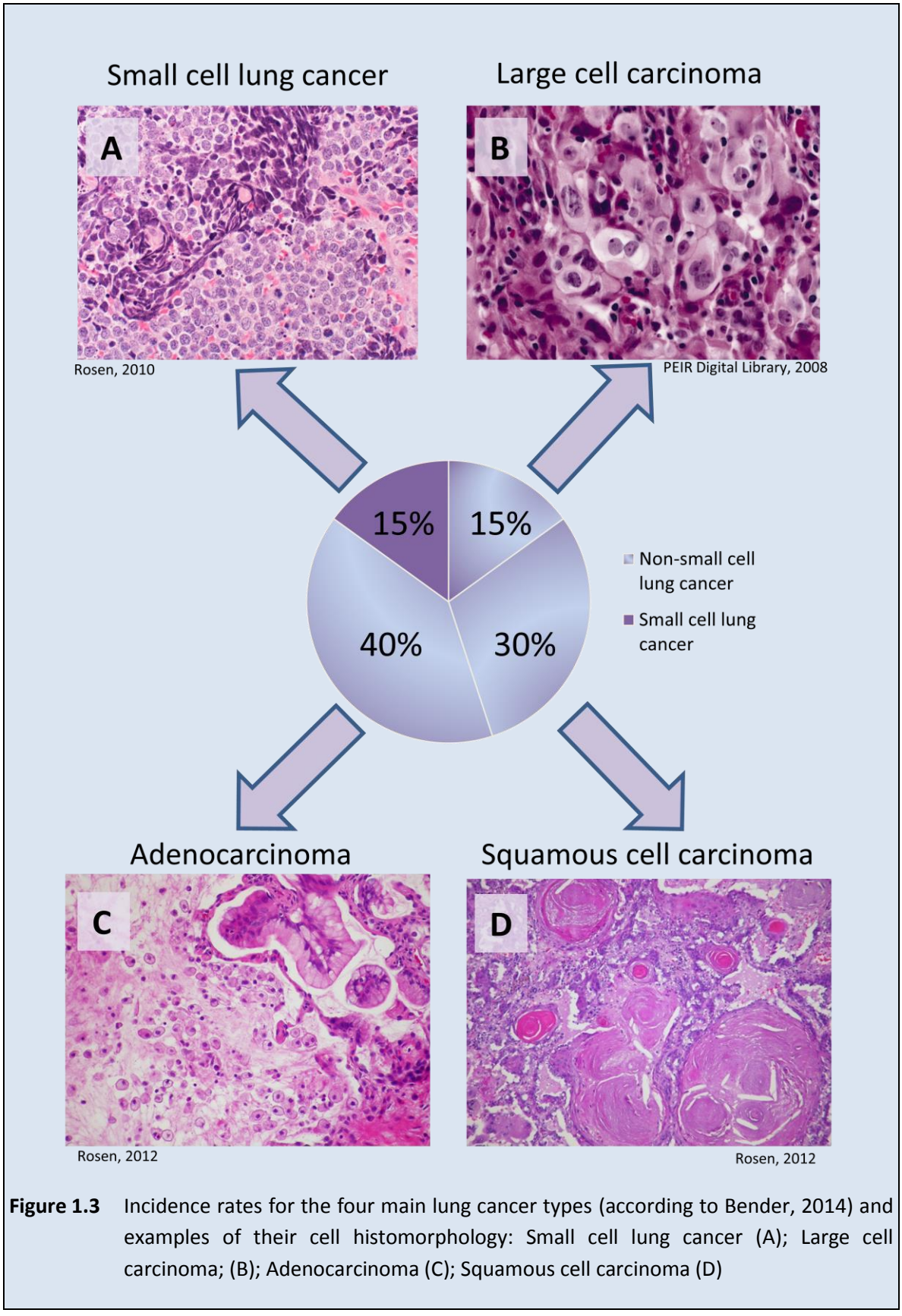

Figure 1.3 Incidence rates for the four main lung cancer types (according to Bender, 2014) and examples of their cell histomorphology: Small cell lung cancer (A); Large cell carcinoma; (B); Adenocarcinoma (C); Squamous cell carcinoma (D)

Squamous cell carcinoma appears most frequently in the centre of the lung, near or in bronchi (within the first four branches of the bronchus) in the flat cells lining the inside of the airways (squamous epithelium). Its occurrence has again been very strongly linked to smoking. It grows slowly, as it requires 3-4 years to grow from the *in situ* lesion to an apparent tumour (WHO, 2008). Cell morphology of squamous cell carcinoma is characterised by keratinisation (so called 'keratin pearls') or intercellular bridges. Poorly differentiated squamous cell tumors may be less keratinised with polygonal shape cells present in a mosaic pattern (NRC, 1991; Petersen, 2011). A microscopic picture of squamous cell carcinoma with large masses of intra-alveolar keratin is shown in Figure 1.3 (D).

1.1.3 Prevalence and risk factors

Lung cancer is the most common cancer in the world and is the main cause of death from cancer in men worldwide. In less developed countries it is the leading cause of death from cancer in men and the second most fatal cancer among women (after breast cancer). It is the second leading cause of death in men (after prostate cancer) and the leading cause of death in women in economically developed countries (Ferlay *et al.*, 2015). Until the beginning of the last century lung cancer was a rare disease. Since then its incidence has increased dramatically (WHO, 2008). It has been estimated that there were 1.6 million fatal cases of lung cancer (19.4% of all deaths caused by cancer) and around 1.8 million new lung cancer cases worldwide in the year 2012 (13% of all cancer diagnoses). Approximately 58% of cases of lung cancer occur in low-income countries (Ferlay *et al.*, 2015).

The geographical and time patterns of lung cancer incidence have been related to tobacco consumption. The link between smoking and lung cancer was identified in the 1950s, and has been accepted as fact by public health and regulatory authorities since the mid-1960s (WHO, 2008). The results of studies conducted in Europe, Japan and North America, indicated that approximately 90% of lung cancers in men, and 57 - 86% of lung cancers in women, are attributable to cigarette smoking (Boyle and Smans, 2008). Different factors contribute to this overall risk, such as the age at which smoking is started, average tobacco consumption, smoking duration, type of tobacco product smoked and pattern of inhalation (WHO, 2003). Although the risk of developing lung tumour decreases with time after smoking cessation, it never levels up with that of a person who has never smoked. Passive

smoking causes an increased risk of developing lung cancer and depends on the level and duration of exposure to smoke (WHO, 2008). Pooled evidence indicates an increase of 20 - 30% in the risk of developing lung cancer from passive smoking associated with living with a smoker (The US Surgeon General, 2006).

Other identified risk factors of lung cancer include occupational and environmental exposures, genetic predispositions, chronic obstructive pulmonary disease (COPD), diet, gender and ethnicity. The main occupational agents causing a high risk of lung cancer are asbestos, arsenic and chromium. Occupational exposure is estimated to cause 2 - 9% of all lung cancer cases (Mazzone *et al.,* 2014). The mechanism of genetic predispositions appears to be driven by a single genetic event, in comparison to many genetic and epigenetic changes observed in lung cancer related to smoking. This is why lung cancer in never-smokers is considered to be a distinct disease characterised by unique carcinogenesis, pathology and response to treatment (Lee Y. *et al.,* 2011). For non-smokers, the risk of lung cancer may be higher in women, since it occurs on average 2.5 times more in women than men (Siegfried, 2001). The accumulating data suggests that women are more likely to develop lung cancer and are more vulnerable to tobacco smoke, possibly due to the influence of hormones and differences in the metabolism of tobacco carcinogens (Kiyohara and Ohno, 2010).

1.1.4 Detection and diagnosis

The earlier the lung cancer is detected, the better the chances of the patient recovering, as appropriate treatment can be applied in time. In the majority of patients, lung cancer is diagnosed at an advanced or metastatic stage, where often therapy is only palliative (Bepler *et al.,* 2003). As a result, lung cancer survival rate depends on the stage of the tumour at diagnosis. For example, in the years 2003-2006 in the UK the 1-year survival rate was 71% for stage I and 14% for stage IV. The 5-year survival rate was 35% for people with stage I lung cancer at the time of diagnosis and 6% for stage III. A very small percentage of people with stage IV survived for 5 years after diagnosis (Cancer Research UK, 2014).

There are two components to efforts to detect cancer early: individual frequent medical check-ups and population screening programmes aimed at a certain age group (WHO, 2003). The only non-invasive methods of lung cancer detection are radiology (chest X-ray

and computed tomography) and sputum cytology. However, there is no evidence of a reduction in mortality related to lung cancer resulting from the screenings employing regiments of X-ray and sputum cytology in Western countries. What is more, frequent radiography of the chest might be harmful to the patient (Manser *et al.,* 2003). New methods of screening such as spiral computed tomography have been shown to detect cancer that is curable by surgery (I-ELCAP, 2006; Kondo *et al.,* 2011). However, there are also limitations to this method such as exposure to radiation, high false-positive rates, and the possibility of overdiagnosis. Currently there is no practicable and effective population screening method for lung cancer (Aberle *et al.,* 2013).

Early diagnosis is difficult for each type of cancer. In lung cancer, there is usually a lack of early clinical symptoms that could lead to early detection of the disease. The symptoms develop as the condition progresses; however they may still not be clear enough to aid in diagnosis (WHO, 2003). This underlines the need for the investigation of new methods of early lung cancer detection. In this search all the "omics" approaches (genomics, proteomics and metabolomics) have been applied (Beger, 2013; Brooks *et al.,* 2012; Honda *et al.,* 2013; Khadir and Tiss, 2013). Proteomics-based efforts in particular have been promising in discovering the potential biomarkers of lung cancer. Potential biomarkers have been analysed in tissue samples, serum, plasma, sputum, and exhaled breath condensate (EBC). Several cancer protein biomarkers in serum have been studied comprehensively, and are currently available for blood testing, such as cytokeratin 19 fragment, carcinoembryonic antigen, and pro-gastrin-releasing peptide. None of them, however, is wholly sufficient for the diagnosis of early stage lung cancer, because of their relatively low sensitivity and specificity in detecting the presence of a tumour (Bastawisy *et al,* 2014; Grunnet and Sorense, 2012; Mattei *et al.,* 2014). The discovery of lung cancer biomarkers is a field of research in its early stages of development and currently no biomarker for the detection of lung cancer has been validated (Xiang *et al.,* 2013).

With the rapid advances in spectrometric techniques, the analysis of volatile organic compounds (VOCs) as potential biomarkers of lung cancer, one of the metabolomic approaches, has become very popular in recent years. However, the 'smell of the disease' is essentially nothing new. The sweet, fruity breath of a diabetic person, the ammonia-like smell of the breath of an uremic patient or the sulfuric breath odour of a patient suffering from cirrhosis, are well known to medicine. In fact, the earliest studies of breath metabolites were investigating correlations between the increased concentrations of some

VOCs and diabetes, cirrhosis and renal disease (Chen S. *et al.*, 1970; Rooth and Östenson, 1966; Simenhoff *et al.*, 1977). Lung cancer was examined in this context for the first time by Gordon *et al.* in 1985. The group identified several candidate biomarkers of lung cancer and pioneered the study in this field. The first studies analysing the volatiles emitted by cells *in vitro* were conducted at the beginning of 2000. Kato *et al.* (2000) investigated formaldehyde concentration in the background of lysed breast cancer cells and D. Smith *et al.* (2003) measured the level of acetaldehyde generated by lung cancer cells. Exciting implications resulting from both studies have pioneered a new era in the analysis of VOCs as potential biomarkers of lung cancer.

1.2. Techniques used for potential VOC biomarkers studies

1.2.1 Extraction techniques

Concentrations of most of the VOCs present in biological matrices are low: in the µmol l^{-1} - pmol l^{-1} (ppm - ppt) range in exhaled human breath, blood and urine (Abbott *et al.* 2003; Guadagni *et al.* 2011; Hakim *et al.* 2012; Mochalski *et al*. 2013a; 2014; Pandey *et al.* 2013). In addition, VOCs are analytes of interest to be extracted from complex mixtures. Therefore, prior to the assay, a pre-concentration step is required, which is the most labour-intensive part of the analysis and is the primary source of errors influencing the reliability and accuracy of analysis (Kataoka and Saito, 2011). Increased reproducibility and elimination of interfering compounds can be achieved by minimising the number of steps. The ideal properties of a sample-preparation device include simplicity, high extraction capacity and selectivity, efficiency, speed, possible automation and miniaturisation, compatibility with a range of separation and detection methods, and safety in use for the operator and environment (Kataoka, 2011; Zhang Z. *et al*., 2013). Microextraction methods employ some of these features the best, when compared to the traditional sampling techniques of liquid-liquid extraction and solid-phase extraction.

Dynamic headspace extraction (also called purged and trap: P&T) on thermal desorption (TD) sorbent traps and solid phase microextraction (SPME) are the two most common extraction techniques used for the collection of VOCs in studies of potential cancer biomarkers. They are presented in Figures 1.4 and 1.5 respectively.

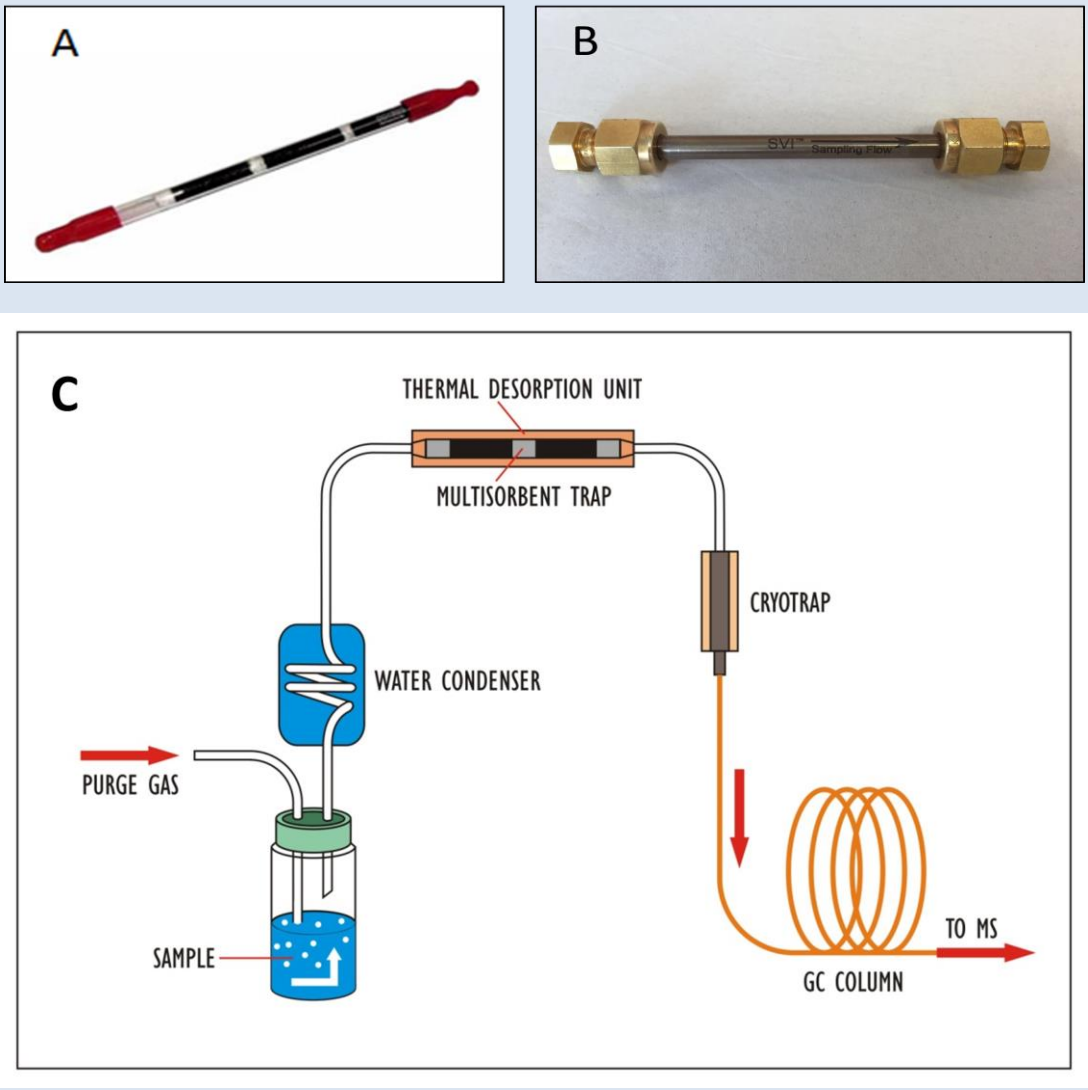

Figure 1.4 Multi-bed glass TD tube (A); stainless steel TD tube (B) and diagram of analysis with online P&T-GC-MS (purge and trap - gas chromatography - mass spectrometry) (C) [Pan *et al.*, 2008 (A) Schmidt and Podmore, 2015a (C)].

Sorbent traps are adsorption materials contained in a 15 cm long glass or stainless steel tube which are shown in Figure 1.4 (A and B respectively). The most commonly used adsorbents for the analysis of VOCs employ charcoal (e.g. Carbotrap®) or porous polymer [e.g. Tenax®: poly(2,6-diphenyl-p-phenylene oxide)] as a trapping material with varying degrees of selectivity. In P&T, the gas sample is purged through the tube by an inert gas and the VOCs are retained on the adsorbent bed. Next they are thermally desorbed with the use of an on-line TD device or extracted with small amounts of solvents (liquid desorption: LD). P&T is shown in Figure 1.4 (C). TD may cause degradation reactions of

sensitive compounds and some column types degradation, as some sorbents have a high affinity to water. LD is a milder technique, however it is less sensitive (Kleeberg *et al.*, 2007). In studies of potential VOC cancer biomarkers TD of sorbent tubes with P&T were employed with cryofocusing to enhance resolution (Phillips M. *et al.*, 1999a; Filipiak *et al.*, 2010).

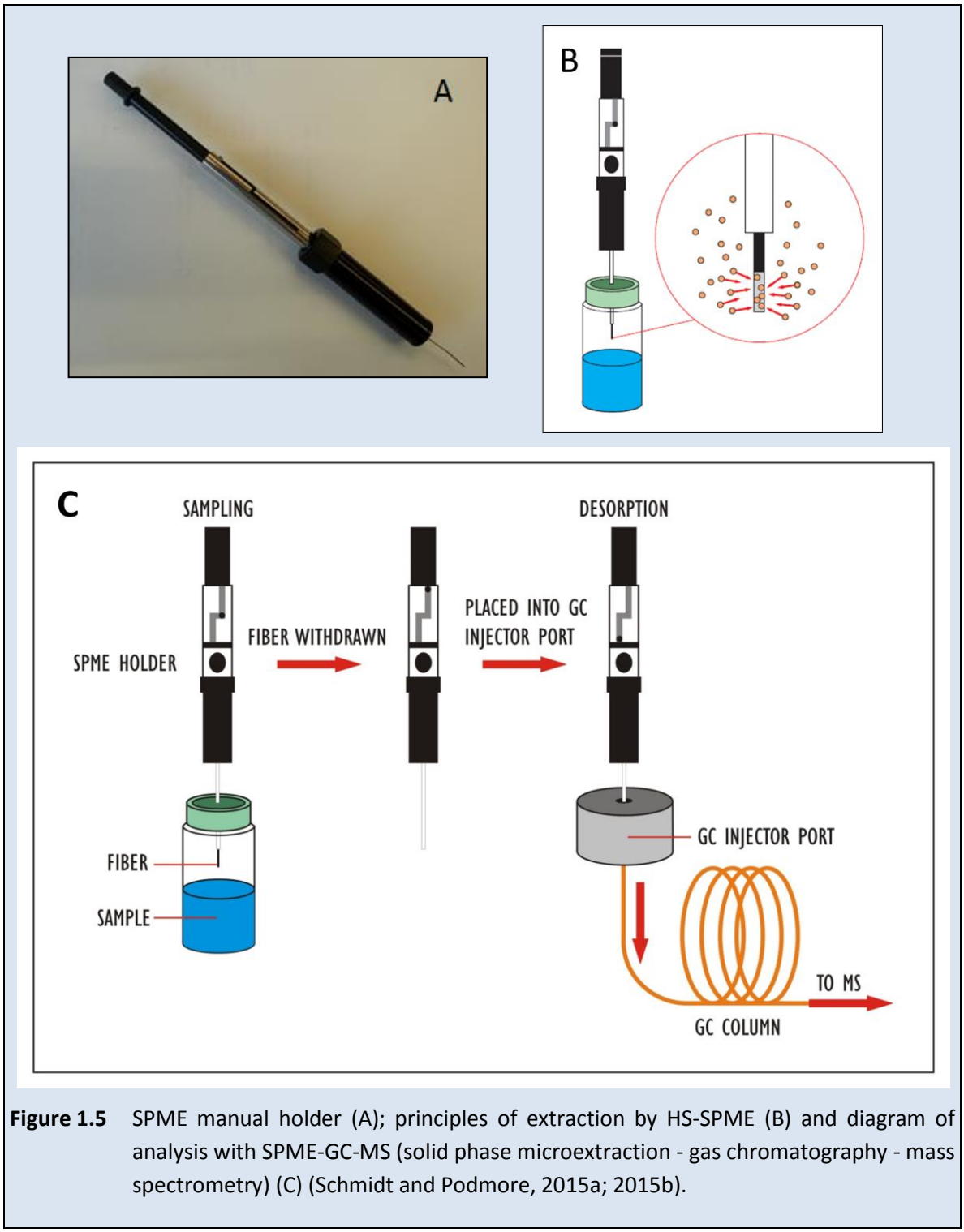

Figure 1.5 SPME manual holder (A); principles of extraction by HS-SPME (B) and diagram of analysis with SPME-GC-MS (solid phase microextraction - gas chromatography - mass spectrometry) (C) (Schmidt and Podmore, 2015a; 2015b).

Figure 1.5 (A) shows: a manual SPME device (A), principles of extraction by headspace (HS) SPME and a diagram of SPME analysis (C). SPME is an extraction technique where an extraction phase is dispersed on a fine rod made of fused silica, StableFlex^{TM} (fused silica with plastic polymer) or metal alloy. The SPME device consists of two parts: the holder, and contained in it, the fiber assembly. There are two versions of the SPME holder: one for manual use and one for use with autosamplers or with a high performance liquid chromatography (HPLC) SPME interface. The fiber unit consists of a fiber core attached via a hub to a stainless steel guiding rod, which is contained in a hollowed needle that pierces the septum. The fiber is withdrawn from this needle when sampling and the needle is removed when not in use. The fiber core is 1 or 2 cm long, and is coated with stationary phase. The fiber is immersed in the liquid sample in the case of direct immersion (DI-SPME) or suspended in the headspace above the sample (HS-SPME).

During extraction, sample molecules preferentially partition from matrix to stationary phase as a result of adsorption or absorption (Kudlejova *et al.*, 2009). After sampling, the analytes are thermally desorbed in the injector port with no use of solvents.

There are several commercially available SPME fibers for sampling a wide range of compounds that employ four polymers as stationary phases: divinylbenzene, polydimethylsiloxane, polyacrylate and polyethyleneglycol. They are used on their own as a coat (available in different thicknesses) or in combination blended with carboxen. The coatings differ by polarity (polar, bipolar, non-polar) and extraction mechanism (absorbent or adsorbent). The choice of fiber coating depends on the polarity of analytes and their molecular weight (MW).

In comparison, P&T is an exhaustive extraction technique, due to chemical reactions between the stationary phase and the analytes, whereas SPME is a non-exhaustive (passive) equilibrium technique where the amounts of VOCs extracted are controlled by the series of distribution constants between the gaseous, liquid and coating phases. Sorbent trapping is based on an adsorption process, where the extraction of the analytes occurs only on the surface of the porous material. The analytes are physically trapped or retained by the stationary phase via chemical reactions. The amount of analyte adsorbed depends on the initial concentration of the analyte as well as on the concentration of the competitive analyte (competitive displacement reactions). SPME, depending on the fiber used, is an absorption (dissolution) technique, or utilises absorption and adsorption

properties simultaneously. During the absorption process the analytes partition preferentially at equilibrium in the porous material of the liquid polymeric phase. The absorption process also depends on the initial concentration of the analyte in the sample. Again, displacement processes may take place during the absorption, but some studies in water analysis indicate that this is a minor concern in these types of SPME coating (Pawliszyn, 1999).

Sorbent trapping is a four-step process (extraction of the analytes to the solid sorbent, primary desorption, cold focusing and secondary desorption), whereas SPME is more simple in use (sorption of analytes onto the fiber then desorption). The simplicity of SPME facilitates the development of normalised methods and standardisation (Di Francesco *et al.*, 2005). On the other hand, limits of detection (LODs) obtained in studies analysing VOCs as potential cancer biomarkers showed that P&T sorbent trap extraction technique yielded better sensitivity (low ng l^{-1} in full scan (FS) mode) than SPME (μg l^{-1} in FS mode) (Filipiak *et al.*, 2010; Ligor T. *et al.*, 2007).

Other variations of SPME techniques such as stir bar sorptive extraction (SBSE), thin-film microextraction (TFME) (or solid phase microextraction membrane), and needle trap device (NTD) have been successfully used for the collection of VOCs, and so may be used in cancer studies (Zhang Z. *et al.*, 2013). NTD has been already used by Mochalski *et al.* (2013b) for analysis of VOCs in the HS of liver cancer cell line. Another microextraction method, single drop microextraction (SDME), was also introduced for the HS analysis of VOCs in cancerous blood (Li N. *et al.*, 2005). The NTD contains a sorbent trap inside a needle. The analytes present in a liquid or a gas sample can be actively drawn into and out of the needle using a syringe or a pump, or passively be introduced via the diffusion process. This technique (as in the case of P&T) is exhaustive and can achieve similar limits of detection to SPME (high-medium ppt range in selected ion monitoring (SIM) mode) (Ligor T. *et al.*, 2007; Mochalski *et al*., 2013a). SDME, in which a small drop of solvent (around 2 μl) is suspended from the tip of the needle where the compounds are extracted from the headspace, offers relatively low costs, simplicity and elimination of carry over. With the use of in-drop derivatisation, SDME offers limits of detection in low ppb range (in SIM mode) (Deng *et al*. 2005). Other extraction techniques used in the analysis of VOCs in cancer studies include the TFME and diffusion badges (Nozoe *et al.*, 2015; Barash *et al.*, 2009). In TFME a thin layer of sorbent is dispersed on the surface of

the aluminium rod. The device is then put into the analysed liquid and the VOCs are extracted (Nozoe *et al.,* 2015). Barash *et al.* (2009; 2012) used Ultra II SKC passive (no purge) diffusion badges for the pre-concentration of VOCs from the HS of the cell culture media. In this type of sampler sorbent traps serve as adsorption material, and extraction is based, as in SPME, on the equilibrium principles (Vrana *et al.,* 2005). Off-line sorbent trapping was also used by Amal *et al.* (2012) (with the use of TD). Finally, cryoconcentration was also used prior to analysis in a study, in order to investigate VOCs produced by leukaemia cell line. The VOCs were quantified in trace levels (low ppb). Separation of the analytes was achieved here by the use of multi-capillary column - gas chromatography (MCC-GC) (Shin *et al.,* 2009).

1.2.2. Detection techniques

The detection techniques that have been employed in cancer biomarker studies are spectroscopic techniques such as gas chromatography - mass spectrometry (GC-MS), proton transfer reaction - mass spectrometry (PTR-MS), selected ion flow tube - mass spectrometry (SIFT-MS), ion mobility spectrometry (IMS), laser absorption spectroscopy (LAS) and a non-spectroscopic technique using an electronic nose (e-Nose). Spectroscopic techniques offer advantages over nanosensors for the identification and quantification of VOCs. There are three major types of spectroscopic techniques used in VOC studies: MS, IMS and LAS. MS measures the mass-to-charge (m/z) ratio of charged molecules; LAS techniques measure the photon absorption properties of a molecule and IMS the mobility of the gas (Chow *et al*., 2012). The main parameters of the six techniques are presented in Table 1.1. Principles of GC-MS technique are described in Appendix A.

Mass spectrometry-based techniques

GC-MS is the most commonly used analytical technique for the investigation of potential VOC cancer biomarkers, due to its sensitivity and reliability in analyte identification. It gives the most detailed analytical information and identifies analytes with the most certainty, when compared to PTR-MS. The identification of VOCs with the use of PTR-MS can be tentative only as it is not possible to discriminate between compounds with the same MW (Bajtarevic *et al*., 2009; Brunner *et al*., 2010; Wehinger *et al.*, 2007). On the other hand, PTR-MS is the most sensitive method of all, with the LOD for aromatic compounds is low as a few ppt levels (Lindinger *et al.,* 1998) or even as low as 200 ppq

(for compounds with MW around 180 Da) (Jordan *et al.*, 2013). It has been demonstrated to be more sensitive than GC-MS measurement by a factor of ~20 (Kushch *et al.,* 2008). GC-MS was shown to have sensitivity for VOC analysis at the ppb and low ppt levels but it needs a further preconcentration step (Fuchs *et al.,* 2010; Ligor T. *et al.,* 2008). SIFT-MS allows for the measurement of trace gases at sub-ppb and low ppb levels, but it is also reliable in the identification of compounds (Kushch *et al.,* 2008; Milligan *et al*., 2007; Ross, 2008). The advantage of PTR-MS and SIFT-MS over GC-MS is that they do not require a preconcentration step and can work in on-line (real time) mode. Therefore, they are better techniques for the quantification of VOCs, as they provide instant quantification of all the analytes in the sample (Smith D. and Španěl, 2005; Wehinger *et al.*, 2007). In comparison, SPME-GC-MS measures analytes semi-quantitatively, as it involves competitive absorption/adsorption of the compounds on the fiber (Pawliszyn, 2000; Chow *et al*., 2012). Because of the preconcentration step, GC-MS is also time consuming when compared to other MS-based techniques for breath analysis (Chow *et al*., 2012). GC-MS instruments are also more expensive. Nevertheless, instruments for all the techniques are not easy to use in clinical settings in terms of portability or transport (Wehinger *et al.*, 2007). Although the easily transportable SIFT (TransSIFT) and PTR (PTR-QMS 300) instruments have been introduced commercially (Kassebacher *et al.,* 2012; Smith D. and Španěl, 2005), their small sizes compromise their sensitivity.

Ion mobility spectrometry

Another detection technique, IMS, is not very common yet in the studies of VOCs as potential cancer biomarkers, but already has shown promising results in differentiation between lung cancer patients and healthy individuals when VOCs in breath were studied (Westhoff *et al*, 2009). The IMS detector is characterised by low selectivity. Therefore, complex mixtures are analysed with the use of a separation technique such as MCC-GC or GC (Lord *et al.*, 2002; Ulanowska *et al.,* 2008). Mainly IMS coupled with MCC has been used for breath analysis in the studies performed to date (Darwiche *et al.,* 2011; Handa *et al.*, 2014; Ulanowska *et al.,* 2008; Westhoff *et al*, 2009). The advantages of MCC-IMS include: very fast analysis (500 s for the breath sample), no need for preconcentration, and on-line analysis. In contrast to other analytical techniques, the use of MCC-IMS allows for the detection of all the analytes in a breath sample through their separation by retention time (RT), mobility, and concentration and by creating a 3D visualisation of each

compound in the chromatogram (Westhoff *et al*, 2009). A downside of IMS is its limited sensitivity, as it is not operated under high vacuum conditions which leads to ion-molecule collisions. This results in a lower number of ions reaching the detector than anticipated (Lourenço and Turner, 2014). Furthermore, it does not allow for the reliable identification of analytes. Nevertheless, IMS is a comparatively cheap detection technique with a potential for miniaturisation and is therefore one of the most promising, next to e-noses, candidates to be used in a clinical setting (Ulanowska *et al.*, 2008).

Table 1.1 Main characteristics of analytical techniques used in the studies of VOCs as potential cancer biomarkers [1] Fuchs *et al.,* 2010; [2] Ligor T. *et al.,* 2008; [3] Lindinger *et al.,* 1998; [4] Jordan *et al*., 2013; [5] Ross, 2008; [6] Milligan *et al*., 2007; [7] Ulanowska *et al.*, 2008; [8] Menzel *et al*., (2001); [9] Halmer *et al*., 2005; [10] Oh *et al.,* 2011.

Analytical technique	Limit of detection	Quantification	Mode	Compound identification
GC-MS	sub-ppb - low ppt* [1, 2]	Semi-quantitative	Off-line	Reliable
PTR-MS	low ppt - sub-ppq [3, 4]	Absolute	Real-time	Tentative
SIFT-MS	sub-ppb - low ppb [5, 6]	Absolute	Real-time	Reliable
IMS	ppm - ppb [7]	Absolute	Real-time	Tentative
LAS	low ppb - sub-ppt [8, 9]	Absolute	Real-time	Tentative
E-nose	low ppb [10]	Semi-quantitative	Real-time	N/A
*** with preconcentration**				

Laser absorption spectrometry

Recently, LAS techniques have been attracting interest as analytical tools in the study of VOCs in exhaled breath, due to the rapid development of photodetectors and semiconductor lasers. LAS advances include cavity ring-down spectroscopy, tunable

diode laser absorption spectroscopy and photoacoustic spectroscopy (Chow *et al*., 2012). The LAS techniques offer relatively fast and easy analysis, LODs in low-ppb – sub-ppt ranges (depending on the molecule and technique used), on-line measurement and the potential for miniaturisation (Mürtz and Hering, 2008). It has been used recently for VOC detection patterns in the breath of lung cancer patients and showed a high potential for screening diagnosis (Kistenev *et al*., 2016). However, the main disadvantage of LAS techniques is that they operate in a narrow spectrum range, limiting detection to a few VOCs (Chow *et al*., 2012). Methods for the screening of VOC profiles require the measurement of a wide-band frequency. Such methods have only started to emerge (Kistenev *et al*., 2016; Mastrigt *et al*., 2016).

Electronic nose (nanosensors)

Compared to these methods, the use of e-noses does not require skilled personnel and is less time-consuming (Peng *et al.*, 2010). These features, as well as the potential miniaturisation of such devices (Hagleitner *et al.*, 2001), make them ideal potential diagnostic tools to be used by general practitioners or even as devices for personal use. There have been several types of e-noses used in the studies of VOCs in cancer: nanomaterial-based chemical sensor arrays (such as organically stabilised gold nanoparticles and single walled carbon nanotubes) (Barash *et al.*, 2009; Peng *et al.*, 2008), colormetric sensor arrays (Mazzone *et al.*, 2007), organic vapour sensors (Khalid *et al.*, 2013a) and quartz microbalance gas sensors (Di Natale *et al.*, 2003). They are designed to recognise VOC patterns emitted from the analysed samples, but not to identify these VOCs (Dragonieri *et al*., 2009). Generally, e-noses have not been designed to quantify analyte intensity (Röck *et al.*, 2008). However, construction of calibration curves allows for the semi-quantitative detection of VOCs (Gao *et al.*, 2012). Quantification of VOCs with the use of an e-nose has not been performed in any studies of cancer.

In terms of breath testing, such sensor systems could be cheap, rapid and simple to use when they have been tailored for a specific use (Di Francesco, 2005). However, electronic noses are highly sensitive to moisture, relatively less sensitive (1-5 ppb) (Oh *et al.*, 2011), and their effectiveness needs more validation studies as they have shown poor linearity and reproducibility (Biasoli *et al.*, 2011). Nevertheless, e-noses are a very promising research area and many such devices have already shown very good accuracy, for example in diagnosing bladder cancer (Khalid *et al.*, 2013a).

1.3 VOC collection and available matrices in lung cancer

In 1989 the WHO established a classification scale for organic indoor pollutants based on their boiling point (BP) ranges. Compounds with a BP from < 0°C to 50 - 100°C are defined as very volatile (gaseous) organic compounds, compounds with a BP from 50 - 100°C to 240 - 260°C are classified as VOCs, and compounds with a BP in a range of 240 - 260°C to 380°C are semi-volatiles. No clear borders, however, exist between the three groups.

VOCs are emitted from the body in exhaled breath, and are present in biofluids such as blood, faeces, saliva, sweat, urine, and therefore may be collected from the HS of these matrices, but also from the HS of cells *in vitro*. Different patterns of VOCs have been correlated with various diseases such as cancer (O'Neill *et al.*, 1988), asthma (Ibrahim *et al.*, 2010), cystic fibrosis (Barker *et al.*, 2006), diabetes (Phillips M. *et al*., 2004a), tuberculosis (Phillips M. *et al*., 2007a), COPD (Phillips C.O. *et al.*, 2012) and heart allograft rejection (Phillips M. *et al.*, 2004b). The presence of different patterns of VOCs between a healthy person and a person suffering from a disease is based on the hypothesis that pathological processes, occurring as a consequence of disease, can generate new VOCs that the body does not produce during normal physiological processes, and/or alter the concentrations of VOCs. These new VOCs, or VOCs that are produced in significantly higher or lower levels than normal, may therefore serve as biomarkers for the assessment or detection of disease.

In order to investigate VOCs as potential cancer biomarkers, analysis of the exhaled breath or EBC of cancer patients has become very popular in recent years. Alternative approaches include the HS analysis of cancer cells, tissues or body fluids. All sample matrices have their advantages and disadvantages.

1.3.1 Breath analysis

Exhaled breath consists of a gaseous phase that contains VOCs and a liquid phase, which can be chilled into EBC. EBC is made up of aerosol particles or droplets originating from the airway lining fluid, condensed water vapour and water soluble VOCs that are absorbed into the EBC (Hunt, 2007). Studies have shown that chemical changes in blood due to the presence of cancer are echoed in an alteration of the composition of VOCs in the patients'

breath (Preti *et al.,* 1988). Therefore, it is hypothesised that abnormal VOCs produced by cancer cells are discharged via the blood stream into the endobronchial cavity. Then, depending on the blood:air partition coefficient ($\lambda_{b:a}$), they are exchanged in alveoli (compounds with low solubility in blood, with $\lambda_{b:a} < 10$; mainly non-polar VOCs), in the airways (gases with high solubility in blood, with $\lambda_{b:a} > 100$; mainly polar VOCs) or in both (compounds with medium solubility in blood, $10 < \lambda_{b:a} < 100$) and finally exhaled with breath (Hakim *et al.,* 2012).

Breath and EBC analysis, compared to blood and urine tests, is non-invasive and a sample may be easily collected at any point and in varying quantities, which makes it easy to repeat (Buszewski *et al.,* 2012a). Furthermore, it eliminates the need for the handling and storage of biofluids. Breath analysis does not require skilled staff to collect a sample or any work-up after collection, and gives direct information about the function of the respiratory tract, which is especially important in cases of lung cancer (Manolis, 1983; Spinhirne *et al.,* 2003). In addition, the breath matrix is a less complex mixture than urine or blood, so complete analysis of all the VOCs (approximately 200) present in a sample is possible (Manolis, 1983; Prado *et al.,* 2003). In spite of this less complex composition, over 3000 different VOCs were detected at least once in the breath of a larger group of subjects, with approximately half of the compounds being of possible endogenous and half of possible exogenous origin (Phillips M. *et al.*, 1999b). New volatile compounds are still being identified. Some argue that only compounds produced inside the body can be considered as biomarkers, which is problematic as the origin of most volatile metabolites is still unknown or remains the subject of speculation (Hakim *et al.,* 2012; Kwak and Preti, 2013). The presence of both endo- and exogenous VOCs in exhaled air is one of the biggest limitations of breath analysis. Another limitation is qualitative and quantitative inter-individual and intra-individual variability. The majority of breath VOCs were detected only once, being unique to a particular individual (Phillps M. *et al.,* 1999b); this may be the result of the patterns of VOCs being altered by food consumption, smoking, gender, age etc. (Di Francesco *et al.,* 2005; Kischkel *et al.,* 2010).

There are different opinions about how detailed knowledge is required for a successful breath diagnostic test. Some argue that there is no need to know the origin of a volatile compound biomarker, as long as it can be used to distinguish disease from a healthy state (Di Natale *et al.,* 2003; Machado, 2009). Others simultaneously measure exhaled and inspired air since environmental contaminant VOCs may be incorrectly assigned as

endogenous compounds (Phillips M. *et al.*, 1997; Schubert *et al.*, 2005). Finally, the last approach requires knowledge about the metabolic pathway of the compound, as well as about normal concentration ranges of a compound in relation to inter-individual variability, before including it into the predictive model of the disease (Kwak and Preti, 2013; Risby, 2008). Moreover, since the beginning of breath analysis in the 1970s (Pauling *et al.,* 1971), standardisation and reproducibility of the sample collection method has been an issue which has resulted in the variability of quantitative information (Manolis, 1983; Montuschi, 2007). The interpretation of VOC levels in EBC is not straightforward either and includes consideration of such factors as water solubility, gas-liquid partition coefficients, the temperature of the airway lining fluid and the condenser, the pH of the airway lining fluid and the EBC, and the possibility of reactions within the EBC matrix (Hunt, 2007). Standardisation is easier to achieve for serum or urine than for breath collection (Manolis, 1983), which is a big advantage of these matrices. Furthermore, equipment for exhaled breath collection is relatively expensive and may thus not be easy to apply widely (Cao and Duan, 2006).

1.3.2 Breath analysis versus other matrices

Although VOCs detected in blood and urine are "in the body" analytes, it still does not mean they are of endogenous origin. Some inhaled VOCs may bind to or dissolve in blood (Pouling and Krishnan, 1996), be stored in body compartments and later excreted through urine (Silva *et al.*, 2011). In addition, it is not known which volatile compounds are produced or consumed by tumour cells as they may also be generated (or consumed) by non-cancerous cells (such as surrounding tissue cells or other regions of the body) (Horvath I. *et al.,* 2009; Phillips M. *et al.* 1999a), immune-competent cells (Aksenov *et al.,*. 2012; Brandacher *et al.,* 2002), human symbiotic bacteria (Bunge *et al.,* 2008; Garner *et al.,* 2007) and infectious pathogens (Brandacher *et al.,* 2002; Bunge *et al.,* 2008; Jünger e*t al.,* 2012; Syhre and Chambers, 2008). Furthermore, VOC patterns differ between individuals because of uncontrolled variables such as genetic differences, environmental settings (Matsumura *et al.,* 2010), diet, drug ingestion, smoking and cosmetics (Ligor T. *et al.,* 2008), which makes VOC analysis a challenge regardless of the matrix used. Nevertheless, there is growing evidence that VOCs that are potentially clinically relevant may be found in breath and other matrices. Dogs were reported to discriminate between patients with or without cancer (breast, bladder, colorectal, lung, ovarian, prostate and

skin) by sniffing breath, blood, feaces, skin, tissue or urine samples of cancer patients, which suggests that characteristic VOC signatures of cancer exist (Buszewski *et al*., 2012b; Cornu *et al.,* 2011; Horvath G. *et al.,* 2008; 2010; McCulloch *et al.,* 2006; Pickel *et al.,* 2004; Sonoda *et al*, 2011; Willis *et al.,* 2004). Sensor mice were also trained to distinguish mice with experimentally-induced cancer from mice without it (Matsumura *et al.,* 2010).

Blood

Blood transports essential compounds to the cells and metabolic waste away from them and as such is a more direct means of identification of potential volatile biomarkers. Blood was used as a matrix for VOC collection in a number of studies of lung cancer (Deng *et al.,* 2004a; 2004b; Deng and Zhang, 2004), childhood forms of cancer (Yazdanpanah *et al.,* 1997) and liver cancer (Xue *et al.,* 2008). The disadvantages of blood as a matrix include invasiveness, and careful handling and work-up after collection as temperature and pH changes, can alter VOC profile (Manolis, 1983, Kouremenos *et al.,* 2012). Moreover, there are difficulties in the collection of arterial blood. When there is a necessity to collect many of such samples, breath analysis would be a better alternative, especially as it closely mirrors the arterial concentrations of metabolites (Cao and Duan, 2006). In theory, the composition of volatile compounds in breath is related to the composition of these compounds in blood (Deng *et al.,* 2004a; Cao and Duan, 2006). This needs to be addressed in studies comparing VOC composition in blood and breath samples. Such an investigation concerning cancer was performed by Deng *et al.* (2004a). The study showed that 23 VOCs found in blood were also present in the exhaled breath of lung cancer patients. Therefore, there are characteristic compounds that identify cancer presence. Among these 23, hexanal and heptanal were detected only in cancerous blood and breath samples and were not found in controls. However, more study is required to compare VOC patterns in both matrices, where ideally the blood and breath samples from the same patient would be investigated.

Urine

Many studies have also investigated volatile biomarkers in urine samples of patients with various cancers such as breast (Silva *et al.,* 2012), lung (Hanai *et al.,* 2012a), leukaemia, colorectal, lymphoma (Silva *et al.,* 2011), childhood leukaemia (Yazdapanah *et al.,* 1997),

bladder cancer (Jobu *et al.*, 2012) and prostate cancer (Khalid *et al*., 2015). Apart from its non-invasive nature, urine as a matrix for VOC analysis has an advantage over other biofluids in that analytes are concentrated by the kidney before being excreted from the body. In addition, when compared to blood, the use of urine usually results in better detection limits as matrix effects may interfere with the release of the VOC's into HS in blood sampling (Mills and Walker, 2000). On the other hand, VOCs in urine may be affected by the drugs administered to a patient and therefore the products of particular drugs must be known as well as determining their effect on the VOCs produced (Jobu *et al.*, 2012). Also standardisation for urine dilution, such as the measurement of urinary creatinine or urine osmolality is needed (Khalid *et al.*, 2015).

Saliva

As a matrix for VOCs studies, an evident advantage of saliva over blood is its non-invasiveness. It also offers an easy and cost-efficient collection as well as low-cost storage (Lima *et al*., 2011). As compositional and concentration correlations of various biochemical and immunological parameters between blood and saliva have been found, saliva mirrors the biochemical and metabolic status of blood (Nagler *et al*., 2002). However, saliva is a more complex matrix containing VOCs also originating from food deposits, gastrointestinal reflux, microorganisms, upper airways, nasal cavities and gingival sulcus fluid (space between tooth and gums) which may complicate the interpretation of VOCs found (Kaufman and Lamster, 2002; Lima *et al*., 2011). Nevertheless, saliva is a promising, currently underused matrix for the study of VOCs as potential biomarkers of cancer. Initial methods for the determination of VOCs in saliva samples coming from lung cancer patients have been proposed based on HS-GC-MS, offering the advantages of no pre-concentration and no manipulation of the sample (Sánchez *et al*., 2012; 2014).

Hybrid volatolomics

An important characteristic of body volatolome (all the VOCs emanating from the human body) is the partition between different body fluids and compartments such as breath, blood, liver and fat. This is driven by blood:air ($\lambda_{b:a}$) and fat:blood ($\lambda_{f:b}$) partition coefficients. The partition coefficient can be very different for different VOCs and this results in different VOC concentrations in different storage tissues, in different rates of

their synthesis and metabolism and in different exhalation kinetics (Amann, 2014) (see Chapter 5.7.1 for further discussion). Therefore, recently a new concept of 'hybrid volatolomics' has been proposed which claims that the most comprehensive profile of human VOCs can be provided only by combining research from different body matrices (Broza *et al.,* 2015). However, before it will be possible to conduct research using predictive models of disease showing VOC patterns complementarily in different matrices *in vivo*, standardised collection methods must be introduced and more research performed regarding the origin of VOCs.

1.3.3 Analysis of targeted VOCs

A different approach to the issue of the possible exogenous origin of proposed VOC biomarkers focuses on the detection of aldehydes (Deng and Zhang, 2004; Deng *et al.* 2004b; Fuchs *et al*. 2010; Guadagni *et al*., 2011) or hydrocarbons (Gaspar *et al.,* 2009; Phillips M. *et al*., 2003a; Yu H. *et al.,* 2005) only as markers of cancer. Studies have shown that oxidative stress is one of the main sources of developing cancer via the overproduction of reactive oxygen and nitrogen species resulting in mutations (Toyokuni, 2008). Some aldehydes are known to be related to oxidative stress as they are products of lipid peroxidation, but the exact mechanism of their presence in breath and body fluids is not known (Eggink *et al.* 2010; Hakim *et al.,* 2012; Romero *et al.,* 1998). The same mechanism underlies the emission of saturated hydrocarbons in the body. They are products of lipid peroxidation of polyunsaturated fatty acids (PUFA) (Hakim *et al.,* 2012). As aldehydes are highly reactive so can easily decompose or react while the sample is prepared for analysis or storage, a chemical derivatisation has been introduced (Fuchs *et al.,* 2010). One of the most common derivatisation methods for aldehyde determination is the reaction of aliphatic aldehydes with PFBHA (O-(2,3,4,5,6-pentafluorophenyl) methylhdroxylamine hydrochloride) to produce stable oximes (Cancilla and Hee, 1992). Different studies that employed different techniques of extraction demonstrated this as an effective method for aldehyde analysis in various matrices (Deng and Zhang, 2004; Li N. *et al.*, 2005; Yazdapanah *et al*., 1997).

Higher concentrations of straight C1-C9 aldehydes, with the exception of acetaldehyde, as well as some unbranched hydrocarbons were identified among VOCs in the breath and blood of cancer patients in many studies (Buszewski *et al.,* 2012a; Deng and Zhang, 2004;

Fuchs *et al.*, 2010; Kischkel *et al.*, 2010; Li N. *et al.* 2005; Lilli *et al.*, 2010; Phillips M. *et al.*. 1999a; 2003a; Poli *et al.*, 2005; 2010).

1.3.4 Analysis of VOCs emitted by cancer cells in vitro

An *in vitro* investigation of the VOCs produced by cancer cells as the source of biomarkers should hypothetically help with the dilemma of their origin. In fact, in contrast to body fluids or breath analysis, this is the only matrix where one can be sure of the endogenous origin of the analytes and that they are produced by cancer cells themselves. Advantages of *in vitro* studies over other matrices include easier control of experimental variables, lower cost, better reproducibility and more easily interpreted results due to the absence of factors such as gender, age and inter-individual variation (with the exception of primary cell cultures) (León *et al.*, 2013).

The cell metabolome is comprised of the endometabolome, which is represented by all metabolites inside the cell, and the exometabolome, which is made up of all metabolites present in extracellular cell culture medium. The profile of these metabolites in the surrounding medium depends on the uptake and extraction of the compounds by the cells and reflects their metabolic activity via their response to experimental variables. *In vitro* studies aiming to find potential volatile markers of cancer essentially apply the extracellular metabolite investigative approach. Endometabolomic studies require cell disruption, and then concentration of the extracted compounds (mainly with the use of evaporation). VOCs could be easily lost during these steps (León *et al.*, 2013).

A number of studies have been performed to investigate potential VOC cancer biomarkers *in vitro* in different types of cancer and using different techniques, and in all of them there were differences observed in the composition of volatile metabolites produced by cancer and normal cells when compared to control. These studies are listed in Table 1.2 together with the analytical technique used, cancer cell lines studied, type of matrix, control, cell culture medium and type of cell culture vessel used in each study. However, some studies found differences in VOC levels, or VOCs produced, between not only different cell lines of the same cancer but also between the same cell line (Filipiak *et al.*, 2008; 2010; Hanai *et al.*, 2012b; Smith D. *et al.*, 2003). While the first observation may be explained by genetic and phenotypic differences and the fact that each cell line is representative of only a small part of a primary tumour, the reasons for the second are unclear (Filipiak *et al.*,

2008; 2010). It may be due to the high and low passage number of cell line. The study of Sponring *et al.* (2009) showed the possibility of a change of released volatile metabolites with increasing passage number. Cells should not be passaged for a long period of time to ensure they have not mutated, as mutation would cause them to no longer reflect the properties of the tumour of origin. The fact that there were significant experimental differences in many studies between the cell cultures that had been passed a low to those that had been passed a high number of times, and that there were studies conducted on cross-contaminated cell lines, makes a compelling case for the use of certified cell lines with defined passage numbers (Hughes *et al.,* 2007).

In the cell/tissue HS analysis of VOCs there is also a variation in the techniques used, and a lack of standardisation and normalisation of the data even when the same technique is used, which may influence variations in VOC patterns between different studies. The aspects to be considered (apart the technique used) in terms of *in vitro* studies of VOCs include: the analysis of different matrices, the use of different media, the periods of cells cultivation, the different cell controls used (in some cases only medium control with no reference to non-cancerous cells and vice versa), the different statistical methods used and finally the differing methodology.

Length of incubation periods, cell senescence, differing types of culture (in monolayer, matrix immobilized cultures or 3D cultures), as well as supplementation of cell culture medium have been shown to have an influence on the composition of the VOCs in the samples (Acevedo *et al.,* 2007; 2010; Hanai *et al.,* 2012b; Hartmann *et al.,* 2008; Rutter *et al.,* 2013).

The three matrices analysed to study VOCs generated by cells are:

i) HS of the cell-free culture medium of a target cell collected after cell cultivation
ii) HS of the medium still containing the cells
iii) cell lysate or preconcentrated supernatant of the lysed cells.

Table 1.2 Analytical technique used, cancer cell lines studied, type of matrix, control, cell culture medium and type of culture vessel used in *in vitro* studies aiming to investigate VOCs as potential cancer biomarkers. CCM: cell culture medium with cells; CFCM: cell-free culture medium; DNTD: dynamic needle trap device; EGF: epithelial growth factor; ESI: electrospray ionisation; FBS: fetal bovine serum; GC-MS: gas chromatography-mass spectrometry; GNs: gold nanoparticles; MCC: multi-capillary column; Mm: metastatic melanoma cell; ns: not specified; MWNTs: multi-wall carbon nanotubes; NSCLC: non-small cell lung cancer; L-g: L-glutamine; QMB: quartz microbalance; p: preconcentration; P&T: purge and trap; pen/strep: penicillin & streptomycin; PTR-MS: proton transfer reaction-mass spectrometry; RGP: radial growth phase cell; SCLC: small cell lung cancer; SIFT-MS: selected ion flow tube - mass spectrometry; SPME: solid phase microextraction; SWNTs: single wall nanotubes; TFME: thin-film microextraction; VGP: vertical growth phase cell.

Analytical technique used	Cancer type	Cell lines studied	Control	Type of matrix	Medium used	Cell culture vessel	Reference
SPME-GC-MS	Lung	A549	OUS11, WI-38 VA 1 Pure medium (some VOCs only)	CFCM	DMEM (high-glucose), 10% FBS, pen/strep	Culture dish (100 mm x 20 mm)	Hanai *et al*. 2012b
SPME-GC-MS	Lung	A549, SK-MES-1, NCI-H446	BEAS-2B	CFCM	ns	T-75	Yu J. *et al*., 2009
SPME-GC-MS	Lung	A549, SK-MES-1, NCI-H446	BEAS-2B, Pure medium	CCM	RPMI 1640, 10% FBS, pen/strep SK-MES-1: MEM, 10% FBS, pen/strep	T-25	Wang Y. *et al*., 2012
SPME-GC-MS	Lung	A549, Lu7466, Lu7387	Pure medium	CCM CFCM	**A549:** Ham's F12, L-g, 10% FBS **Lu:** DMEM/Ham's F12,L-g, 20% FBS	T-150	Schallschmidt *et al.* 2015a; 2015b
SPME-GC-MS	Lung	Primary lung cancer cells	Primary normal cells (human lung cells, lipocytes, osteogenic cells and rat tastebud cells)	CFCM	DMEM-H, 10% FBS, pen/strep	Glass culture dish (70 mm) [poly-D-lysine: 0.01%]	Chen X. *et al*., 2007
SPME-GC-MS	Lung	A549	Pure medium	CCM	RPMI 1640, 10% FBS, pen/strep	Culture flask (size ns)	Pyo *et al*., 2008

Table 1.2 Cont'd

Analytical technique used	Cancer type	Cell lines studied	Control	Type of matrix	Medium used	Cell culture vessel	Reference
SPME-GC-MS	Breast	MCF-7, MDA-MB-231	CCD-1095Sk, Pure medium	CFCM	DMEM, 10% FBS, pen/strep	Cell culture dish	Huang Y. *et al,* 2016
SPME-GC-MS	Colon	SW1116, SW480	NCM460, pure medium	CCM	DMEM/F12, 10% FBS	glass vials [poly-D lysine: 0.01%]	Zimmerman *et al*., 2007
SPME-C-MS	Liver	HepG2	Pure medium	CCM	RPMI 1640, 10% FBS, pen/strep	250 ml cell flasks	Castaneda *et al.,* 2007
SPME-GC-MS	Skin	**RGP:** M35, WM3211, Sbcl2 **VGP:** WM115, WM983A **Mm:** WM983B, WM1158	FOM136, FOM191, Pure medium	CFCM	**Cancer cells:** Tu 2% medium with Leibovitz's L-15, 1.68 mM $CaCl_2$, 2% FBS, 5 µg/ml insulin **Normal cells:** 254 with human melanocyte growth supplement	T-25	Kwak *et al*., 2013
Nanosensors (QMB) SPME-GC-MS	Skin, thyroid, synovial sarcoma	Primary cells	Pure medium	CCM	RPMI 1640, 10% FBS, pen/strep	Cell culture dish (75 mm)	Bartolazzi *et al*., 2010
Ultra II SKC & SPME-GC-MS Nanosensors (GNs)	Lung	**NSCLC:** A549, Calu-3, H1650, H4006, H1435, H820, H1975	Pure medium	CCM	RPMI 1640, 10% FBS	Cell culture dish (100 mm)	Barash *et al*., 2009
Ultra II SKC & SPME-GC-MS Nanosensors (GNs)	Lung	**NSCLC:** A549, Calu-3, H1650, H4006, H1435, H820, H1975, H2009, HCC95, HCC15, H226, NE18 **SCLC:** H774, H69, H187, H526	IBE, Pure medium	CCM	**NSCLC :** RPMI 1640, 10% FBS **IBE:** BEBM 1640, 10% FBS	Cell culture dish (100 mm)	Barash *et al*., 2012

Table 1.2 Cont'd

Analytical technique used	Cancer type	Cell lines studied	Control	Type of matrix	Medium used	Cell culture vessel	Reference
Ultra II SKC & SPME-GC-MS Nanosensors (GNs)	Lung	**$EGFR_{mut}$:** H3255,H820, H1650, H1975, HCC4006, HCC2279 **$KRAS_{mut}$:** A549, H2009, H460, NE18 **$EML4\text{-}ALK_{mut}$:** H2228 **WT:** H322, H1703, H125, H1435, Calu-3, HCC15, H520, HCC193	Pure medium	CCM	RPMI 1640, 10% FBS	Cell culture dish (100 mm)	Peled *et al*., 2013
Nanosensors Ultra II SKC & TD-GC-MS	Lung	**$TP53_{mut}$:** HBEC-3KT53 **$KRAS_{mut}$:** HBEC-3KTR **$TP53_{mut}$ & $KRAS_{mut}$:** HBEC-3KTR53	HBEC-3KT parental cell line Pure medium	CCM	K-SFM, 50ug/l bovine pituitary extract, 5 ng/l EGF	Cell culture dish (100 mm)	Davies *et al*., 2014
Nanosensors (MWNTs) SPME-GC-MS	Gastric	MGC-803	GES-1 (gastric mucosa cells), Pure medium	CFCM	RPMI 1640, serum-free	T-75	Zhang Y. *et al*., 2014
Nanosendors (metal-oxide semiconductor gas sensor) SPME-GC-MS	Breast	MDA-231, MCF-7, SKBR3, BT-474, ZR75-1	MCF-10A, Pure medium	CFCM	**MCF-10A:** DMEM/F12, 5% FBS, insulin, hydrocortisone, growth factor, pen/strep **Cancer cells:** DMEM (high glucose), 10% FBS, pen/strep	T-25	Lavra *et al*., 2015
P&T- GC-MS Nanosensors (GNs; SWNTs)	Liver	MHCC97-H, MHCC97-L;, HepG2, SMMC-7721, BEL-7402	L-02	CCM	DMEM, 10% FBS	T-25	Amal *et al*., 2012

Table 1.2 Cont'd

Analytical technique used	Cancer type	Cell lines studied	Control	Type of matrix	Medium used	Cell culture vessel	Reference
P&T-GC-MS	Lung	Calu-1	Pure medium	CCM	DMEM (high-glucose, red phenol-free), 10% FBS, pen/strep	Cell culture fermenter	Filipiak *et al*., 2008
P&T-GC-MS	Lung	NCI-H2087, NCI-H1666	Pure medium	CCM	RPMI 1640, red phenol free, 5% FBS, pen/strep	Cell culture fermenter	Sponring *et al*., 2009; 2010
P&T-GC-MS	Lung	A549	HBEC, hFB, Pure medium	CCM	**A549 & hFB:** DMEM (high-glucose, red phenol-free), 10% FBS, 293 mg/l L-G, pen/strep **HBEpC:** Airway Epithelial Cell Growth Medium	Cell culture fermenter	Filipiak *et al*., 2010
P&T-GC-MS	Colon	Caco-2	Pure medium	CCM	DMEM , 4.5 g glucose, L-g, sodium pyruvate, 10% FBS, 1% non-essential amino acids, pen/strep	T-75	Baranska *et al*., 2015
DNTD-GC-MS	Liver	HepG2	Pure medium	CCM	DMEM(high-glucose, red-phenol free)	Glass bottle (1 l) with Teflon plug, poly-lysine	Mochalski *et al*., 2013b
TFME-GC-MS	Cervical	HeLa	Pure medium	CCM	DMEM (high-glucose), 10% FBS, pen/strep	Culture dish (100 mm)	Nozoe *et al.*, 2015
pMC-GC-MS (p: cryogenic)	Leukemia	HL60	Pure medium	CCM	RPMI 1640, serum-free	Teflon vial	Shin *et al*., 2009
SIFT-MS	Lung	Calu-1	NL20, Pure medium	CCM	**Calu-1**: DMEM with glucose, 10% FBS **NL20:** Ham's F12, 4% FBS, pen/strep, with glucose	Culture flask (size ns)	Rutter *et al*., 2013

Table 1.2 Cont'd

Analytical technique used	Cancer type	Cell lines studied	Control	Type of matrix	Medium used	Cell culture vessel	Reference
SIFT-MS	Lung	Calu-1, SK-MEM-1	Pure medium	CCM	DMEM, 1% L-g, 10% FBS, antibiotics	Glass bottles (150 ml)	Smith D. *et al.*, 2003
SIFT-MS	Lung	Calu-1	NL20, 35FL121 Tel+, Pure medium	CCM	**Calu-1 & 35FL121:** DMEM, glucose, 10% FBS, antibiotics **NL20:** Ham's 12, 4% FBS, gluose, antibiotic	Glass bottles (150 ml)	Sulé-Suso *et al.*, 2009
SIFT-MS	Breast	MCF-7, MCF-7Adr	Drug untreated cells	Cell lysate	RPMI 1640, HEPES, 10% FBS, 2mM L-g, pen/strep	T-175	Kato *et al.,* 2000
p-SIFT-MS (p: distillation)	Breast, leukemia, cervical, prostate	MCF-7, MCF-7Adr, HeLa S3, K562, LNCaP, DU-145	Solid residue left after centrifugation	Cell lysate	RMI 1640, HEPES, 10% FBS, 2 mM L-g, pen/strep	T-175	Kato *et al.*, 2001
p-SIFT-MS (p: distillation)	Breast	MCF-7, MCF-7Adr	Solid residue left after centrifugation	Cell lysate	RMI 1640, HEPES, 10% FBS, 2 mM L-G, pen/strep	T-175	Kato *et al.*, 2002
PTR-MS	Lung	A549, EPLC	RPE, BEAS-2B, Pure medium	CCM	**RPE:** DMEM/F12, 2.5 mM L-g, 10% FBS, 0.25% sodium bicarbonate, pen/strep **A549:** DMEM (high-glucose), 10% FBS, wmM L-g, pen/strep BEAS-2B&EPLC: RPMI 1640, 2 mM L-g, 10%FBSpen/strep	T-175	Brunner *et al.*, 2010
On-line (ESI)MS	Breast cancer	T47D, SKBR-3, MDA-MB-231	HMLE, Pure medium	CFCM	DMEM	Culture dish (100 mm)	He *et al.*,2014

The analysis of cell lysate or preconcentrated supernatant of the lysed cells has only been used in a few studies solely for the determination of targeted VOCs produced by cancer cells treated with drugs (Kato *et al.*, 2000; 2001; 2002).

There are some substantial differences in terms of the extraction procedure details for the main two matrices. For example, analysis of culture media with cells usually takes place at 37ºC (physiological conditions), while analysis of media only may employ a higher temperature. Also, the efficiency of analysis of media only samples can be improved by the addition of salts or by a change of pH, while such changes are not possible when cells are present. On the other hand, the analysis of media with cells ensures that no VOCs are lost during storage. Finally, the vessel used for cell culture is of great importance as standard plastic flasks and dishes for cell culture may release contaminants disturbing the mass spectra (Schallschmidt *et al.*, 2015a; Zimmermann *et al.*, 2006) (for further discussion see Chapter 5.2).

1.4 Studies of VOCs as potential cancer biomarkers

Studies *in vivo* aiming to investigate VOCs as potential biomarkers of lung cancer are described briefly below, with an emphasis on the GC-MS analytical technique. Both canine sniffing studies for the discrimination of cancer patients, and the use of electronic devices in diagnosis are not described unless the study was performed with the use of GC-MS as well. In most of these studies statistical analysis was performed. Some of the research includes data classification into the relevant groups by applying principal component analysis (PCA) and/or factor analysis without the accuracy and precision determination of the test. Others included a measure of diagnostic accuracy of the VOC predictive model of the disease, and its ability to correctly classify subjects into the relevant alternative groups. However, the concepts and the measures of the test performance vary from study to study. Therefore, it is difficult to compare the results between studies (Zweig and Campbell, 1993). In general, studies presented the values for sensitivity and specificity and/or predictive values only, or applied differentiation between the groups by applying a receiver operating characteristic (ROC) curve. Sensitivity relates to the percentage of subjects who are correctly identified as having the disease and specificity relates to the percentage of the subjects who were correctly identified as not having the disease (Zhu *et al.*, 2010). Positive predictive value (PPV) is the ratio of

subjects having the disease to the number of patients with a positive test result, while negative predictive value (NPV) represents the proportion of patients without the disease to the number of subjects with a negative test result (Parikh *et al.*, 2008). A ROC curve is a tool for the determination of the test accuracy which represents the whole spectrum of sensitivities and specificities for each decision cut-off in the diagnostic test. Each point of the plot represents a pair of sensitivity and specificity values corresponding to a particular decision cut-off of the variables (Zweig and Campbell, 1993). The area under the ROC curve (AUC) measures how well the test distinguishes the alternative states. The diagnostic test with an AUC in the range of 0.9 - 1.0 is considered to have an excellent accuracy classification (Zhu *et al.*, 2010).

All research to date that investigated VOCs produced or consumed by cells *in vitro* in studies of different types of cancer with the use of various analytical techniques is also described below.

1.4.1 Studies of VOCs in lung cancer in vivo

GC-MS studies

Analysis of VOCs produced by humans was pioneered by Pauling *et al*. in 1971, who quantitatively determined (but did not identify) approximately 250 substances in a breath sample and about 280 compounds in a urine vapour sample using GC.

The first studies of lung cancer conducted in the 1980s identified candidate biomarkers of this disease with the use of GC-MS, showing that VOCs may have a great potential in lung cancer diagnosis (Gordon *et al.*, 1985; O'Neill *et al*., 1988; Preti *et al.*, 1988).

The first predictive model of lung cancer was created by M. Phillips *et al.* (1999a). It employed 22 discriminately chosen VOCs, mainly alkanes, methylated alkanes and derivatives of benzene, compounds which were similar to those found by O'Neill *et al.* (1988). The sensitivity rate of the test was 71.7% and the specificity rate was 66.7%. The team improved these results in their next studies by the incorporation of improved study design, statistical methodology and a greater number of subjects (Phillips M. *et al.*, 2003a; 2007b; 2008). For example, the latter study yielded a ROC curve AUC = 0.90, sensitivity of 84.5% and specificity of 81.0% (Phillips M. *et al.*, 2008). Although the candidate VOC

biomarkers were not identical, they were primarily alkanes and benzene derivatives and secondarily alcohols, esters and ketones in these studies. The team employed sorbent traps as a method of extraction. The desorbed VOCs were concentrated by two sequential cryotraps and then desorbed again directly into GC-MS (Phillips *et al.* 1997; 1999a). A drawback of these studies is the identification of VOCs by spectral library matching only.

The use of HS-SPME for the extraction of VOCs in studies of cancer was used for the first time by Deng *et al.* (2004a). This study investigated volatile biomarkers in the HS of blood samples from cancer and control patients and compared the VOCs found to the breath samples of lung cancer patients. The levels of hexanal and heptanal were found to be significantly elevated in blood samples of cancer patients, as well as in the exhaled air of cancer subjects, indicating that these compounds may be potential markers of lung cancer. The elevated levels of the two aldehydes were confirmed by this group in further studies using SPME-on-fiber derivatisation-GC-MS (Deng and Zhang, 2004; Deng *et al.*, 2004b) and SDME with droplet derivatisation (Li N. *et al.*, 2005).

Guadagni *et al.* (2011) measured the levels of hexanal and heptanal in the urine of lung cancer patients without derivatisation. The results showed differences in the median values of the two VOC concentrations in the cancer samples when compared to controls (heptanal 0.41 and 0.22 pg μl^{-1}; hexanal 1.04 and 0.28 pg μl^{1} in cancerous urine and control urine, respectively).

SPME-GC-MS was applied in a number of studies which sought to identify VOC biomarkers in the breath of people suffering from lung cancer. Good sensitivity and specificity (72.2% and 93.6%, respectively) were obtained by Poli *et al.* (2005) with a predictive model composed of 13 VOCs which classified the subjects into four groups: lung cancer patients, COPD sufferers, smokers and healthy controls. Ten of these VOCs were previously used in the predictive model of lung cancer built by M. Phillips *et al.* (1999a). The concentrations of 10 VOCs were at significantly higher levels in the samples of cancer subjects when compared to controls. It was also the case when follow-up studies were conducted 15-30 days after surgery. This would suggest that the compounds are not biomarkers of cancer but an "epiphenomenon of lung-cancer development" and are not recommended for monitoring a patient condition after surgery (Poli *et al.*, 2005; Luque de Castro and Fernández-Peralbo, 2012). The same group later used SPME-GC-MS (with PFBHA derivatisation) for targeting aldehydes (C3-C9) in the breath samples of patients

suffering from NSCLC. The concentrations of all aldehydes were increased when compared to the samples of healthy controls, and what is more, without significant influence of smoking or age. Multivariate discriminant analysis (MDA) correctly identified lung cancer patients in 90% of cases and healthy persons in 92.1% of cases (Poli *et al.*, 2010).

Also with the use of SPME-GC-MS, Song *et al.* (2010) identified two single VOCs, 1-butanol and 3-hydroxy-2-butanone, as potential biomarkers of lung cancer with high rates of sensitivity and specificity (> 93% and > 85 respectively), as they were significantly elevated in the breath samples of cancer patients. The drawback of the study is again the identification of the compounds based on spectral library matching only.

X. Chen *et al.* (2007) determined 11 VOCs that could be used to distinguish lung cancer patients from healthy controls and patients with chronic bronchitis. Some of them, such as decane, isoprene, hexanal and heptanal were reported as candidate biomarkers of lung cancer both before and after this study (Bajtarevitz *et al*., 2009; Deng and Zhang, 2004; Deng *et al.*, 2004a; Phillips *et al.*, 1999a; Poli *et al.*, 2005). Statistical analysis allowed them to correctly classify lung cancer patients in 86.2% of cases, healthy controls in 69.2% of cases and patients suffering from chronic bronchitis in 71.4% of cases. The PPV of the test was 80.6% while the NPV was 72.8%. The same study also compared the patterns of VOCs in breath samples to the VOCs emitted by primary cancer cells *in vitro* (see Chapter 1.4.10). The team employed SPME-GC-MS for their studies.

Another study utilising SPME (with on-fiber derivatisation) focused on the detection and comparison of aldehydes present in the exhaled air of lung cancer patients, smokers and healthy controls, and found four potential biomarkers that could be used to discriminate cancer patients from healthy controls and smokers. Pentanal, hexanal, octanal, and nonanal were found in significantly higher concentrations in the breath of cancer patients when compared to smoking and healthy controls. Sensitivity and specificity varied for each VOC. The study was performed on a relatively small number of patients and most of them were in the late stages of cancer. Therefore, a study examining a larger number of subjects, and using patients in earlier stages of the disease is needed to evaluate these results (Fuchs *et al.*, 2010).

Another study of the VOCs present in the exhaled breath of lung cancer patients, which was performed by the use of SPME-GC-MS, was an attempt to distinguish between

smoking and non-smoking lung cancer patients both with or without chemotherapy treatment, healthy smokers and healthy non-smokers (Gaspar *et al.*, 2009). Even though the number of subjects in each category was very small, the study showed that patterns of VOCs were different in each group, discriminating them from one another, except for smoking and non-smoking cancer patients (smoking had no effect in the patterns of VOCs in this group). Ten VOCs were used in the discrimination model, all of which were either linear or branched hydrocarbons. The peak purity and peak identification of the linear hydrocarbons was confirmed by analysis of standard solutions, while branched hydrocarbons were identified only tentatively.

A study by Bajtarevitz *et al.* (2009), which was performed using a combination of SPME-GC-MS and PTR-MS techniques, showed that they complement each other. SPME-GC-MS identifies compounds with greater certainty than PTR-MS, but the latter yields far more reliable quantitative information. Concentrations of more than 50 VOCs, analysed with the use of GC-MS, were different in the exhaled breath of lung cancer patients (however not in all samples) when compared to the healthy controls. VOCs used in three predictive models were alcohols, aldehydes, ketones, and hydrocarbons. The study showed that the sensitivity of the predictive model for cancer detection grows with the increased number of VOCs included in the set. Sets containing 4 VOCs gave a sensitivity of 52% and 21 VOCs a sensitivity of 80%. Specificity of 100% was achieved with the use of both sets.

Rudnicka *et al.* (2011) applied SPME-GC-MS for discrimination between the breath of lung cancer patients and healthy controls. Here, statistical analysis extracted six compounds that allowed separation (although not complete) of the samples into two groups (healthy and lung cancer subjects). These compounds were carbon disulfide, ethylbenzene, 2-propanol (IPA), propane, 2-propenal and styrene (Rudnicka *et al.*, 2011). Carbon disulfide and IPA were VOCs found at higher levels in the breath of patients suffering from lung cancer when compared to healthy controls in another study by this group, which also investigated VOCs emitted from lung tissue samples (Buszewski *et al*., 2012a) (see chapter 1.4.10). Ethylbenzene, IPA and 2-propenal (plus 9 other VOCs) were found at elevated levels in the breath of lung cancer patients in another study (Buszewski *et al*., 2012b). Finally, propane and IPA plus 18 other VOCs were included in the set of VOCs used to differentiate between the breath of lung cancer patients and healthy controls in a recent study by this group (Rudnicka *et al*., 2015). The study also involved the

evaluation of sensitivity and specificity obtained by sniffing dogs for the differentiation between cancerous breath and normal breath. They were 85.5% and 71.8% respectively.

In a study conducted by Hanai *et al.* (2012a), nine VOCs (mainly alcohols and ketones) were determined at significantly higher concentrations in the HS of lung cancer urine samples, when compared against the urine of healthy controls. The sensitivity and specificity rates were found to be very high (> 95%) for all of the compounds. The AUC for the PCA model was 0.955. Moreover, the group found 2-pentanone as a potential biomarker that could be used to discriminate between adenocarcinoma and squamous cell lung cancers.

Another study notes the importance of the normalisation of data obtained from the collection of VOCs, and points out that there is no generally accepted method of normalisation at present. Kischkel *et al.* (2010) analysed breath samples from lung cancer patients, healthy smokers and healthy non-smokers using SPME-GC-MS. The study yielded different statistical information depending on the type of normalisation used for the 14 VOCs showing significant differences between the groups. Some of these compounds were previously reported as candidate biomarkers of lung cancer (such as acetone and hexanal) (Phillips M. *et al.,* 1999a; Fuchs *et al.,* 2010). When physiology and variables such as age, gender, or inspired concentration of VOCs were included in the analysis, it was not possible to find any unique potential biomarker of lung cancer. The results indicated that the levels of VOCs exhaled in breath may depend on a variety of factors other than the examined disease, and these factors must be taken into account. It also underlined the problem of contaminant exogenous VOCs present in the breath samples that should be excluded from the analysis regardless of the statistical method used (Kischkel *et al.,* 2010).

An interesting study by C. Wang *et al.* (2014) investigated differences in VOCs patterns between the cancerous and healthy lung of the same patient during resection surgery (n = 18). SPME-GC-MS analysis detected significant differences between the healthy and affected lungs before and after surgery and between blood samples before and after the operation. However, some of the discriminating VOCs (straight and branched hydrocarbons) may have been products of lipid peroxidation due to ischemia-reperfusion processes, making their use as biomarkers unclear. The group proposed caprolactam and propanoic acid as potential biomarkers of lung cancer as these VOCs were found in higher

levels in the air of the affected lung when compared to the healthy lung before the operation, and no differences were found after the surgery.

An initial method employing monolithic material sorptive extraction (MMSE) as a preconcentration technique combined with GC-MS in the study of volatile biomarkers of lung cancer has been proposed recently by W. Ma *et al.* (2015). It was applied for the analysis of BTEX (benzene, toluene, ethylbenzene and xylenes) compounds in the breath of lung cancer patients and healthy controls (both n = 10). The group have not performed, however any statistical analysis on the data obtained. BTEX compounds were detected previously in lung cancer breath in other studies (Poli *et al*., 2005; Buszewski *et al.*, 2012b). The MMSE-GC-MS method showed low LODs and low precision values, therefore it has the potential to be applied in future case studies of lung cancer detection.

GC-MS and nanosensor studies

More recent studies of VOCs as potential biomarkers of cancer more often applied e-noses rather than GC-MS, or tended to use both approaches complementarily (Krilaviciute *et al.*, 2015). As mentioned before, GC-MS allows for the identification of VOCs and the determination of their relative composition, aiding studies into their fate *in vivo*. However, e-noses can be more easily introduced into clinic. Studies employing e-noses did not only show discrimination between lung cancer patients and healthy controls, but were also used for discrimination between different cancer types, in post-surgery follow-up studies, and treatment monitoring. Some examples are discussed below.

A study by Broza *et al.* (2013) investigated follow-up VOC patterns of pre-surgery and post-surgery lung cancer patients. SPME-GC-MS analysis showed that five VOCs (2-hexanone, 2-heptanone, 2-methyl-1-pentene, styrene and 2,2,4-trimethylhexane) were significantly reduced in the breath of lung cancer patients after surgery. An e-nose could differentiate between lung cancer pre-surgery and post-surgery states (83% sensitivity, 75% specificity) and between lung cancer and benign states before surgery (100% sensitivity, 80% specificity), but not between lung cancer and benign conditions after the surgery, indicating that the VOC patterns were associated with the presence of the tumour.

The analysis of breath based on nanosensors (gold nanoparticles) allowed for differentiation between not only patients with different types of cancer and healthy controls, but also between patients suffering from breast, lung, colon and prostate cancers.

In contrast, GC-MS analysis applied in the same study could distinguish between cancerous breath and healthy breath, but not between different types of cancer. On the other hand, SPME-GC-MS analysis identified six discriminant VOCs in lung cancer, six in colon cancer, six in breast cancer and four in prostate cancer breath which could be investigated further (Peng *et al*., 2010).

In a recent study by Nardi-Agmon *et al.* (2016) GC-MS and an e-nose were used for monitoring responses to treatment in patients with advanced lung cancer. GC-MS analysis of VOCs identified three compounds discriminating between the responses studied (partial response, stable disease, and progressive disease). 4-Methyldodecane, α-phellandrene and styrene were identified as potential markers of stable disease and partial response. Styrene allowed for discrimination between breath samples of partial response and stable disease from progressive disease. However, GC-MS analysis failed to discriminate between partial response and stable conditions. Nanosensors allowed for the monitoring of changes in responses to treatment with a relatively good level of accuracy (> 89%).

A study by Capuano *et al.* (2015) investigated VOC patterns in breath and in the air inside both lungs (cancerous and healthy) during bronchoscopic examinations. The main finding of this study was that the identification of lung cancer with an e-nose was not dependent on the lung from which the sample was collected, while SPME-GC-MS showed a small difference between the air of the two lungs of the same patient with a set of 20 VOCs discriminating between them (with 76% sensitivity). Nevertheless, an e-nose was able to distinguish with 90% sensitivity the presence of lung cancer when compared to non-cancer breath samples.

1.4.2 Studies of VOCs in lung cancer in vitro

There have been several studies performed to investigate the volatolomes of the lung cancer cell lines (Tab. 1.2). Some of them also investigated VOCs in normal lung cell lines. It can be concluded from these studies that there are differences in VOC production or consumption between cell lines, regardless of their cancerous or non-transformed origin. The reasons may lie in phenotypic and/or genotypic differences and require further study (Filipiak *et al.*, 2010). Nevertheless, all the studies found differences between cancerous and normal cells, which underline the importance of studies of VOC patterns at the microcellular level.

Filipiak *et al.* (2010) presented an overview of the VOCs produced or consumed by three lung adenocarcinoma cell lines (A549, NCI-H2087, NCI-H1666), one squamous carcinoma cell line (Calu-1), as well as two control cell lines: human bronchial epithelial cells (HBECs), and human fibroblasts derived from dermis (hFBs). In general, more metabolites were released in significantly higher concentrations by normal cells than by cancer cells, such as hydrocarbons, methylated hydrocarbons and alcohols, indicating possible tumour suppression of some metabolic pathways. Also, normal cells were shown to degrade only aldehydes (except an ester: n-butyl acetate and a ketone: 3-penten-2-one for hFBs), while tumour cells also consumed nitrogen-containing compounds (pyrrole by A549 cells and acetonitrile (ACN) by Calu-1 cells), a ketone and ethers (2-butanone, methyl *tert*-butyl ether, and ethyl *tert*-butyl ether, by Calu-1 cells). Only a few VOCs were found to be unique to cancer cells and not determined in the HS of the control cell culture medium. For example, 4-mehyloctane was observed to be produced exclusively by Calu-1 or ethanol (EtOH) by A549 cells.

The study of Brunner *et al.* (2010) clearly distinguished two lung cancer cell lines (A549 and squamous lung carcinoma EPLC) from healthy control cell lines (retinal pigment epithelium RPE and BEAS-2B) by the means of PTR-MS. The group used linear discriminant analysis with the use of 42 selected VOCs for this analysis. Two experimental set-ups, on-line (continuous drawing of the gas from the cell culture flask into the PTR-MS) and off-line (accumulation of the metabolites in the container before analysis) were used. These two set-ups showed the cancer cell lines to significantly degrade acetaldehyde in comparison to control cells. Other results of the study of Brunner *et al.* (2010) was consumption of butanal exclusively by the A549 cell line and degradation of other aldehydes (pentanal, hexanal and heptanal) in different degrees by the four cell lines. The drawback of this study was a tentative identification of the compounds.

Degradation of acetaldehyde by lung cancer cells is in agreement with the study of Filipiak *et al.* (2010) and Sponring *et al.,* (2009). However, another group that investigated the emission of acetaldehyde by two squamous cell carcinoma cell lines (Calu-1 and SK-MES) by the use of different analytical means (SIFT-MS) obtained contradictory results. They found that acetaldehyde was produced by both the cell lines in proportion to the number of cells in the culture medium (Smith D. *et al.,* 2003). The group repeated the study, which also included quantification of the VOCs released by normal lung cell lines, NL20 and 35FL121Tel+ (telomerase positive lung fibroblast cells). The

production of acetaldehyde by the Calu-1 cells was confirmed. The NL20 cell line was shown to generate the compound as well, while fibroblasts consumed it (Sulé-Suso *et al.*, 2009). The researchers repeated the study once again, with Calu-1 and NL20 cell lines, this time cultured in 3D models which mimic more accurately *in vivo* growth of cells in the organism than 2D standard models. Interestingly, the study showed that both cell lines grown in 3D models released more acetaldehyde than the cell lines grown in monolayer. And what is more, in some cases (depending on the cell concentrations) Calu-1 produced more than 3 fold higher amounts of the VOC than the non-transformed cells (Rutter *et al.*, 2013). Therefore, the use of acetaldehyde as a potential lung cancer biomarker needs further evaluation. The study underlines the problem of standard cell culture conditions, which may have a great impact on the metabolic behaviour of the cells, thereby losing accuracy when looking for biomarkers. The cell volatolome may also change with different experimental design as the above studies showed.

A unique approach to studying the VOC signatures of lung cancer was taken in the study by Hanai *et al.* (2012b), where metabolites in the HS of culture medium of the A549 cell line were compared to VOCs in the HS of the urine of mice implanted with these cells. They also recorded the VOCs emitted/consumed by this cell line after three different periods of incubation (one, two and three weeks). Several compounds (mainly ketones and alcohols) were determined at significantly altered concentrations in the HS of the A549 culture medium when compared to the culture medium of two non-transformed lung cell lines (OUS-11 and WI-38VA13). The length of the incubation period had an influence on the composition of VOCs in the A549 culture medium. A comparison of the VOC profiles determined in the HS of the urine of transformed mice to the urine of normal mice also showed several metabolites with different patterns. Finally, comparison of the VOCs present in the HS of the A549 cell medium to the urine of implanted mice showed seven VOCs at higher concentrations in both samples: acetophenone, 2-butanone, dimethyl succinate, 2-hexanone, 2-methylpyrazine, 2-pentanone and phenol.

SPME-GC-MS was used to analyse VOCs emitted by seven lung cancer cell lines and develop an array of nanosensors for distinguishing NSCLC patients from healthy subjects. There were 15 metabolites identified in the HS of all cancer cell lines but not in control medium (saturated and unsaturated hydrocarbons, oxygen compounds and benzene derivatives). The e-nose was composed of 18 chemiresistors (gold nanoparticles), which were molecularly modified to sense and recognise the VOCs identified previously in

cancer cell medium, thereby recognising patterns of cancer biomarkers. Multidimensional PCA of HS of cancerous cells and pure medium, as well as of breath samples of lung cancer patients and healthy controls tested by the sensors, showed 100% separation (Barash *et al.,* 2009). The same group later conducted a similar study, in which they analysed VOC patterns in the HS of 18 lung cancer cell lines and a control cell line. A SPME-GC-MS analysis showed only patterns of decanal to be significantly different between lung cancer and control cells but it allowed for a 100% separation between the two groups. Decanal was consumed by the cancer cells. Three VOCs (acetophenone; 1,3-bis(1,1-dimethylethyl)-benzene and decanal) were identified as main contributors to the separation between the NSCLC and SCLC (100% sensitivity, 75% specificity). VOCs such as 2-ethylhexanol, 1,3-dimethylbenzene, and 1,3-bis(1,1-dimethylethyl)-benzene were found at higher levels in adenocarcinoma cells when compared to squamous carcinoma cells (100% sensitivity, 67% specificity). Analysis with the use of an e-nose designed on the basis of SPME results also allowed for the discrimination not only between the normal cell line and lung cancer cell lines, but also between the NSCLC and SCLC cell lines, as well as between the adenocarcinoma and squamous cell carcinoma cells in the group of NSCLC cells with very good sensitivities (> 86%) and good specificities (> 75%) (Barash *et al.,* 2012). Both studies applied a unique method of extracting VOCs from the HS of cultured cells with Ultra II SKC badges (with either Chromosorb 116 or Tenax TA® sorbents). Then the HS of the sorbents was sampled by SPME fiber in the TD device.

A study by Pyo *et al.* (2008) was performed in order to detect potential VOC biomarkers of Cisplatin-induced apoptosis and necrosis in the A549 cell line. Three compounds (nonanal; 1,3-bis(1,1-dimethylethyl)-benzene and 2,6-bis(1,1-dimethylethyl)-2,5-cyclohexadiene-1,4-dione) were found at higher concentrations in the medium with cells treated with Cisplatin, in comparison to medium with control cells, and therefore were proposed as markers of apoptosis.

Schallschmidt *et al*. (2015a) investigated VOC patterns in three lung adenocarcinoma cell lines (A549, Lu7466 and Lu7387) with the use of SPME-GC-MS, using three different matrices: HS of the medium cultured with cells (*in situ* sampling), HS of the collected cell culture medium and air collected from the flask. They found differences in concentration trends for the same VOC between the three sampling methods. For example, hexanal and 2,4-dimethyl-1-heptene were detected only with the use of the collected medium, while 3-

methylnonane was detected only *in situ*. All analysed cell lines produced 1-propanol and consumed aldehydes (2-methyl-2-propenal, butanal, pentanal and 2-methyl-2-butenal). The group in another study (Schallschmidt *et al.*, 2015b) investigated the ability of sniffing dogs and honey bees to differentiate between gas samples from the HS of medium cultured with cells and pure medium controls. Neither bees nor dogs could discriminate between the samples. The reason behind this may be the very slight differences in VOC concentrations that differentiate these samples which cannot be picked up by the animals. Similarly, some studies showed that e-noses also had problems with differentiation where GC-MS analysis has shown differences in VOC patterns (Capuano *et al.*, 2015; Peng *et al*., 2010).

Finally, the association of VOC patterns with specific mutations of lung cancer were also analysed *in vitro*. A study by Peled *et al.* (2013) investigated three, usually mutually exclusive, lung cancer mutations: EGFR (epidermal growth factor receptor), KRAS (Kirsten rat sarcoma viral oncogene homolog) and EML4-ALK (echinoderm microtubule-associated protein-like gene fused to the anaplastic lymphoma kinase gene) as well as wild type cell lines. Five VOCs were found to be altered due to the presence of specific oncogenes. Triethylamine was depleted in EGFR mutated cells and wild type cells when compared to the pure medium controls. Benzaldehyde was depleted only in cells carrying KRAS mutations. Decanal was depleted in the EGFR and EML4-ALK cells. Styrene was found at increased levels in the EGFR mutated cells while toluene exclusively in the EML4-ALK fusion cell line. The study also employed an e-nose for discrimination between the cell lines. Very good sensitivity and specificity were obtained for the differentiation of mutations. A recent study by the same group (Davies *et al*., 2015) investigated two mutations in a genetically manipulated HBEC cell line. The derived cell lines were carrying either the KRAS or TP53 (tumour protein p53) knockdown mutation, or both. These gene mutations were found to be the most frequent in lung cancer (TP53 in NSCLC and SCLC, and KRAS in adenocarcinoma and squamous cell carcinoma) in the recent genomic-based human lung cancer classification (CLCGP and NGM, 2013). The levels of benzaldehyde, 2-methylpropene, tridecane and 1,2,3-trimethylbenzene were found to be different between the mutations. ROC-AUC on the set of 20 VOCs resulted in good separation between the cell lines (0.87-1.0, sensitivities 70-100% and specificities 83-100%). The nanosensor analysis also provided a good AUC of 0.87 when comparing all three mutated cells and the parental cell line (sensitivity 70%, specificity 92%).

1.4.3 Studies of VOCs in breast cancer in vitro

First *in vitro* studies of VOCs produced by breast cancer cell lines (MCF-7) were related to the determination of concentrations of formaldehyde (Kato *et al.,* 2000; 2001) and acrolein (Kato *et al.,* 2002) in order to test drug cytotoxicity and resistance mechanisms. While elevated levels of formaldehyde were observed in Doxorubicin sensitive cells compared to resistant cells, there was no change in the concentration of acrolein. In these studies, preconcentration (sorbent traps) and SIFT-MS were used for the examination of drug-treated and untreated breast cancer cell cultures. A study from 2001 (Kato *et al.,* 2001) also included cell lines of different types of cancer such as leukaemia (K562), cervical cancer (HeLa53) and prostate cancer (LNCaP and DU-145). The first two cell lines were shown to have elevated concentrations of intracellular formaldehyde while the prostate cancer cell lines showed the presence of formaldehyde at the levels of reference water.

Untargeted *in vitro* VOC pattern analysis in breast cancer has not been undertaken until recently. He *et al.* (2014) with the use of on-line electrospray ionisation (ESI)-MS studied three breast cancer cell lines (T47D, SKBR-3, MDA-MB-231) and the non-transformed human mammary epithelial cell line (HMLE). They found seven fatty acids produced and five other VOCs consumed by cancer cells when compared to normal cells. PCA allowed for a clear distinction between the three cancer cell lines and the non-transformed cell line (90% correct classification).

Lavra *et al.* (2015) investigated five breast cancer cell lines (MDA-231, MCF-7, SKBR3, BT-474, ZR75-1) and one normal cell line (MCF-10a) with the use of SPME-GC-MS, in order to find differences in VOC patterns associated with the rate of proliferation and the expression of some breast prognostic factors: estrogen receptor (ER), progesterone receptor (PR) and human epithelial growth factor receptor 2. The same samples were also analysed with nanosensors. Eight VOCs were found to be increased in breast cancer cell lines when compared to MCF-10a cells. VOCs such as 2,4-dimetyl-1-heptene and cyclohexanol were reported in other *in vitro* studies of cancer (Filipiak *et al.,* 2010; Huang Y. *et al*., 2016; Schallschmidt *et al*., 2015a; Sponring *et al*., 2009). Moreover, the group observed differences in VOC signatures between the cells with a high and those with a low replicating rate, and between the cell lines expressing the receptors and the cell lines that did not express them. Nanosensors also allowed for the discrimination between these

four variables. This study is a first step in aiding decisions about the best treatment for different breast cancer types with the use of VOCs as potential biomarkers.

Recently Huang Y. *et al.* (2016) analysed two breast cancer cell lines (MCF-7 and MDA-MB-231) and a normal mammary cell line (CCD-1095Sk) employing SPME-GC-MS. Four VOCs were found to discriminate between the breast cancer cells and the normal cells. 2-Ethylhexanol was found at significantly higher levels and 2,4-dimethyl-benzaldehyde, cyclohexanol and p-xylene at significantly lower levels in breast cancer samples when compared to the non-transformed cell samples. In addition, the differences were significant for the three metabolised VOCs between ER and PR positive MCF-7 cells, when compared to ER and PR negative MDA-MB-231 cells.

1.4.4 Studies of VOCs in cervical cancer in vitro

The only study to date investigating VOC patterns in cervical cancer cells was performed by Nozoe *et al*. (2015). The extraction of VOCs from the cell culture medium cultured with HeLa cells was accomplished via DI of a homemade TFME device in the cell culture medium. After the required period of incubation, TFME was analysed by TD-GC-MS. Four VOCs were observed to be produced (2-undecanone, 2-tridecanone, 2-pentadecanone and 2-octyl benzoate) and 18 VOCs to be consumed by the cells, when compared to the pure medium control. The metabolised compounds were aldehydes, alcohols and carboxylic acids.

1.4.5 Studies of VOCs in colon cancer in vitro

The first *in vitro* study conducted to investigate VOC biomarkers in colon cancer was undertaken by Zimmermann *et al.* (2007) who applied SPME-GC-MS. The researchers profiled volatolomes of two colon cancer cell lines in different stage of the disease (SW480 and SW1116), and compared them to the VOCs produced by the normal colon epithelial cell line (NCM460), and also to the VOCs present in the HS of the pure medium. 2-Pentadecanone was found to be produced only by the SW480 cell line, 3-methylbutan-1-ol and 1-heptanol exclusively by the SW1116 cell line and 1-octanol by both cancer cell lines.

The VOC patterns associated with oxidative stress in adenocarcinoma colorectal Caco-2 cells induced by 1h exposure to medium containing 100 μM H_2O_2 were investigated by Baranska *et al.* (2015). The levels of 15 VOCs were observed to be altered in the HS of Caco-2 samples when compared to normal medium controls, and the levels of 10 VOCs were altered in the HS of the H_2O_2-treated cells when compared to the H_2O_2-treated medium controls. Not all the altered compounds were identified. The first comparison showed that phenol was consumed while 1-nonene, 2-nonene and heptane were produced by the analysed cells. The second analysis showed that compounds such as 3-dimethoxybenzyl alcohol, 4-methyl-2-pentene and undecanal were produced by the cells and 1-butene (or 2-butene), 2-octene and octadecane isomer $C_{18}H_{38}$ were consumed by the cells, suggesting that these VOCs could be potential biomarkers of oxidative stress. P&T-GC-MS was an analytical technique used in this study.

1.4.6 Studies of VOCs in gastric cancer in vitro

The only study to date investigating VOC fingerprints of gastric cancer cells was performed by Y. Zhang *et al*. (2014) with the use of SPME-GC-MS. The levels of eight VOCs were found to be different between gastric cancer MGC-803 cells and normal gastric GES-1 cells. Butanone and 3-octanone were found to be present exclusively in the gastric cell samples, while formic acid propyl ester, 1,4-butanediol and 2,6,11-trimethyldodecane were observed solely in the GES-1 samples. 4-Buoxy-1-butanol, 4-isoproxybutanol and nonanol were observed at higher levels in the normal cells when compared to cancer cells.

1.4.7 Studies of VOCs in leukaemia in vitro

To investigate whether human immune cells can produce detectable VOCs *in vitro*, an HL60, promyelocytic human leukaemia cell line was studied. The HS of cells cultured in a bioreactor and incubated for three different durations was cryogenically concentrated and analysed by MCC-GC-MS. There were significantly increased levels of acetaldehyde and hexanal. The amounts of detected compounds increased with an increase of culture time (Shin *et al.,* 2009).

1.4.8 Studies of VOCs in liver cancer in vitro

A study by Castaneda *et al.* (2007) investigated volatile metabolites of HepG2 hepatocyte cancer cells treated with low concentrations of EtOH (1 mM). The GC-MS analysis showed that 2-undecanone was significantly produced by EtOH exposed cells when compared to untreated cells. The study also observed an increased apoptotic rate in EtOH exposed HepG2 cells.

Amal *et al.* (2012) investigated VOC patterns in six liver cancer cell lines and one normal liver cell line, employing sorbent tubes with TD-GC-MS complementarily with nanosensors. The group also investigated differences in VOC patterns between liver cancer cells with a high metastatic potential (HCC-HMP) and a low metastatic potential (HCC-LMP). They found significant differences in the levels of methane-sulfonyl chloride when all cancer cell lines were compared to normal cells. The levels of 2,3-di-hydro-benzofuran and acetic acid were found to be higher and levels of EtOH lower in the HCC-HMP when compared to normal cells. 2,3-Dihydro-benzofuran was significantly lower in HCC-LMP cells when compared to normal cells and HCC-HMP cells. Nanosensors were able to differentiate between the groups (liver cancer cell lines versus normal cells; HCC-LMP versus normal cells; and HCC-HMP versus normal cells) with very good sensitivities (> 95%) and specificities (100%). The differentiation between the HCC-HMP and HCC-LMP cells yielded good sensitivity (83%) and excellent specificity (100%).

Another study investigating potential VOC biomarkers of liver cancer was performed by Mochalski *et al*. (2013b), who investigated the VOC profile of HepG2 cells. The metabolites were extracted with the use of HS-NTD and analysed applying GC-MS (with the majority of compounds identified against standards). Nine compounds were found to be consumed and 12 compounds to be released by the hepatocytes. The first group consisted of aldehydes (benzaldehyde, 2-ethylacrolein, n-hexanal, 2-methylpropanal, 2-methyl-2-propenal, 3-methylbutanal), isoprene, n-propyl proprionate, and EtAc. The VOCs produced by the cells comprised mainly of ketones (2-pentanone, 2-heptanone, 3-heptanone, 3-octanone and 2-nonanone) and sulfur-containing compounds (methyl sulfide, dimethyl sulfide, 3-methylthiophene, 2-methyl-1-(methylthio)-propane and 2-methyl-5(methylthio)furan). While ketones were previously reported to be emitted by cancer cells, this is the first report of sulfur compounds released by cancer cells. The remaining two VOCs detected as metabolites emitted by the cells were heptane and propyl acetate. As the

study was one of the first towards investigation of the volatolomes of liver cancer cells, the obvious limitation of the study is the lack of non-transformed cells control.

1.4.9 Studies of VOCs in skin cancer in vitro

An *in vitro* study on skin cancer cell lines was performed by Kwak *et al.* (2013). They investigated the profiles of VOCs detected in the HS of three types of human melanoma cells: RGP (radial growth phase cells: M35, WM3211 and Sbcl2), VGP (vertical growth phase cells: WM115 and WM983A) and Mm (metastatic melanoma cells: WM983B and WM1158) and compared them to the VOC patterns of normal melanocytes (neonatal foreskin melanocytes: FOM136 and FOM191). They found differences in VOC production between melanoma cells and control cells, as well as between the three types of cancer cells. Thirty one VOCs were chosen for quantitative comparison. Compounds found to be unique only to the cancer cells (not found in the medium controls and normal cells medium controls) and therefore candidate biomarkers of melanoma were dimethyl disulfide and dimethyl trisulfide. The remaining 29 compounds were detected in the HS of all samples but at significantly different levels. 3-Methylbutanol was found to be present in significantly higher levels in melanoma cell culture samples (RGP and Mm) when compared to FOM cells and media only. Acetone and 3-hydroxy-2-butanone were detected at higher concentrations in VGP cell cultures only, dimethylsulfone in Mm cell samples only and benzyl alcohol in RGP samples only, when compared to both types of the controls. However, the authors note that the differences in 3-hydroxy-2-butanone may exist due to the higher amounts of this compound in melanoma medium from the start. In contrast, 3-methylbutyric acid was detected at lower concentration in all melanoma cell lines when compared to FOM cell lines. Finally, benzaldehyde was found at higher levels in medium only. Interestingly, there were no differences found in the VOC patterns between the cell lines of the same type aiding the possibility of the determination of a tumour stage. In addition, the team conducted nanosensor tests on the samples gaining clear differentiation in responses for melanoma versus normal cells and among different types of skin cancer cells.

SPME-GC-MS was also used to complement gas sensor array studies on the three primary melanomas, a primary synovial sarcoma and a primary thyroid cancer cell lines. Fourteen VOCs were identified in the total ion chromatogram (TIC), however this was on the basis of spectra library matches only. No statistical analysis to compare the identified VOC

levels between the cell lines was performed. PCA analysis of the gas sensor data clustered the cell lines according to cancer type (Bartolazzi *et al.*, 2010).

1.4.10 Complementary studies in vivo, ex vivo and in vitro

Without doubt, there is a need for a simultaneous investigation of the correlation of the VOC pattern in exhaled breath (and other sample types) collected from a patient and an *in vitro* and/or *ex vivo* analysis of the VOCs produced by the cancer cells or emitted from the cancer tissues (ideally of the same patient). This approach eliminates analytical technique and, in the case of the samples coming from the same patient, factors such as gender, age and inter-individual variation as the sources of possible differences in VOC patterns between *in vivo*, *in vitro* and *ex vivo* samples. Some studies already have been conducted specifically in order to simultaneously compare VOCs produced by cancer cells *in vitro* and *ex vivo* to the ones found in breath from the patient. They are described below.

X. Chen *et al.* (2007) investigated VOCs in the HS of culture media of four types of primary lung cancer cells (squamous cell carcinoma, adenocarcinoma, bronchiole-alveolar carcinoma, and NSCLC) and four control cell lines (HBEC, rat tastebud cells, osteogenic cells, and lipocytes). This study aimed also to compare VOCs produced by four types of primary lung cancer cells to VOCs found in cancer breath samples. Eleven VOCs were found in breath samples and chosen for PCA in order to discriminate cancer patients from healthy controls, and two compounds were shared with lung cancer cells excised from the patients (namely isoprene and undecane). These VOCs were used in the differentiation models for lung cancer detection in other studies (Philips *et al*. 1999a; Buszewski *et al*. 2012a). Comparison between the chromatograms of cancer cells, culture medium controls and non-cancer cell lines revealed four VOCs unique to the cancer cells: isoprene, undecane and two other unidentified VOCs. Interestingly, X. Chen *et al*. (2007) showed that cancer may be detected at a microcellular level. Microscopic examination of the apparently normal tissue they wanted to use as a control did not show signs of cancer. However, its VOC composition was nearly the same as that of lung cancer cells (it differed only in concentration). After two weeks cancer cells could be observed under the microscope in this tissue. Moreover, each cancer cell line studied had unique VOCs (not identified, however), suggesting that different types of lung cancer may be distinguished at the VOC level.

Another study compared volatile metabolites determined in a culture medium of lung cancer cell line A549 to the VOC composition in the HS of urine of mice implanted with these cells. There were seven VOCs found at significantly higher levels in both sample types when compared to normal cancer cell lines (acetophenone, 2-butanone, dimethyl succinate, 2-pentanone, phenol, 2-methylpyrazine, 2-hexanone, 2-butanone and acetophenone) (Hanai *et al*., 2012b).

The study performed by Buszewski *et al.* (2012a) involved quantitative VOC measurement in the HS of healthy and lung cancer tissues and comparison of these results to the ones obtained from the breath samples of the healthy individuals and lung cancer patients. Twenty seven VOCs were detected in the air above cancerous tissues, cutting down the number of potential biomarkers that need to be considered when breath samples were analysed. Twenty two out of these 27 VOCs (mainly alcohols, aldehydes, ketones, aromatic and aliphatic hydrocarbons) were found in the breath samples, just as in the HS of lung tissues. Quantitative analysis of VOCs emitted by lung cancer tissues showed higher levels of EtOH, acetone, ACN, 1-propanol, IPA, carbon disulfide, dimethyl sulfide, 2-butanone and 2-pentanone when compared to control lung tissues. The same compounds were detected in increased concentrations in the breath samples of patients suffering from lung cancer when compared to healthy controls. Using discriminant analysis for all the compounds found in the tissue and breath samples, the researchers discriminated three groups: lung cancer patients, lung cancer tissues and healthy tissues. Here benzaldehyde, 2-butanol and pyridine had the highest discrimination power. With the use of factor analysis they managed to distinguish breath samples of lung cancer patients from tissue samples, but the analysis did not allow discrimination between cancer and healthy tissues. Here 2-butanol, pyridine but also carbon disulfide, 2-methylpenatne, 4-methyloctane and pentane appeared to be important VOCs that could be used to discriminate between the samples. Some of them were detected in the HS of cancer cells in previous studies (Barash *et al*.,2009; Filipiak *et al*., 2008, 2010; Pyo *et al*., 2008; Sponring *et al.*, 2009).

The exhaled breath of lung cancer patients was compared not only to the breath of healthy controls, but also to the compounds detected in the HS of lung tissues (cancerous and healthy), again in the recent study by Filipiak *et al.* (2014). They detected 39 VOCs in both type of samples, tissue specimens and exhaled breath (with different occurrence ranging 8 - 100%). Over half of the detected compounds were previously reported in the HS of cancer cells *in vitro* in different studies (Filipiak *et al.*, 2008, 2010; Sponring *et al*.,

2009, 2010). Although approximately half of the VOCs in the breath samples had negative alveolar gradient (alveolar gradient: abundance in breath minus abundance in the air), suggesting their exogenous origin, these findings show common VOCs in all three sample types. Out of 39 detected, they found 30 VOCs at higher concentrations in cancerous lung tissue, when compared to the healthy tissue controls. Six were elevated at the chosen level of significance: EtOH, pyridine, 4-methylheptane, acetaldehyde, n-octane in the HS of lung cancer tissues, n-hexanone in the HS of healthy tissues. EtOH and octane were also found at significantly higher levels in the breath of lung cancer patients. What is more, these compounds were previously detected in the HS of lung cancer cells *in vitro* (Filipiak *et al*., 2010). Acetaldehyde and 4-methylheptane were also found in the HS of cultured cancer cells. Other VOCs found in higher levels in the cancerous lung tissue (but not at significant levels) such as 2-methyl-1-pentene, 4-methyloctane, 2,4-dimethylheptane, hexane and acetic acid were also previously detected in the HS of different cancer cell lines (Filipiak *et al*., 2010; Sponring *et al*., 2009).

J. Yu *et al.*, (2009) determined five VOCs in their predictive model of lung cancer detection (decane, eicosane, heneicosane, 2-nonadecanone and 5-methylundecane) by applying SPME-GC-MS with relatively good levels of sensitivity and specificity (76.7% and 96.7% respectively). They simultaneously determined the VOCs produced by three lung cancer cell lines (A549, SK-MEM-1 and NCIH 446) and a normal lung BEAS-2B cell line. They found one distinctive VOC (2-tridecanone) in the HS of three cancer cell lines, which was not found in the control cell line. Another study by the same group investigating the same cell lines observed two VOCs (2-pentadecanone and nonadecane) in all three cancer cell lines, and one VOC (eicosane) in two cancer cell lines (A549 and NCI-H446), which were not found in the control cell line. Moreover, these three compounds were also found in the HS of lung cancer tissue samples examined in the same study. In addition, the group also analysed breath samples from lung cancer patients and healthy controls in the study. The discriminant analysis yielded very good sensitivity and specificity with a 23 VOC set (96.5% and 97.5% respectively). 2-Pentadecanone and nonadecanone were also included in this discriminating set (Wang Y. *et al*., 2012).

1.5 Rationale, hypothesis and aims of the research

1.5.1 Rationale

To aid the early detection of lung cancer which may help in fighting the disease, the discovery of biomarkers is required. The analysis of VOCs as potential biomarkers of cancer appears to be a very promising approach as it is fast, non-invasive and the cost of sample collection and assay is potentially low. Review of the current literature clearly shows that there are differences in VOC patterns between the breath of lung cancer patients and healthy individuals. Such differences were also visible in the body biofluids of healthy and diseased people. However, despite many efforts, none of the proposed VOC biomarker sets have yet been established for clinical use. A good biomarker of disease relates to the biochemical or molecular processes underlying the disease. However, there is an absence of a well-understood biochemical foundation for most of the VOCs observed in exhaled breath and body biofluids. VOCs detected in these matrices can be biochemically altered before excretion and/or not be related to the disease because of many other confounders. *In vitro* investigations of the VOCs released and consumed by cell cultures could give valuable information about the biochemical origin and physiological role of VOCs. *In vitro* studies of VOCs as potential biomarkers of cancer explicitly show different VOC fingerprints between numerous cancer and non-transformed cell lines. This suggests that potential cancer-specific biomarkers exist. Clearly, more research regarding the VOC patterns generated by many cell lines and primary tumour samples is needed in order to profile as many cells as possible, so that an attempt can be made to firstly identify the biochemical background of the VOCs and secondly, to find the common VOCs for particular types of cancer and then relate these to the VOCs found in breath and biofluids.

1.5.2 Hypothesis

It is proposed that due to pathological processes occurring as a consequence of lung carcinoma, lung cancer cells cultured *in vitro* can generate new VOCs that non-cancer cells do not produce, and/or can alter the levels of VOCs that are produced by non-cancer cells. These new VOCs, or VOCs that are produced in significantly higher or lower levels

than normal, may therefore serve as potential biomarkers for the assessment or detection of the disease.

1.5.3 Aims of the research

• To develop an MMSE-GC-MS method for the capture and semi-quantification of VOCs present in the HS of the cell culture medium.

• To detect and identify VOCs produced or consumed by the adenocarcinoma human alveolar epithelial A549 cell line and the normal human lung fibroblasts (NHLFs) cell line control.

• To develop a TD-GC-MS method with the use of an EasyVOC^{TM} pump as a sampling tool for the capture and semi-quantification of VOCs present in the HS of the cell culture medium.

• To detect and identify VOCs produced or consumed by the A549 cell line and the normal human bronchial epithelial BEAS-2B cell line control.

• To perform, interpret and evaluate statistical analysis of the data obtained in the MonoTrap (MT) and thermal desorption (TD) experiments.

• To establish the use of the VOCs detected at altered levels in the HS of cell samples in comparison to the controls as potential biomarkers of lung cancer.

• To propose possible metabolic pathways for the production and/or metabolism of the VOCs detected at altered levels in the MT and TD experiments.

• To compare the techniques of MMSE-GC-MS and TD-GC-MS for the application of VOC collection from the HS of the cell culture medium.

• To examine the inconsistency in the VOC patterns between the MT, TD and other *in vitro* studies.

• To examine the inconsistency in the VOC patterns between the MT, TD, other *in vitro* studies and *in vivo* studies.

Chapter 2

Materials and Methods

2.1 Materials and chemicals

2.1.1 Chemicals

Acetophenone*; dichloromethane (DCM); EtOH*; tetrahydrofuran; toluene were obtained from Fisher Scientific (UK). ACN*; alkane standard solution C_8-C_{20} in hexane*; acetone; benzaldehyde*; benzene; cyclohexanol*; decanal*; 2,6-di-*tert*-butyl-1,4-benzoquinone; dimethoxyethane (DME)*; EtAc*; IPA*; 2-nitrophenol; 2-pentanone; d-limonene; 2-fluorop[1]henol* (2-F); 2-ethylhexanol*; 2-methylbutanal; methanol* (MeOH); nonanal; pentane; 1-phenylehtanol* and sodium chloride (NaCl) were purchased from Sigma-Aldrich (UK). 2-Bromobenzyl alcohol* (2-B); chloroform* (CHF); geranyl acetone; hexane*; styrene* were obtained from Alfa Aesar (UK). Benzothiazole; cyclohexanone; 2,4-dimethylfuran; ethylbenzene*; hexanal; heptanal; 1-hexanol; methylcylohexane; 2-methylfuran; 3-methylpentane; octanal; 1-octanol were bought from Acros Organics. 2-Methylpentane was purchased from Lancaster Synthesis (UK) and phenol from BDH Limited (UK). All standards were used without previous purification.

2.1.2 Materials and reagents

Materials and reagents for cell culture such as Roswell Park Memorial Institute (RPMI) 1640 medium, RPMI 1640 medium with 25 mM HEPES buffering agent (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), Dulbecco's Modified Eagle Medium (DMEM) medium, L-glutamine (200 mM), fetal bovine serum (FBS), phosphate buffered saline (PBS), hemocytometer slides and trypan blue dye (4%) were obtained from LabTech (UK). 0.25 % Trypsin-EDTA (1x) was purchased from Lonza (UK). Cell culture flasks (T-75), tubes for medium storage (50 ml), dimethyl sulfoxide (DMSO), universal tubes (20 ml), cryopreservation vials, serological pipettes, 0.22 μm filters, sterile needles (23 gauge, 0.6

* Chemicals purchased at the highest purity available.

x 25 mm), sterile syringes (1 ml, 10 ml, 20 ml) and Suba-Seals® (no 53) for sealing cell culture flasks in the TD experiment were bought from Fisher Scientific (UK). The MT disks (DCC18 and DSC18), MT holders, MT stand, clean pin hole septums with sample vials (40 ml) and extraction caps with vials (20 ml) were purchased from Hichrom Ltd (GL Sciences, UK). The GC vials with inserts and 10 ml and 20 ml glass vials with polytetrafluoroethylene-lined (PTFE) screw caps were obtained from Fisher Scientific (Chromacol brand, UK). The TD Soil Vapour Intrusion (SVI™) and Air Toxic™ sorbent tubes were obtained from Perkin Elmer (UK). The Easy-VOC™ pump was purchased from Markes International (UK). The bubble flowmeter was obtained from Sigma-Aldrich (UK). The polyvinyl chloride (PVC) laboratory tubing (ID x OD 1.4 in. x 3.8 in.) was purchased from Scientific Laboratory Supplies (UK).

2.2 Cell culture

2.2.1 Cell lines studied

The human lung adenocarcinoma epithelial A549 cell line (passage 9) (86012804, Sigma Aldrich UK), the immortalised human bronchial epithelial BEAS-2B cell line (passage 10) (95102433, HPA UK) and the normal human lung fibroblasts NHLFs (passage 11) (CC-2512 Clonetics®, Lonza USA) were obtained from the Cell Bank of the Biomedical Research Centre at the University of Salford. The main characteristics of the three cell lines are presented in Table 2.1. The A549 cell line is a widely used *in vitro* model of type II alveolar epithelium cells (Foster *et al*., 1998). It is the best characterised NSCLC cell line to date in terms of VOC patterns. The semian-virus-40-transformed BEAS-2B cell line was isolated from a normal (non-cancerous) lung tissue and the cell line, when injected into mice, did not show tumorigenicity (Reddel *et al*., 1989). The cell line displays the ability to squamous differentiation in response to FBS (Zhao F. and Klimecki, 2014). Both cell lines show physical characteristics of human epithelial cells. The NHLFs are primary cells obtained from a non-diseased patient, which in contrast to the two other immortalised cell lines have a finite proliferating lifespan in cell culture, which depends on the age of the donor and number of passages (Kaji *et al*., 2009). *In vitro* they undergo transdifferentiation into myofibroblats induced by FBS (Fang *et al*., 2009).

Table 2.1 Main characteristics of the three cell lines studied: A549, normal lung fibroblasts (NHLFs) and BEAS-2B. References: [1] ATCC, 2014a; [2] Mio *et al*., 1992; [3] Costa *et al*., 2010; [4] Fang *et al.*, 2009; [5] Zhao and Klimecki, 2014

	A549	NHLF	BEAS-2B
Organism	human	human	human
Lifespan	immortalised	primary (finite lifespan)	immortalised (virus transformed)
Tissue	lung	lung	lung
Cell type	alveolar basal epithelial	fibroblast	bronchial epithelial
Morphology	epithelial-like (polygonal-shaped)	fibroblast-like (spindle-shaped)	epithelial-like (polygonal-shaped)
Culture properties	adherent	adherent	adherent
Disease	adenocarcinoma	normal	normal
Doubling time	~22 hours [1]	32 ± 6 hours (depending on a donor) [2]	~ 22 hours [3]
Differentiation	no	yes (in response to FBS they transdifferentiate into myofibroblats) [4]	yes (in response to FBS they undergo squamous terminal differentiation) [5]

Figure 2.1 presents the pictures of confluent A549 cells (A), confluent NHLF (B) cells, sub-confluent BEAS-2B cells (C) and confluent BEAS-2B cells (D). The three cell lines were grown in 75 cm^2 cell culture flasks (T-75) with a filter cap. In the MT experiment, the vent/screw caps were used to obtain a complete seal of the flask. In the TD experiments, 25.5 mm diameter Suba-Seals® (a flanged stopper with a hollow plug and a sleeve extension) were used to enable penetration of the flask with a needle. RPMI 1640 medium containing 10% FBS and 2mM L-glutamine was used for cultivation of the A549 cells and the NHFL cells. BEAS-2B cells were propagated in DMEM medium supplemented with 10% FBS and 2mM L-glutamine. For the VOC measurements in both MT and TD experiments, the cells were cultured in RPMI 1640 with 25 mM HEPES, (containing 10% FBS and 2mM L-glutamine) to maintain physiological pH, despite

changes in CO_2 (due to tightly closed caps). For sampling purposes, the sub-confluent cells were trypsynised, counted, tested for viability and then an appropriate amount of the cell suspension was transferred into the cell culture flasks. The flasks containing the cells were kept in an incubator with a humidified atmosphere containing 5% CO_2 at 37ºC. Counting and viability tests were performed with the use of hemocytometer slides and trypan blue dye (4%).

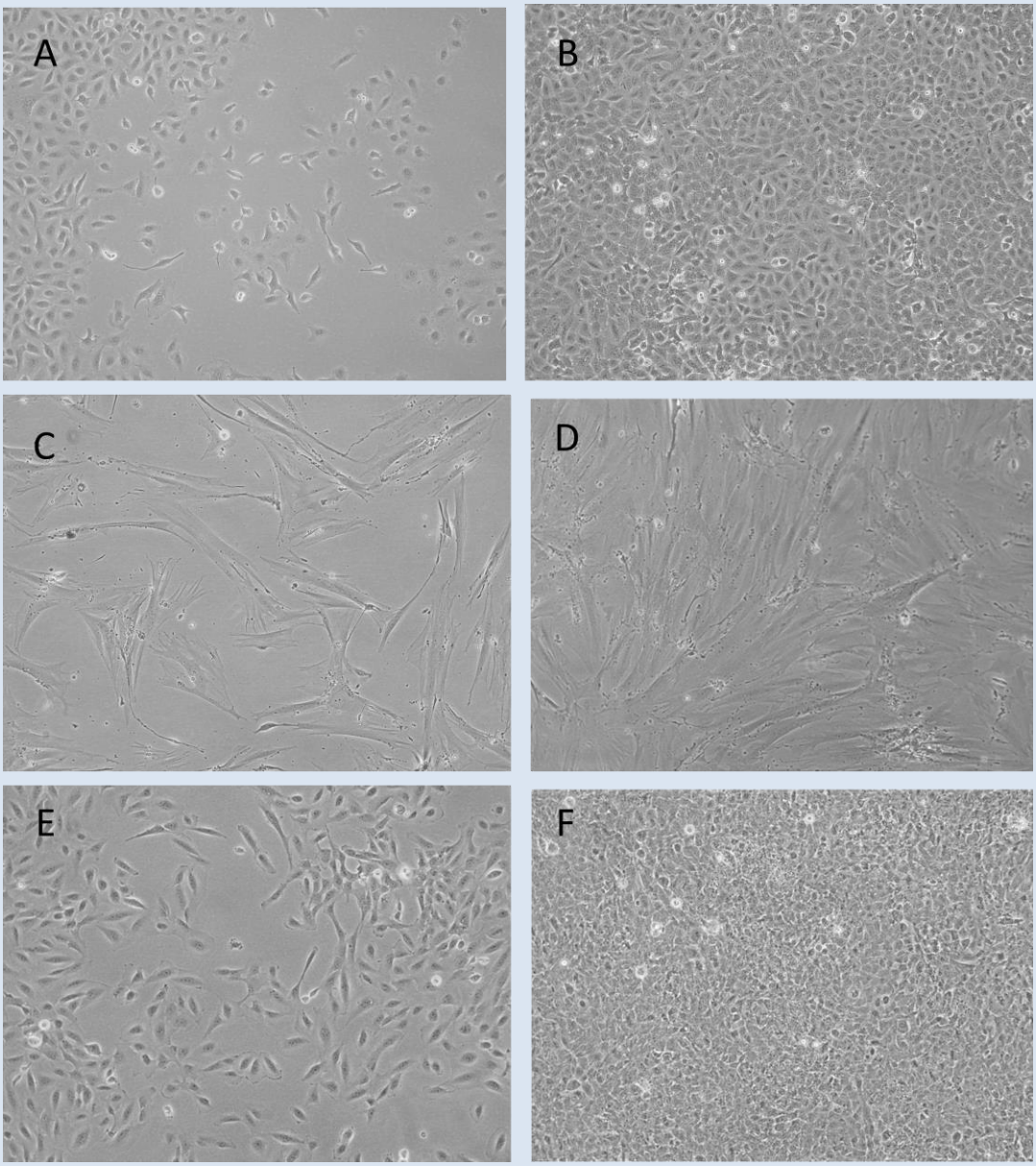

Figure 2.1 Microscopic pictures of sub-confluent (A) and confluent A549 cells (B), sub-confluent (C) and confluent NHLF cells (D), sub-confluent (E) and confluent BEAS-2B cells (F); 10 x magnification.

2.2.2 Cell seeding density

For the MT experiment, the A549 cell line was seeded at a density of 1 x 10^4 viable cells cm^{-2} and the NHLFs at 2.5 x 10^3 viable cells cm^{-2}. For the TD experiment both the A549 and BEAS-2B cell lines were seeded at 1 x 10^4 viable cells cm^{-2}. The seeding densities did not exceed recommendations for starting new cultures with these cell lines given by American Type Culture Collection (ATCC, 2014b) for A549 cells, Lonza (2010) for NHLFs and European Collection of Authenticated Cell Cultures (ECACC, no date) for BEAS-2B cells.

2.2.3 Period of incubation

For the MMSE method development, the cell-free RPMI 1640 culture medium was incubated for 7 days (30 ml of the medium per T-75 flask). After this time the medium was collected into 50 ml tubes and stored in a -80°C freezer. On the day of the analysis the medium was thawed (about 30 min in the incubator) and then prepared for sampling (see Chapter 2.3.2, Method development sample preparation).

In the MT experiment, the cells were incubated for 7, 14 (A549 cells and NHLFs) and 21 days (NHLFs only). Before sample collection, the cells were examined under an inverted microscope for possible contamination. After these periods of time had elapsed, the medium was collected, centrifuged for 5 min at 1500 rpm in order to remove dead cells, and transferred into a medium pot (150 ml). The sample and control medium were prepared for sampling directly after collection i.e. samples were not supplied to freezing (see Chapter 2.3.3, sample preparation). When ready, the vial was placed in a water bath set at the selected extraction temperature. The MT disks were exposed to the HS above the cell culture medium at the appropriate depth for the selected extraction time.

In the TD experiment, the A549 and BEAS-2B cells were incubated for both 7 and 14 days. Before sample collection, the cells were examined under a microscope for possible contamination. After these periods of incubation, 200 ml of HS air above the cell culture medium was collected with the use of an Easy-VOC™ pump (See Fig. 2.5).

2.2.4 Cell cryopreservation

For cell cryopreservation, the sub-confluent cells (~80%) were trypsinsed and pelleted by centrifugation. Next the culture medium was removed and the cells re-suspended in freezing solution (FBS with 10% of DMSO). The freezing solution with cells was transferred into cryopreservation vials (1 ml per vial), and the vials were then placed in a "Mr. Frosty" freezing container containing IPA, in order to achieve a rate of cooling very close to -1°C min^{-1} (the optimal cell freezing temperature rate). The "Mr. Frosty" container was placed in the -80ºC freezer. On the next day, the vials were transferred into a liquid nitrogen tank for long-term storage.

2.3 HS-MMSE procedure

2.3.1 The workflow of the HS-MMSE

The MT equipment and accessories are shown in Figure 2.2. They consist of MT disks, a 40 ml sampling vial with clean pinhole septum, an extract cup, an extraction vial (20 ml), an MT stand and an MT holder.

The workflow of the HS-MMSE using the MT disk is shown in Figure 2.3. The cell culture medium incubated either with or without the cells was collected and put into a pre-baked sampling vial. A pH of the collected medium was measured and adjusted in a sampling vial. An appropriate amount of salt was weighed and added into the beaker and a portion of a sample was transferred into this beaker. When the salt had dissolved, the sample was transferred into the volumetric flask. In the MT experiment the ISTD was added to the sample at this step. The MT method development was performed without the ISTD as the variable factors tested would have had an unknown impact on the ISTD adsorption onto the MT. Finally, the appropriate concentration of salt and ISTD was made by filling up the volumetric flask to the mark. The entire contents of the volumetric flask were transferred into a fresh pre-baked sampling vial, a stirring bar was added and the vial was placed in a water bath set at the required temperature and equilibrated for 1 hour.

For the MT sampling, an MT holder was placed in an MT stand. A new MT disk was put on the MT holder with the use of tweezers cleaned with acetone. Then the holder was pushed through the predrilled septum in the cap and the cap was immediately screwed onto the sampling vial containing a previously prepared and equilibrated cell culture

medium sample and the stirring bar. The vial was placed back in the water bath and the magnetic stirrer was switched on (set to 700 rpm) for the required period of sampling time.

For the solvent extraction of the VOCs from the MT the extract cap was previously fitted into the extraction vial filled with 17 ml of distilled water required for sonication. After sampling, the cap with the MT holder with the MT disk was removed and screwed onto and extraction vial. Immediately prior to this step the extract cap was filled with the required volume of the extraction solvent. The extraction vial was placed in a sonicator for 4 min in order to accelerate the extraction (Jang *et al*., 2011). After solvent extraction, the extract was transferred into a 2 ml GC glass vial with a PTFE screw cap and insert (200 µl), and was stored in a -80°C freezer until analysis.

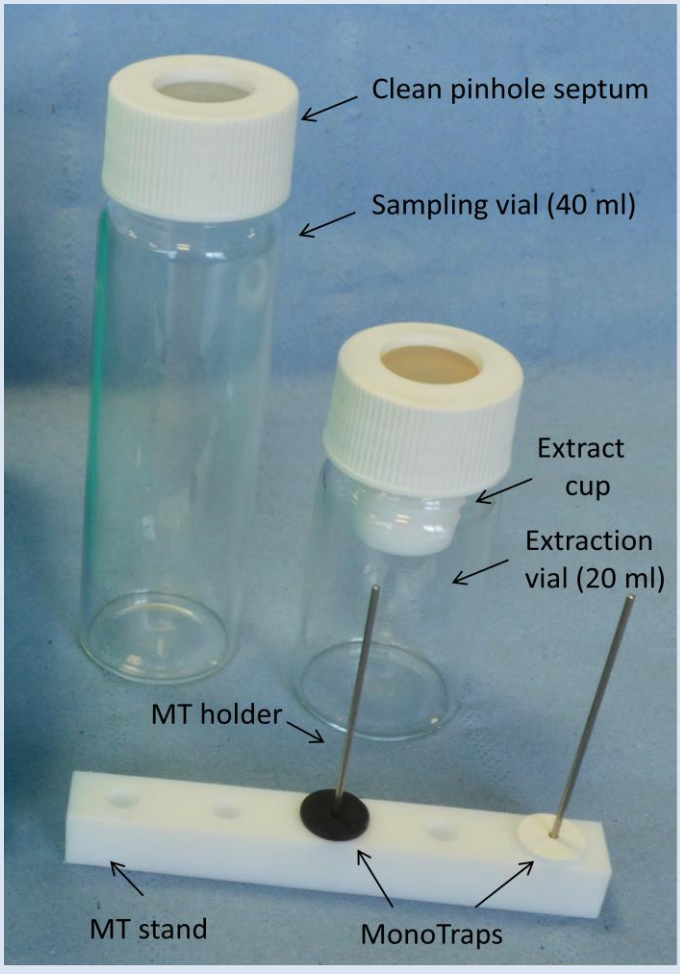

Figure 2.2 The equipment required for the HS-MMSE method with the use of the MonoTrap (MT) disk.

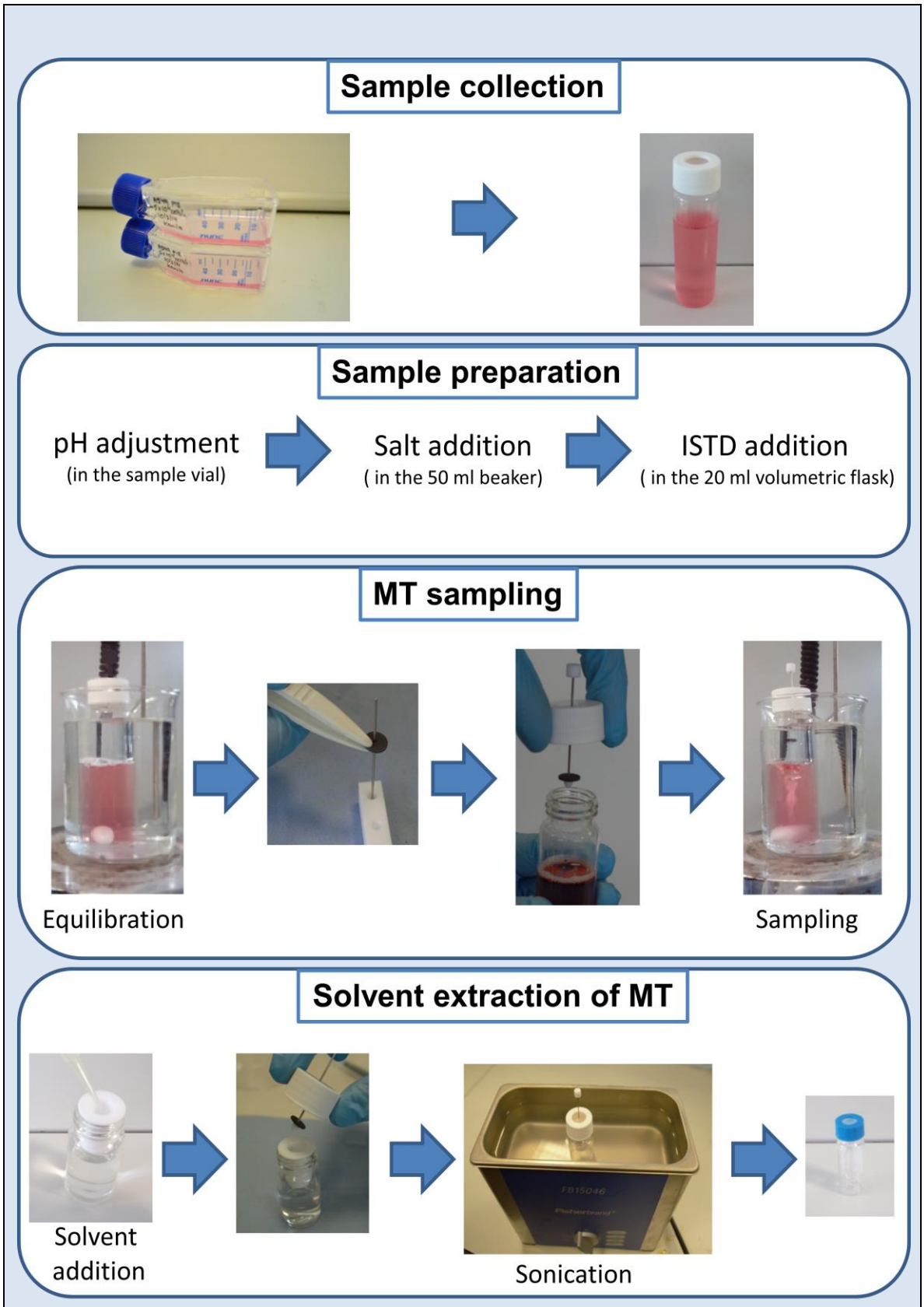

Figure 2.3 The workflow of the HS-MMSE method with the use of the MonoTrap disk. ISTD: internal standard, MT: MonoTrap.

2.3.2 Method development

Variable factors

In order to optimise the extraction of VOCs by MMSE, several factors were varied. They included: the type of the MT disk used (DSC18 or DCC18), the sampling mode (static HS, HS and floating mode), the extraction time (60 min, 90 min, 120 min and 150 min), the extraction temperature (45°C, 60°C and 75°C), the ionic strength of the sample (0%, 15% and 30% NaCl), the pH of the sample (pH 3, 7.5 and 10), the solvent volume (70 µl, 100 µl, 130 µl and 160 µl) and the solvent type (ACN, CHF_3, DCM, EtAc, EtOH, Hexane and IPA). In static HS mode an MT disk was suspended in the HS of the medium sample and left in the oven. In HS mode a stirring bar was introduced into the sample vial, an MT disk was suspended above the medium and sampling was performed with agitation in a water bath. A floating method applied the same conditions as for HS, except that the disk was floating on the surface of the medium.

Method development sample preparation

Complete RPMI 1640 culture medium was prepared as usual for cell culture purposes and was kept in the incubator at 37°C for 7 days (30 ml per T-75 flask). The medium collected after 7 days was put into the -80°C freezer. On the day of analysis the medium was thawed (about 30 min in the incubator). The required amount of NaCl was weighed in a 50 ml beaker. Next, approximately 15 ml of medium was transferred into the beaker, a stirring bar was added, and the medium was left for around 1 min in order to dissolve the salt. Next, the medium was transferred into a 25 ml volumetric flask. Then the volume was made up to the mark with medium to give the correct concentration. Finally, the entire contents of the volumetric flask were transferred into a sampling vial with a screw cap, a stirring bar was added and the vial equilibrated in a water bath at 60°C (or any other required temperature) for 60 minutes. For ionic strength experiments, to give 30% NaCl solution in 25 ml, 7.5 g of NaCl was weighed and 3.75 g for 15% NaCl solution. For the pH experiments, 26 ml of thawed medium was placed into a 40 ml vial, its pH adjusted to pH 3 or 10 (with the use of 1M HCl or 1M NaOH) and then transferred into a beaker with weighted salt. All glassware was washed with acetone and baked-out overnight at 70°C.

A few blank water samples were prepared in order to determine any contaminant compounds originating from the sampling vial. Blank samples were prepared in the same

way as cell-free medium samples. First 3.75 g of NaCl was weighed in the beaker, about 15 ml of distilled water was added and after the salt had dissolved the contents were transferred into a 25 ml volumetric flask. Finally, the volumetric flask was filled up with water to the mark.

2.3.3 The MonoTrap experiment

Sample preparation

For the purpose of VOC analysis, the sub-confluent cells were trypsinised, counted as usual (with the use of trypan blue for the viability test) and seeded in 30 ml of the complete RPMI 1640 HEPES per T-75 flask. After 60 min of equilibration of the flasks in the incubator, the caps were closed and the flasks incubated for 7 days (the time of the closing of the cap was noted).

One cell sample and one medium control were prepared together on the same day (always using the same batch of cell culture medium). Three GC-MS runs per sample and three runs per control were treated as 'experiment a'. Three experiments (a, b, c) were conducted on the three different days, so one cell collection had n = 9 samples and one control collection had n = 9 samples.

On the day of HS-MMSE sampling, the medium was collected, centrifuged for 5 min at 1500 rpm in order to remove dead cells, and transferred into a sampling vial. The pH of the cell samples and their controls was adjusted to 7.00 (± 0.03) with the use of 1M NaOH and 1M HCl. The measured pH for the cell samples and controls analysed in the MT experiment are shown in Table. 2.2. The amounts of acid and base added never exceeded 1 % of the total sample volume (20 ml) and the differences were levelled up with distilled water.

Once the salt was dissolved and 15 ml of the sample transferred into the 20 ml volumetric flask, the appropriate volumes of the internal standards (ISTDs) were added with the use of a 10 µl gas tight syringe. 2-F and 2-B were used as ISTDs at the concentration levels of 0.25 mg l^{-1} and 0.50 mg l^{-1} respectively for all of these experiments. Then the proper concentration was made up with the appropriate volume of medium to the mark.

Table 2.2 Measured pH (n=3) for each cell sample and control analysed in the MT experiment. C: control, Col: collection, S: sample, SD: standard deviation.

	pH									
	A549 Col 1		**A549 Col 2**		**NHLF Col 1**		**NHLF Col 2**		**NHLF Col 3**	
	C	S	C	S	C	S	C	S	C	S
mean	7.52	6.89	7.57	6.89	7.56	7.50	7.53	7.50	7.49	7.47
SD	± 0.04	± 0.02	± 0.06	± 0.06	± 0.05	± 0.04	± 0.03	± 0.02	± 0.05	± 0.05

Sampling

The MMSE sampling in the MT experiment was conducted according to the optimised HS-MMSE method. The DCC 18 MT disk was used for the extraction. Adsorption time was set at 120 min and temperature at 60°C. Matrix modifications included NaCl addition to make 15% (w/v) salt solution and pH adjustment to 7.0. DCM was used as a solvent at the volume of 100µl (see Chapter 3.4.1).

Blanks

Two types of blanks were used for the MT experiment:

(i) GC run of DCM to be used for extraction to check for carry-overs, and possible contamination of the solvent, blank DCM runs were conducted between each sample run.

(ii) Blank water samples prepared on the day of MMSE analysis (they were not incubated). The blank water samples were prepared in the same way as cell samples and medium controls (according to the optimised MMSE method) in order to determine any contaminant compounds originating from the sampling vial.

Compound confirmation against the chemical standard

For compound confirmation against the chemical standard, a 30 mg l^{-1} solution of the reference chemical standard was prepared in DCM and 1 µl of the solution was run under the same GC-MS experimental conditions as the samples.

GC-MS separation and detection

One µl of the solvent with extracted VOCs was injected onto the 1079 injection port of a Varian CP-3800 gas chromatograph (Varian, USA). The injector port was equipped with a 3.4 mm ID split/splitless tapered focus inlet liner (SGE Analytical Science, UK) and a Merlin Microseal septumless system (a general purpose version) (Sigma-Aldrich, UK). The gas chromatograph was equipped with a non-polar Rxi-5MS column [30 m length x 0.25 mm ID x 0.25 µm film thickness (df), Restek, UK]. Desorption was carried out for 1 min in splitless mode and then in split mode (4 min) at 250ºC. The analysis of the cell culture medium was performed with the following temperature program: 30 ºC for 1 min followed by 5ºC/min ramp up to 70ºC (held for 3 min) and another ramp of 5ºC/min up to 110 ºC (held for 3 min) and a final ramp of 10ºC/min up to 300ºC. This oven program was developed in order to achieve the best possible separation of the peaks present in the chromatogram of the cell culture medium. Solvent delay was set to 5.30 min. Helium carrier gas flow was run in constant flow at 1.0 ml min^{-1}. Compounds eluted from the column were ionised by electron ionisation (EI) and separated by a 1200 MS/MS quadrupole mass spectrometer (Varian, USA). The mass spectrometer was used in FS mode (35-400 m/z). The other parameters of the detector were: EI source and interface both at a temperature of 200ºC. No sample carry-over was observed, which was confirmed by running DCM blanks between each sample run.

Peak integration

The peak integration in the MT experiment was conducted with the use of Varian MS Workstation version 6.4. The settings were as follows: peak width 100 scans, smoothing: average, subtract baseline: 2.0 x noise, S/N ratio: on, compute area down: to baseline minus 0.0 x noise. If S/N was < 10 for a peak or it was co-eluting, then the SIM mode of the instrument was applied to extract the peak from the chromatogram. When S/N was < 3 the peak was reported as 0.

2.4 Thermal desorption sorbent tubes extraction procedure

2.4.1 Method development

Variable factors

In order to optimise the extraction of VOCs by TD sorbent tubes, several factors were varied. They included: the type of the multi-sorbent tubes used (Air Toxic™ or SVI™), the sample air volume (100 ml and 200 ml), the split ratio of the inlet and outlet splits and the time of air flush. A comparison of the peak areas of eight selected VOCs from the TIC of RPMI 1640 medium (n = 3) was undertaken, in order to assess the impact of the variation of the sample air volume and the split ratio on VOC detection. The impact of the type of the TD sorbent tube used and time of air flush were assessed in singular experiments (n = 1).

Method development sample preparation

For the TD method development the full RPMI 1640 medium was prepared as usual for cell culture purposes. Then 30 ml of the medium was added per T-75 flask. Next, the flasks were sealed with the previously autoclaved and oven-dried Suba-Seals®. The flasks were then incubated at 37°C for 7 days. The sampling of the flasks was performed exactly 7 days later.

Carrier gas flow rates

Carrier gas flow settings in the TD system include gas flow during purging of the sample tube, primary and secondary desorption flows, inlet and outlet split flows and the GC column flow rate. Because the volume of a TD tube is approximately 3 ml, a minimum flow rate through the tube of 10 ml min^{-1} is required for efficient tube desorption. Therefore, desorb flow in the TD experiment was set to 20 ml min^{-1}. The ability to use sample split points on the inlet and outlet of the cold trap helps to manage water amounts entering the column during the analysis of humid samples (Perkin Elmer, 2007). As the cell culture media were humid samples, an inlet flow of 20 ml min^{-1} was used in the TD experiment.

During primary desorption:

Sample tube flow = Desorb flow + Inlet split flow **(Equation 1)**

Flow through the cold trap = Desorb flow **(Equation 2)**

Therefore, in the TD experiment, flow through the sample tube was 40 ml min^{-1} and flow through the cold trap was 20ml min^{-1}.

During secondary desorption, sample compounds need to be desorbed from the cold trap and transferred to the GC column as quickly as possible so as to prevent band broadening. Therefore, the carrier gas flow through the cold trap during secondary desorption should be at least 10 ml min^{-1} when using a standard cold trap. Optimum flow rates applied for high resolution capillary GC columns are between 0.5 and 2 ml min^{-1} (Perkin Elmer, 2007). An outlet split flow of 10 ml min^{-1} and a carrier gas flow of 1 ml min^{-1} were used in the TD experiment.

During secondary desorption:

Flow through the heated trap = Column flow + Outlet split flow **(Equation 3)**

Therefore, in the TD experiment flow through the heated trap during secondary desorption was 11 ml min^{-1}.

Sample split ratio

The percentage of the sample from the TD tube which reaches the GC column (sample split ratio) can be calculated as follows (Perkin Elmer, 2007):

$$\frac{\textbf{Column Flow x Desorb Flow x 100}}{\textbf{(Outlet Split Flow + Column Flow) x (Desorb Flow + Inlet Split Flow)}}$$

(Equation 4)

Example: The column flow rate is 1 ml min^{-1}. The desorb flow rate is 20 ml min^{-1}, inlet split flow is 20 ml min^{-1} and the outlet split flow is 10 ml min^{-1}.
In this example 4.5 % of the sample adsorbed onto the TD tube reaches the GC detector.

2.4.2 Thermal desorption experiment

Sample preparation

For the purpose of VOC analysis the sub-confluent cells were trypsinised, counted as usual (with the use of trypan blue for the viability test) and seeded in 30 ml of complete RPMI 1640 HEPES medium. A correct amount of DME as an ISTD was added to each flask to make up a final concentration of 4 mg l^{-1}. Then the right amount of medium was added to make up a total volume of 30 ml per single flask. The flasks were sealed with previously autoclaved Suba-Seals®. After 60 min of equilibration of the flasks in the incubator, each of the flasks was flushed for 15 min with dry air (BOC, UK) with a flow of ~ 90 ml min^{-1}. The dry air flush flow rate was measured by a bubble flowmeter. Figure 2.4 shows dry air flushing of the flask. Next the flasks were incubated for 7 days (the time of the flask put into the incubator after the air flush was noted).

Together there were three cell samples and three medium controls prepared on the same day (always using the same batch of medium) which were treated as 'experiment a'. Three experiments (a, b, c) were conducted on the three different days, so one cell sample collection had n = 9 samples and one medium control collection had n = 9 samples.

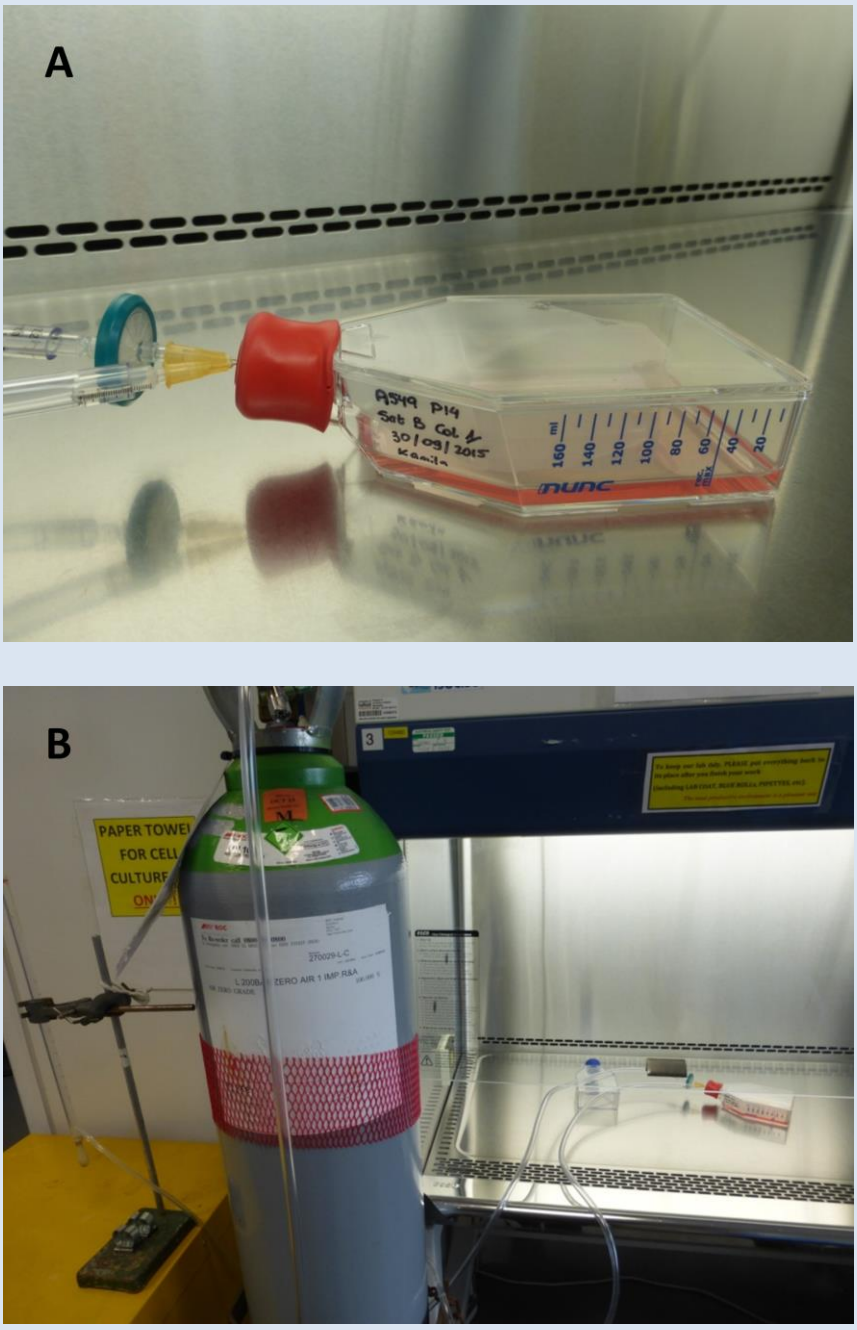

Figure 2.4 Air flushing of the flask (A) and connection to a bubble meter (B).Air was introduced into the flask through an inlet tube connected to a sterile 0.22 µm filter and sterile needle via a cut end of a sterile 1 ml syringe. Air was exiting the flask through an outlet tube connected to a sterile needle via a cut end of a 1 ml syringe. The outlet tube was connected to a bubble flowmeter in order to measure the air flow rate.

Sampling

The headspace of the flasks was sampled with the use of an Easy-VOC™ pump (Markes International, UK). It is shown in Figure 2.5. TD sorbent tubes were conditioned before the analysis and a blank was run before each extraction (on the same day). The volume of the HS air collected was set to 200 ml. After collection the flasks were refilled with 30 ml of the fresh media or water, the ISTD was added and the flasks sealed with the fresh Suba-Seals®. The flasks were left for the next 7 days in the incubator. After this period, the sampling procedure was repeated. Between each sample analysis a blank was run.

Figure 2.5 Sampling with the Easy-VOC™.

Blanks

Four types of blanks were used in the TD experiment:

(i) TD of a tube to be used for sampling to check for carry-overs, a blank TD tube was run between each experiment.

(ii) Empty cell culture flasks, sealed with Suba-Seals®, flushed with dry air and incubated for 7 days at 37°C, n = 6.

(iii) Water samples containing 30 ml of distilled water and 4 mg l^{-1} of DME as an ISTD, sealed with Suba-Seals®, flushed with dry air, incubated for 7 days at 37°C and then sampled as cell culture medium samples (see 'Water experiment');

(iv) Air from the cell culture cabinet where the cell samples and controls were prepared, (sampled overnight ~10 h), n = 6.

Water experiment

Water samples were prepared in the T-75 flasks by adding 30 ml of distilled water and an appropriate amount of DME as an ISTD, to get a final concentration of 4 mg l^{-1}. Next, the flasks were sealed with previously autoclaved Suba-Seals®, flushed with dry air and incubated for 7 days at 37°C. Next, 200 ml of the HS air was loaded onto the TD SVI™ sorbent tube and analysed as usual. The flask was then refilled with 30 ml of fresh distilled water, the freshly prepared ISTD was added and the flask incubated for a further 7 days. Together, there were three water samples prepared on the same day which were treated as 'experiment a'. Three experiments (a, b, c) were conducted on the three different days, so one water sample collection had n = 9 samples.

Compound confirmation against the chemical standard

For compound confirmation against the chemical standard, a 250 ml baked-out conical flask was sealed with a Suba-Seal®. Next 1 µl of the chemical standard was injected into the flask and the flask was equilibrated for 10 min in the oven. Next, 50 ml of the air from the conical flask was loaded onto the TD sorbent tube with the use of the Easy-VOC™ pump and the tube was analysed as usual.

TD-GC-MS separation & detection

TD of the sorbent tubes with extracted VOCs was performed with a Turbomatrix 300 thermal desorber (Perkin Elmer, UK). The sample tube was loaded onto the thermal desorber and purged for 5 min at room temperature. Nitrogen (oxygen-free, BOC, UK) was used as purging gas. Primary desorption of the TD sorbent tube was conducted for 5 min at 330°C. Flows were set as follows: desorption flow to 20 ml min^{-1}, inlet split flow to 20 ml min^{-1} and outlet split flow to 10 ml min^{-1}. Helium was used as a carrier gas (CP

grade, BOC, UK). The temperature of the transfer line was set to 250°C and of the cold trap to 5°C. During secondary desorption the cold trap was rapidly heated to 300°C.

The VOCs were separated with the Clarus 5800 gas chromatograph (Perkin Elmer, UK) equipped with an Rxi-5MS column (30 m length x 0.25 mm ID x 0.25 µm df, Perkin Elmer, UK). The analysis of the volatiles was performed with the following temperature program: 30ºC for 5 min followed by 10ºC/min ramp up to 150ºC (held for 2 min) and another ramp of 10ºC/min up to 300ºC (held for 2 min). Helium carrier gas flow was run in constant flow at 1.0 ml min^{-1}. Compounds eluted from the column were ionised by EI and separated by the Clarus 800 mass spectrometer (Perkin Elmer, UK). The mass spectrometer was used in FS mode (35-400 m/z). The other parameters of the detector were: EI source temperature 180ºC; interface temperature 200ºC.

Peak integration

Peak integration was performed with the use of TurboMass software (Perkin Elmer) with peak-to-peak amplitude set to 300 and threshold (absolute area) to 2000. The S/N ratio of each peak was checked with the use of the 'signal to noise' (peak-to-peak) option in the software. If S/N was < 10 for a peak or it was co-eluting, then the SIM mode of the instrument was applied to extract the peak from the chromatogram. For less intense peaks, Savitzky Golay smoothing (window size: 1, number of smooths: 2) was also applied to further improve S/N ratios. When S/N was < 3 the peak was reported as 0.

2.5 Calibration and precision

2.5.1 Calibration

The ISTD method of calibration was used in the MT and TD experiments in order to semi-quantify the detected analytes. In this method, the same amount of the reference compound is added to each of the analysed samples. This method of calibration involves normalisation of the response of the target analyte to the response of the reference compound. The ratio of the peak area of the analyte in the sample (A_a) to the peak area of the ISTD added to the same sample (A_{ISTD}) is calculated. The ratios for a particular VOC generated during each GC run are compared.

No calibration was applied in the method development of MMSE and TD sorbent tubes extraction. Here a comparison of the peak areas of selected VOCs from the TIC of pure medium was undertaken, in order to assess the impact of the varied factors on VOC detection.

2.5.2 Selection of internal standards

The candidate compounds to serve as ISTDs in the MT experiment were checked against water solubility and MeOH solubility, as water is the main constituent of the cell culture medium. Therefore, for the purpose of quantitation an ISTD should be easily soluble in water or in alcohol (which can be fully dissolved in water), in order to obtain the correct concentration of an ISTD. For the TD experiment only compounds easily soluble in water were tested for use as an ISTD, as MeOH could have cytotoxic effect on the cultured cells. In addition, the compounds serving as ISTDs should not be potential metabolites of the cells cultured *in vitro* and ideally should not be metabolites in pathways of any living organism. The compounds were searched against the KEGG (Kyoto encyclopedia of genes and genomes) database in order to find out if they exist as metabolites in metabolic pathways. KEGG is a free collection of pathway maps representing molecular interactions and reaction networks for metabolomics, among the other "omics" fields. Several compounds that have relatively high water or MeOH solubility and were found neither in the KEGG database, nor by any group investigating VOCs produced by cells *in vitro* (Tab. 1.1), were investigated in terms of their retention times such as: bromoacetonitrile; 2-B; dichloroacetonitrile; 1-chloro-2-methyl-2-propanol; DME; 2-F; 3-nitrobenzaldehyde; 4-nitrobenzaldehyde; 2-nonyn-1-ol; 4-pentyl-1-ol; 1,2-propenediol; pyrrole-2-carboaldehyde; trifluoroacetic acid and 2,2,2-trifluoroethanol.

2.5.3 Preparation of internal standard solutions

Preparation of MT internal standard solutions:

The ISTD solutions to be used in the MT experiment were prepared at approximately 0.5 g l^{-1} concentration. About 15 ml of distilled water was placed in a 25 ml volumetric flask. Approximately 12.5 mg of 2-F was weighed and transferred into this flask. The contents were carefully mixed and the volume made up to the mark with water, to give a proper concentration. The ISTD solution was transferred into the vial with a PTFE-lined screw

cap and stored with minimal headspace in the refrigerator until analysis. The ISTD solution was prepared daily. The same procedure was applied to 2-B (except that MeOH was used instead of water).

Preparation of TD standard solutions:

The ISTD solutions to be used in the TD experiment were prepared at approximately 1.0 g l^{-1} concentration. About 15 ml of distilled, autoclaved water was placed in a 25 ml volumetric flask. Approximately 25 mg of 2-F or DME was weighed and transferred into this flask. The contents were carefully mixed and the volume made up to the mark with water to give a proper concentration. Next, in the cell culture cabinet, the contents were transferred into a universal bottle and filtered with the use of 0.22 μm filter into another universal bottle. A filtered ISTD solution was then put into the refrigerator until the preparation of samples. The standard solution was prepared daily.

2.5.4 Precision calculations

Precision describes the reproducibility or repeatability of the analysed data. It evaluates how closely multiply measurements of a given sample agree with each other. In the case of repeatability the results are obtained by the analysing the same sample under the same conditions (same analytical instrument, operator, laboratory, time intervals etc.). Therefore, if the results of each analysis vary from each other it is because of the experimental errors and/or other causes beyond control. These results will be distributed randomly around a mean value of all repeated analyses (Patnaik, 2010). The reproducibility is demonstrated in different laboratories and more often it is a measurement of bias in data than determination of differences in precision (Green, 1996).

In the MT and TD experiments the precision was evaluated as an intra-batch precision and an inter-batch precision. The intra-batch precision was testing the performance of the analytical instrument via injecting the same sample (n = 3) in the MT experiment or desorbing the sorbent tubes loaded with samples prepared on the same day (n = 3) in the TD experiment. The inter-batch precision was obtained by the analysis of all three experiments (three different days) (n = 9 injections or tube desorptions) in order to asses variability between the different samples (García - Arieta, 2009). Calculations for inter-batch precision for dodecyl acrylate (NHLF, collection 3) and diisobutyl phthalate (NHLF,

collection 1) as well as for dodecyl acrylate (NHLF, collections 1 and 3), 2-nonadecanone and octadecanal (NHLF, collection 2) were performed for n = 7 or n = 8 respectively, as peaks for these VOCs in some chromatograms were not resolved. The peak area ratio for the peak with S/N < 3 was reported as zero. Zero values were not included into the precision calculations.

In both MT and TD experiments the intra- and inter-batch precision were determined as the relative standard deviation (RSD), a ratio of the standard deviation of the measurements to the arithmetic mean of the replicate analyses, expressed as a percent (Patnaik, 2010):

$$\mathbf{RSD = SD/\mu \times 100} \qquad \textbf{(Equation 5)}$$

where: RSD is the relative standard deviation, SD is the standard deviation and μ is the mean of the population.

Intra-batch precision

First the mean of peak area ratios ($\bar{x}\ A_a/A_{ISTD}$) for the three injections per sample (the MT experiment) or the three tube desorptions of the samples prepared on the same day (the TD experiment) and the standard deviation (SD) between the three peak area ratios were calculated. The RSD was calculated for each sample as a ratio of this standard deviation to the mean.

$$\textbf{Intra-batch \% RSD} = \frac{\mathbf{SD}_{\textbf{sample a}}}{(\bar{\mathbf{x}}\ \mathbf{A_a/A_{ISTD}})_{\textbf{sample a}}} \times \mathbf{100\%} \qquad \textbf{(Equation 6)}$$

RSD	is the relative standard deviation
SD	is the standard deviation between the area ratios (n = 3)
$\bar{\mathbf{x}}\ \mathbf{A_a/A_{ISTD}}$	is the mean of peak area ratios (n = 3)

Because there were three samples per collection, three intra-batch precision values were obtained. The highest precision was reported as an intra-batch precision for a particular collection.

Inter-batch precision

First the mean of peak area ratios ($\bar{x}$ A_a/A_{ISTD}) for nine injections (the MT experiment) or tube desorptions (the TD experiment) per collection and the SD between these nine peak area ratios were calculated. The RSD was calculated for each sample according to Equation 7 (García - Arieta, 2009):

$$\text{Inter-batch \%RSD} = \frac{SD\ \bar{x}_{\text{sample a, b, c}}}{\bar{x}\ (\bar{x}\ A_a/A_{ISTD})_{\text{sample a, b, c}}} \times 100\% \qquad \textbf{(Equation 7)}$$

RSD is the relative standard deviation
SD is the standard deviation between the area ratios (n = 9)
$\bar{x}\ A_a/A_{ISTD}$ is the mean of peak area ratios (n = 9)

2.6 VOC identification

In both the MT and TD experiments the identification of peaks was accomplished with manual interpretation of mass spectra, by matching against the National Institute of Standards and Technology (NIST) mass spectral library (NIST 11 version 2.0g for the MT experiment and NIST 14 version 2.2 g for the TD experiment) and comparison of the RTs with the commercial standards when available. For the tentatively assigned compounds, the alternative identifications were also reported. The chemical structures and BPs of the identified VOCs were obtained from the ChemSpider database (2015).

The NIST library search provides a direct match factor and reverse match factor to assess how well the unknown spectrum fits the library spectrum. The first match factor directly compares the search spectrum to the library spectrum. The second is a comparison of the library spectrum against the unknown spectrum, and non-matching peaks in the spectrum are ignored. A match factor of 999 is a perfect hit, while 0 indicates no common peaks between the spectra. Generally it is considered that a match factor > 900 is an excellent match, between 800 and 900 a good match, between 700 and 800 a fairly good match (Wachsmuth *et al.,* 2013).

2.7 Statistical analysis

The statistical analysis of the data was performed using R software environment created by the R Core Team (2015). Statistical tests were run using the base 'stats' package. Summary descriptive were created using the 'describeBy' function in the 'psych' package (Revelle, 2016). The normality test and t-test were conducted in IBM SPSS Statistics (Version 23). The chosen level of significance was $\alpha = 0.05$ for all statistical tests. The tests were performed for n = 9 (except the MT and TD method development data, where n = 3). Statistical analysis for dodecyl acrylate (collection 3 for NHLFs) and diisobutyl phthalate (collection 1 for NHLFs) were performed for n = 7, and for dodecyl acrylate (collections 1 and 2 for NHLFs), 2-nonadecanone and octadecanal (collection 2 for NHLFs) were performed for n = 8, as peaks for these VOCs in some chromatograms were not resolved. The following statistical tests were used:

Shapiro-Wilk test

To test whether the peak area ratio (n = 3 in the MT and TD method development and n = 9 in the MT and TD experiments) observations for the VOC are a population of normally distributed data, the Shapiro-Wilk test was applied. It is a widely used test for normality for observations $n < 100$. When the significance value (p) was $p \geq 0.05$, the data was assumed to have a normal distribution and when $p < 0.05$, the data significantly deviated from a normal distribution.

Wilcoxon signed-rank test

To compare the peak area ratios yielded for a VOC between the sample and its medium control (the 'between sample and control' analysis), the Wilcoxon signed-rank test was conducted in the MT and TD experiments. It is a non-parametric statistical hypothesis test for two groups arranged as paired observations. It is used when the population cannot be assumed to be normally distributed. Here the differences between the n = 9 pairs of observations are ranked from the smallest to the largest. Then separate sums of the positive and negative ranks are computed. The smaller sum is compared with the values in statistical table V for n = 9. There have to be at least six differences in order to conduct the Wilcoxon signed-rank test (Sokal and Rohl, 2012). The null hypothesis of the Wilcoxon signed-rank test assumes that the median difference of the pairs of observations is zero.

When the $p \geq 0.05$, it was assumed that the medians of peak area ratios between the sample and control were similar and the null hypothesis can be accepted. The alternative hypothesis says that the true location shift is not equal to zero.

Kruskal-Wallis

For the comparison of the peak area ratios for a VOC between samples of two different cell lines and between collections of the same cell line (the 'between-sample' analysis), the Kruskal-Wallis analysis was conducted. The test was conducted for the standardised peak area ratios (i.e. control results were subtracted from sample results) of the VOCs detected in the cell samples in the MT experiment. The Kruskal-Wallis is a non-parametric test for differences of location in ranked data, which are grouped by single classification. The null hypothesis of the test is that the compared groups do not differ in 'location'. If the Kruskal-Wallis analysis allows for the rejection of the null hypothesis, it indicates that at least one sample dominates one other sample. However, this does not give an answer as to which sample is different. Therefore, an additional test must be performed following rejection by Kruskal-Wallis (Sokal and Rohl, 2012).

Mann-Whitney U (Wilcoxon ranked-sum test)

The Mann-Whitney-U test was used when the Kruskal-Wallis analysis gave a significant difference in the location of the compared samples in the 'between-sample' analysis. It was also used for the comparison of the peak areas of the VOCs between collection 1 and collection 2 of the water samples in the 'water experiment'. The Mann-Whitney U test is a statistical hypothesis test for two independent samples. Similarly to the Wilcoxon-signed rank test, it involves summation of ranks. After computing the p-values, the Bonferroni correction was applied, which is a method of controlling Type I errors (positive false results) when multiple tests are used (Sokal and Rohl, 2012).

Paired sample t-test

Paired sample student's t-test was used for the pairwise comparisons of the peak area ratios of a VOC between the different factors tested in the MT and TD method development. This is a parametric statistical hypothesis test which is used when the data are normally distributed.

Chapter 3

The MonoTrap Experiment

Aims:

• To develop an MMSE method for capturing and semi-quantification of VOCs present in the HS of the cell culture medium.

• To identify VOCs detected in the HS of the medium cultured with the A549 lung cancer cell line and with the NHLF non-cancerous lung cell line, as well as in the HS of their pure medium controls.

• To determine the precision of the HS-MMSE-GC-MS method used.

• To compare the levels of the VOCs detected in the MT experiment between the A549 and the NHLF cell lines and their pure medium controls.

• To compare the levels of the VOCs detected in the cell samples in the MT experiment between the A549 and the NHLF cell lines, as well as between collections of the same cell line.

3.1 Introduction

3.1.1 *MMSE as an extraction technique*

Monolithic material sorptive extraction (MMSE) is a new alternative technique to SPME and sorbent tubes trapping for the concentration and analysis of VOCs and semi-volatile compounds. It applies MonoTrap™ (MT), a novel adsorbent made of silica. As the MT patent is pending there are only a several applications of MTs reported in the literature and these appeared mainly in the fields of the food industry (Gu *et al*., 2014; Ono *et al*., 2016; Wang S. *et al*., 2016; Wu N. *et al.*, 2016; Zhao L. *et al.*, 2015; Zhou *et al*., 2016) but also for alcoholic beverages analysis (Gamero *et al.*, 2013; Matsui *et al*., 2016), detection of aroma compounds in plants (Dong F. *et al.*, 2012; Jang *et al*., 2011; Kuwahara *et al.*, 2014; Ma W. *et al.*, 2013), behavioural studies of insects (Yaganawa *et al.*, 2012) and human body odour (Ozeki and Moro, 2015). The only study to date where MMSE was used for the determination of volatile composition in breath was conducted by W. Ma *et al*. (2015).

The MT is made of monolithic silica. Its structure is shown in Figure 3.1. The MT surface area for adsorption (150 m^2 g^{-1}) is increased due to the system of through pores and mesopores present in the silica skeleton (GL Sciences, 2014).

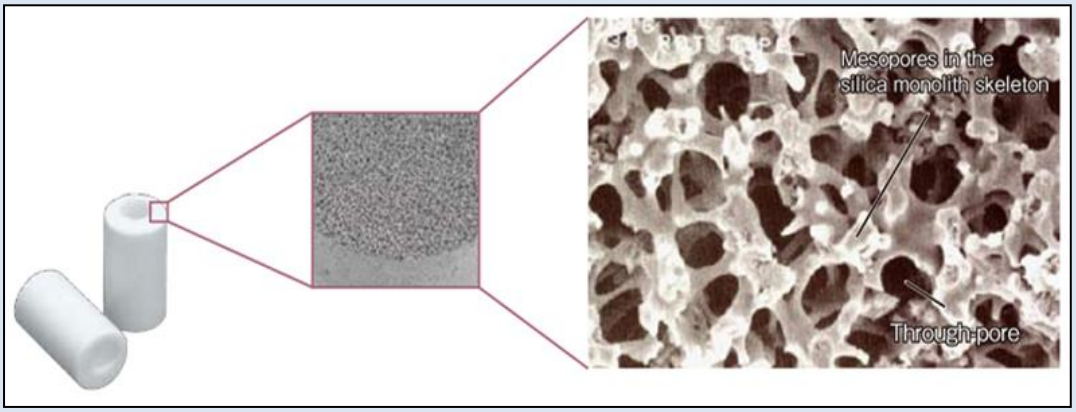

Figure 3.1 Silica monolith structure of MonoTrap™. The high surface area provided by pores and mesopores offers unmatched adsorption and desorption efficiency (reprinted by permission from GL Sciences copyright 2014).

Figure 3.2 presents the mechanism of adsorption by MT. The silica monolith can have octadecyl (ODS) functional groups only, both ODS groups and an activated (or graphite) carbon adsorbent (which has a highly porous structure), or a graphite carbon adsorbent and PDMS. This enables the absorption and/or adsorption of a range of different compounds. Sample molecules passing through the through-pores of monolithic silica are trapped by ODS groups chemically bonded to its surface or by activated carbon present both inside and outside the silica monolith. As ODS groups are hydrophobic the MT does not adsorb water from aqueous samples, making it compatible with GC (GL Sciences, 2014). The introduction of water into the GC column may cause damage to its stationary phase, variability of retention times by overloading, and deterioration of the filament (Ras *et al.*, 2009).

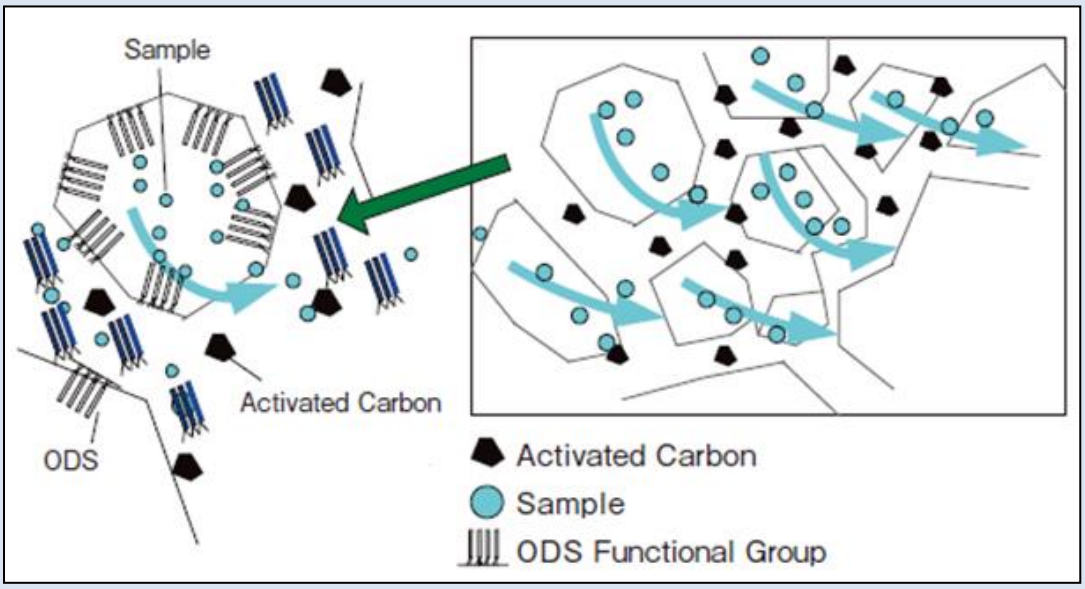

Figure 3.2 Mechanism of adsorption by MonoTrap™. The adsorption properties of a MonoTrap™ are based on octadecyl (ODS) functional group or ODS groups and activated carbon material (reprinted by permission from GL Sciences copyright 2014).

3.1.2 *MMSE versus other extraction techniques*

The comparison of MMSE as a technique of extraction of VOCs from aqueous samples to three competitive extraction techniques i.e. SPME, SBSE and sorbent tubes is shown in Table 3.1. Among these four techniques, only SPME and P&T on sorbent tubes were used in studies where VOCs were collected from cell culture medium. SBSE employs a stirring

bar coated with the sorption medium, and VOCs in an aqueous solution preferentially partition between the liquid and coating phase with the same principle as in SPME (David and Sandra, 2007). In the P&T technique an inert gas is bubbled through the aqueous sample and then passed through the sorbent trap (Madrera *et al.,* 2005) (Fig. 2.1, B).

In general, LD in comparison to TD in all cases (MMSE, SBSE and sorbent tubes) offers cost-effectiveness and possible multiple analyses of one sample. On the other hand, it necessitates an additional step in method development (Prieto *et al.,* 2010). In comparison to other extraction techniques in LD mode, MMSE does not require any additional equipment making it a relatively cheap technique. P&T on sorbent tubes requires additional hardware such as a water trap, portable pump and flow meter or vacuum system (to pass the sample through the sorbent bed). SBSE must be cleaned before each use to avoid carry-overs. To do so, it requires either an additional conditioning unit, or it must be chemically cleaned before each use (Margoum *et al.,* 2013). TD requires an expensive TD unit on the GC setup for SBSE, sorbent tubes and MTs, and in the case of sorbent tubes and SBSE an additional trap/temperature vapouriser for the cryofocusing of the desorbed analytes before they enter the column to enhance resolution (Prieto *et al.,* 2010). SPME differs from these three techniques as it can be used only in TD mode, but without any additional hardware. TD allows for lower detection limits as the sample is completely passed to a GC column and there is no solvent peak masking the presence of early eluting analytes (Ras *et al.,* 2009). A limitation of TD is that it can only be used to analyse thermally stable volatiles. Sensitivity comparison for aqueous samples indicates that TD-SBSE is the most sensitive method of all those described above (David and Sandra, 2007), however this technique is relatively expensive and is the only mode of SBSE that can be used for the analysis of VOCs. Less sensitive but cheaper LD-SBSE is useful mainly for non-volatile compounds due to use of polar solvents which are only compatible with the PDMS coating (Prieto *et al.,* 2010). A new ethylene glycol coating should overcome this limitation. However to date, there is a lack of literature data regarding this coating. The limits of detection for extraction with the use of MT have been reported in the literature at levels of low mg l^{-1} in FS mode and low pg l^{-1} in SIM mode (Jang *et al.,* 2011; Ma *et al*., 2015). The LOD in FS mode, obtained with LD, makes MT less sensitive than other techniques. On the other hand, the LOD in SIM mode, obtained with TD, makes it highly sensitive when compared to other techniques. Nevertheless, because it is a new technique these results need further validation.

Table 3.1 Comparison of monolithic material sorptive extraction (MMSE), stir bar sorptive extraction (SBSE), solid-phase microextraction (SPME) and sorbent tube extraction techniques for the analysis of VOCs. Abbreviations: EG: ethylene glycol, FS: full scan, GC: gas chromatography, HPLC: high performance liquid chromatography, LD: liquid desorption, MW: molecular weight, PA: polyacrylate, P&T: purge and trap, PDMS: polydimethylsiloxane, PDMS/EG: polydimethylsiloxane/ethylene glycol, SIM: selected ion monitoring, TD: thermal desorption. References: [1] GL Sciences, 2014; [2] Kole *et al.,* 2011; [3] Benanou *et al.,* 2003; [4] Sigma-Aldrich, 2014; [5] Jang *et al.*, 2011; [6] Ochiai *et al.,* 2000; [7] David and Sandra, 2007; [8] Ligor *et al.,* 2007;[9] Filipiak *et al.,* 2010; [10] Ma *et al*., 2015 [11] Huybrechts *et al.,* 2000; [12] Heiden *et al.,* 2008.

	MonoTrap™	SBSE	SPME	Sorbent tubes
Surface area or coating volume	150 m^2 g^{-1} [1]	24 - 126 µl (depending on the Twister size) [2]	0.5 µl [3]	5 - 1500 m^2 g^{-1} [4]
Coating variety and analytes range	Three types of traps in two configurations (disk and rod) (recovery of polar and non-polar compounds, concentrated samples and trace analysis, volatiles and semi-volatiles)	PDMS coating (recovery of polar compounds) PDMS/EG (recovery of non-polar compounds)	10 different fiber coatings available (recovery of polar and non-polar compounds, high and low MW compounds, volatiles and semi-volatiles, trace and concentrated samples)	> 20 adsorbent types available that can be packed in multi-bed tubes (recovery of low and high MW compounds, polar and non-polar compounds)
Desorption	TD or LD	TD or LD	TD or HPLC	TD or LD
Carry over	No carry over (disposal)	Carry over	Carry over	Carry over
Conditioning	Not needed	Needed	Needed	Needed
Sensitivity	LD: Low mg l^{-1} (FS)[5] TD: Low pg l^{-1} (SIM) [10]	TD: Low ng l^{-1} (FS) [6] TD: Low pg l^{-1} (SIM) [7]	Low µg l^{-1} (FS) [8] High ng l^{-1} (SIM) [8]	TD: Low ng l^{-1} (FS) [9] TD: High pg l^{-1} (SIM) [11]
Labour	Requires minimal time and labour outlay	Requires minimal time and labour outlay	Requires minimal time and labour outlay	Requires preparation of multi-sorbent bed (in case of selection of own adsorbents)

Table. 3.1 Cont'd

	MonoTrap™	SBSE	SPME	Sorbent tubes
Water trapping	Hydrophobic, no water trapping	PDMS - hydrophobic EG - hydrophilic, highly cross-lined polymer synthesis eliminates water absorption	PDMS - hydrophobic PEG - hydrophilic, highly cross-lined polymer synthesis eliminates water absorption [11] PA - hydrophobic Water may be trapped between the SPME fiber rod and protective sleeve [12]	Water trapping (mainly when activated carbon sorbent used), may require moisture trap or dry purge Tenax® - hydrophobic
Durability	Fragile, easy to break	Not fragile	Fragile, easy to break, easy to strip the coating	Not fragile
GC runs number	LD: Single sample - several GC runs TD: single sample	LD: Single sample - several GC runs TD: single sample	Single sample - no injection volume errors	LD: Single sample - several GC runs TD: single sample
Extraction samples number	Many samples may be sampled at the same time	Many samples may be sampled at the same time	Sample number limited by the number of SPME holders	P&T: one sample can be sampled at the time Easy-VOC™: one sample can be sampled at the time
Additional hardware	Thermal desorber for TD	Conditioning unit, thermal desorber for TD	Not needed	Thermal desorber for TD, pump or vacuum for P&T, cold focusing trap; water trap or dry purge
Cost	LD: no hardware required TD: cost of TD unit initially expensive MonoTrap™: relatively cheap (not reusable)	Hardware: initially expensive (conditioning unit) TD: cost of TD unit initially expensive Twisters very expensive (reusable up to 200 times)	No hardware required, initially cheap Fibers: relatively expensive (reusable up to 100 times)	TD: hardware initially expensive Adorbents: very expensive Ready multi-sorbent beds: very expensive (reusable 100-200 times)

3.1.3 *Principles of HS analysis*

There are two types of HS analysis: static (equilibrium) HS and dynamic HS (P&T). In static HS the sample (liquid or solid) is placed in the sampling vial with gas volume above it (HS) and the vial is tightly closed. Next, the vial is equilibrated at a constant temperature until equilibrium is reached between the two phases. Finally, a defined volume of the HS is introduced either manually (eg. via syringe) or automatically into the GC column and is analysed (Kolb and Ettre, 2006). Instead of introducing an aliquot of the HS directly into the GC column an adsorption device, such as MT, might be introduced into the HS of the vial. Such a device is a means of pre-concentration as it separates the volatiles to be analysed from the excess of the diluted HS gas. The vial is kept at a constant temperature for the time of adsorption. During sampling with MT, only a portion of the target compound is removed from the sample as it is a non-exhaustive equilibrium extraction technique. Sample molecules preferentially partition between the matrix, HS and the MT. This partitioning between the three phases depends on the affinity of the analyte to each of them at equilibrium. The equilibrium of different analytes between the matrix and the adsorption medium is reached at different times. Therefore, a time of extraction is established experimentally for given mixture of analytes. The period after which equilibrium is reached depends on the type of the analytes and extraction conditions (Pawliszyn, 2009).

The efficiency of the HS techniques for the extraction of VOCs depends on the concentration of the analytes in the HS. This concentration is limited by the water:air distribution constant at equilibrium (partition coefficient K) (Eq. 7), the ratio between the volumes of the HS and the liquid sample (phase ratio β) (Eq. 8) and the initial concentration of the analyte in the sample (Eq. 9) (Kolb and Ettre, 2006):

$$\mathbf{K = C_s/C_g} \qquad \textbf{(Equation 7)}$$

K is the partition coefficient
C_s is the concentration of analyte in the sample phase
C_g is the concentration of analyte in the headspace

$$\mathbf{\beta = V_g/V_s} \qquad \textbf{(Equation 8)}$$

β is the phase ratio
V_g is the volume of headspace phase
V_s is the volume of the sample phase

$$C_g = C_0 / (K + \beta) \qquad \textbf{(Equation 9)}$$

C_g is the concentration of analyte in the headspace
C_0 is the initial concentration of analyte in the sample phase
K is the partition coefficient
β is the phase ratio

Compounds with low K values tend to partition into the HS while compounds with high K values favour the liquid phase. Lower values of phase ratio β (larger sample size) give higher responses for VOCs with low K values. If K is high, the adjustment of β will have a small effect on the HS concentration of the analyte. Higher initial concentration of the analyte in the sample will result in the higher concentration of the analyte in the HS (and in a better sensitivity). Aiming for the lowest values for both K and β will also result in higher concentrations of volatile analytes in the gas phase.

$$C_g = 1 / (K + \beta) \qquad \textbf{(Equation 10)}$$

For example, if the initial concentration of the analyte in the sample is constant, and = 1 (arbitrary unit), for K = 0.20, the HS concentration of a VOC for β = 0.25 (80% sample volume) will be almost 10 times of that for β = 4 (20% sample volume). For K = 20 the difference will be only 1.18 between the two β ratios. Finally, when K = 250 the effect of changing sample volume from 80% to 20% will be negligible (1.02) (Kolb and Ettre, 2006).

3.1.4 *Development of MMSE method*

Development of the MMSE method includes several important considerations: the type of MT to be used, optimisation of the extraction and desorption conditions, and finally the employment of an appropriate calibration procedure. Extraction optimisation includes consideration of the mode of sampling (static HS, agitation HS, floating mode), the time and temperature of sampling, the ionic strength and pH of the matrix, the sample volume, the extraction solvent volume, and the solvent type (Jang *et al*., 2011; Kudlejova *et al*., 2009).

Selection of the MonoTrap™ type

There are two types of MT available for LD, one with the characteristics of silica gel, and one with a silica skeleton containing an adsorption medium (activated carbon). The silica monolith of both types is chemically bonded with ODS groups (Fig. 3.2). For non-polar compounds both types have the same adsorption capabilities. For the extraction of more polar compounds, MTs with activated carbon groups are more efficient (GL Sciences, 2014).

MTs are available in two configurations: a disk for trace analysis, as these traps have a high section area, and a rod shape for trapping highly concentrated samples. The two configurations are shown in Figure 3.3. There are four types of MTs to be used with LD: two rod shaped traps (RCC18 and RSC18) and two disk shaped traps (DCC18 and DSC18). There are also three types of MT (RGC18 TD, RSC18 TD and RGPS TD) for direct GC desorption which requires a thermal desorber system (T-Dex 2/ATD, Gerstel or Linex). The types and recommended applications of MTs are shown in Table 3.2.

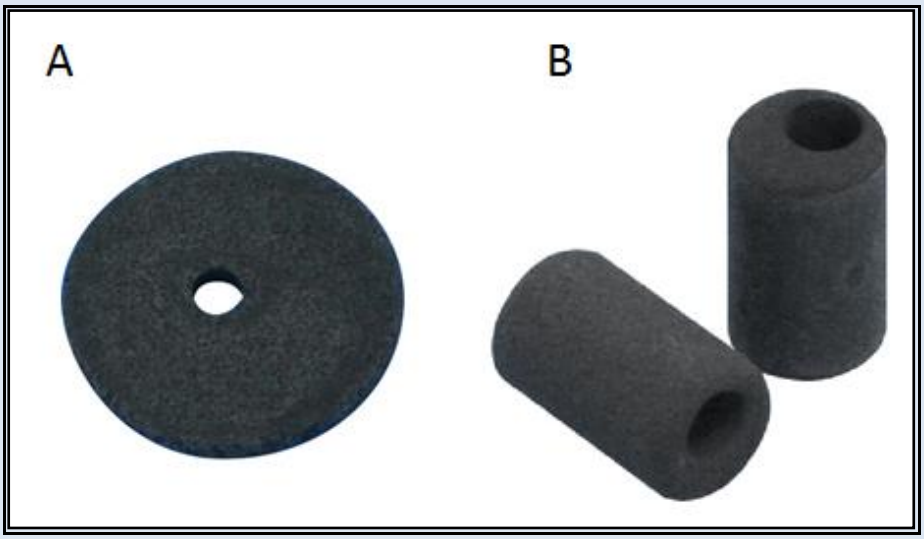

Figure 3.3 MonoTrap™ in two configurations: disk shape (A) and rod shape (B) (reprinted by permission from GL Sciences copyright 2014).

Table 3.2 Summary of available types of MonoTrap™ (Hichrom, 2016). BP: boiling point; ODS: Octadecyl C18; PDMS: polydimethylsiloxane

MonoTrap Type	Target concentration	Target Compounds	Functional Group	Desorption Type
DCC18 disk	Low	Non-polar to highly polar, low-medium BP	ODS, activated carbon	Liquid
RCC18 rod	High	Non-polar to highly polar, low-medium BP	ODS, activated carbon	Liquid
DSC18 disk	Low	Non-polar to intermediate polarity, medium-high BP	ODS	Liquid
RSC18 rod	High	Non-polar to intermediate polarity, medium-high BP	ODS	Liquid
RGC18 TD rod	All	Non-polar to highly polar, low-medium BP	ODS, graphite carbon	Thermal
RSC18 TD rod	All	Non-polar to intermediate polarity, medium-high BP	ODS	Thermal
RGPS TD rod	All	High BP	PDMS, graphite carbon	Thermal

MonoTrap sampling mode

There are two MT extraction modes used for the extraction of VOCs: floating mode and HS mode. In the former, the MT is introduced directly onto the sample, and in the latter the device is placed into the air above the sample. The MT extraction modes are shown in Figure 3.4. In floating mode, because the MT is hydrophobic, it does not sink but floats on the surface of the aqueous sample. In the floating mode, VOCs move between the liquid phase, gaseous phase and the adsorption medium until they reach equilibrium, but hydrophilic compounds are also directly adsorbed by the trap from the liquid. In the HS mode the MT is placed onto the stainless steel holder and suspended in the gaseous phase above the sample. HS mode is considered a more efficient mode for the extraction of VOCs from complex liquids and solid samples, as only analytes of interest are adsorbed. In addition, extraction time is greatly reduced as diffusion constants for the VOCs in the gaseous phase are four orders of magnitude higher than in the liquid phase (Zhang Z. and Pawliszyn, 1993).

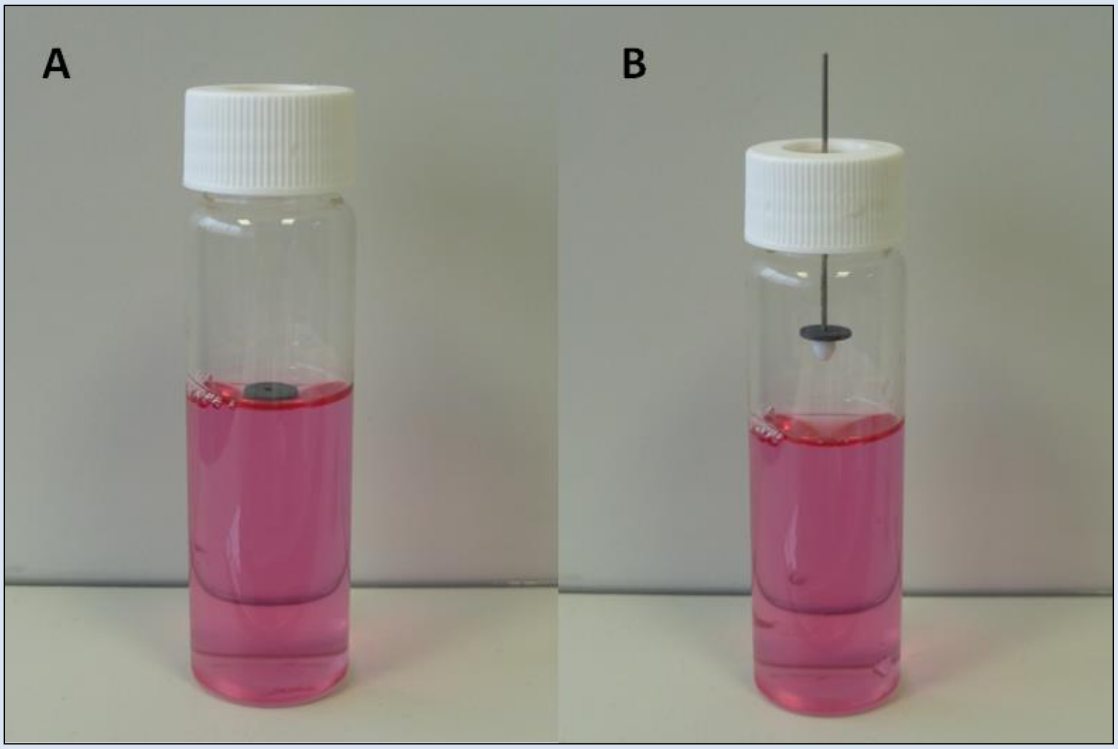

Figure 3.4 Modes of sampling with MonoTrap™: floating (A) and headspace (B).

MonoTrap extraction time & temperature

Extraction time is usually the most time-limiting factor in extraction techniques, and is therefore one of the main parameters to optimise. It might be shortened by efficient agitation of aqueous solutions and/or elevation of temperature. However, although higher temperatures result in the more efficient release of compounds from the matrix (decrease of K values), an increase in temperature simultaneously causes loss of sensitivity as distribution constants decrease i.e. equilibrium is reached faster but the amount of analyte extracted is smaller at this equilibrium than if equilibrium were to be reached at the lower temperature. Therefore, the selection of the temperature of extraction is a compromise between the sensitivity, length and repeatability of the method (Pawliszyn, 2009). Agitation reduces the time it takes for equilibrium to be reached, and improves sensitivity in pre-equilibrium extraction, as it enhances the mass transport between the sample and the adsorption device.

Solvent type

When using the solvent, the recovery rates of extraction of analytes from the MTs may depend on the type of solvent used and on its volume. Extraction of the analytes into solvents is accelerated by the use of a sonicator. However, short times should be used to avoid solvent evaporation due to heat and to help prevent the loss of the VOCs (GL Sciences, 2014).

Matrix conditions

The efficiency of the HS techniques for the extraction of VOCs depends on the concentration of the analytes in the HS. One of the factors influencing this concentration is the water:air distribution constant (K). This constant depends on the characteristics of the analytes (water solubility, vapour pressure) as well as on the optimisation of the matrix conditions which include sample volume, temperature, pH, ionic strength, sample agitation and the addition of an organic modifier (Kudlejova *et al*., 2009; Tipler, 2013).

Ionic strength

The ionic strength of the sample is modified by the addition of salt and it may influence the adsorption in two ways: modifying the properties of the phase boundary and a "salting-out" effect. The latter refers to the process of decreasing the solubility of hydrophobic analytes in the aqueous phase, and is more often observed. The sensitivity of HS analytical methods is widely enhanced by the "salting-out" effect (Kolb and Ettre, 2006; Yang and Peppard, 1994). The process improves sensitivity through the formation of hydration spheres by water molecules with salt ions. This effect drives the additional sample molecules into the HS due to a reduction in the concentration of water molecules available for dissolving analysed compounds. However with a higher concentration of salts an opposite process may occur. Electrostatic interactions of analyte molecules with the ionic salt molecules in the solution may reduce their movement into the HS (Lord and Pawliszyn, 2000; Lord, 2009). In general, salt addition increases the extraction of polar compounds. However, it has no significant effect on non-polar compounds.

Effects of pH

The adjustment of the pH of the sample solution is important in the case of acidic and basic compounds, as in HS methods only neutral/undissociated compounds are adsorbed onto the adsorption medium. Low pH values will result in better extraction of acidic compounds, while high values of pH will improve adsorption efficiency for basic compounds (Kudlejova *et al*., 2009).

Sample volume

Phase ratio β is the proportion of gaseous volume to the sample volume in the vial; the lower the values of phase ratio, the better the sensitivity of the HS method. Therefore, HS volume should be as small as possible in order to achieve higher sensitivity, as equilibrium time is reduced and the mass of compound extracted by adsorption medium increases, thereby improving detection limits (Zhang Z. and Pawliszyn, 1993). The larger the HS volume, the more of the compound to be analysed goes into the HS, and the less that goes onto the adsorption medium and remains in the liquid phase (Yang and Peppard, 1994). However, a decrease of HS volume does not result in better adsorption of all the VOCs; when the phase ratio is reduced by using a larger sample volume it has a minimal impact on the HS concentrations of compounds with high K values (Tipler, 2013). Therefore, when the amount of sample is an issue, the optimal HS volume should be established experimentally for a given system and application.

Desorption

Desorption times used in VOC studies of cancer vary greatly from 20 s (at 200ºC) to 10 min (at 260ºC) (Chen X. *et al*., 2007; Zimmermann *et al*., 2007). In general, an increase of the injector temperature reduces desorption time. Care must be taken when a small volume GC inlet is used, as different solvents have different vapour points and with higher temperatures it can cause overloading of the sample. The focus liner used in the MT experiment was checked for use with DCM as a solvent and the possibility of overloading against the liner-selection tool on the SGE website.

Calibration

HS-MMSE as a non-exhaustive method needs careful selection of calibration for quantitative analysis**.** Studies of VOCs as cancer biomarkers with the use of SPME use the traditional calibration method i.e. external standards as long as blank sample matrices are available (Ouyang, 2009). These blank samples are breath, blood and urine from healthy patients and pure culture medium for *in vitro* studies. Quantification of analytes in SPME is based on the principle that the amount of compound extracted onto the trap is linearly proportional to the compound concentration in the sample. LD, as in the case of MT, introduces the potential error of injection variations due to differences in sample volumes. Another potential error in the analytical method is the instability of the detection system (e.g. accumulation of siloxanes coming off the column on the ion volume, reducing its sensitivity). To overcome these limitations an internal calibration may be used. Here a known amount of ISTD is added into the sample and calibration mixture. Then if all the samples contain the same ISTD concentration, a calibration curve of analyte response divided by ISTD response versus analyte concentration may be constructed (Flanagan *et al.*, 2007).

Method validation

Once the analytical method's parameters are optimised, the method should be tested for a particular application. If quantification is going to be performed, the analytical method should be tested for specificity, linearity, accuracy, precision, LOD and limit of quantification (LOQ), and range (HimaBindu *et al.*, 2013).

3.2 MonoTrap method development

The method development of MMSE included investigation of the effects of differences in sampling mode, time, temperature, solvent volume and type of solvent used for extraction, and changes to the sample such as ionic strength, pH, and volume. All of these experiments allowed the detection of VOCs, but in differing amounts and numbers. The results of these studies are presented below. A comparison of the peak areas of eight selected VOCs from the TIC of RPMI 1640 medium was undertaken, in order to assess the impact of the variation of these factors on VOC detection. The method was not

optimised on the cell medium incubated with cells, in order to exclude potential VOC variability arising from the presence of cells. A range of compounds from different areas of the chromatogram (i.e. VOCs with the low, medium or high RTs), with well separated peaks and with a relatively high intensity, was chosen. Compounds that eluted after 20 min of the GC run were not selected as their peaks were not resolved, they were possible siloxane contaminants coming off the column and/or their identity was unknown. The TIC of the RPMI 1640 cell culture medium incubated for seven days at 37°C is shown in Figure 3.5. The compounds selected as model compounds are marked on this chromatogram. Freshly prepared medium and medium incubated for shorter periods of time (e.g. 3, 4 days) did not show intensive peaks (data not shown).

The selected VOCs represented four chemical groups commonly detected in studies of VOCs *in vitro* i.e. alcohols, aldehydes, ketones and aromatic hydrocarbons. Identification of acetophenone, benzaldehyde, cyclohexanol, 2-ethylhexanol, heptanal, nonanal and styrene was confirmed against the RTs of standards (data not shown). Identification of 1-octanol was based on spectral matching with the NIST library. All VOCs were detected in cell-free RPMI 1640 cell culture medium by others, but also in other types of cell culture media (Barash *et al.*, 2009, 2012; Davies *et al*., 2014; Filipiak *et al.*, 2008; 2010; Hanai *et al.*, 2012b; Kwak *et al.*, 2013; Sponring *et al.*, 2009; Zimmermann *et al.*, 2007).

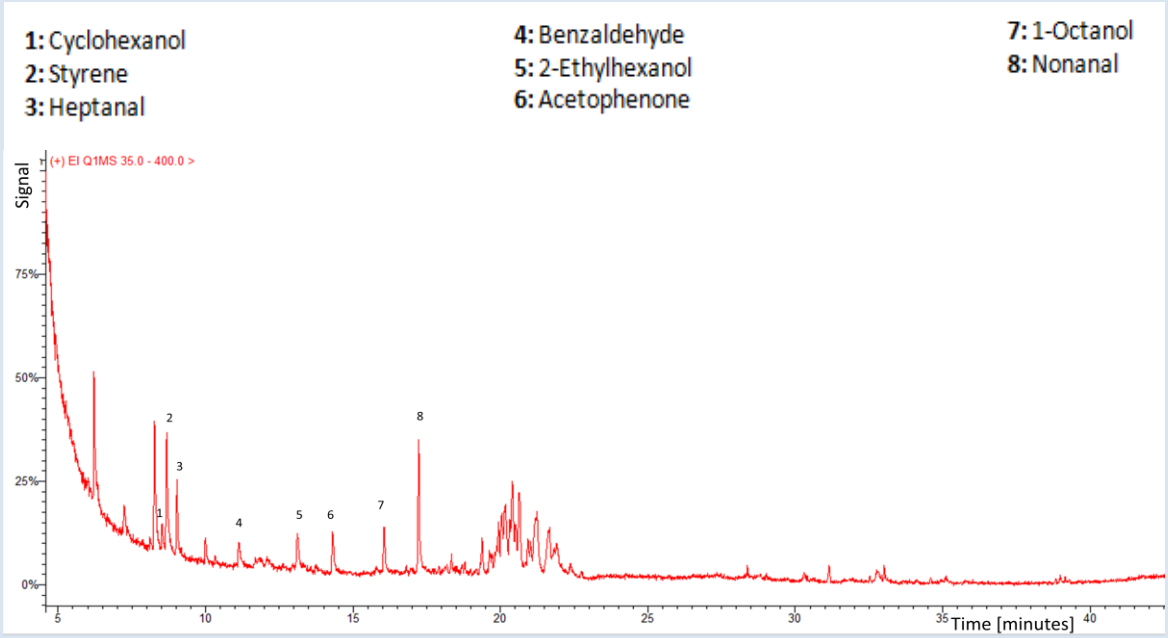

Figure 3.5 Total ion chromatogram (TIC) of the pure medium control obtained in full scan mode in the MonoTrap method development.

It must be noted that the collected amounts of some compounds (e.g. acetophenone, 2-ethylhexanol or nonanal) varied between different batches of the complete medium (i.e. prepared on different days). This might be due to changes in some VOC concentrations depending on the period of storage of open bottles of the cell culture medium. Therefore, a set of experiments investigating the effects of a particular factor were planned in order to use the same batch of the RPMI medium for a more direct comparison.

The graphs presented in this chapter show peak areas (detector's response) for each of the selected compounds obtained with various experimental settings. Each bar represents a mean of peak areas (n = 3) for a particular analyte obtained from three injections of the same sample. Error bars are marked on the graphs for each analyte and represent one standard deviation (SD) of uncertainty. The differences in peak areas were compared with the use of the t-test (paired sample, 2-tailed) for two reasons. Firstly, because the data obtained for < 5% of VOCs in the MT method development failed the assumption of normality, which indicated that the results were approximately normally distributed (Shapiro-Wilk, $\alpha = 0.05$) (Appendix B, Tab. B1). Secondly, because the Wilcoxon signed-rank test (a non-parametric equivalent to paired t-test) requires at least 6 differences ($n \geq$

6) and in the MT method development n = 3. The differences in peak areas were considered significant when p value of the t-test < 0.05.

3.2.1 MonoTrap disk type

The use of the two types of disk-shaped MTs: DCC18 and DSC18 was investigated in the experiment shown in Figure 3.6. DCC18 was determined to be more efficient for the collection of the VOCs of interest. DCC18 MTs are more efficient for the adsorption of polar compounds because of the presence of activated carbon, in comparison to DSC18

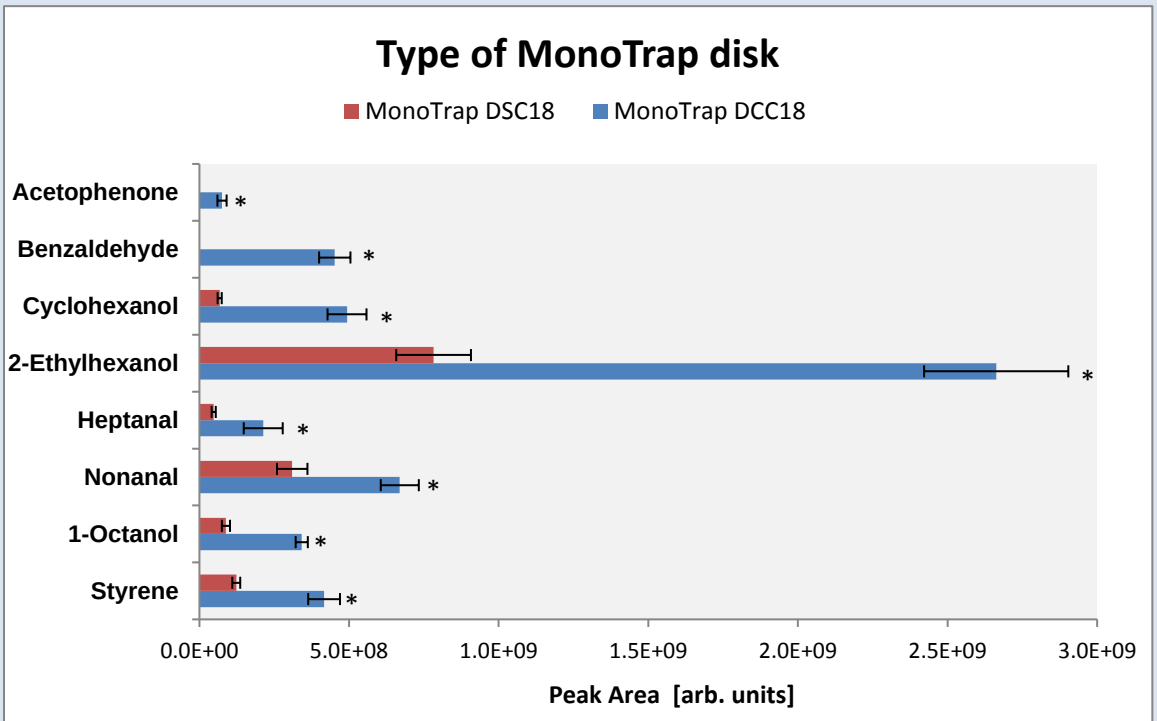

Figure 3.6 Influence of the type of the MonoTrap (MT) disk used on the efficiency of MT adsorption of acetophenone, benzaldehyde, cyclohexanol, 2-ethylhexanol, heptanal, nonanal, 1-octanol and styrene. The VOCs were detected in RPMI 1640 complete cell culture medium incubated for 7 days at 37°C. Bars represent the means of peak areas for n = 3. MMSE conditions: 60°C, 2 hours, 15% NaCl, 700 rpm. MT extraction conditions: 100 µl of DCM, 4 min sonication. Difference is significant between the bar with an asterix and the other tested MT disk type at $p < 0.05$ (paired sample t-test, 2-tailed).

traps which have only hydrophobic ODS groups. Polar acetophenone, benzaldehyde, cyclohexanol, 2-ethylhexanol, heptanal, 1-octanol and nonanal were significantly better extracted with the use of a carbon activated trap. The peak area of styrene was significantly higher for the sampling performed with the use of the DCC18 trap, even though styrene is a relatively non-polar compound and theoretically should be adsorbed equally by the two MTs.

3.2.2 Sampling mode

The results of the experiment testing three different modes of sampling using MT are shown in Figure 3.7. The three modes tested were: HS with agitation, floating with agitation and static HS (without agitation). HS with agitation appeared to be the most efficient mode of sampling the model VOCs with the use of MT. The difference was significant for all the analytes, except cyclohexanol and styrene which were collected at similar amounts with the use of the HS with stirring and floating with stirring modes. The peak areas of benzaldehyde, 2-ethylhexanol and styrene were significantly higher with the use of floating with agitation mode, than with static HS mode. There was no difference for acetophenone between these two modes. Cyclohexanol was collected at similar levels in all three tested modes. Static HS was shown to be the least efficient sampling option. This was expected, as agitation speeds up the transfer of VOCs from the aqueous phase into the HS, by generating a continuously fresh surface (Zhang Z. and Pawliszyn, 1993). For MT used in the floating mode, this is probably also the case for the top of the disk facing the HS. However, the bottom of the MT was immersed in the solution. Here a thin, static water layer surrounding the extraction device limits the transfer of analytes from the sample, thereby extending the equilibrium times. Therefore, this is probably why the amounts of the analytes collected in floating mode were lower than those obtained in HS with stirring mode. HS with agitation was chosen as the mode of sampling for further experiments.

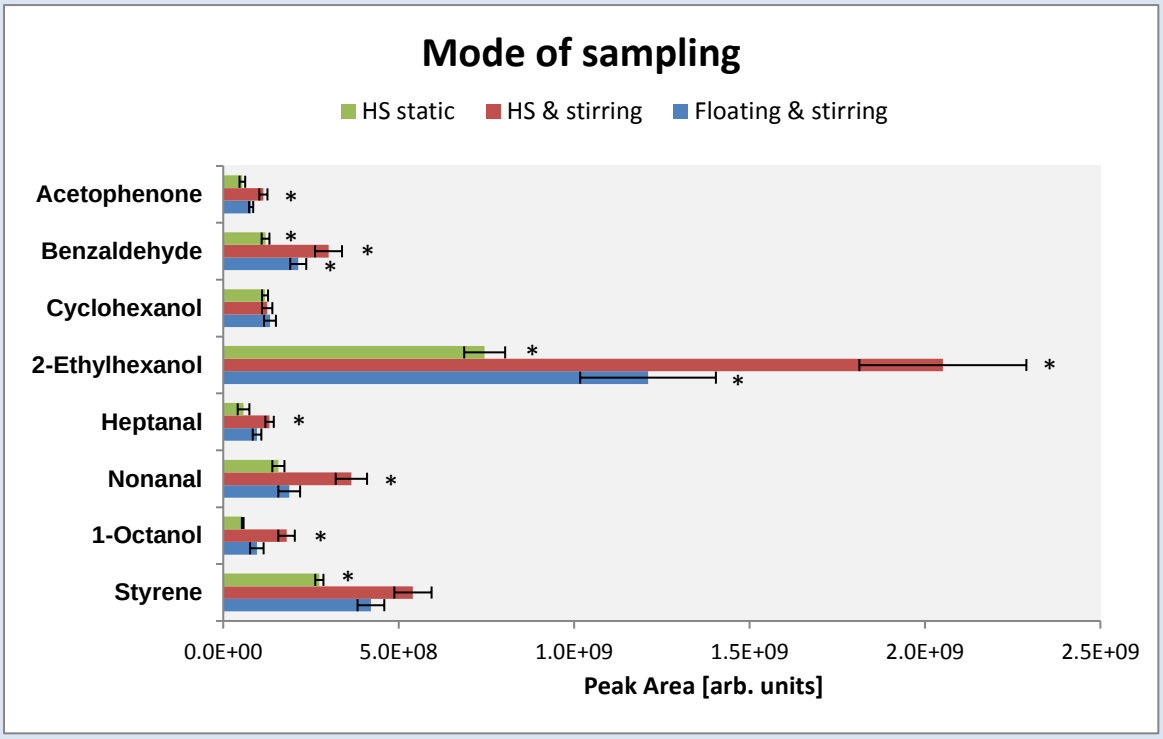

Figure 3.7 Influence of the sampling mode on the efficiency of MonoTrap adsorption of acetophenone, benzaldehyde, cyclohexanol, 2-ethylhexanol, heptanal, nonanal, 1-octanol and styrene. The VOCs were detected in RPMI 1640 complete cell culture medium incubated for 7 days at 37°C. Bars represent the means of peak areas for n = 3. MMSE conditions: 60°C, 2 hours, 15% NaCl, 700 rpm. MT extraction conditions: 100 µl of DCM, 4 min sonication. Differences are significant between the bars with an asterix and all other tested sampling modes at $p < 0.05$ (paired sample t-test, 2-tailed).

3.2.3 Sampling time and temperature

Time effects

Figure 3.8 shows the extraction time profiles for the selected VOCs. Adsorption of the compounds onto MT disks was stopped after 60, 90, 120 and 150 minutes. In general, the amounts of analytes adsorbed onto the traps increased with a higher adsorption time, the highest peak areas being reached for most of the compounds at 120 min (acetophenone, benzaldehyde, cyclohexanol, 2-ethylhexanol, nonanal and 1-octanol). The peak areas of cyclohexanol, 2-ethylhexanol and nonanal significantly decreased after 150 min, while the

amounts of benzaldehyde and 1-octanol remained roughly the same. Acetophenone, styrene and heptanal were extracted equally efficiently with 90 min and 120 min sampling times and their adsorption dropped after 150 min (except acetophenone).

Trapping using the MMSE technique is based on the partitioning of the sample molecules into ODS groups and on the intermolecular interactions (adsorption) between the sample molecules and activated carbon. The number of micropores formed by activated carbon is limited so that no more analyte molecules can be adsorbed when all micropores are occupied. The adsorption of the sample onto activated carbon is a competitive process, which means that a molecule with a lower affinity for the surface can be replaced by a molecule with a higher affinity (Câmara *et al.,* 2006). In equilibrium based systems such as HS-MMSE, the amount of the compound adsorbed by the adsorption medium increases, until the equilibrium distribution of an analyte between the sample phase, gas phase and adsorption medium is reached. The equilibrium may be reached at different times by different compounds, depending on their partition coefficient K (Pawliszyn, 2009). Therefore, the compounds reaching equilibrium later and that have a higher affinity to the adorption sites may replace the analytes with lower affinity. In these experiments, the amounts of some compounds decreased with 150 min when compared to 120 min sampling, probably due to the replacement of these analytes by the compounds reaching equilibrium later. A similar phenomenon was observed by Jang *et al.* (2011) who reported that the analytes were adsorbed the most efficiently after 90 min and after 120 min their peak areas significantly decreased. The 120 min sampling time was selected, because differences in the amounts of VOCs extracted for most of the analytes were significant between this time and the other three sampling times tested. Other extraction times employed for the analysis of VOCs by MT found in literature were 30 min (Wu N. *et al*., 2016), 60 min (Ma W. *et al.,* 2013), 90 min (Jang *et al.,* 2011); 3 hours (Dong F. *et al.*, 2012), 16 hours (Gamero *et al.,* 2013) and 20 hours (Yanagawa *et al*., 2012).

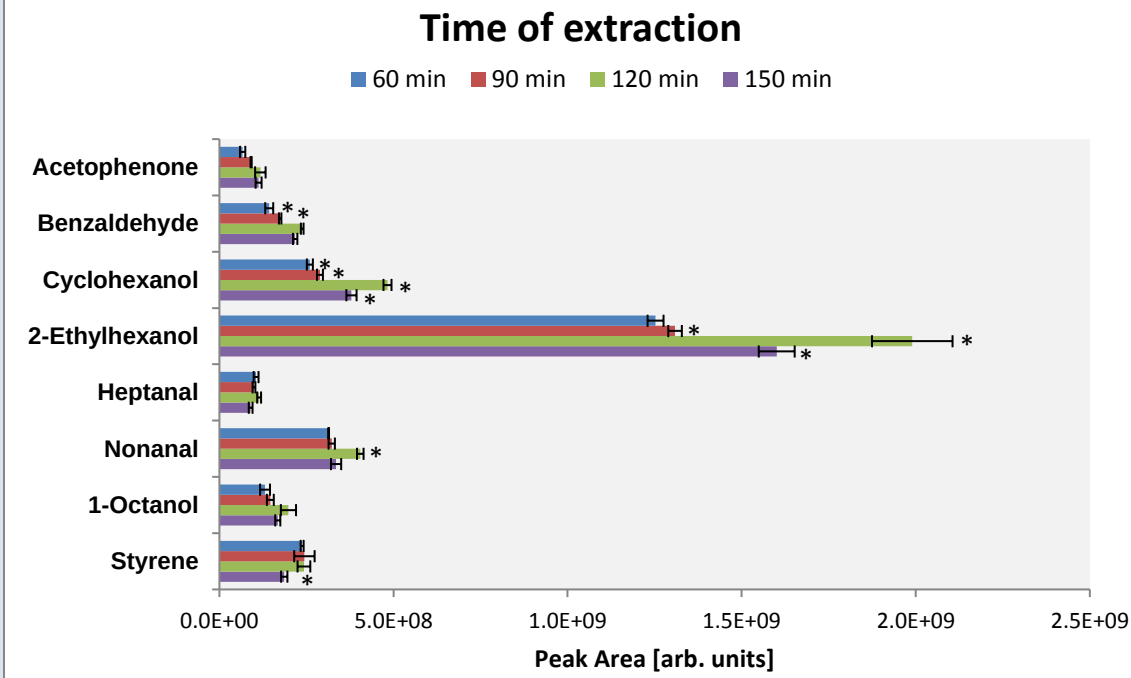

Figure 3.8 Influence of different times of sampling on the efficiency of MonoTrap adsorption of acetophenone, benzaldehyde, cyclohexanol, 2-ethylhexanol, heptanal, nonanal, 1-octanol and styrene. The VOCs were detected in RPMI 1640 complete cell culture medium incubated for 7 days at 37°C. Bars represent the means of peak areas for n = 3. MMSE conditions: 60°C, 15% NaCl, 700 rpm. MT extraction conditions: 100 µl of DCM, 4 min sonication. Difference is significant between the bar with an asterix and all other tested sampling times at $p < 0.05$ (paired sample t-test, 2-tailed).

Temperature effects

The influence of three temperatures (45°C, 60°C and 75°C) on the amounts of the selected VOCs adsorbed onto MT disks is shown in Figure 3.9. Extraction time is usually the most time-limiting factor in analysis by HS techniques, and is therefore one of the main parameters to optimise. It might be shortened by efficient agitation of aqueous solutions and/or elevation of temperature (Wang Y. *et al.*, 2005). However, although higher temperatures result in the more efficient release of compounds from the matrix, an increase in temperature simultaneously causes loss of sensitivity as distribution constants decrease i.e. equilibrium is reached faster but the amount of analyte extracted is smaller at

this equilibrium (Pawliszyn, 2009). Therefore, the selection of the temperature of extraction is a compromise between the sensitivity and length of the method.

The profiles of VOC adsorption at the three different temperatures, shown in Figure 3.9, indicate that most of the compounds were significantly better adsorbed onto traps at 60°C and 75°C than at 45°C. The results obtained for acetophenone, benzaldehyde, 2-ethylhexanol, heptanal, nonanal and 1-octanol indicated that both higher temperatures are equally efficient for their collection. Only cyclohexanol was collected in the highest amount at 45°C, showing that this temperature is too low to saturate the HS with the remaining VOCs. There were no significant differences in the tested sampling temperatures for styrene. Temperature of 60°C was selected for further experiments.

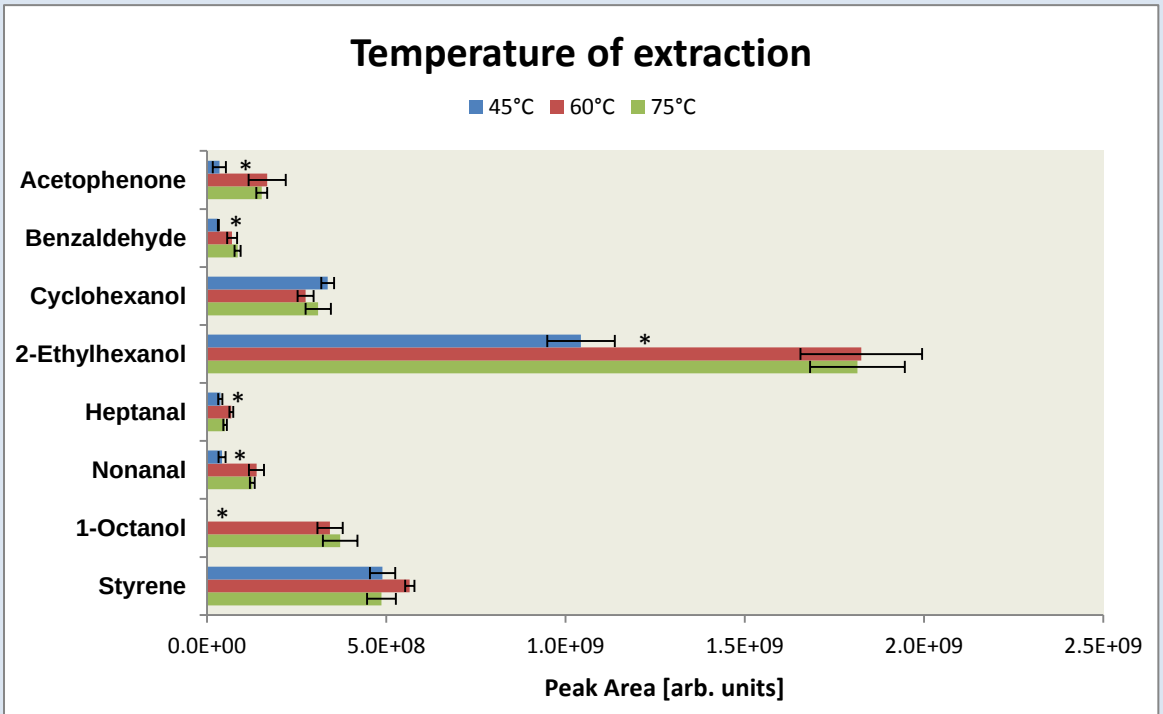

Figure 3.9 Influence of different temperatures of sampling on the efficiency of MonoTrap adsorption of acetophenone, benzaldehyde, cyclohexanol, 2-ethylhexanol, heptanal, nonanal, 1-octanol and styrene. The VOCs were detected in RPMI 1640 complete cell culture medium incubated for 7 days at 37°C. Bars represent the means of peak areas for n = 3. MMSE conditions: 2 hours, 15% NaCl, 700 rpm. MT extraction conditions: 100 µl of DCM, 4 min sonication. Difference is significant between the bar with an asterix and all other tested sampling temperatures at $p < 0.05$ (paired sample t -test, 2-tailed).

The extraction temperature of 60°C is recommended by GL Sciences (2014) as the most efficient temperature for collection of VOCs by HS-MMSE. The temperatures used in the literature where MMSE has been used are 60°C for the extraction of aroma compounds from a plant extract (Jang *et al.*, 2011) and 80°C, 90°C or 100°C where a solid sample was investigated (Ma W. *et al.*, 2013; Wang S. *et al*., 2016; Zhou *et al*., 2016). Temperatures applied with HS-SPME for the investigation of VOCs in cell culture medium in the literature vary greatly from room temperature (Yu *et al.*, 2009), 37°C (Chen X. *et al*., 2007, Zimmermann *et al*., 2007), 45°C (Hanai *et al.*, 2012b), 60°C (Acavedo *et al.*, 2007; 2010) to 80°C (Poli *et al*., 2004).

3.2.4 Matrix conditions

Better sensitivity of the HS method may be achieved by optimisation of the matrix conditions which include sample volume, temperature, pH, ionic strength, sample agitation and the addition of an organic modifier. Salt addition (increase of ionic strength) and pH adjustment are common techniques for the enhancement of the extraction efficiency of organic analytes from aqueous solutions (Kudlejova *et al.*, 2009).

Salt addition

Figure 3.10 shows the influence of the addition of salt on the amounts of collected compounds. For acetophenone, benzaldehyde, cyclohexanol, 2-ethylhexanol, nonanal and 1-octanol the addition of NaCl improved adsorption onto the MT in comparison to the sample with no salt added. Among these VOCs a 15% solution of NaCl appeared to be significantly more effective than a 30% salt solution for benzaldehyde, 2-ethylhexanol, nonanal and 1-octanol. For cyclohexanol the differences were not significant between 15% and 30% salt solutions. The adsorption efficiency for acetophenone was better with the addition of a higher amount of salt (30% solution). The differences in the peak areas of heptanal were not significant between 0% and 15% salt solutions, but they were significantly higher for the 15% than for the 30% NaCl concentration. The extraction of styrene was not affected by the salt addition in any way.

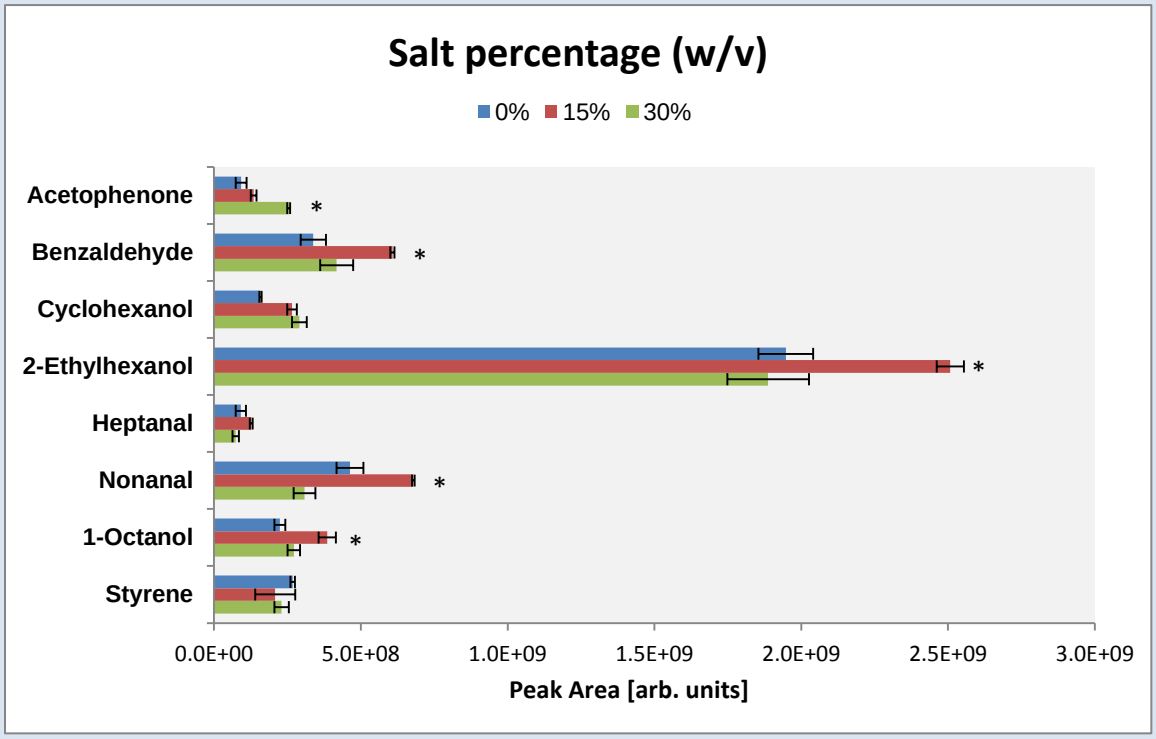

Figure 3.10 Influence of the different salt percentage (w/v) of the sample on the efficiency of MonoTrap adsorption of acetophenone, benzaldehyde, cyclohexanol, 2-ethylhexanol, heptanal, nonanal, 1-octanal and styrene. The VOCs were detected in RPMI 1640 complete cell culture medium incubated for 7 days at 37°C. Bars represent the means of peak areas for n = 3. MMSE conditions: 60°C, 2 hours, 700 rpm. MT extraction conditions: 100 µl of DCM, 4 min sonication. Difference is significant between the bar with an asterix and all other tested salt concentrations at $p < 0.05$ (paired sample t-test, 2-tailed).

In general, salt addition increases the extraction of polar compounds. However, it has usually no significant effect on non-polar compounds (Lord and Pawliszyn, 2000). And here, in this experiment, non-polar styrene was extracted in similar amounts in all three salt percentage variants. All polar compounds were better adsorbed with a 15% or 30% salt addition than in the medium with not added salt. The results obtained are in agreement with SPME experiments investigating effects of salt addition on the extraction of VOCs from various matrices. Peak areas of the investigated compounds increased in these studies in most cases with a rise of salt concentration reaching a maximum value and then dropping or remaining constant with a further addition of salt although these values could be different for different VOCs (Cassada *et al.*, 2000; Guadagni *et al.*, 2011; Rocha *et al.*, 2001; Yang and Peppard, 1994). A 15% salt solution was selected for further studies, as

this appeared to be the most effective salt percentage for the extraction of the selected analytes.

pH effects

The effect of pH on the amount of adsorbed VOCs is shown in Figure 3.11. Three pH values were investigated: pH 3.0, pH 7.6 (unmodified pH of incubated medium samples) and pH 10.0. Acetophenone, benzaldehyde and styrene were adsorbed in significantly higher amounts at the unchanged pH. Dropping the pH to 3.0 significantly decreased the adsorption of these compounds. Extraction efficiency of the remaining compounds were not affected by the reduction of pH from 7.6 to 3.0, as they were collected at roughly equal amounts in both pH environments. Changing the sample pH to 10.0 reduced the peak areas for all of the investigated VOCs (not significantly in the case of 2-ethylhexanol).

In HS methods only the neutral/undissociated species of compounds are adsorbed onto the trap. Adjustment of pH may improve method sensitivity through conversion of the ion species into neutral forms. Low pH values will benefit the extraction of acidic analytes, and high pH values will increase the extraction efficiency of basic compounds. Optimum sensitivities for the collection of some VOCs with the solid-phase extraction cartridge method (for styrene among others) at neutral pH was shown by Pandey and Kim (2012). Polar compounds investigated by this group were extracted with less efficiency at pH 10 than at neutral pH. In the MT method development experiment the extraction of polar VOCs (except 2-ethylhexanol) were also significantly reduced at pH 10. Another explanation for this phenomenon is that compounds like alcohols may behave as weak acids, which in strong bases ionise, reducing the pool of neutral species that can be adsorbed onto the disk. However, pKa values for 2-ethylhexanol and 1-octanol are 15.8 and 16.8 respectively, suggesting that if dissociation of these VOCs at pH 10.0 occurs, it is stronger for 2-ethylhexanol. Because the adsorption of 2-ethylhexanol was not affected by altering the pH to 10.0, but for 1-octanol it was, it is not clear what mechanism drove their adsorption onto the MT. The extraction of alcohols was not affected at low pH values as the differences in peak areas between acidic and neutral conditions are not significant. They apparently do not exhibit strong enough acidic intramolecular bonding forces in aqueous solution (Friant and Suffet, 1979). It must be noted however, that more acidic compounds (e.g. carboxylic acids) were detected in cell culture medium as cancer cell metabolites by others (Smith *et al.*, 2003; Barash *et al.*, 2009; Hanai *et al.*, 2012b, Kwak *et*

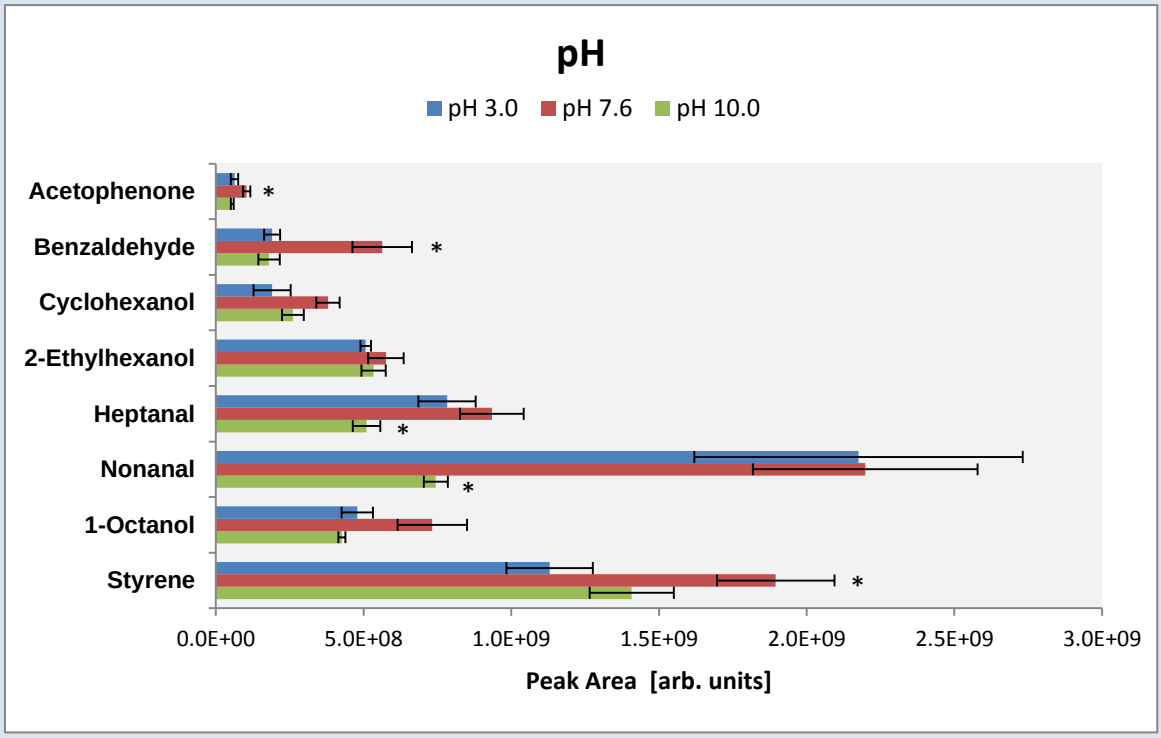

Figure 3.11 Influence of pH of the sample on the efficiency of MonoTrap adsorption of acetophenone, benzaldehyde, cyclohexanol, 2-ethylhexanol, heptanal, nonanal, 1-octanol and styrene. The VOCs were detected in RPMI 1640 complete cell culture medium incubated for 7 days at 37°C. Bars represent the means of peak areas for n = 3. MMSE conditions: 60°C, 2 hours, 15% NaCl, 700 rpm. MT extraction conditions: 100 µl of DCM, 4 min sonication. Difference is significant between the bar with an asterix and all other tested pH values at $p < 0.05$ (paired sample t-test, 2-tailed).

al., 2013). Kwak *et al.* (2013) reported that some organic acids were major VOCs detected at a low pH value and were barely visible in the chromatogram obtained in neutral conditions. On the other hand, the intensities of many other compounds decreased with a reduction of pH. This was also the case for some compounds in the MT method development experiment (acetophenone, benzaldehyde and styrene). Carboxylic acids are a relatively non-abundant group of metabolites of cells that were reported in the literature (Filipiak *et al.*, 2016). A neutral pH seems to be a better compromise. Utilising the unmodified pH of the sample does not require any additional work and the volume of the sample is not changed by the addition of acid or base. However, the unmodified pH of the sample could not have been applied in the MT experiment because the presence of A549

cells resulted in a slight drop of the medium pH to ~ 7.0 when compared to the medium control (see Table 2.2). Therefore, neutral pH was chosen for future experiments.

Sample volume

Figure 3.12 shows the influence of the sample volume on the amounts of collected VOCs. Four sample volumes were tested: 15 ml, 20 ml, 25 ml and 30 ml. As the volume of the sample vial was 40 ml, the phase ratio β was 1.6, 1.0, 0.6 and 0.3 for these sample volumes respectively. The use of a 15 ml sample size resulted in the extraction of significantly lower amounts of four VOCs (benzaldehyde, 2-ethylhexanol, heptanal and styrene) than the application of the other three set-ups. There were no significant differences between the peak areas of the majority of compounds, when comparing the use of a 20 ml, 25 ml and 30 ml sample volume. The peak area of cyclohexanol was significantly smaller between a 30 ml sample and other sample sizes. Sample volume size had no significant effect on the extraction of acetophenone.

The experiment testing variations in the sample volume showed that 20 ml, 25 ml, and 30 ml sample sizes are similarly efficient for the collection of the selected VOCs. A 15 ml sample volume was significantly less efficient. This is because the larger is the volume of the HS, the less sensitive is the method as less analytes goes onto the adsorption medium and more remains in the HS and liquid phase (Yang and Peppard, 1994; Zhang Z. and Pawliszyn, 1993). However, a larger sample volume does not result in better sensitivity for all the VOCs; it has a small impact on the HS concentration of VOCs with high K values (see Chapter 3.1.3). This was the case for the majority of compounds. On the other hand, it is not clear why a 30 ml sample volume resulted in smaller levels of cyclohexanol in the HS when compared to the other three sample volumes.

In the method development experiment, the use of the 20 ml and 25 ml sample volumes was similarly efficient for the collection of all the selected VOCs. A 20 ml volume was selected as a sample size for further experiments, as it required less of the sample than a 25 ml volume.

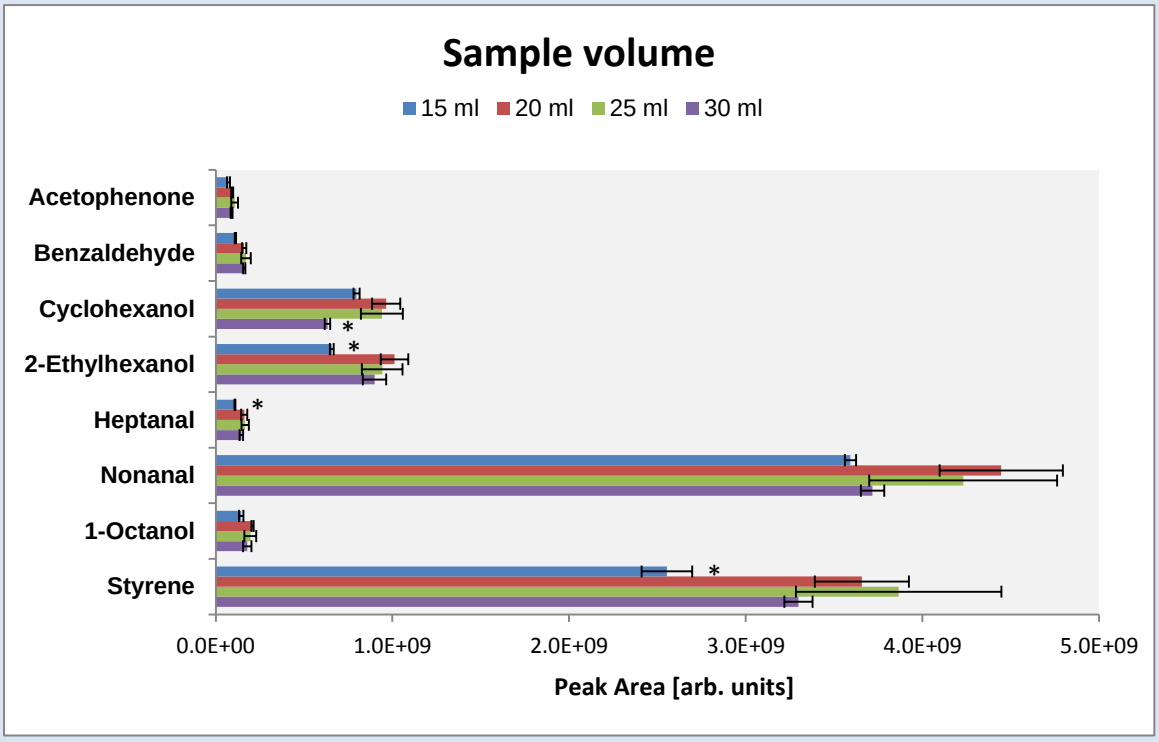

Figure 3.12 Influence of the sample volume on the efficiency of MonoTrap adsorption of acetophenone, benzaldehyde, cyclohexanol, 2-ethylhexanol, heptanal, nonanal, 1-octanol and styrene. The VOCs were detected in RPMI 1640 complete cell culture medium incubated for 7 days at 37°C. Bars represent the means of peak areas for n = 3. MMSE conditions: 60°C, 2 hours, 15% NaCl, 700 rpm. MT extraction conditions: 100 µl of DCM, 4 min sonication. Difference is significant between the bar with an asterix and all other tested sample volumes at $p < 0.05$ (paired sample t-test, 2-tailed).

3.2.5 Solvent volume and type

Solvent volume effects

The effects of solvent volume on the release of VOCs adsorbed onto the MT disk were determined in four experiments using 70 µl, 100 µl, 130 µl or 160 µl of DCM presented in Figure 3.13. In general, peak areas decreased with a higher solvent volume, due to the increasing dilution of VOCs. There were no significant differences in peak areas between the chromatograms obtained with the use of 70 and 100 µl of DCM for all the VOCs. Peak areas of all the investigated VOCs were significantly larger when 100 µl of solvent was used, in comparison to the results obtained with 130 µl and 160 µl of solvent volume.

Acetophenone was not detected at all with the use of higher volumes of DCM. There were no significant differences for half of the compounds when 130 µl and 160 µl solvent volumes were compared. Because some volume of DCM evaporates during sonication (regardless of the solvent volume used), the sample volume collected with 70 µl of DCM was not enough for three injections and gave relatively high SDs. Therefore, 100 µl solvent volume was selected for further studies. The same volume of DCM was used by Jang *et al.* (2011).

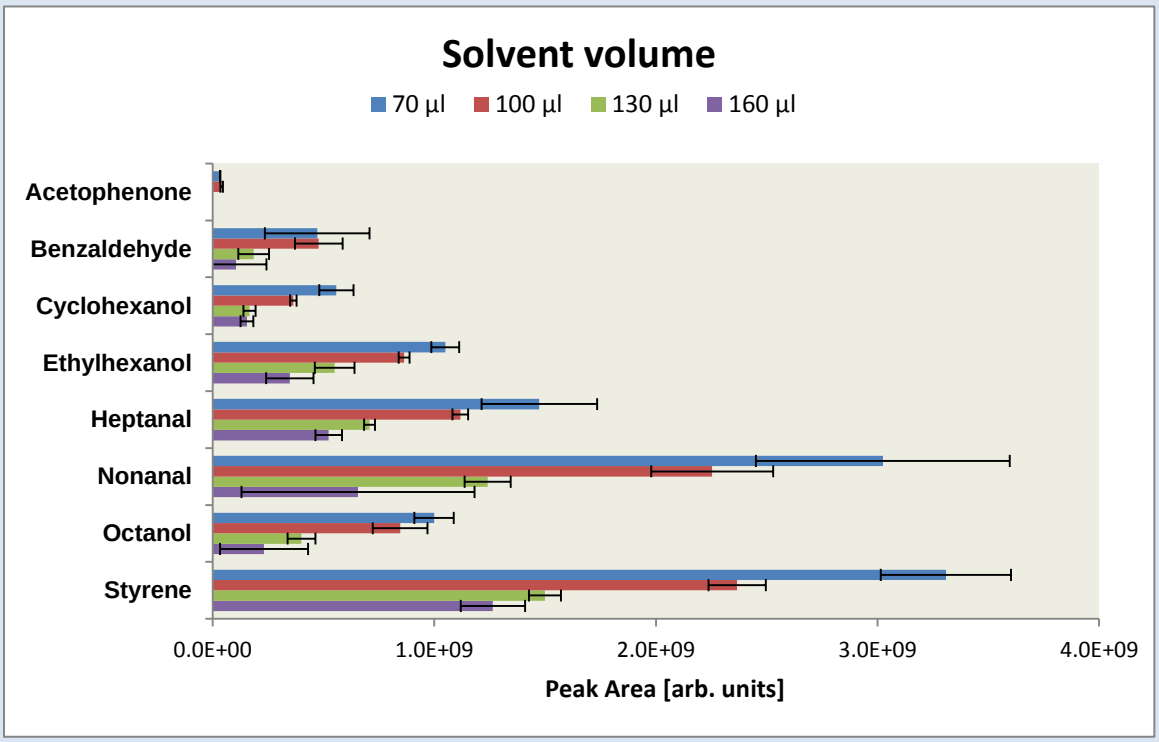

Figure 3.13 Influence of the solvent volume on the efficiency of extraction of acetophenone, benzaldehyde, cyclohexanol, 2-ethylhexanol, heptanal, nonanal, 1-octanol and styrene. The VOCs were detected in RPMI 1640 complete cell culture medium incubated for 7 days at 37°C. Bars represent the means of peak areas for n = 3. MMSE conditions: 60°C, 2 hours, 15% NaCl, 700 rpm. MT extraction conditions: 4 min sonication.

Solvent type effects

Figures 3.14 and 3.15 show an evaluation of the effects of different solvents used in the extraction of VOCs from the MT disks. Only the use of DCM, CHF or EtAc allowed for the extraction of all the investigated compounds. Among them, DCM appeared to be the most efficient as the peak areas were significantly higher in the cases of benzaldehyde, 2-ethylhexanol, heptanal and styrene. The remaining analytes were extracted with similar efficiency by the three solvents (except octanol as the efficiency of EtAc was significantly lower than that of the other two solvents). The use of hexane did not allow for the detection of acetophenone and benzaldehyde and yielded significantly lower amounts of cyclohexanol, 2-ethylhexanol and styrene than the previously mentioned solvents. The use

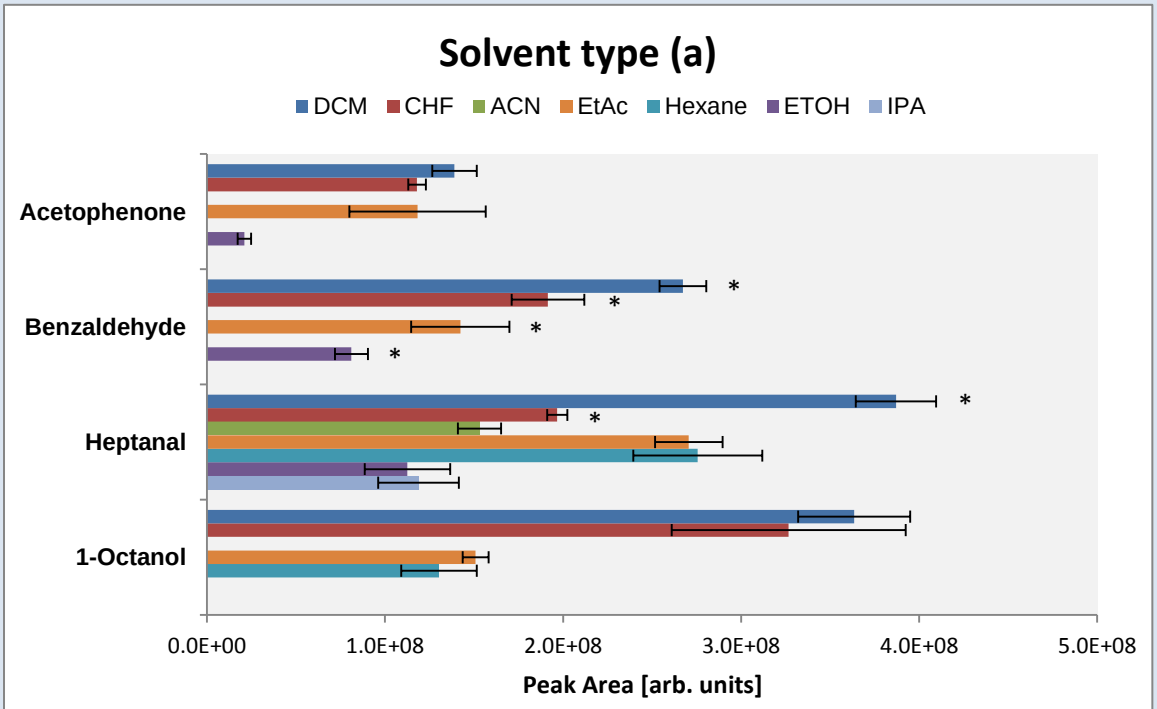

Figure 3.14 Influence of the solvent type on the efficiency of extraction of acetophenone, benzaldehyde, heptanal and 1-octanol. The VOCs were detected in RPMI 1640 complete cell growth medium incubated for 7 days at 37°C. Bars represent the means of peak areas for n = 3. MMSE conditions: 60°C, 2 hr, 15% NaCl, 700 rpm. MT extraction conditions: 100 µl of solvent, 4 min sonication. Difference is significant between the bar with an asterix and all other tested solvents at $p < 0.05$ (paired sample t-test, 2-tailed).

of ACN enabled extraction of relatively high amounts of some of the compounds (2-ethylhexanol and cyclohexanol) but did not allow the extraction of acetophenone, benzaldehyde and 1-octanol. The lowest amounts of VOCs were extracted with the use of alcohols EtOH and IPA. IPA appeared to be the least efficient, as acetophenone, benzaldehyde, 1-octanol and nonanal were not detected at all in the experiment with its use as a solvent.

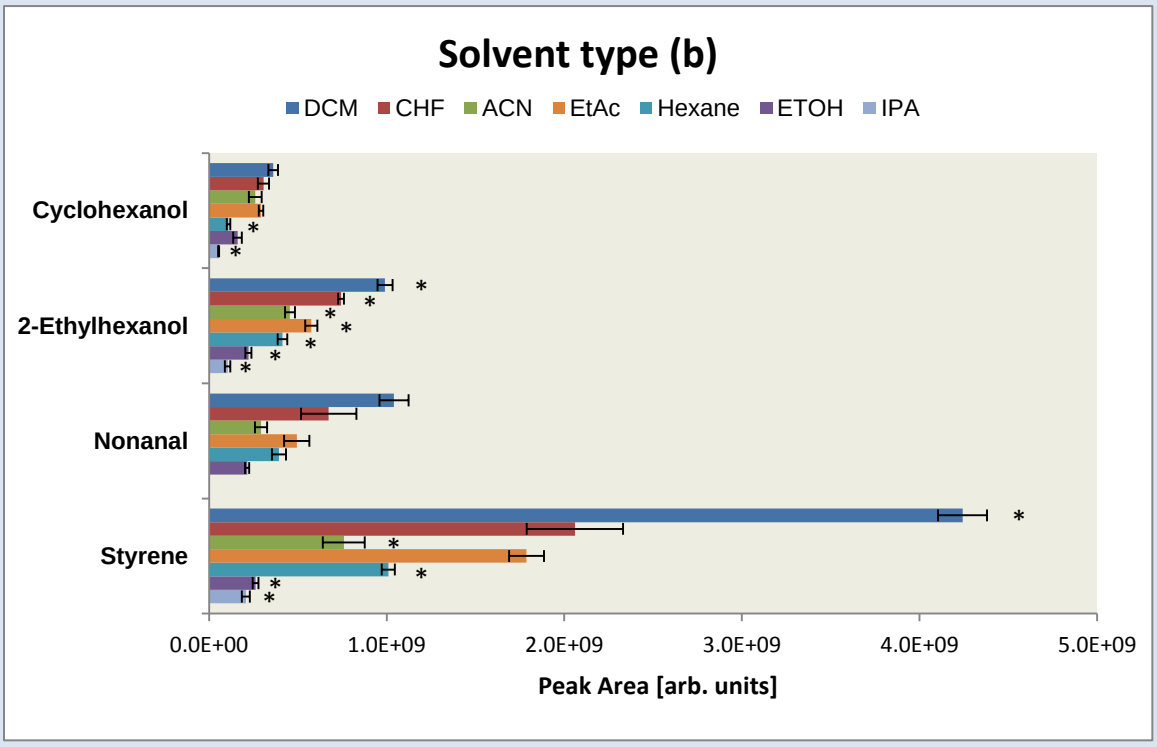

Figure 3.15 Influence of the solvent type on the efficiency of extraction of cyclohexanol, 2-ethylhexanol, nonanal and styrene. The VOCs were detected in RPMI 1640 complete cell growth medium incubated for 7 days at 37°C. Bars represent the means of peak areas for n = 3. MMSE conditions: 60°C, 2 hours, 15% NaCl, 700 rpm. MT extraction conditions: 100 µl of solvent, 4 min sonication. Difference is significant between the bar with an asterix and all other tested solvents at $p < 0.05$ (paired sample t-test, 2-tailed).

The seven types of solvent investigated belong to four groups: non-polar (hexane, CHF), "borderline" polar aprotic (DCM, EtAc), polar aprotic (ACN) and polar protic (EtOH, IPA). The dielectric constant order for the solvents used in the experiments (which roughly provides polarity of the solvent and rises with an increase of polarity) is as

follows: hexane < CHF < EtAc < DCM < IPA < EtOH < ACN. The most efficient solvents appeared to be DCM, CHF and EtAc as they extracted all the investigated compounds and with relatively higher levels than the rest of the solvents. DCM was significantly more efficient in the extraction of four of the VOCs in comparison to CHF and EtAc. DCM is a "borderline" polar solvent and may be considered as slightly polar. EtAc and CHF are solvents with similar dielectric constants, and for the investigated VOCs they may be too non-polar. The use of alcohols as solvents (EtOH and IPA) yielded the weakest peak areas for the range of investigated VOCs, possibly due to high values of their dielectric constants. ACN appeared to be too polar and hexane too non-polar for this application. Consequently, DCM was chosen as a solvent for future experiments.

3.2.6 Selection of internal standard

Over twenty compounds were tested for potential use as an ISTD for the analysis of VOCs from the cell culture medium. From these 2-Fluorophenol (2-F) and 2-Bromobenzyl alcohol (2-B) were selected for this purpose, as they did not co-elute with any VOC detected in medium during the MT method development, they are soluble in water (2-F) and in MeOH (2-B) and were not found in the KEGG database as an intermediate or final metabolite produced by any living organism present in the database. The selected ISTDs were used at the final concentrations of 0.25 mg l^{-1} of 2-F and 0.50 mg l^{-1} of 2-B. The peak areas for both compounds were in the middle range of the peak areas of the analytes in the TIC.

3.3 MonoTrap cell culture conditions

3.3.1 Cell seeding density

Previous studies have shown that the concentrations of VOCs emitted by cells *in vitro* are low and that a higher amount of cells allowed for the detection of a higher number of altered VOC levels in cell samples when compared to pure medium controls (Filipiak *et al*., 2010; Hanai *et al*., 2012b). Therefore, the highest possible recommended seeding density was used for the cells. The recommended ratio of medium volume to growing surface area is in the range of 0.2 - 0.5 ml cm^{-2} (Freshney, 2005). This makes 15 ml the lower limit of the medium volume in a T-75 flask. A volume of 30 ml was used in the MT and TD experiments as the seeding densities used were the highest recommended and the medium was not changed for a week. Therefore, this higher amount of medium is sufficient for supplying the essential nutrients and supplements to the cells for longer. Additionally, a single T-75 was sufficient to yield the 20 ml of sample required for a single MT experiment, making it less laborious in terms of cell culture. The ratio of 30 ml medium volume to 75 cm^2 surface area is 0.4 ml cm^{-2}, which is in the recommended range.

3.3.2 Time of incubation

Initial MT experiments showed that a freshly opened cell culture medium and medium cultivated for 3 - 4 days, did not allow for the detection of VOCs at high intensities. The A549 cells were confluent on the sixth day of culturing, while the fibroblasts on around the 11^{th}-12^{th} day of growth. Therefore, the A549 cell culture medium was collected after a week and again after two weeks of cell cultivation, to evaluate the VOCs from the growing cells and from the fully confluent cells respectively. As the fibroblasts are primary, much slower growing cells, the NHLF cell line was cultured for three weeks and the medium was collected three times during this period (after the first, second and third weeks of cell culture).

3.3.3 Cell culture medium

Different types of media are recommended for the cultivation of A549, NHLF and BEAS-2B cell lines. As different media types are likely to emit different VOCs, for a more direct

comparison of the VOCs being released or consumed by the analysed cell lines, the same type of medium, RPMI 1640, was used for the cultivation of all the cell lines. A general purpose medium, RPMI 1640 has a wide range of applications for the culture of mammalian cells and it has been widely used in the literature for the culture of A549 and BEAS-2B cell lines, also in studies of VOCs (Barash *et al.* 2009; 2011; Brunner *et al.* 2010; Pyo *et al.* 2009; Wang Y. *et al*., 2012). Also commonly, it has been used for the culture of the NHLFs (e.g. Krasnodembskaya *et al*., 2010; Liu X. *et al.*, 2004; Probst *et al.*, 2015). In the MT and TD experiments, a HEPES modification of the RPMI 1640 medium was used. HEPES is a buffer, stabilising the pH in the range of 7.2 - 7.6 which is required for cell culture at high cell densities, when the pH otherwise would fall, and for sealed culture systems with depleted levels of exogenous CO_2, which helps to regulate physiological pH (Freshney, 2005).

3.3.4 Cell viability

Cell viability (trypan blue exclusion) tests were performed on each of the sampled flasks after the period of incubation had finished. After two weeks of cultivation the average viability of the A549 cells was 98.8 ± 0.9 % (n = 3) and after three weeks of incubation of the NHLF cells the average viability was 94.8 ± 3.6 % (n = 3). High viabilty percentages for both cell lines indicated that the cell culture conditions were appropiate for the analysed cell lines and that the analysed VOCs were potentially coming from living cells and were not due to processes associated with cell death.

3.4 *In vitro* MMSE-GC-MS analysis of VOCs

3.4.1 Optimised method for HS-MMSE

The method that was developed after looking through the variety of effects of different factors on the extraction of VOCs found in the RPMI 1640 culture medium with the use of MMSE is shown in Figure 3.16. The DCC18 MT with activated carbon was selected for this application. Sampling time was set at 2 hours and temperature at 60°C. Matrix modifications included NaCl addition to make a 15% salt solution and pH adjustment to 7.0. The most efficient solvent for the release of VOCs from MT appeared to be DCM and it was used at the volume of 100 µl.

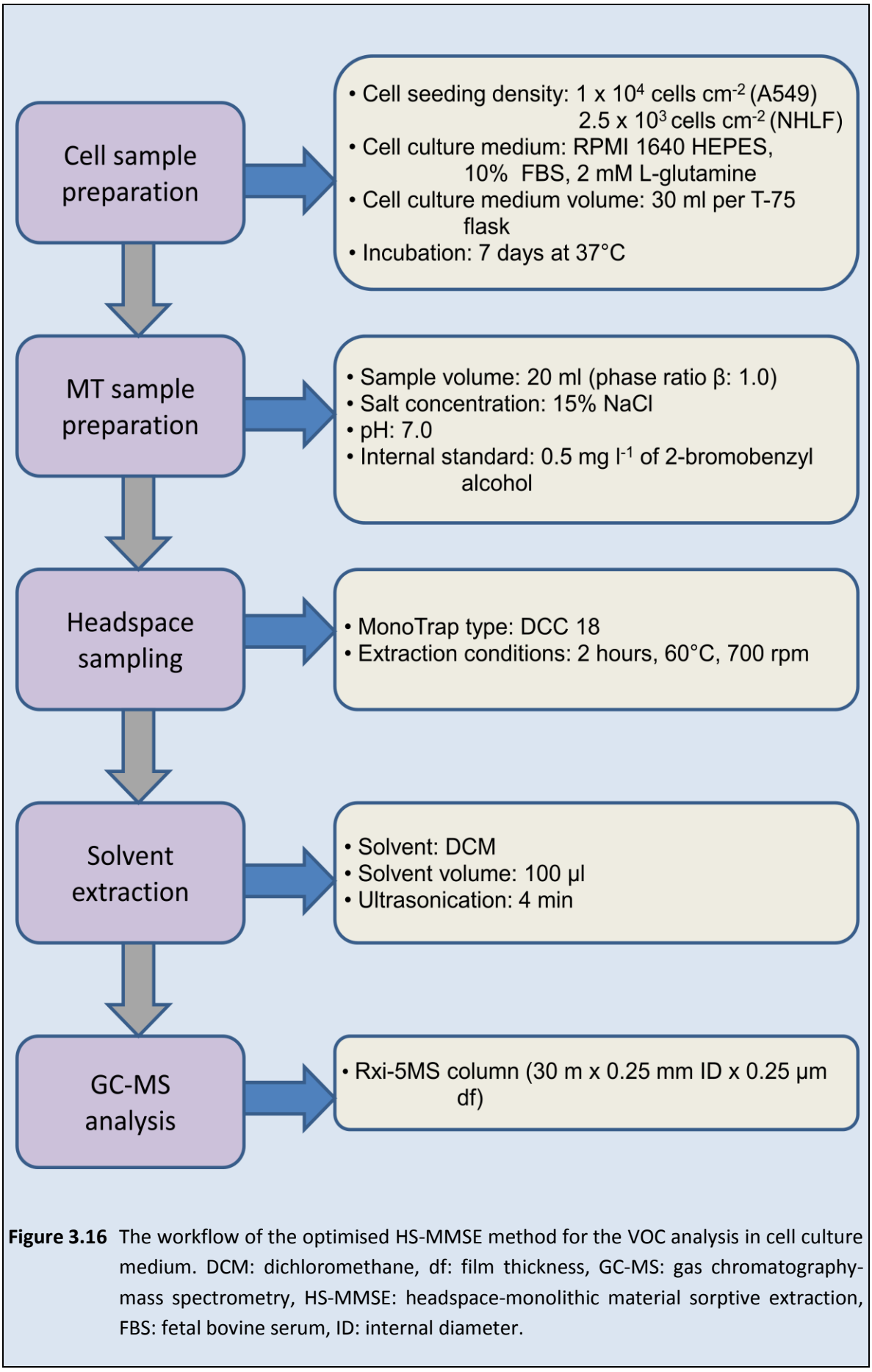

Figure 3.16 The workflow of the optimised HS-MMSE method for the VOC analysis in cell culture medium. DCM: dichloromethane, df: film thickness, GC-MS: gas chromatography-mass spectrometry, HS-MMSE: headspace-monolithic material sorptive extraction, FBS: fetal bovine serum, ID: internal diameter.

3.4.2 *Identification of detected VOCs*

In the MT experiment the two analysed cell lines, A549 and NHLF, were incubated for seven days. After one week, the cell culture medium from the flasks with the cells and from the flasks with the cell-free control medium were collected, fresh medium was added and the flasks were incubated for a further seven (A549) or 14 (NHLF) days. After the collection of the cell culture medium on the 14th day of incubation, the A549 cells in each sampled flask were tested for viability. The fibroblasts were incubated for an additional week because they had been reaching confluency five-six days later than the cancer cells. In this way the cell culture medium was collected from the proliferating cells (collection 1 for the A549 and collections 1 and 2 for the NHLF cell line) and from the confluent cells (collection 2 for the A549 and collection 3 for the fibroblasts). This experimental design addresses not only the differences in the VOC patterns between the cell samples and the medium controls, and between the samples of the two cell lines, but also the potential differences between the VOC patterns of growing cells and confluent cells of the same cell line.

Figure 3.17 shows a typical chromatogram of the pure cell culture medium obtained in FS mode in the MT experiment. The peaks of all identified VOCs (except siloxanes) are numbered in the chromatogram and their identification is presented above it. Peaks of compounds such as 2,3-dimethylheptane, dodecyl acrylate, 3-heptanol, geranyl acetone, 2-ethenyl-2-butenal, 2-methoxydimethylmethane, 4-methylundecane, 2-nitrophenol and 2,3,5-trimethyldecane were usually visible in the FS mode at S/N < 10 and, therefore, analysed in SIM mode. The RTs of these VOCs are marked with blue arrows.

1: Hexanal
2: Hexane,2,3,5-trimethyl-
3: Heptane,2,4-dimethyl-
4: Heptene, 2,4-dimethyl, 1-
5: Heptane,2,3-dimethyl-
6: Octane, 4-methyl-
7: Ethylbenzene
8: p-Xylene
9: 2-Fluorophenol (ISTD)
10: Cyclohexanol
11: Styrene
12: 2-Ethenyl-2-butenal
13: Cyclohexanone
14: 3-Heptanol
15: Heptanal
16: Ethanol, 2-butoxy-
17: Benzaldehyde,
18: Pentanoic acid, 2,4-dimethyl-3-oxo-, methyl ester
19: Heptanol
20: Phenol
21: Decane
22: Octanal
23: 2-Ethylhexanol
24: Benzyl alcohol
25: 1-Phenylethanol
26: Acetophenone
27: 1-Octanol
28: Octane, 2,3,6,7-tetramethyl-
29: α-Cumyl alcohol
30: Undecane
31: Nonanal
32: Undecane, 4-methyl-
33: 2-Nitrophenol
34: 1-Nonanol
35: 4-Decanol
36: Dodecane
37: Decanal
38: Benzene, 1,3-di-*tert*-butyl-
39: Tridecane
40: 2-Bromobenzyl alcohol (ISTD)
41: Decane, 2,3,5-trimethyl-
42: Propanoic acid, 2-methy- (2,2-dimethy-1-(2-hydroxy-1-methylethyl) propyl ester
43: Propanoic acid, 2-methyl-, 3-hydroxy-2,4,4-trimethylpentyl ester
44: 6-Dodecanol
45: Tetradecane
46: Dodecanal
47: Geranyl acetone
48: 2,6-Di-*tert*-butyl-1,4-benzoquinone
49: Pentadecane
50: Tridecanal
51: 2,4-Di-*tert*-butylphenol
52: 2-Tetradecanone
53: Propanoic acid, 2-methyl-, 1-(1,1-dimethyl)-2-methyl-1,3-propanediyl ester
54: Tetradecanal
55: 2-Methoxy-diphenylmethane
56: Dodecyl acrylate
57: 2-Pentadecanone
58: Pentadecanal
59: Benzene, 1,1'-(1,2-cyclobutanediyl)bis-, cis
60: 1-Nonadecene
61: Octadecane
62: Hexadecanal
63: Diisobutyl phthalate
64: Nonadecane
65: Heptadecanal
66: Eicosane
67: 2-Nonadecanone
68: Octadecanal
69: Dodecanoic acid, isooctyl ester

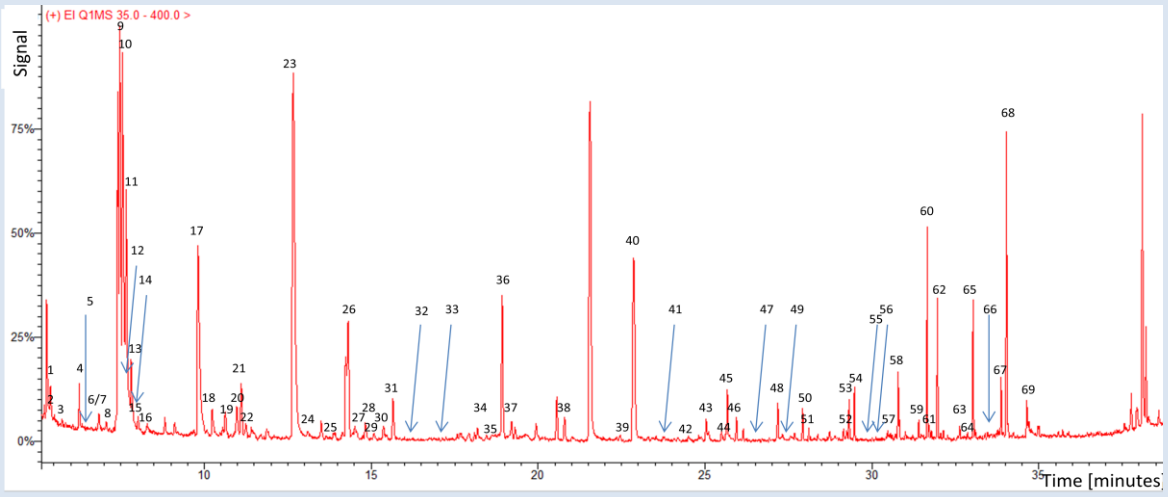

Figure 3.17 TIC (total ion chromatogram) of the pure medium control. Arrows indicate compounds usually visible in TIC at S/N < 10.

All the VOCs (except siloxanes) that were detected in the HS of the cell culture medium and identified in the MT experiment are presented in Table 3.3. The table shows the analytes in order of their RTs. The VOCs identified by confirming their RTs against the RTs of chemical standards are noted. The remaining compounds have been identified by

means of spectral match with the NIST library. The quantified ions used for the peak integration are also shown. There were a few VOCs present in the chromatogram that were identified, however the peak integration was not possible due to a lack of resolution or an unusual rise in the local baseline.

NIST library search

Over 110 peaks were present in each of the chromatograms obtained for the MT experiment. Among them 25 - 30 compounds (depending on the chromatogram) were identified as siloxanes and 69 were identified as analytes of other chemical groups. Around 10 VOCs could not be identified because of a low library match and therefore they were disregarded from the analysis (NIST library match factor < 700). Compounds that eluted closely and compounds detected at S/N < 10 in the TIC were identified by the use of SIM mode. Table 3.3 lists all the identified VOCs extracted from the cell culture medium with the use of MMSE. Among them, the main detected chemical groups were aliphatic hydrocarbons, alcohols and aldehydes. Some aromatic hydrocarbons, ketones, esters, ethers and phenols were also found. The molecular weights of the analysed compounds were in the range of 94 - 312 Da. The BPs of the detected VOCs were between 130°C and 354°C.

The compounds identified in the MT experiment listed in Table 3.3 had NIST match factors > 750. Where possible, the analyte reference standards were run to verify their identification. The majority of the compounds detected in the MT experiment had a match factor > 900. What is more, the first three or four hits for these compounds were the same, indicating an excellent match. Top five hits of search NIST libraries for tentatively assigned peaks are given in Table 3.3. For some peaks (the esters of pentanoic acid and propanoic acid) namely pentanoic acid, 2,4-dimethyl-3-oxo-, methyl ester; propanoic acid, 2-methy- (2,2-dimethy-1-(2-hydroxy-1-methylethyl) propyl ester and propanoic acid, 2-methyl-, 3-hydroxy-2,4,4-trimethylpentyl ester and propanoic acid, 2-methyl-, 1-(1,1-dimethyl)-2-methyl-1,3-propanediyl ester) NIST library search gave less than five hits.

The identification of the analytes in many studies of VOCs has been based on a library match only (Basanta *et al*., 2012a; Huang Y. *et al*., 2016; Huang Z. *et al*., 2016; Phillips M. *et al*., 1999a; 1999b; 2008; 2013; Tisch *et al*., 2013; Wang Y. *et al*., 2012). While this is a very reliable way of identifying peaks, sometimes it can be very misleading. For

example, tridecane appeared 7th on the list of the best hits, for the peak with the RT 22.50 min, with a match factor of > 850. Its homologous compounds, such as heptadecane and pentadecane, were higher on the hit list than tridecane and with better match factors (heptadecane was 1st). Without confirmation of the RT against the chemical standard, it would not be possible to correctly identify tridecane.

The compounds that were assigned tentatively, but with a high probability are marked in bold in Table 3.3. These compounds occupied the 1st position only on the hit list or the 1st and 2nd - 4th positions on the list. These VOCs had relatively higher match factors in comparison to other best hits and/or the BPs of other best hits did not fit into the pattern. These compounds were: benzyl alcohol; 2-butoxyethanol; diisobutyl phthalate; 2,4-dimethylheptane; 2,4-dimethyl-1-heptene; 2,3-dimethylheptane; dodecanal; dodecyl acrylate; 4-methyloctane; 2,4-di-*tert*-butylphenol; 2-ethenyl-2-butenal; 6-dodecanol; heptanol; 3-heptanol; 4-methylundecane; 1-nonanol; 1-octanol; 1,3-di-*tert*-butylbenzene; 1,1'-(1,2-cyclobutanediyl)bis-, cis- benzene; propanoic acid, 2-methyl-, 1-(1,1-dimethyl)-2-methyl-1,3-propanediyl ester; 2-tetradecanone; 2-pentadecanone; tridecanal; tetradecanal and 2,3,5-trimethylhexane. The possible alternative identification for these compounds is given below the name of the VOC. The compounds that were assigned tentatively, but with a lower probability, are underlined in Table 3.3. These compounds were first on the hit list, having the highest match factor, but they occupied first position only and the other best hits had a similar match factor value. These compounds were: 2-methoxy-diphenylmethane; 2-nonadecanone; 1-nonadecene; pentanoic acid, 2,4-dimethyl-3-oxo-, methyl ester; 2,3,6,7-tetramethyloctane; 2,3,5-trimethyldecane and p-xylene. Although it was also the case for some C12 - C18 aldehydes (pentadecanal; hexadecanal and heptadecanal), their assigned identity fits into the BP pattern of C6 - C10 aldehydes confirmed with standards.

Table 3.3 Volatile organic compounds detected in the MonoTrap experiment listed in retention time order. Top five hits of search NIST libraries for tentatively assigned peaks are given. Number of top positions (out of 5) occupied by a particular hit (if > 1) is shown in the brackets after the factor match for tentatively assigned VOCs. BP: boiling point at 760 mmHg; CAS: chemical abstract service; FM: NIST library search factor match; ID: identification; ISTD: internal standard; LOD: limit of detection (S/N < 3); MW: molecular weight; NR: not resolved; RS: compound identified through the comparison of the retention time and mass spectrum with a reference standard; RT: retention time; QI: quantification ion; T: tentatively assigned compounds; TIC: total ion chromatogram; WB: water blank; ✓: compound also detected in a "water control" sample. **Bold:** compound that was assigned tentatively, but with a high probability i.e. it occupied the 1st position only or 1st and 2nd - 4th position on the hit list, had relatively higher factor match in comparison to other best hits and/or BP of other best hits did not fit into the pattern. Underlined: compound that was assigned tentatively, but with a lower probability i.e. it had the highest match factor but it occupied 1st position only, other hits had similar match factor and their BP fitted into the pattern.

No.	RT [min]	Volatile organic compound	WB	ID	FM	QI	CAS number	MW	BP [°C]
1	5.40	Hexanal	✓	RS	> 900	56, 57	66-25-1	100	130-131
2	5.55	**Hexane,2,3,5-trimethyl-**	✓	T	> 900	57, 71	1069-53-0	128	132 ± 7
		"			> 850(2)		"	"	"
		Heptane,2,3-dimethyl-			> 850		3074-71-3	128	141 ± 7
		Pentane, 3-ethyl-2,4-dimethyl-			> 850		1068-87-7	126	136 ± 7
3	5.75	**Heptane, 2,4-dimethyl-**	✓	T	> 900	57, 71	2213-23-2	128	134 ± 7
		"			> 850(2)		"	"	"
		Hexane, 2,4-dimethyl-			> 800		589-43-5	114	109 ± 7
4	6.25	**Heptene, 2,4-dimethyl, 1-**	✓	T	> 900(2)	55, 126	19549-87-2	126	137 ± 7
		1-Hexanol, 5-methyl-			> 750		627-98-5	116	161 ± 8
		1-Heptene, 5-methyl-			> 750		13151-04-7	112	112 ± 7
		1-Fluorooctane			> 750		463-11-6	132	142 ± 3
5	6.60	**Heptane, 2,3-dimethyl-**	✓	T	> 900(2)	57, 69	3074-71-3	128	141 ± 7
		Decane, 5,6-dimethyl-			> 850(2)		1636-43-	170	204 ± 7
		Hexane, 2,3,5-trimethyl-			> 850		1069-53-0	128	132 ± 7
6	6.70	**Octane, 4-methyl-**	✓	T	> 900(2)	85, 128	2216-34-4	128	142 ± 7
		"			> 850		"	"	"
		Hexane, 3-ethyl-			> 850		619-99-8	114	118 ± 7
		Hexane, 2,3,4-trimethyl-			> 800		921-47-1	128	139 ± 7

Table 3.3 Cont'd

No.	RT [min]	Volatile organic compound	WB	ID	FM	QI	CAS number	MW	BP [°C]
7	6.70	Ethylbenzene		RS	> 900	91, 106	100-41-4	106	136 ± 3
8	6.80	p-Xylene o-Xylene "		T	> 850(2) > 850(2) > 800	91, 106	106-42-3 95-47-6 "	106 106 "	137-138 144 ± 1 "
9	7.50	2-Fluorophenol		ISTD	-	92, 112	367-12-4	112	150-152
10	7.60	Cyclohexanol		RS	> 800	100	108-93-0	100	160-161
11	7.70	Styrene	✓	RS	> 900	78, 104	100-42-5	104	145-146
12	7.70	**2-Ethenyl-2-butenal** 2,4-Dimethylfuran 3-Ethylcyclopentene		T	> 800(2) > 750(2) > 700	95, 96	20521-42-0 3710-43-8 694-35-9	96 96 96	148 ± 9 95 ± 9 98 ± 0
13	7.80	Cyclohexanone		RS	> 900	55, 98	108-94-1	98	154-156
14	7.90	**3-Heptanol** " " " 3-Hexanol, 5-methyl-	✓	T	> 900 > 850(3) > 750	59, 87	589-82-2 " 623-55-2	116 " 116	157 ± 0 " 153 ± 8
15	8.00	Heptanal		RS	> 850	44, 70	111-71-7	114	153 ± 1
16	8.30	**Ethanol, 2-butoxy-** Ethylene glycol monoisobutyl ether 1,2-Dibutoxyethane		T	> 850(3) > 800 > 750	NR	111-76-2 4439-24-1 112-48-1	118 118 174	171-173 159 ± 8 204 ± 8
17	9.80	Benzaldehyde		RS	> 950	TIC	100-52-7	106	178-179
18	10.20	Pentanoic acid, 2,4-dimethyl-3-oxo-, methyl ester Pentane,1-butoxy- sec-Butyl nitrite		T	> 850 > 800 > 700	43, 71	5942-51-7 18636-66-3 924-43-6	158 144 103	193 ± 8 164 ± 8 70.5 ± 9
19	10.30	**Heptanol** " " " Formic acid, heptyl ester Heptyl pentafluoropropanoate		T	> 850 > 800(2) > 750 > 750	56, 70	111-70-6 " 112-23-2 959033-16-0	116 " 144 262	175-176 " 177-178 211 ± 35
20	11.00	Phenol		RS	> 850	94, 66	108-95-2	94	181± 0
21	11.10	Decane	✓	RS	> 900	57, 112	124-18-5	142	172-174
22	11.40	Octanal	✓	RS	> 900	56, 69	124-13-0	128	169-171

Table 3.3 Cont'd

No.	RT [min]	Volatile organic compound	WB	ID	FM	QI	CAS number	MW	BP [°C]
23	12.70	2-Ethylhexanol		RS	> 850	TIC	104-76-7	130	185 ± 0
24	13.10	**Benzyl alcohol**		T	> 900	79, 108	100-51-6	108	205 ± 0
		" " "			> 850(2)		"	"	"
		4-Nitrophenyl N-[(benzyloxy)carbonyl]glycylglycinate			> 800		13574-81-7	387	676 ± 55
		Nα,Nω-Dicarbobenzoxy-L-arginine			> 800		53934-75-1	442	-
25	14.10	1-Phenylethanol		RS	> 950	79, 107	98-85-1	122	204 ± 1
26	14.20	Acetophenone		RS	> 900	105, 120	98-86-2	120	202 ± 0
27	14.50	**1-Octanol**		T	> 900(2)	55, 56	111-87-5	130	193-195
		" " "			> 850(2)		"	"	"
		Cyclopropane, pentyl-			> 850		2511-91-3	112	127 ± 3
28	15.10	α-Cumyl alcohol		RS	> 850	105, 121	617-94-7	136	215 ± 0
29	15.20	Octane, 2,3,6,7-tetramethyl-		T	> 880	71, 127	52670-34-5	170	200 ± 7
		Octane, 6-ethyl-2-methyl-			> 860		62016-19-7	156	181 ± 7
		Sulfurous acid, nonyl pentyl ester			> 850		no CAS	278	345 ± 11
		Decane, 2,4,6-trimethyl-			> 850		62108-27-4	184	212 ± 7
		Tridecane			> 850		629-50-5	184	235 ± 0
30	15.40	Undecane	✓	RS	> 800	57, 112	1120-21-4	156	196 ± 0
31	15.70	Nonanal	✓	RS	> 900	57, 82	124-19-6	142	191 ± 3
32	15.95	**Undecane, 4-methyl-**		T	> 850(2)	71, 127	2980-69-0	170	209 ± 7
		Sulfurous acid, nonyl pentyl ester			> 850(2)		no CAS	278	345 ± 11
		Tridecane			> 800		629-50-5	170	235 ± 0
33	16.70	2-Nitrophenol		RS	> 800	109, 139	88-75-5	139	214-216
34	18.20	**1-Nonanol**		T	> 900(2)	56, 147	143-08-8	144	214-216
		1-Decanol			> 850(2)		112-30-1	158	230-232
		1-Undecanol			> 800		112-42-5	172	243 ± 0
35	18.70	**4-Decanol**		T	> 800	83, 140	2051-31-2	158	210-211
		" " "			> 700(3)		"	"	"
		Dodecyl acrylate			> 700		2156-97-0	240	306 ± 11
36	18.90	Dodecane		RS	> 900	57, 170	112-40-3	170	216 ± 1

Table 3.3 Cont'd

No.	RT [min]	Volatile organic compound	WB	ID	FM	QI	CAS number	MW	BP [°C]
37	19.20	Decanal	✓	RS	> 850	57, 82	112-31-2	156	208 ± 1
38	20.80	**Benzene, 1,3-di-*tert*-butyl**		T	> 900(3)	TIC	1014-60-4	190	224 ± 10
		Benzene, 1,4-di-*tert*-butyl			> 800(2)		1012-72-2	190	236 ± 0
39	22.50	Tridecane		RS	> 850	57, 85	629-50-5	184	235 ± 0
40	22.80	2-Bromobenzyl alcohol		ISTD	-	TIC	18982-54-2	187	262 ± 15
41	24.10	Decane, 2,3,5-trimethyl		T	> 850	71, 141	62238-11-3	184	217 ± 7
		Dodecane, 4-methyl			> 800		117-97-1	184	228 ± 7
		Heptadecane, 2,4,10,14-tetramethyl			> 800		18344-37-1	296	257 ± 9
		Hexadecane			> 800		544-76-3	226	287 ± 3
		Pentadecane			> 800		629-62-9	212	269-270
42	24.50	**Propanoic acid, 2-methy- (2,2-dimethy-1-(2-hydroxy-1-methylethyl) propyl ester**	✓	T	> 900	TIC	74367-33-2	216	249± 13
		2,2,4-Trimethyl-1,3-pentadienol diisobutyrate			> 800		6846-50-0	286	280 ±0
		Propanoic acid, 2-methyl-, 2-(hydroxymethyl)-1-propylbutyl ester			> 700		74367-32-1	216	259 ± 13
43	25.10	**Propanoic acid, 2-methyl-, 3-hydroxy-2,4,4-trimethylpentyl ester**	✓	T	> 850	71, 89	74367-34-3	216	249 ± 13
		Propanoic acid, 2-methyl-, 2,2-dimethyl-1-(2-hydroxy-1-methylethyl)propyl ester			> 750		74367-33-2	216	249 ± 13
		Propanoic acid, 2-methyl-, 2-ethyl-3-hydroxyhexyl ester			> 700(2)		74367-31-0	216	259 ± 13
44	25.50	**6-Dodecanol**		T	> 800	TIC	6836-38-0	186	246 ± 8
		1-Nitrododecane			> 700		16891-99-9	215	285 ± 3
		5-Tetradecanol			> 700		21078-83-1	214	276 ± 8
		8-Heptadecanol			> 700		219-820-7	256	318 ± 10
		7-Tetradecanol			> 700		3981-79-1	214	276 ± 8
45	25.70	Tetradecane		RS	> 900	TIC	629-59-4	198	253 ± 1
46	26.00	**Dodecanal**	✓	T	> 900(2)	57, 82	112-54-9	184	242 ± 3
		Tridecanal			> 850		10486-19-8	198	257 ± 3
		Tetradecanal			> 850		124-25-4	212	272 ± 3
		E-2-Tetradecen-1-ol			> 850		75039-86-0	212	294 ± 8
47	26.90	Geranyl acetone		RS	> 900	43, 151	3796-70-1	194	256 ± 2

Table 3.3 Cont'd

No.	RT [min]	Volatile organic compound	WB	ID	FM	QI	CAS number	MW	BP [°C]
48	27.20	2,6-Di-*tert*-butyl-1,4-benzoquinone		RS	> 900	TIC	719-22-2	220	285 ± 15
49	27.80	Pentadecane		RS	> 800	<LOD	629-62-9	212	271 ± 3
50	27.95	**Tridecanal**	✓	T	> 900(2)	TIC	10486-19-8	198	257 ± 3
		Pentadecanal			> 850		2765-11-9	226	285 ± 3
		Tetradecanal			> 850		124-25-4	212	272 ± 3
		Octadecanal			> 850		638-66-4	268	321 ± 5
51	28.10	**2,4-Di-*tert*-butylphenol**		T	> 900(2)	191, 206	96-76-4	206	266 ± 9
		"			> 850		"	"	"
		2,5-Di-*tert*-butylphenol			> 850		5875-45-6	206	283 ± 9
		2,6-Di-*tert*-butylphenol			> 800		128-39-2	206	252 ± 9
52	29.30	**2-Tetradecanone**		T	> 800(2)	58, 59	2345-27-9	212	279 ± 3
		2-Pentadecanone			> 700		2345-28-0	226	293 ± 3
		2-Dodecanone			> 700(2)		6175-49-1	184	248 ± 3
53	29.35	**Propanoic acid, 2-methyl-, 1-(1,1-dimethyl)-2-methyl-1,3-propanediyl ester**	✓	T	> 900	TIC	74381-40-1	286	325 ± 15
		Pentanoic acid, 2,2,4-trimethyl-3-carboxyisopropyl, isobutyl ester			> 750		no CAS	286	270 ± 8
54	29.50	**Tetradecanal**	✓	T	> 900(2)	57, 82	124-25-4	212	272 ± 3
		Octadecanal			> 850		638-66-4	268	321 ± 5
		Dodecanal			> 850		112-54-9	184	242 ± 3
		Pentadecanal			> 850		2765-11-9	226	285 ± 3
55	30.00	2-Methoxy-diphenylmethane		T	> 900	165, 198	883-90-9	198	291 ± 9
		3-Methoxy-diphenylmethane			> 850		23450-27-3	198	302 ± 11
		p-Benzylanisole			> 750		834-14-0	198	302 ± 11
		1,3,5-Cycloheptatriene,7-(4-methoxyphenyl)-			> 750		29304-87-8	198	317 ± 21
		Azulene, 1,4-dimethyl-7- (1-methylethyl)-			> 700		489-84-9	198	305 ± 22
56	30.55	**Dodecyl acrylate**		T	> 900(2)	55, 127	2156-97-0	240	306 ± 11
		2-Propenoic acid, tridecyl ester			> 850		221-351-8	254	323 ± 11
		2-Propenoic acid, pentadecyl ester			> 800		43080-23-5	282	355 ± 11
		3-(Prop-2-enoyloxy)tridecane			> 800		no CAS	254	317 ± 11

Table 3.3 Cont'd

No.	RT [min]	Volatile organic compound	WB	ID	FM	QI	CAS number	MW	BP [°C]
57	30.60	**2-Pentadecanone**		T	> 900	58, 59	2345-28-0	226	293 ± 3
		" " " "			> 850		"	"	"
		2-Nonadecanone			> 850		629-66-3	283	344 ± 5
		2-Hexadecanone			> 750		8787-63-8	240	318 ± 0
		2-Heptadecanone			> 750		2922-51-2	254	320 ± 5
58	30.80	**Pentadecanal**	✓	T	> 900	57, 82	2765-11-9	226	285 ± 3
		Tridecanal			> 900		10486-19-8	198	257 ± 3
		Octadecanal			> 900		638-66-4	268	321 ± 5
		Hexadecanal			> 850		629-80-1	240	298 ± 3
		Tetradecanal			> 850		124-25-4	212	272 ± 3
59	31.40	**Benzene, 1,1'-(1,2-cyclobutanediyl)bis-, cis-**		T	> 900(2)	78, 104	7694-30-6	208	309 ± 22
		Cyclobutane, 1,2-diphenyl-			> 850(2)		7694-30-6 & 20071-09-4	208	309 ± 22
		Benzene, 1,1'-(1,2-cyclobutanediyl)bis-, trans-			> 800		20071-09-4	208	309 ± 22
60	31.65	1-Nonadecene	✓	T	> 950(2)	NR	18435-45-5	266	328 ± 5
		3-Eicosene, (E)-			> 950		74685-33-9	280	354 ± 9
		5-Eicosene, (E)-			> 900		74685-30-6	280	354 ± 9
		E-15-Heptadecenal			> 900		no CAS	252	340 ± 11
61	31.70	Octadecane	✓	RS	> 800	NR	593-45-3	254	316 ± 5
62	32.00	**Hexadecanal**	✓	T	> 900	NR	629-80-1	240	298 ± 3
		Tetradecanal			> 900 (2)		124-25-4	212	272 ± 3
		Octadecanal			> 850		638-66-4	268	321 ± 3
		E-2-Tetradecen-1-ol			> 850		no CAS	212	294 ± 8
63	32.65	**Diisobutyl phthalate**	✓	T	> 950	57, 149	84-69-5	278	337 ± 10
		" " " "			> 900(2)		"		
		Phthalic acid, isobutyl 4-octyl ester			> 900		no CAS	334	342 ± 10
		Butyl ethylhexyl phthalate			> 850		85-69-8	334	360 ± 10
64	32.70	Nonadecane		RS	> 750	57, 85	629-62-9	268	330 ± 5

Table 3.3 Cont'd

No.	RT [min]	Volatile organic compound	WB	ID	FM	QI	CAS number	MW	BP [°C]
65	33.00	**Heptadecanal**	✓	T	> 850	57, 82	629-90-3	254	310 ± 5
		Octadecanal			> 800		628-66-44	268	321 ± 5
		Z-2-Octadecen-1-ol			> 800		no CAS	268	351 ± 10
		Oxirane, hexadecyl-			> 800		7390-81-0	268	358 ± 0
66	33.80	Eicosane		RS	-	< LOD	112-95-8	286	343 ± 0
67	33.90	2-Nonadecanone	✓	T	> 870	58, 59	629-66-3	283	344 ± 5
		2-Pentadecanone			> 850		2345-28-0	226	293 ± 3
		2-Dodecanone			> 850				248 ± 3
		2-Tridecanone			> 850				264 ± 3
		2-Tetradecanone			> 850				279 ± 3
68	34.00	**Octadecanal**	✓	T	> 900(3)	57, 82	638-66-4	268	321 ± 5
		Hexadecanal			> 900		629-80-1	240	298 ± 3
		Oxirane, hexadecyl-			> 900		7390-81-0	268	358 ± 0
69	34.80	**Dodecanoic acid, isooctyl ester**	✓	T	> 820	70, 112	84713-06-4	312	354 ± 0
		Lauric acid, 2-methylbutyl ester			> 700		9385-53-3	270	312 ± 10
		Isoamyl laurate			> 700(3)		6309-51-9	270	312 ± 10

Boiling point

Selectivity of the column is the ability of the stationary phase to separate two compounds by different interactions via exploring differences in their physical and chemical properties. For polysiloxane columns there are three main interactions possible: dispersion, dipole and hydrogen bonding. The dominant interaction for polysiloxane stationary phases is dispersion, i.e. the volatility of the compound. The lower the BP of the compound, the shorter is its RT. This is the case for compounds with the same functional groups and in the homologous series and is a useful principle to help with compound identification when the NIST library match is used. However, when the sample is a mixture of different groups of chemical compounds, this major effect can be altered by the polar interactions and/or hydrogen bonding between the compound and the stationary phase, and the BP order often fails (Sliepcevich and Gelosa, 2009). As the Rxi-5MS is considered as effectively non-polar column, the compounds in the homologous series, as expected, were separated by their BPs. When it comes to the RT due to different chemical groups, the order of the BP is not so useful anymore and for example cyclohexanol with a BP of 161°C eluted at 7.60 min, earlier than heptanal with a BP of 153°C which eluted at 8.00 min.

In such mixed samples Kovats Retention Index might be very useful to help with the identification of the compounds. It is based on the relative retentions of the analytes to 'marker compounds'. Kovats used homologous series of n-alkanes as marker compounds and assigned a value of 100 to C1 alkane, 200 to C2 alkane and so on. The series of n-alkanes are run under the same conditions as the compound of interest and then its index value is calculated. Kovats retention index tables for different stationary phases and temperatures were developed (McNair and Miller, 1998). Kovats Retention Index was not applied in the MT experiment because of the small variations in the RTs caused by manual injection. An autosampler could not be used due to the small amounts of the sample in the GC vial (< 80 µl).

Some VOCs in Table 3.3 can be disregarded as an alternative identification because the BPs do not fit the pattern. For example, it is rather unlikely that the peak eluting at 10.20 min is sec-butyl nitrite, as its BP of 70.5°C is too low. The BPs of two other hits for this peak (pentanoic acid, 2,4-dimethyl-3-oxo-, methyl ester and 1-butoxypentane) fit better into the pattern.

Co-eluting peaks

Some peaks were found to elute very closely to each other. This was the case for ethylbenzene and 4-methyloctane; 2-fluorophenol, cyclohexanol and styrene; styrene, 2-ethenyl-2-butenal and cyclohexanone; cyclohexanone and 3-heptanol; acetophenone and methyltris(trimethylsiloxy)silane; octadecane and 2-nonadecene. Co-eluting peaks can be identified by the selection of mutually exclusive ions present for each analyte, so they can be quantified. The ions monitored for the compounds detected in the MT experiment are presented in Table 3.3. The ions quantified for acetophenone were m/z 120 and m/z 105 which were not present in the mass spectra of siloxanes identified as best hits for the co-eluting peak by the NIST library. Octadecane and 1-nonadecene could not be properly quantified because of the lack of exclusive ions and therefore these peaks were excluded from the MT analysis. 2-Butoxyethanol and hexadecanal could not be properly quantified because of the co-eluting unidentified peaks and therefore the peaks were also excluded from the MT analysis.

Rxi-5MS columns and similar phases are commonly used in the HS analysis of VOCs in various fields, including breath and *in vitro* analysis (Poli *et al.*, 2010; Pyo *et al*., 2008; Song *et al*., 2010; Wang C. *et al.*, 2014; Zhang Y. *et al*., 2014). However, because this is a general purpose column, more selective stationary phases could help to achieve better separation of the co-eluting peaks.

Blanks

Two types of blank were conducted during the MT experiment:

(i) blank GC runs of the solvent between the sample runs;
(ii) blank water sample extractions *i.e*. water samples prepared as cell culture medium samples and subjected to MMSE under the same conditions.

Regarding the first type of blank, no carry-overs were observed for any of the analytes during the MT experiment.

One third of the detected VOCs in the MT experiment were also detected in water blanks (Tab. 3.3). Their origin in water blanks is not known as they may be released from the water, from the sample vial, from the vial septum, from the MT and/or may originate from the ambient air during the experiment.

Deionised autoclaved water was used for water blanks. The water was autoclaved in GL 45 reagent bottles. A higher temperature could potentially result in release of contaminant VOCs from the plastic lid. The MT sampling vials were cleaned and baked out before use. The baking out oven temperature of 70°C, however, might have been too low to get rid of all the contaminants absorbed during shipping. Finally, the MT sampling vials may absorb contaminant VOCs from the lab air/oven and flushing them with dry air/nitrogen before introducing the sample may help to further reduce the contaminant background. Running an experiment looking at the ambient air only, sampling de-ionised water that has not been autoclaved and flushing MT sample vials with nitrogen before sampling are steps that could help in identifying the origins of contaminant VOCs in future experiments.

MonoTrap^{TM} manuals state that the background of a blank MT is very low and they can be used without conditioning (GL Sciences, 2014). However, conditioning was performed on MTs in some studies. For example, Jang *et al*. (2011) applied a procedure of washing MT disks in DCM and baking them at 100°C for 30 min prior to use. Others baked MT rods at 250°C for 30 or 60 min (Wu *et al*., 2016; Zhao *et al*., 2015). As it is not known how baking could potentially affect adsorption efficiency of the traps, no conditioning was applied in the MT experiment. Nevertheless, conditioning should be introduced as another step in the MT method development. In addition, a third type of a blank could be introduced in order to find out about a potential origin of some VOCs from the MTs: solvent extraction of a fresh MT (without previous sampling) from each new batch.

3.4.3 Precision

Table 3.4 presents the intra-batch precision (within the same sample; n = 3) and inter-batch precision (between the different samples; n = 9) expressed as % RSD calculated for each VOC analysed in each collection of the cell samples and their controls for the MT experiment. The VOCs are listed in RT order. It must be noted that the inter-batch precision is not the inter-day precision, as the latter should be determined by GC-MS analysis of the same sample run on the different days (García - Arieta, 2009). Calculations for inter-batch precision in Table 3.4 for dodecyl acrylate (collection 3 for NHLFs) and diisobutyl phthalate (collection 1 for NHLFs) were performed for n = 7, and for dodecyl acrylate (collections 1 and 2 for NHLFs), 2-nonadecanone and octadecanal (collection 2 for NHLFs) were performed for n = 8, as peaks for these VOCs in some chromatograms were not resolved.

In general, the intra-batch precision was better than the inter-batch precision for most of the analytes, which was to be expected as more variables were involved between the different samples. For compounds such as acetophenone; benzaldehyde; 1,3-di-*tert*-butylbenzene; 1,1'-(1,2-cyclobutanediyl) bis-,cis-benzene; cyclohexanone; dodecane; dodecanal; 2-ethylhexanol; 1-heptanol; hexanal; nonanal; octanal; 1-octanol; propanoic acid, 2-methyl-, 1-(1,1-dimethyl)-2-methyl-1,3-propanediyl ester; styrene and tetradecane the intra-batch precision was good, < 20% for all the collections. Other VOCs yielded precision < 25% for all the collections: cyclohexanol; 2,6-di-*tert*-butyl-1,4-benzoquinone; decane; decanal; 6-dodecanol; 2-ethenyl-2-butenal; 3-heptanol; pentanoic acid, 2,4-dimethyl-3-oxo-, methyl ester; 2-methoxy-diphenylmethane; propanoic acid, 2-methy-(2,2-dimethy-1-(2-hydroxy-1-methylethyl) propyl ester; propanoic acid, 2-methyl-, 3-hydroxy-2,4,4-trimethylpentyl ester; phenol; 1-phenylethanol; 2,3,6,7-tetramethyloctane; tridecanal; tetradecanal and undecane. The higher values of the inter-batch precision for these VOCs reflected most likely variability in cell culture sample preparation (different cell passage used, differences in cell seeding density and different medium batches used that can differ between each other in their VOC content and concentration) and in MT sample preparation (salt addition, pH adjustment, variability in ISTD stock solution preparation etc.).

A poorer intra-batch precision, 20 - 50%, was yielded for some VOCs probably because of their relatively low signal intensity. This was the case for benzyl alcohol; α-cumyl alcohol; 4-decanol; 2,4-dimethylheptane; 2,4-di-*tert*-butylphenol; 2,4-dimethyl-1-heptene; dodecyl acrylate; ethylbenzene; geranyl acetone; 4-methyloctane; 4-methylundecane; 2-nitrophenol; tridecane; 2,3,5-trimethylhexane; 2,3,5-trimethyldecane and p-xylene. The same compounds had poor inter-batch precision, for some collections > 100%. Values of inter-batch precision, > 35%, obtained for diisobutyl phthalate; dodecanoic acid, isobutyl nonyl ester; heptadecanal; 2-nonadecanone; octadecanal; pentadecanal and 2-pentadecanone were caused by reasons other than low S/N ratio, as they were relatively intensive peaks in the chromatogram.

In the MT experiment the injections were performed manually. This might be a cause of the variability in the size of peak areas, especially for the peaks with low signal intensity, resulting in poorer precision (Barwick, 1999). However, it can be seen that both inter- and intra-batch precision are good for some VOCs and worse for others (Tab. 3.4). In general, the RSD values are poorer with the increase in BP. The injector port temperature may

have an impact on precision particularly for higher BP compounds. A general rule is to set the injector port temperature around 50°C higher than the sample BP (McNair and Miller, 1998). As the MT sample was a mixture of VOCs, it would mean setting the injector port to 50°C higher than the highest BP *i.e.* ~400°C. Regardless of the fact that setting the injector temperature > 350°C would be above the maximum operating temperature of the GC column used and that such a high temperature may cause thermal degradation of some compounds or their chemical rearrangements (McNair and Miller, 1998), the injector port temperature was not optimised during the MT method development as high-boiling compounds (> 200°C) were not identified at that time because of the lower intensity and/or poor resolution of these peaks. The method development and the MT analysis of the cell samples were performed at different times, which involved disconnecting the GC column. This could have a big impact on the sensitivity of the method, resulting in the VOCs showing lower intensity in the MT method development when compared to the MT experiment.

A literature review of SPME methodology for *in vitro* studies of VOCs showed that injector temperature was most commonly set to 250°C (Schmidt and Podmore, 2015b) and this temperature was used for the MT experiment. Therefore, the VOCs with a BP > 250°C may have accumulated in the GC inlet, although no carry-overs of any VOCs were observed in the blank runs between the sample injections for the MT experiment.

The poorer precision obtained for lower and higher BP VOCs may be also due to only one ISTD being used in the MT experiment. It is a common practice in GC-MS analysis to employ ISTDs that elute at the beginning, middle and end of the chromatogram which have a similar molecular structure and physical properties to the analysed compounds, in order to give similar responses from the detector. The ideal solution is the use of stable isotope-labelled versions of the compounds of interest as ISTDs (Bouchonnet, 2013). 2-B eluted approximately in the middle of the chromatogram (22.80 min). 2-F, which was meant to be used as an ISTD for the earlier eluting peaks, appeared to co-elute with cyclohexanol. This is probably the reason why the precision obtained with 2-F as an ISTD was poorer than the precision obtained with 2-B (data not shown). Moreover, 2-B as an alcohol may differ in response to other chemical group compounds, adding to the poorer precision obtained for some VOCs. Ideally, isotopically-labelled analogues of the targeted VOCs should be used as ISTDs, however they are not available for all the compounds and their cost is high (Bouchonnet, 2013).

Finally, compounds with a high BP may remain in the needle because of fractionation of the sample from the needle, as losses on the needle wall rise with the BP of the analyte. Losses of 80% are common (Grob, 1994). The use of a hot needle for sample injection reduce sample discrimination (Barswick, 1999). However, this could pose problems for the precision of the injection of low BP analytes, as the evaporation of solutions often begins in the needle. Precision could be lost via injection of varying amounts of the solution, as the volume read on the barrel would be lower than the real volume injected (Grob, 1994).

Table 3.4 Evaluation of the intra-batch and inter-batch precision, expressed as % relative standard deviation (RSD) of the monolithic material sorptive extraction - gas chromatography - mass spectrometry (MMSE-GC-MS) method used in the MonoTrap experiment. Col: collection; Intra: intra-batch precision RSD (%) for n= 3; inter: inter-batch precision RSD (%) for n = 9, n = 7 or n = 8; ND: not detected. Sample: medium incubated with cells at 37°C for 7days; Control: cell-free medium incubated at 37°C for 7 days.

Volatile organic compound	RSD (%)	A549 Col 1		A549 Col 2		NHLF Col 1		NHLF Col 2		NHLF Col 3	
		Sample	Control	Sample	Control	Sample	Control	Sample	Control	Sample	Control
Hexanal	Intra	7.7	4.2	9.4	15.2	11.9	13.5	5.7	9.8	5.7	10.0
	Inter	21.7	11.0	18.2	12.1	8.4	40.8	4.2	19.5	37.2	31.9
Hexane, 2,3,5-trimethyl	Intra	38.9	18.9	18.6	ND	ND	ND	ND	ND	ND	ND
	Inter	57.6	17.2	109.9	ND	ND	ND	ND	ND	ND	ND
Heptane, 2,4-dimethyl	Intra	10.2	18.9	21.3	9.5	ND	ND	20.7	20.2	12.8	20.2
	Inter	67.2	17.2	145.0	102.5	ND	ND	44.1	39.9	47.2	45.3
Heptene, 2,4-dimethyl, 1-	Intra	27.1	25.1	3.8	16.4	ND	ND	20.8	12.4	2.1	29.8
	Inter	70.0	34.9	-	-	ND	ND	37.6	15.8	-	-
Heptane, 2,3-dimethyl	Intra	27.1	37.6	1.7	7.4	ND	ND	ND	ND	ND	ND
	Inter	72.7	38.2	-	28.7	ND	ND	ND	ND	ND	ND
Octane, 4-methyl	Intra	11.2	13.4	39.4	35.0	ND	ND	29.3	14.0	39.9	30.4
	Inter	77.9	51.4	105.5	83.5	ND	ND	24.0	19.2	37.6	51.8
p-Xylene	Intra	11.4	11.4	13.4	38.3	23.2	11.7	8.6	12.9	20.6	10.7
	Inter	46.5	56.0	40.0	47.5	31.2	23.8	9.0	45.8	74.4	31.0
Ethylbenzene	Intra	13.3	3.7	9.8	32.7	4.2	6.4	7.8	11.4	20.0	14.9
	Inter	16.2	13.3	32.8	23.8	9.2	19.0	6.5	23.5	15.3	12.3
2-Ethenyl-2-butenal	Intra	ND	15.9	ND	22.2	20.1	10.3	23.3	6.6	10.7	11.4
	Inter	ND	26.7	ND	19.1	78.9	68.3	41.9	49.3	-	65.8
Cyclohexanol	Intra	18.6	7.6	3.9	14.3	13.4	16.2	14.7	13.5	19.3	22.2
	Inter	18.2	28.0	32.6	6.4	10.1	35.3	26.9	53.1	16.2	25.5
Styrene	Intra	7.8	6.5	6.6	15.5	6.2	16.0	4.6	10.4	13.7	12.9
	Inter	7.8	9.8	11.6	14.1	9.2	17.0	3.7	10.9	13.4	14.0

Table 3.4 Cont'd

Volatile organic compound	RSD (%)	A549 Col 1		A549 Col 2		NHLF Col 1		NHLF Col 2		NHLF Col 3	
		Sample	Control	Sample	Control	Sample	Control	Sample	Control	Sample	Control
Cyclohexanone	Intra	ND	9.3	ND	14.1	6.4	9.3	10.3	9.0	15.8	11.6
	Inter	ND	28.5	ND	25.7	34.8	15.3	46.6	65.0	11.7	9.3
3-Heptanol	Intra	7.20	24.0	3.1	5.4	16.3	10.1	22.7	18.7	13.7	22.0
	Inter	17.0	19.2	23.5	51.2	25.8	46.5	20.3	20.6	16.8	25.1
Benzaldehyde	Intra	12.6	9.0	3.2	8.2	4.3	9.3	6.1	3.1	11.6	5.5
	Inter	25.4	7.9	15.2	5.2	17.9	7.8	6.2	5.5	20.6	17.3
Pentanoic acid, 2,4-dimethyl-3-oxo-, methyl ester	Intra	17.3	ND	ND	22.6	12.3	16.2	21.2	11.0	13.6	3.2
	Inter	152.2	ND	ND	31.4	42.8	52.3	28.7	37.7	49.6	72.1
2-Ethylhexanol	Intra	5.3	6.2	8.4	14.3	6.1	8.2	2.4	4.8	9.1	13.8
	Inter	4.8	8.8	61.9	8.1	6.0	15.5	8.2	8.6	10.0	7.5
1-Heptanol	Intra	9.2	13.5	ND	ND	ND	ND	ND	ND	ND	ND
	Inter	33.6	20.7	ND	ND	ND	ND	ND	ND	ND	ND
Phenol	Intra	13.2	5.6	11.7	17.2	4.5	13.1	7.1	11.3	21.1	10.6
	Inter	19.9	13.7	30.3	9.8	14.9	28.0	8.3	18.5	36.7	37.6
Octanal	Intra	10.9	6.2	13.1	14.8	7.0	11.2	13.8	10.2	11.8	20.1
	Inter	37.7	12.4	29.8	15.1	25.7	42.1	20.1	29.3	34.2	26.4
Decane	Intra	22.7	15.2	3.9	7.1	3.2	8.2	5.6	3.9	8.5	9.0
	Inter	60.4	62.7	12.9	17.1	5.7	15.2	15.8	16.8	12.6	11.9
Benzyl alcohol	Intra	11.5	28.5	34.9	16.7	8.1	16.9	9.00	17.2	18.2	12.9
	Inter	10.5	34.6	57.6	19.1	57.3	105.6	34.2	51.7	20.7	34.6
1-Phenylethanol	Intra	5.0	3.9	6.5	25.0	8.8	9.5	5.2	14.5	18.8	10.8
	Inter	10.4	15.4	24.0	17.5	15.9	38.2	10.6	23.2	41.5	43.7
Acetophenone	Intra	11.5	6.0	2.5	9.4	5.4	12.6	2.3	6.1	12.5	16.9
	Inter	8.3	8.8	24.4	5.6	8.5	7.8	3.6	10.6	11.9	22.0
1-Octanol	Intra	8.6	3.2	10.1	11.3	10.6	17.1	6.5	12.3	12.4	12.7
	Inter	29.3	10.3	15.6	17.7	17.7	8.6	16.4	17.1	20.1	11.8

Table 3.4 Cont'd

Volatile organic compound	RSD (%)	A549 Col 1		A549 Col 2		NHLF Col 1		NHLF Col 2		NHLF Col 3	
		Sample	Control	Sample	Control	Sample	Control	Sample	Control	Sample	Control
Octane, 2,3,6,7-tetramethyl-	Intra	22.9	11.7	ND	ND	ND	ND	ND	ND	ND	ND
	Inter	88.31	11.7	ND	ND	ND	ND	ND	ND	ND	ND
α-Cumyl alcohol	Intra	5.22	4.6	15.8	33.5	7.6	15.7	5.6	16.6	29.5	11.6
	Inter	4.12	17.3	23.9	23.4	57.5	65.5	9.5	26.0	69.0	69.7
Undecane	Intra	12.2	17.7	5.6	20.9	7.2	11.7	7.8	9.4	16.5	15.1
	Inter	19.6	38.0	44.6	18.7	8.9	21.4	27.7	16.6	22.9	15.9
Nonanal	Intra	5.8	4.2	3.1	10.3	4.0	13.6	6.3	5.3	13.5	8.7
	Inter	46.0	26.2	50.4	21.8	30.1	50.3	23.6	25.4	87.0	50.3
Undecane,4-methyl	Intra	15.4	33.4	4.4	14.7	17.8	40.6	35.7	24.9	50.8	51.9
	Inter	87.0	50.3	65.2	37.3	27.9	45.8	28.5	20.6	46.6	35.5
2-Nitrophenol	Intra	20.5	7.3	1.9	19.7	9.5	14.7	14.9	21.6	27.5	18.4
	Inter	17.8	5.9	-	27.6	8.5	11.6	19.3	19.3	22.5	18.3
1-Nonanol	Intra	14.5	10.8	14.6	25.9	14.9	15.6	16.4	10.2	24.7	18.2
	Inter	28.3	10.3	34.7	13.8	16.4	19.0	35.4	12.3	25.6	17.9
4-Decanol	Intra	20.1	11.3	15.5	25.2	14.0	23.3	25.2	20.1	23.5	15.6
	Inter	24.1	23.1	24.8	25.5	20.5	33.2	28.7	18.1	14.9	18.7
Dodecane	Intra	6.81	6.6	6.4	13.4	4.5	8.7	4.9	5.9	9.5	3.8
	Inter	29.3	25.2	11.3	8.4	12.6	17.8	7.6	6.3	11.6	10.2
Decanal	Intra	9.8	12.8	5.4	24.0	18.3	11.2	4.3	13.3	14.1	13.1
	Inter	26.9	16.9	70.3	56.8	31.3	52.1	11.8	26.0	27.4	28.7
Benzene, 1,3-di-*tert*-butyl	Intra	15.3	15.6	9.9	9.9	15.7	15.0	11.8	7.3	14.4	11.7
	Inter	21.2	16.7	11.5	11.6	25.1	23.7	19.1	15.8	12.0	14.3
Tridecane	Intra	16.2	16.6	7.0	22.2	9.3	24.1	13.8	26.3	19.4	11.4
	Inter	97.5	28.1	123.9	15.6	8.6	24.0	29.1	17.9	23.0	18.6
Decane, 2,3,5-trimethyl	Intra	11.5	8.6	10.6	16.1	ND	ND	ND	ND	7.3	41.9
	Inter	93.8	8.6	89.6	-	ND	ND	ND	ND	92.2	41.9

Table 3.4 Cont'd

Volatile organic compound	RSD (%)	A549 Col 1		A549 Col 2		NHLF Col 1		NHLF Col 2		NHLF Col 3	
		Sample	Control	Sample	Control	Sample	Control	Sample	Control	Sample	Control
Propanoic acid, 2-methy-2,2-dimethy-1-(2-hydroxy-1-methylethyl)-, propyl ester	Intra	12.2	8.5	24.2	23.3	9.6	12.3	6.3	24.1	18.0	16.8
	Inter	25.2	11.8	102.4	29.5	26.1	21.2	5.4	19.5	21.1	38.0
Propanoic acid, 2-methyl-, 3-hydroxy-2,4,4-trimethylpentyl ester	Intra	14.0	10.7	5.8	24.5	8.6	13.1	15.6	9.6	19.2	15.4
	Inter	30.8	11.9	22.5	16.1	21.8	21.2	13.1	7.2	29.2	43.5
6-Dodecanol	Intra	18.1	5.5	7.0	21.6	17.2	14.2	16.8	13.2	18.2	13.3
	Inter	23.5	34.2	28.1	23.2	15.7	20.8	11.6	14.2	21.5	30.2
Tetradecane	Intra	10.9	10.8	4.4	12.8	8.9	15.6	9.6	8.4	7.3	11.9
	Inter	32.8	21.0	21.7	10.4	8.9	19.0	11.8	9.6	7.2	13.3
Dodecanal	Intra	10.6	10.4	8.0	17.7	7.4	18.3	11.1	9.0	8.2	14.7
	Inter	21.5	12.9	26.9	18.6	8.3	28.5	17.3	30.5	28.2	25.1
Geranyl acetone	Intra	10.3	12.3	32.9	15.5	24.7	16.2	35.8	37.1	22.3	22.0
	Inter	18.9	49.2	34.6	15.6	28.5	26.8	24.4	32.5	39.5	38.7
2,6-Di-*tert*-butyl-1,4-benzoquinone	Intra	11.7	8.4	6.1	24.5	12.0	15.7	15.2	14.2	15.4	14.4
	Inter	21.3	13.5	24.6	19.4	7.2	13.6	12.0	14.1	18.2	12.6
Tridecanal	Intra	11.2	17.2	2.5	13.8	7.7	22.0	8.9	13.8	11.4	10.6
	Inter	23.0	15.6	39.5	13.2	13.2	35.7	32.3	41.5	29.3	31.1
2,4-Di-*tert*-butylphenol	Intra	7.3	4.6	7.4	29.3	11.5	16.3	7.5	5.8	17.3	8.0
	Inter	25.4	30.5	48.9	35.6	50.4	57.4	42.0	49.1	48.4	38.5
2-Tetradecanone	Intra	12.8	9.8	9.5	15.4	15.6	25.0	22.0	19.0	21.7	29.4
	Inter	36.7	8.2	83.6	57.8	22.4	42.3	19.7	25.5	20.3	27.0
Propanoic acid, 2-methyl-, 1-(1,1-dimethyl)-2-methyl-1,3-propanediyl ester	Intra	8.6	10.8	6.3	13.9	4.7	10.0	6.6	7.4	12.0	10.0
	Inter	43.7	67.9	30.8	13.0	92.3	38.7	34.3	20.1	17.3	19.7
Tetradecanal	Intra	12.6	12.9	9.7	13.8	14.2	22.7	14.1	17.4	10.8	16.4
	Inter	26.6	21.3	32.0	15.2	21.6	43.4	39.8	44.9	31.1	34.5
2-Methoxy-diphenylmethane	Intra	10.0	5.0	9.6	13.4	21.1	4.8	ND	7.3	22.5	25.9
	Inter	52.9	79.2	12.6	85.5	25.7	27.6	ND	41.1	38.3	45.2

Table 3.4 Cont'd

Volatile organic compound	RSD (%)	A549 Col 1		A549 Col 2		NHLF Col 1		NHLF Col 2		NHLF Col 3	
		Sample	Control	Sample	Control	Sample	Control	Sample	Control	Sample	Control
Dodecyl acrylate	Intra	22.5	25.9	11.1	33.3	46.6	53.9	29.2	2.2	9.2	19.4
	Inter	84.3	83.0	78.9	90.8	103.4	113.9	31.8	39.6	24.4	24.6
2-Pentadecanone	Intra	12.3	16.3	13.3	21.2	25.7	27.2	29.5	23.1	17.8	15.7
	Inter	41.0	11.8	46.0	28.1	31.7	37.9	33.7	23.6	26.0	34.9
Pentadecanal	Intra	12.5	13.8	11.3	26.9	23.4	22.9	40.7	22.5	26.8	28.6
	Inter	31.6	19.5	41.3	19.5	28.0	46.7	55.8	60.2	45.4	46.4
Benzene, 1,1'-(1,2-cyclobutanediyl)bis-, cis-	Intra	9.3	8.3	8.0	14.9	6.6	10.5	7.6	16.6	15.5	13.4
	Inter	6.1	8.1	15.7	10.4	26.2	9.7	10.8	11.5	47.6	9.2
Diisobutyl phthalate	Intra	24.2	24.0	61.7	44.5	47.4	11.5	65.1	47.9	20.1	16.0
	Inter	51.7	22.6	46.0	23.3	53.0	62.4	62.9	44.9	57.4	22.7
Heptadecanal	Intra	15.2	14.3	18.0	35.8	26.9	35.9	43.3	48.3	60.4	47.5
	Inter	30.4	25.0	32.1	25.3	34.7	40.5	64.7	77.9	61.6	58.5
2-Nonadecanone	Intra	54.1	30.9	11.8	48.2	40.1	8.0	45.7	38.7	62.0	76.9
	Inter	36.3	26.1	36.6	32.9	54.7	43.5	44.2	57.7	43.5	51.8
Octadecanal	Intra	14.1	19.9	38.0	5.9	8.1	8.5	32.1	12.8	51.4	54.6
	Inter	15.3	17.7	24.0	12.4	14.8	10.7	47.5	102.7	59.3	59.5
Dodecanoic acid, isooctyl ester	Intra	14.5	8.5	ND	ND	13.3	24.8	98.1	5.7	ND	ND
	Inter	68.2	47.7	ND	ND	77.9	64.8	51.9	39.1	ND	ND

3.4.4 'Between sample and control analysis'

The data obtained for around 30% of VOCs in the MT experiment failed the assumption of normality (Shapiro-Wilk, $\alpha = 0.05$) (Appendix B, Tab. B2). Therefore, for the comparison of VOCs present in the cell culture medium incubated with or without the cells, the Wilcoxon signed-rank test ($\alpha = 0.05$) was used. It is a non-parametric test so it does not assume a normal distribution of the population, and it is more stable to outliers. It is a method used for examining the median difference in observations for two samples that are paired. The experiment was designed to use the same batch of the medium (the same bottle) for both the sample and its paired control, in order to address the issue of possible between-day variability in VOC concentrations. This potential variability could be due to the loss of VOCs during storage, pre-warming the medium (although this was done to aliquots, not to the whole bottle) and finally different batches of the cell culture media being used in experiments on different days.

The null hypothesis of the Wilcoxon signed-rank test assumes that the median difference of the pairs of observations is zero. Therefore when the p-value is greater than the chosen level of significance, it can be concluded that the medians are similar and the null hypothesis can be accepted. The alternative hypothesis says that the true location shift is not equal to zero.

Part A of tables 3.5 and 3.6 alphabetically lists the VOCs that have been found in elevated or decreased levels in the cell culture medium incubated with the A549 or NHLF cells respectively, in comparison to their pure medium controls in the MT experiment. Chemical structures of the VOCs found at altered levels in the MT experiment can be found in Appendix C1. Part B of tables 3.5 and 3.6 lists the compounds that were found at similar levels for both cancer and normal cell samples and their controls. In both tables the median peak area ratios for the sample and its control of each compound are presented, as well as V statistics and p-values of the Wilcoxon-signed rank test ($n = 9$). V statistics is the sum of the ranks of those pairs for which sample ratio > control ratio. The chosen level of significance to create the tables 3.5 and 3.6 was $p < 0.05$. It must be noted, however, that p-values are approximate tools for interpretation of how likely the observed difference could occur by chance only. With the 0.05 level of significance there is a 1 in 20 chance that the observed extreme has happened due to random variation. Calculations in Table 3.6 for dodecyl acrylate (collection 3) and diisobutyl phthalate (collection 1) were performed

Table 3.5 Comparison of the peak area ratios (peak area of an analyte/peak area of an internal standard) between the A549 samples (n = 9) and their pure medium controls (n = 9) for VOCs found in the MT experiment. NS: not significant; S: significant; ND: not detected. **Part A**: VOCs for which the Wilcoxon signed rank test detected the median difference between the pairs of observations not equal to zero at a level of significance α = 0.05. These VOCs were observed at increased or decreased levels in the A549 cell samples when compared to their pure medium controls. **Part B**: VOCs for which the Wilcoxon signed rank test detected the median difference between the pairs of observations equal to zero at a level of significance α = 0.05. These VOCs were observed at similar levels in the A549 cell samples and their pure medium controls. Chemical structures of the VOCs found at altered levels in the MT experiment can be found in Appendix C1.

Part A								
Volatile organic compound	**Sample Median**	**Control Median**	**V**	**p-value**	**Sample Median**	**Control Median**	**V**	**p-value**
	A549 collection 1				**A549 collection 2**			
Increased VOCs								
1,1'-(1,2-cyclobutanediyl)bis-,cis-benzene	3.171E-02	1.490E-02	45	0.003906	2.496E-02	1.353E-02	45	0.003906
Cyclohexanol				NS	7.519E-03	5.466E-03	45	0.003906
2,4-Dimethylheptane	4.333E-02	1.335E-02	45	0.003906	2.047E-03	7.278E-04	45	0.003906
2,4-Dimethyl-1-heptene	4.941E-03	1.786E-03	43	0.01172		ND		
2,6-Di-*tert*-butyl-1,4-benzoquinone	1.891E-01	1.230E-01	45	0.003906				NS
3-Heptanol	3.093E-02	5.698E-03	45	0.003906	1.130E-02	3.562E-03	45	0.003906
4-Methyloctane	1.322E-02	3.291E-03	45	0.003906	1.101E-03	6.179E-04	21	0.03603
4-Methylundecane	4.463E-03	1.046E-03	45	0.003906				NS
2-Pentadecanone	1.197E-02	2.538E-03	45	0.003906				NS
1-Phenylethanol	1.096E-01	2.010E-02	45	0.003906	5.333E-02	1.139E-02	45	0.003906
2,3,6,7-Tetramethyloctane	4.118E-03	0.000E+00	45	0.003906		ND		
2,3,5-Trimethyldecane	6.364E-03	0.000E+00	45	0.003906				NS
2,3,5-Trimethylhexane				NS	2.073E-03	0.000E+00	45	0.003906
Decreased VOCs								
Acetophenone	5.389E-02	2.366E-01	0	0.003906	3.902E-02	1.536E-01	0	0.003906
Benzaldehyde	3.179E-02	4.015E-01	0	0.003906	3.645E-02	2.845E-01	0	0.003906
Cyclohexanone	0.000E+00	5.032E-02	0	0.003906	0.000E+00	4.376E-02	0	0.003906

Table 3.5 Cont'd

Volatile organic compound	Sample Median	Control Median	V	p-value	Sample Median	Control Median	V	p-value
	A549 collection 1				A549 collection 2			
Decreased VOCs (Cont'd)								
4-Decanol	6.203E-03	7.719E-03	3	0.01953				NS
Diisobutyl phthalate				NS	2.973E-02	3.487E-02	0	0.003906
1,3-Di-*tert*-butylbenzene	9.642E-03	1.199E-02	0	0.003906				NS
2,5-Di-*tert*-butylphenol				NS	5.252E-03	9.529E-03	3	0.01953
Dodecane	8.690E-02	9.963E-02	0	0.003906				NS
Dodecanal				NS	5.318E-03	6.782E-03	5	0.03906
6-Dodecanol	7.241E-03	1.560E-02	0	0.003906	3.624E-03	8.088E-03	1	0.007812
Ethylbenzene	2.362E-02	3.711E-02	0	0.003906				NS
2-Ethenyl-2-butenal	0.000E+00	1.907E-03	0	0.003906	0.000E+00	2.353E-03	0	0.003906
2-Ethylhexanol	1.696E+00	1.937+00	0	0.003906	3.094E-01	1.991E+00	0	0.003906
Geranyl acetone				NS	1.357E-03	2.651E-03	1	0.03461
Heptanal				NS	5.421E-03	8.706E-03	0	0.003906
Heptanol	4.417E-03	9.567E-03	4	0.02734		ND		
Hexanal	6.120E-03	1.573E-02	0	0.003906	8.116E-03	1.848E-02	0	0.003906
2-Methoxy-diphenylmethane				NS	1.488E-03	1.285E-03	4	0.02734
2-Nitrophenol	2.192E-03	4.550E-03	0	0.003906	0.000E+00	1.958E-03	3	0.01953
1-Nonanol	2.973E-03	7.041E-03	0	0.003906	1.541E-03	4.416E-03	0	0.003906
Pentanoic acid, 2,4-dimethyl-3-oxo-, methyl ester		ND			0.000E+00	1.455E-02	0	0.003906
Phenol	1.019E-02	5.494E-02	0	0.003906	3.583E-03	4.742E-02	0	0.003906
Octanal				NS	4.235E-03	6.597E-03	1	0.007812
1-Octanol	9.948E-03	2.380E-02	0	0.003906				NS
Tetradecanal				NS	1.021E-02	1.235E-02	5	0.03906
Tetradecane				NS	8.618E-02	1.275E-01	1	0.01172
2-Tetradecanone	2.658E-03	4.696E-03	1	0.007812	2.001E-03	4.150E-03	1	0.007812
Styrene	2.166E-01	2.514E-01	1	0.007812				NS

Table 3.5 Cont'd

Part B								
Volatile organic compound	**Sample Median**	**Control Median**	**V**	**p-value**	**Sample Median**	**Control Median**	**V**	**p-value**
	A549 collection 1				**A549 collection 2**			
Benzyl alcohol	2.573E-03	1.894E-03	39	0.05469	1.090E-03	1.805E-03	15	0.4258
α-Cumyl alcohol	4.195E-03	3.997E-03	24	0.9102	3.731E-03	3.530E-03	26	0.7344
Cyclohexanol	6.430E-03	6.358E-03	25	0.8203				S
Decanal	1.882E-02	2.480E-02	12	0.25	8.291E-03	9.665E-03	13	0.3008
Decane	5.521E-02	5.600E-02	6	0.05469	4.182E-02	5.080E-02	15	0.4258
4-Decanol				S	2.688E-03	3.415E-03	13	0.3008
Diisobutyl phthalate	1.923E-02	3.481E-02	8	0.09766				S
2,3-Dimethylheptane	3.403E-03	9.237E-04	39	0.05469	0.000E+00	0.000E+00	9	0.2012
1,3-Di-*tert*-butylbenzene				S	1.007E-02	1.172E-02	7	0.07422
2,6-Di-*tert*-butyl-1,4-benzoquinone				S	1.257E-01	1.019E-01	26	0.4961
2,5-Di-*tert*-butylphenol	1.216E-02	1.158E-02	26	0.7344	5.252E-03	9.529E-03	3	0.01953
Dodecanal	6.576E-03	7.716E-03	19	0.7344				S
Dodecane				S	6.159E-02	6.604E-02	13	0.3008
Dodecanoic acid, isooctyl ester	8.450E-03	9.728E-03	30	0.4258		ND		
Dodecyl acrylate	1.205E-02	1.093E-02	18	0.6523	1.021E-02	1.082E-02	22	1.0000
Ethylbenzene				S	2.889E-02	3.388E-02	24	0.9102
Geranyl acetone	6.725E-03	9.628E-03	12	0.25				S
Heptadecanal	3.005E-02	2.917E-02	26	0.7344	3.314E-02	3.432E-02	19	0.7344
Heptadecane	5.431E-03	5.819E-03	24	0.9102		ND		
Heptanal	8.308E-03	1.229E-02	7	0.07422				S
2-Methoxy-diphenylmethane	1.553E-02	7.963E-03	39	0.05469				S
4-Methylundecane				S	0.000E+00	1.174E-03	24	0.9102
Nonadecane	7.451E-03	5.212E-03	35	0.1641		ND		
2-Nonadecanone	2.865E-02	2.297E-02	32	0.3008	2.103E-02	2.249E-02	23	1.0000

Table 3.5 Cont'd

Part B (cont'd)								
Volatile organic compound	**Sample Median**	**Control Median**	**V**	**p-value**	**Sample Median**	**Control Median**	**V**	**p-value**
	A549 collection 1				**A549 collection 2**			
Nonanal	4.897E-02	3.247E-02	29	0.4961	4.104E-02	5.618E-02	21	0.9102
Octadecanal	7.642E-02	9.036E-02	16	0.4961	6.186E-02	6.697E-02	33	0.25
Octanal	7.102E-03	9.835E-03	8	0.09766				S
1-Octanol				S	5.247E-03	8.938E-03	18	0.6523
Pentadecanal	1.346E-02	1.419E-02	26	0.7344	1.494E-02	1.781E-02	16	0.4961
2-Pentadecanone				S	2.338E-03	2.331E-03	33	0.25
Propanoic acid,2-methy-(2,2-dimethy-1-(2-hydroxy-1-methylethyl)propyl ester	2.813E-02	3.027E-02	28	0.5703	1.588E-02	1.72E-02	25	0.8203
Propanoic acid,2-methyl-,3-hydroxy-2,4,4-trimethylpentyl ester	8.026E-03	9.970E-03	17	0.5703	7.327E-03	8.863E-03	12	0.25
Propanoic acid,2-methyl-,1-(1,1-dimethyl)-2-methyl-1,3-propanediyl ester	3.119E-02	3.078E-02	15	0.4258	3.029E-02	2.78E-02	30	0.4258
Styrene				S	2.385E-01	2.313E-01	27	0.6523
Tetradecanal	9.297E-03	1.093E-02	25	0.8203				S
Tetradecane	7.943E-02	9.085E-02	16	0.4961				S
Tridecanal	5.216E-02	5.041E-02	28	0.5703	0.047234	0.057957	19	0.7344
Tridecane	6.505E-03	4.297E-03	37	0.09766	4.918E-03	5.735E-03	24	0.9102
2,3,5-Trimethyldecane				S	4.855E-04	0.000E+00	15	0.05906
2,3,5-Trimethylhexane	5.836E-03	4.054E-03	35	0.1641				S
Undecane	8.801E-03	9.791E-03	24	1	7.712E-03	9.475E-03	25	0.8203
p-Xylene	5.253E-03	6.333E-03	15	0.4258	6.572E-03	4.151E-03	39	0.05469

for n = 7, and for dodecyl acrylate (collections 1 and 2), 2-nonadecanone and octadecanal (collection 2) were performed for n = 8.

A549 cell line

Overall, 13 VOCs were found at increased and 28 at decreased levels in the cell culture medium incubated with the A549 cells in comparison to the pure medium. After one week of the incubation 11 VOCs were found to be produced and 18 consumed by the A549 cells. The compounds found at significantly higher levels in the A549 cell samples in comparison to the medium controls were mainly hydrocarbons (2,4-dimethyl-1-heptene, V = 43, p = 0.0117; 2,4-dimethylheptane; 4-methyloctane; 4-methylundecane; 2,3,6,7-tetramethyloctane and 2,3,5-trimethyldecane, V = 45, p = 0.0039). Other compounds included alcohols such as 3-heptanol and 1-phenylethanol (V = 45, p = 0.0039), an aromatic hydrocarbon 1,1'-(1,2-cyclobutanediyl)bis-,cis-benzene (V = 45, p = 0.0039), and ketones (2,6-di-*tert*-butyl-1,4-benzoquinone and 2-pentadecanone, V = 45, p = 0.0039). The significantly decreased compounds were alcohols (4-decanol; V = 3, p = 0.01953; heptanol; V = 4; p = 0.02734; 6-dodecanol; 2-ethylhexanol; 1-nonanol; 1-octanol, V = 0 p = 0.0039); an alkane (dodecane, V = 0; p = 0.0039); aldehydes (benzaldehyde; 2-ethenyl-2-butenal and hexanal, V = 0, p = 0.0039); ketones (acetophenone; cyclohexanone, V = 0, p = 0.0039; 2-tetradecanone, V = 1, p = 0.0078); phenols (phenol and 2-nitrophenol, V = 0, p = 0.0039) and aromatic hydrocarbons (1,3-di-*tert*-butylbenzene; ethylbenzene, V = 0; p = 0.0039; styrene, V = 1, p = 0.0078).

The number of VOCs produced or consumed in the A549 samples changed after an additional week of incubation. There were seven significantly elevated and 21 significantly decreased compounds detected. The cancer cells were observed to continue the release of 1,1'-(1,2-cyclobutanediyl)bis-,cis-benzene; 2,4-dimethylheptane; 3-heptanol; 1-phenylethanol (V = 45, p = 0.0039) and 4-methyloctane (V = 21; p = 0.03603). Additionally, 2,3,5-trimethylhexane and cyclohexanol were noted at higher levels in the samples than in controls (V = 45; p = 0.003906). The A549 cells stopped to emit 2,6-di-tert-butyl-1,4-benzoquinone; 4-methylundecane; 2-pentadecanone and 2,3,5-trimethylhexane. 2,4-dimethyl-1-heptene was not detected in collection 2 at all, neither in the samples nor in the controls. Most of the consumed VOCs were found to be the same as for collection 1 (acetophenone; benzaldehyde; cyclohexanone; 2-ethylhexanol; 2-ethenyl-2-butenal; hexanal; 1-nonanol; phenol (V = 0; p = 0.0039); 6-dodecanol; 2-tetradecanone;

(V = 1, p = 0.0078) and 2-nitrophenol (V = 3, p = 0.01953). Differences in medians between the cell samples and controls were found to be no longer significant for 1,3-di-*tert*-butylbenzene; ethylbenzene; 4-decanol; dodecane; 1-octanol and styrene. Heptanol was not detected at all in collection 2, neither in the samples nor in the controls. Finally 10 new VOCs were found at lower levels in the A549 culture medium when compared to the control medium, namely 2,5-di-*tert*-butylphenol (V = 3, p = 0.01953); heptanal; pentanoic acid, 2,4-dimethyl-3-oxo-, methyl ester and diisobutyl phthalate (V = 0; p = 0.0039); dodecanal; tetradecanal (V = 5; p = 0.03906); geranyl acetone (V = 1, p = 0.03461); octanal; tetradecane (V = 1, p = 0.0078) and 2-methoxy-diphenylmethane (V = 4; p = 0.02734).

NHLF cell line

For the fibroblasts, 17 VOCs were found at increased and 11 at decreased levels in the cell culture medium incubated with the NHLFs in comparison to the pure medium. The first collection yielded six analytes at significantly increased and four at significantly decreased levels. The normal cells were found to release benzyl alcohol (V = 44; p = 0.007812); 1,1'-(1,2-cyclobutanediyl)bis-, cis-benzene; 2-ethylhexanol; octadecanal; propanoic acid, 2-methyl-,1-(1,1-dimethyl)-2-methyl-1,3-propanediyl ester (V = 40; p = 0.03906) and 2-pentadecanone (V = 45; p = 0.003906) and to consume benzaldehyde; ethylbenzene; 2-ethenyl-2-butanal (V=45; p=0.003906) and 1,3-di-*tert*-butylbenzene (V = 3, p = 0.01953).

During the second week of cultivation, there were eight VOCs at significantly elevated and seven at significantly reduced levels found in the NHLF samples when compared to their pure medium controls. 2-Ethylhexanol; propanoic acid, 2-methyl-,1-(1,1-dimethyl)-2-methyl-1,3-propanediyl ester (V = 44; p = 0.007812); 1,1'-(1,2-cyclobutanediyl)bis-,cis-benzene and 2-pentadecanone continued to be produced by the fibroblasts (V = 45; p = 0.003906). The other four VOCs observed at higher concentrations were 2,4-dimethylheptane (V = 42; p = 0.01953); 2,6-di-*tert*-butyl-1,4-benzoquinone (V = 40; p = 0.03906); 6-dodecanol and dodecanoic acid, isooctyl ester (V = 43; p = 0.01172). Volatiles that were found to be metabolised by the NHLF cells during the second week of incubation were again benzaldehyde and 2-ethenyl-2-butanal (V = 0; p = 0.003906), but also dodecane (V = 5; p = 0.03906); hexanal; 1-nonanol; octanal (V = 45; p = 0.003906) and tetradecanal (V = 1; p = 0.007812). In comparison to collection 1, the differences were no longer significant for benzyl alcohol and ethylbenzene.

Table 3.6 Comparison of the peak area ratios (peak area of an analyte/peak area of an internal standard) between the NHLF samples (n = 9) and their pure medium controls (n = 9) for VOCs detected in the MonoTrap experiment (n = 9). NS: not significant; S: significant; ND: not detected; n = 7: detected only in seven samples; n = 8: detected only in eight samples. **Part A**: VOCs for which the Wilcoxon signed rank test detected the median difference between the pairs of observations not equal to zero at a level of significance α = 0.05. These VOCs were observed at increased or decreased levels in the NHLF cell samples when compared to their pure medium controls. **Part B**: VOCs for which the Wilcoxon-signed rank test detected the median difference between the pairs of observations equal to zero at a level of significance α = 0.05. These VOCs were observed at similar levels in the A549 cell samples and their pure medium controls. Chemical structures of VOCs found at altered levels in the MT experiment can be found in Appendix C1.

Part A												
Volatile organic compound	**Sample Median**	**Control Median**	**V**	**p-value**	**Sample Median**	**Control Median**	**V**	**p-value**	**Sample Median**	**Control Median**	**V**	**p-value**
	NHLF collection 1				**NHLF collection 2**				**NHLF collection 3**			
Increased VOCs												
Benzyl alcohol	3.151E-02	1.405E-02	44	0.007812				NS				NS
1,1'-(1,2-cyclobutanediyl)bis-, cis-benzene	5.666E-02	3.567E-02	40	0.03906	4.267E-02	2.397E-02	45	0.003906				NS
2,4-Dimethylheptane		ND			1.922E-03	8.325E-04	42	0.01953	2.351E-03	1.161E-03	42	0.01953
2,6-Di-*tert*-butyl-1,4-benzoquinone				NS	1.199E-01	1.097E-01	40	0.03906				NS
2,5-Di-*tert*-butylphenol				NS				NS	9.343E-03	7.791E-03	45	0.003906
Dodecanal				NS				NS	8.129E-03	6.423E-03	43	0.01172
Dodecanoic acid, isooctyl ester				NS	1.547E-03	7.352E-04	43	0.01172		ND		
6-Dodecanol				NS	3.737E-02	2.991E-02	43	0.01172	3.582E-02	2.529E-02	45	0.003906
2-Ethylhexanol	2.184E+00	2.037E+00	40	0.03906	2.069E+00	2.047E+00	44	0.007812				NS
Geranyl acetone				NS				NS	3.865E-03	3.343E-03	45	0.003906
4-Methyloctane		ND						NS	1.045E-03	3.670E-04	21	0.03603
Octadecanal	6.777E-02	6.442E-02	40	0.03906				NS				NS
2-Pentadecanone	3.528E-03	2.208E-03	45	0.003906	4.172E-03	1.469E-03	45	0.003906	5.779E-03	1.853E-03	45	0.003906
1-Phenylethanol				NS				NS	9.714E-03	8.808E-03	44	0.007812

Table 3.6 Cont'd

Volatile organic compound	Sample Median	Control Median	V	p-value	Sample Median	Control Median	V	p-value	Sample Median	Control Median	V	p-value
	NHLF collection 1				NHLF collection 2				NHLF collection 3			
Increased VOCs (Cont'd)												
Propanoic acid,2-methyl-,1-(1,1-dimethyl)-2-methyl-1,3-propanediyl ester	2.921E-02	3.043E-02	40	0.03906	2.772E-02	2.522E-02	44	0.007812				NS
Tetradecane				NS				NS	1.030E-01	8.565E-02	41	0.02734
2-Tetradecanone				NS				NS	3.339E-03	2.574E-03	44	0.007812
Decreased VOCs												
Benzaldehyde	1.386E-01	3.872E-01	0	0.003906	6.798E-02	3.380E-01	0	0.003906	5.09E-02	3.462E-01	0	0.003906
Diisobutyl phthalate				NS				NS	7.969E-03	1.583E-02	5	0.03906
1,3-Di-*tert*-butylbenzene	4.151E-03	5.392E-03	3	0.01953				NS				NS
Dodecane				NS	6.941E-02	7.014E-02	5	0.03906				NS
2-Ethenyl-2-butenal	1.014E-03	2.679E-02	0	0.003906	7.209E-04	2.751E-02	0	0.003906	0.000E+00	1.718E-02	0	0.003906
Ethylbenzene	2.157E-02	2.877E-02	0	0.003906				NS				NS
Heptanal				NS	5.953E-03	6.429E-03	0	0.003906				NS
Hexanal				NS	1.481E-02	2.102E-02	0	0.003906	1.387E-02	1.805E-02	0	0.003906
1-Nonanol				NS	1.894E-03	3.731E-03	0	0.003906	1.976E-03	3.585E-03	0	0.003906
Octanal				NS	3.662E-03	4.723E-03	0	0.003906				NS
Tetradecanal				NS	0.007762	0.009304	1	0.007812				NS
Styrene				NS				NS	2.625E-01	2.785E-01	5	0.03906
Part B												
Acetophenone	2.054E-01	2.014E-01	26	0.7344	1.492E-01	1.556E-01	12	0.25	1.152E-01	1.352E-01	13	0.3008
Benzyl alcohol				S	3.332E-02	3.290E-02	33	0.25	1.796E-02	2.073E-02	17	0.5703
α-Cumyl alcohol	1.948E-02	2.080E-02	13	0.3008	2.016E-02	1.936E-02	28	0.5703	1.216E-02	1.166E-02	32	0.3008
1,1'-(1,2-cyclobutanediyl)bis-,cis-benzene				S				S	1.781E-02	1.604E-02	29	0.4961

Table 3.6 Cont'd

Part B (cont'd)												
Volatile organic compound	**Sample Median**	**Control Median**	**V**	**p-value**	**Sample Median**	**Control Median**	**V**	**p-value**	**Sample Median**	**Control Median**	**V**	**p-value**
	NHLF collection 1				**NHLF collection 2**				**NHLF collection 3**			
Cyclohexanol	7.868E-03	6.603E-03	21	0.9102	8.250E-03	7.389E-03	25	0.8203	6.896E-03	6.645E-03	26	0.7344
Cyclohexanone	3.731E-02	3.717E-02	29	0.4961	3.579E-02	2.566E-02	29	0.4961	4.068E-02	4.015E-02	30	0.4258
Decanal	7.287E-03	6.239E-03	1	23	5.505E-03	5.254E-03	28	0.5703	7.140E-03	7.565E-03	8	0.09766
Decane	4.137E-02	4.267E-02	18	0.6523	2.938E-02	3.023E-02	17	0.5703	2.046E-02	2.365E-02	13	0.3008
4-Decanol	4.137E-03	4.029E-03	26	0.7344	2.329E-03	2.698E-03	9	0.1289	2.986E-03	2.687E-03	30	0.1289
2,4-Dimethyl-1-heptene	ND				0.000E+00	0.000E+00	11	0.4185	0.000E+00	0.000E+00	4	0.7893
Diisobutyl phthalate	1.176E-02	1.747E-02	7	0.236724	7.862E-03	1.191E-02	14	0.3594				S
1,3-Di-*tert*-butylbenzene				S	8.007E-03	9.837E-03	21	0.9102	1.063E-02	1.089E-02	21	1.0000
2,6-Di-*tert*-butyl-1,4-benzoquinone	1.622E-01	1.460E-01	35	0.1641				S	1.015E-01	1.031E-01	23	1.0000
2,5-Di-*tert*-butylphenol	2.130E-02	2.166E-02	19	0.7344	2.629E-02	2.729E-02	20	0.8203				S
Dodecanal	6.794E-03	8.209E-03	7	0.07422	4.512E-03	4.743E-03	9	0.1289				S
Dodecane	0.081546	0.075591	24	0.9102				S	6.645E-02	7.462E-02	21	0.9102
Dodecanoic acid, isooctyl ester	8.238E-04	7.892E-04	25	0.07593				S	ND			
6-Dodecanol	6.576E-02	6.735E-02	25	0.8203				S				S
Dodecyl acrylate	1.964E-03	1.594E-03	30	0.092892	1.073E-03	1.189E-03	6	0.345448	1.311E-03	1.263E-03	12	0.735317
Ethylbenzene				S	2.315E-02	2.203E-02	21	0.9102	1.895E-02	1.925E-02	21	0.9102
2-Ethylhexanol				S				S	2.295E+00	2.164E+00	30	0.4258
Geranyl acetone	3.759E-03	3.527E-03	25	0.8203	2.971E-03	2.028E-03	35	0.1641				S
Heptadecanal	2.948E-02	2.751E-02	19	0.7344	1.854E-02	1.620E-02	25	0.8203	2.347E-02	1.870E-02	39	0.05469
Heptanal	6.524E-03	7.225E-03	10	0.1641				S	7.837E-03	6.773E-03	28	0.5703
3-Heptanol	4.563E-03	4.273E-03	24	0.9102	2.519E-03	2.707E-03	21	0.9102	2.786E-03	2.104E-03	36	0.1289
Hexanal	1.372E-02	1.555E-02	11	0.2031				S				S
4-Methyloctane	ND				7.317E-04	0.000E+00	18	0.1422				S

Table 3.6 Cont'd

Part B (cont'd)												
VOC	**Sample Median**	**Control Median**	**V**	**p-value**	**Sample Median**	**Control Median**	**V**	**p-value**	**Sample Median**	**Control Median**	**V**	**p-value**
	NHLF collection 1				**NHLF collection 2**				**NHLF collection 3**			
4-Methylundecane	0.000E+00	4.917E-04	12	0.4412	9.223E-04	7.775E-04	31	0.3594	6.620E-04	1.011E-03	19	0.7344
2-Methoxy-diphenylmethane	4.140E-04	3.458E-04	21	0.7263	0.000E+00	3.644E-04	0	0.05906	4.958E-04	2.828E-04	22	0.6241
2-Nitrophenol	4.071E-03	4.160E-03	14	0.3594	1.518E-03	1.444E-03	27	0.6523	1.019E-03	1.078E-03	16	0.4961
2-Nonadecanone	1.760E-02	2.498E-02	19	0.7344	1.468E-02	1.067E-02	29	0.123485	1.867E-02	1.315E-02	37	0.9766
Nonanal	1.411E-02	1.479E-02	13	0.3008	1.580E-02	1.377E-02	19	0.7344	1.188E-02	1.273E-02	32	0.3008
1-Nonanol	3.735E-03	4.319E-03	6	0.05469				S				S
Octadecanal				S	6.611E-02	6.060E-02	6	0.092892	7.377E-02	6.100E-02	33	0.25
Octanal	4.374E-03	4.195E-03	13	0.3008				S	4.505E-03	4.151E-03	25	0.8203
1-Octanol	1.202E-02	1.242E-02	28	0.5703	1.108E-02	1.146E-02	18	0.6523	1.126E-02	1.187E-02	28	0.5703
Pentadecanal	1.604E-02	1.455E-02	15	0.4258	1.019E-02	1.205E-02	12	0.25	1.205E-02	1.179E-02	36	0.1289
Pentanoic acid, 2,4-dimethyl-3-oxo-, methyl ester	2.157E-02	2.018E-02	17	0.5703	2.460E-02	1.889E-02	23	1.0000	1.360E-02	1.460E-02	16	0.2945
Phenol	5.065E-02	4.838E-02	19	0.7344	3.642E-02	3.778E-02	20	0.8203	3.603E-02	3.445E-02	24	0.9102
1-Phenylethanol	2.284E-02	2.040E-02	28	0.5703	1.381E-02	1.204E-02	35	0.1641				S
Propanoic acid,2-methy-(2,2-dimethy-1-(2-hydroxy-1-methylethyl) propyl ester	2.553E-02	2.617E-02	23	1.0000	2.205E-02	2.599E-02	39	0.05469	2.519E-02	2.387E-02	25	0.8203
Propanoic acid,2-methyl-,3-hydroxy-2,4,4-trimethylpentyl ester	8.896E-03	1.119E-02	15	0.4258	7.152E-03	7.652E-03	14	0.3594	7.439E-03	6.100E-03	19	0.7344
Propanoic acid,2-methyl-,1-(1,1-dimethyl)-2-methyl-1,3-propanediyl ester				S				S	4.463E-02	3.853E-02	34	0.2031
Styrene	2.763E-01	2.781E-01	19	0.7344	2.842E-01	2.820E-01	28	0.5703				S
Tetradecanal	1.200E-02	1.142E-02	11	0.2031				S	1.040E-02	1.017E-02	32	0.3008

Table 3.6 Cont'd

Part B (cont'd)												
Volatile organic compound	**Sample Median**	**Control Median**	**V**	**p-value**	**Sample Median**	**Control Median**	**V**	**p-value**	**Sample Median**	**Control Median**	**V**	**p-value**
	NHLF collection 1				**NHLF collection 2**				**NHLF collection 3**			
Tetradecane	1.148E-01	1.237E-01	14	0.3594	9.545E-02	9.701E-02	17	0.5703				S
2-Tetradecanone	4.209E-03	3.409E-03	33	0.25	2.988E-03	2.338E-03	36	0.1289				S
Tridecanal	4.866E-02	5.581E-02	7	0.07422	3.429E-02	3.656E-02	11	0.2031	4.368E-02	4.338E-02	37	0.09766
Tridecane	5.015E-03	5.380E-03	16	0.4961	4.017E-03	3.855E-03	23	1.0000	4.392E-03	4.208E-03	37	0.09766
Undecane	9.540E-03	9.661E-03	10	0.1641	7.445E-03	7.749E-03	28	0.5703	7.337E-03	6.901E-03	33	0.25
p-Xylene	3.665E-03	3.718E-03	6	0.05469	3.553E-03	8.721E-03	6	0.05469	6.862E-03	6.469E-03	37	0.09766

In the third NHLF collection 10 VOCs were observed at higher and six at lower levels in the fibroblast samples when compared to the medium controls. Three volatiles were continued to be released by the fibroblasts from collection 2, namely 2,4-dimethyl-heptane (V = 42, p = 0.01953); 6-dodecanol and 2-pentadecanone (V = 45; p = 0.003906). Seven new VOCs appeared to be produced by the NHLFs such as dodecanal (V = 43; p = 0.01172); geranyl acetone; 2,5-*tert*-butylphenol (V = 45; p = 0.003906); 4-methyloctane (V = 21; p = 0.03603); 1-phenylethanol; 2-tetradecanone (V = 44; p = 0.007812) and tetradecane (V = 41; p = 0.02734). In comparison to collection 2, the differences between peak area ratios were no longer significant for 1,1'-(1,2-cyclobutanediyl)bis-,cis-benzene; 2,6-di-*tert*-butyl-1,4-benzoquinone; 2-ethylhexanol and propanoic acid, 2-methyl-,1-(1,1-dimethyl)-2-methyl-1,3-propanediyl ester. Diisobutyl phthalate and styrene (V = 5; p = 0.03906) were the new volatiles found at significantly lower levels during the third week of the NHLF incubation alongside the previously observed benzaldehyde; 2-ethenyl-1-butenal; hexanal and 1-nonanol (V = 0; p = 0.003906).

A549 versus NHLFs

Overall, in the MT experiment six VOCs detected at increased levels were common to both cell lines, namely 1,1'-(1,2-cyclobutanediyl)bis-,cis-benzene; 2,6-di-*tert*-butyl-1,4-benzoquinone; 2,4-dimethylheptane; 4-methyloctane, 2-pentadecanone and 1-phenylethanol. All 12 compounds found in reduced levels in the NHLF cell samples were also decreased in the A549 samples (benzaldehyde; diisobutyl phthalate; 1,3-di-*tert*-butylbenzene; dodecane; ethylbenzene; 2-ethenyl-2-butenal; hexanal; heptanal; 1-nonanol; octanal; tetradecanal and styrene). Such VOCs as cyclohexanol; 2,4-dimethyl-1-heptene; 3-heptanol; 4-methylundecane; 2,3,6,7-tetramethyloctane; 2,3,5-trimethyldecane and 2,3,5-trimethylhexane were observed at increased levels only in the A549 cell samples while benzyl alcohol; dodecanoic acid isooctyl ester and octadecanal were found at higher levels solely in the NHLF samples. The cancer cells were found to exclusively metabolise acetophenone; cyclohexanone; 4-decanol; pentanoic acid, 2,4-dimethyl-3-oxo-, methyl ester; heptanol; 2-methoxy-diphenylmethane; phenol; 2-nitrophenol and 1-octanol. Finally, 2-ethylhexanol; dodecanal; 6-dodecanol; 1,3-di-*tert*-butylbenzene; 2,5-di-*tert*-butylphenol; tetradecane and 2-tetradecanone were detected at decreased levels in the cancer cell samples and at increased levels in the fibroblast samples.

Each of the VOCs found to be produced or consumed by the analysed cell lines are discussed in more detail in Chapter 5.4 in relation to the other *in vitro* studies of the VOCs in cancer as well as the *in vivo* studies.

3.4.5 'Between-sample analysis'

Table 3.7 presents the results of the Kruskal-Wallis followed by Mann-Whitney U comparative analysis conducted for the standardised peak area ratios of the VOCs detected in the cell samples in the MT experiment. This is a comparison of the VOC levels between the A549 and the NHLF cell lines, as well as between the collections of the same cell line. The analysis gives an extended view of the uptake and release of the VOCs by the studied cells. The paired Wilcoxon analysis compared the levels of the analytes in the samples to the pure medium. Here the VOC levels in the samples are discussed in relation to the other sample collections.

The Kruskal-Wallis statistical analysis showed significant differences ($p< 0.05$) in median peak area ratios for 41 VOCs and no differences for 21 analysed compounds in the MT experiment. The p-values for these compounds are shown in Table 3.7 (part A and B respectively). For the VOCs which the Kruskal-Wallis test gave a p-value at the level of significance $p< 0.05$, the Mann-Whitney U pairwise analysis of medians was conducted. For five compounds, namely 4-decanol; heptanol; 2-methoxy-diphenylmethane; styrene and p-xylene, the Kruskal-Wallis p-value was found to be significant, but the Mann-Whitney U test showed no significant differences in the median peak area ratios between the samples. This is most likely because the Mann-Whitney U test does not explicitly tie-in with the Kruskal-Wallis test. The Dunn's post-hoc test is more favourable in these terms as it is based on the same assumptions and employs the same rankings as the Kruskal-Wallis test (Dunn, 1964). However, because in the 'between-sample analysis' the stress was put on how the comparison relates to the Wilcoxon signed rank test ('between sample and control analysis') rather than on interpreting the results on their own, the Mann-Whitney U test was used which employs a similar approach of the Wilcoxon paired test (Whitley and Ball, 2002). The Mann-Whitney U test as a post-hoc after Kruskal-Wallis rejection is still widely recommended (Dytham, 2011; Ruxton and Bauchamp, 2008).

Table 3.7 Comparison of volatile organic compound peak area ratios (peak area of an analyte/peak area of an internal standard) between the A549 cell samples (n= 9) and the NHLF cell samples (n = 9), as well as between the collections of the same cell line for VOCs found in the MT experiment. **Part A**: VOCs for which the Kruskal-Wallis analysis found differences in peak area ratios when all the samples were compared, at a level of significance α = 0.05 and then the Mann-Whitney U test was conducted to compare the peak area ratios between each of the analysed samples. **Part B**: VOCs for which the Kruskal-Wallis analysis found no differences in peak area ratios when all the samples were compared, at a level of significance α = 0.05. A1: collection 1 of the A549 cells; A2: collection 2 of the A549 cells; N1: collection 1 of the NHLF cells; N2: collection 2 of the NHLF cells; N3: collection 3 of the NHLF cells; •1: VOC detected only in the A1 sample; •2 VOC detected only in the A2 sample; ○1 VOC detected only in the N1 sample; ○2: VOC detected only in the N2 sample; ○3: VOC detected only in the N3 sample. Colour codes: green: the level of the VOC was found higher for the earlier collection of the same cell line; orange: the level of the VOC was found lower for the earlier collection of the same cell line; blue: the level of the VOC was found higher for the A549 cell line; purple: the level of the VOC was found lower for the A549 cell line.

Part A											
Volatile organic compound	**A1 vs A2**	**N1 vs N2**	**N1 vs N3**	**N2 vs N3**	**A1 vs N1**	**A1 vs N2**	**A1 vs N3**	**A2 vs N1**	**A2 vs N2**	**A2 vs N3**	**Kruskal-Wallis p-value**
	Mann-Whitney U p-value										
Aliphatic hydrocarbons											
2,4-Dimethylheptane	1.0000	0.0345○2	0.0345○3	1.0000	0.0016•1	0.0029↑	0.0123↑	0.0016•2	1.0000	1.0000	1.883e-05
2,4-Dimethyl-1-heptene	1.000	1.000○2	1.000○3	1.000	0.035•1	0.265	0.118	0.772	1.000	1.000	0.01979
Dodecane	0.00288↓	1.00000	1.00000	1.00000	0.00041↓	0.00041↓	0.00165↓	1.00000	1.00000	1.00000	0.000313
4-Methyloctane	1.0000	0.6568○2	0.0532○3	1.0000	0.0016•1	0.1029	0.2141	0.0532•2	1.0000	1.0000	0.001057
4-Methylundecane	1.0000	1.0000	1.0000	1.0000	0.0016↑	0.0016↑	0.0029↑	1.0000	1.0000	1.0000	0.006574
Tetradecane	0.188	1.000	0.770	0.770	1.000	1.000	0.939	0.244	0.078	0.019↓	0.005844
2,3,6,7-Tetramethyloctane	0.00064•1	-	-	-	0.00064•1	0.00064•1	0.00064•1	-	-	-	9.02e-09
2,3,5-Trimethyldecane	1.0000	-	0.1256○3	0.1256○3	0.0014•1	0.0014•1	1.0000	0.1256•2	0.1256•2	1.0000	0.0001868
2,3,5-Trimethylhexane	1.0000	-	-	-	1.0000•1	1.0000•1	1.0000•1	0.0011•2	0.0011•2	0.0011•2	0.0001306

Table 3.7 Cont'd

Volatile organic compound	A1 vs A2	N1 vs N2	N1 vs N3	N2 vs N3	A1 vs N1	A1 vs N2	A1 vs N3	A2 vs N1	A2 vs N2	A2 vs N3	Kruskal-Wallis p-value
	Mann-Whitney U p-value										
Aromatic hydrocarbons											
1,1'-(1,2-Cyclobutanediyl) bis-,cis-benzene	0.1419	1.0000	1.0000	0.6253	1.0000	0.1419	1.0000	1.0000	0.0049↓	1.0000	0.02961
1,3-Di-*tert*-butylbenzene	1.000	1.000	1.000	1.000	0.040↓	0.316	0.056	1.000	1.000	1.000	0.02609
Ethylbenzene	1.000	0.503	0.028↓	1.000	1.000	0.188	0.028↓	1.000	1.000	1.000	0.01622
Styrene	0.142	1.000	1.000	0.625	0.503	0.078	1.000	1.000	1.000	0.625	0.03185
p-Xylene	1.000	1.000	0.503	0.142	1.000	1.000	1.000	0.078	0.106	1.000	0.01582
Alcohols											
Benzyl alcohol	0.77	0.14	0.04↑	1.00	0.04↓	1.00	1.00	0.04↓	1.00	1.00	0.008021
4-Decanol	0.63	1.00	1.00	0.24	0.50	0.63	0.19	1.00	1.00	1.00	0.04107
6-Dodecanol	1.00000	1.00000	0.93912	1.00000	0.02756↓	0.00082↓	0.00041↓	0.05636	0.00165↓	0.00165↓	1.107e-05
2-Ethylhexanol	0.00041↑	1.00000	1.00000	1.00000	0.00041↓	0.00041↓	0.00494↓	0.00041↓	0.00041↓	0.00041↓	1.069e-06
Heptanol	0.15•1	-	-	-	0.15•1	0.15•1	0.15•1	-	-	-	0.009643
3-Heptanol	0.00041↑	1.0000	1.0000	0.39984	0.00041↑	0.00041↑	0.00041↑	0.00082↑	0.00041↑	0.00041↑	5.512e-07
1-Nonanol	0.93912	1.00000	0.10613	1.00000	0.00041↓	0.02756↓	0.01851↓	0.00782↓	0.18758	0.39984	6.888e-05
1-Octanol	0.24434	1.00000	1.00000	1.00000	0.00082↓	0.00041↓	0.00082↓	1.00000	1.00000	1.00000	0.0004864
1-Phenylethanol	0.00041↑	1.00000	1.00000	1.00000	0.00041↑	0.00041↑	0.00041↑	0.00041↑	0.00041↑	0.00041↑	7.257e-07
Phenols											
2,4-Di-*tert*-butylphenol	0.2443	1.0000	0.1876	0.2443	1.0000	1.0000	0.0276↓	0.3998	0.3998	0.0078↓	0.002062
2-Nitrophenol	0.77005	1.00000	1.00000	1.00000	0.00041↓	0.00041↓	0.00041↓	0.31469	0.07775	0.31469	7.926e-05
Phenol	1.00000	1.00000	1.00000	1.00000	0.00041↓	0.00041↓	0.00041↓	0.00041↓	0.00041↓	0.00041↓	1.9e-06
Aldehydes											
Benzaldehyde	0.00041↓	1.00000	1.00000	1.00000	0.00082↓	0.00288↓	0.00288↓	1.00000	1.00000	1.00000	0.000198

Table 3.7 Cont'd

Volatile organic compound	A1 vs A2	N1 vs N2	N1 vs N3	N2 vs N3	A1 vs N1	A1 vs N2	A1 vs N3	A2 vs N1	A2 vs N2	A2 vs N3	Kruskal-Wallis p-value
	Mann-Whitney U p-value										
Dodecanal	1.000	1.000	0.040↓	0.040↓	1.000	1.000	1.000	1.000	1.000	0.012↓	0.0174
2-Ethenyl-2-butenal	0.07775	1.00000	1.00000	0.50309	0.00041↑	0.00041↑	0.00041↑	0.14192	0.00041↑	0.00041↑	2.187e-06
Heptanal	1.0000	1.0000	1.0000	0.0185↓	1.0000	1.0000	1.0000	1.0000	1.0000	0.0016↓	0.04063
Hexanal	0.3147	1.00000	1.0000	0.3998	1.0000	1.0000	0.1419	1.0000	1.0000	0.0029↓	0.02465
Octanal	1.0000	0.9391	1.0000	0.0185↓	1.0000	1.0000	1.0000	0.3998	1.0000	0.0049↓	0.01733
Ketones											
Acetophenone	0.00041↓	1.00000	1.00000	1.00000	0.02756↓	0.00041↓	0.00041↓	0.03990↓	0.00041↓	0.00041↓	5.025e-06
Cyclohexanone	1.00000	1.00000	1.00000	1.00000	0.00041↓	0.00041↓	0.00041↓	0.00041↓	0.00041↓	0.00041↓	1.931e-06
2,6-Di-*tert*-butyl-1,4-benzoquinone	0.0029↑	1.0000	1.0000	1.0000	0.0049↑	0.0049↑	0.0029↑	1.0000	1.0000	1.0000	0.0008138
Geranyl acetone	1.000	0.5031	0.0777	1.000	1.000	1.000	1.000	0.6355	0.1040	0.0057↓	0.008812
2-Pentadecanone	0.00041↑	0.14192	0.18758	0.00082	0.00041↑	0.00041↑	0.00082↑	0.93912	0.01234↓	0.00082↓	2.796e-07
2-Tetradecanone	1.00000	1.00000	1.00000	1.00000	0.01851↓	0.00288↓	0.00082↓	0.00288↓	0.00165↓	0.00082↓	1.275e-05
Esters											
Dodecanoic acid isooctyl ester	nr	0.51938	0.00773↑	0.00082↑	1.00000	1.00000	0.93912	nr	nr	nr	0.0004115
Pentanoic acid, 2,4-dimethyl-3-oxo-, methyl ester	0.00161•**2**	1.00000	1.00000	1.00000	1.00000○1	1.00000○2	1.00000○3	0.00041	0.00041	0.00401	0.0001756
Ethers											
2-Methoxy-diphenylmethane	0.77	0.20	1.00	0.50	1.00	1.00	1.00	0.24	1.00	0.24	0.03584

Table 3.7 Cont'd

Part B		
Volatile organic compound	**Comments**	**Kruskal-Wallis p-value**
α-Cumyl alcohol		0.6738
Cyclohexanol		0.1149
Decanal		0.6065
Decane		0.3808
Diisobutyl phthalate		0.3251
2,3-Dimethylheptane		0.4234
Dodecyl acrylate		0.240
Heptadecanal		0.6755
Heptadecane	• 1	-
Nonanal		0.3216
Nonadecane	• 1	-
2-Nonadecanone		0.4154
Octadecanal		0.1272
Pentadecanal		0.6776
Propanoic acid,2-methy-(2,2-dimethy-1-(2-hydroxy-1-methylethyl)propyl ester		0.5513
Propanoic acid,2-methyl-,3-hydroxy-2,4,4-trimethylpentyl ester		0.7848
Propanoic acid,2-methyl-,1-(1,1-dimethyl)-2-methyl-1,3-propanediyl ester		0.5745
Tetradecanal		0.0785
Tridecanal		0.230
Tridecane		0.3592
Undecane		0.6443

Comparison of the VOC trend levels between the cell lines

Aliphatic hydrocarbons

Most of the aliphatic hydrocarbons with significant Kruskal-Wallis p-values were not detected in all the analysed sample groups. 2,3,6,7-Tetramethyloctane was detected only in the A1 sample. 2,4-Dimethylheptane, 2,4-dimethyl-1-heptene and 4-methylocatne were not found in the N1 sample. 2,3,5-Trimethyldecane was not present in the N2 and N3 samples. Finally 2,3,5-trimethylhexane was present only in the cancer cell samples. Additionally, it was observed for these VOCs that they were not present in all the experiments (n = 3) within the sample (within one collection); for example, 2,4-dimethyl-1-heptane was found only in the experiment b of the A2 sample. Because this was the case for both samples and their pure medium controls, these results most probably reflect different initial concentrations of the analytes between different batches of the cell culture medium or different initial concentrations of the VOCs in different cell culture flasks (see Chapter 4.4.7, 'water experiment'). This phenomenon was often observed in the 'between-sample analysis'.

Differences in the median peak area ratios for 2,4-dimethyl-1-heptene were found to be significant only between the A1 and N1 samples ($p = 0.035$). Even though the VOC was observed to be produced by the A549 cells in collection 1, there were no differences found between the A1 and other fibroblast samples. Similarly, tetradecane was observed to be consumed by the A549 cells during the second week of incubation. However, the 'between-sample analysis' only detected significant differences in its levels between the A2 and N3 samples ($p = 0.019$). Both cases were most likely caused by the different batches of the media used or due to different initial concentrations of the analytes in different cell culture flasks.

Since 4-methylundecane was found to be produced exclusively by the A549 cells during the first week of incubation (when compared to the medium controls), it was found at increased levels in the cancer cell samples after the first week of cell culture in comparison to the fibroblast samples ($p < 0.003$). No significant differences were observed between the A2 cancer samples and any of the NHLF samples for 4-methylundecane.

2,4-Dimethyheptane was found to be produced by the A549 cells during the first and second week of incubation, and by the fibroblasts during the last two weeks of cell culture (when compared to the medium controls). The VOC was found at increased levels in the A1 sample in comparison to all fibroblast samples ($p < 0.015$), suggesting that the growing A549 cells produced the VOC at a higher rate than the growing (collection 2) and confluent (collection 3) fibroblasts. No significant differences in the levels of 2,4-dimethyheptane were found between the A2 sample and the last two collections for the NHLF cells where the fibroblasts produced the VOC. This suggests that 2,4-dimethyheptane was produced at a similar rate by the confluent cancer cells and by the growing and confluent fibroblasts. The significant difference found between the A1 and N1 samples ($p = 0.0016$) was due to the absence of 2,4-dimethylheptane in the latter.

The levels of dodecane were found to be significantly lower for the A549 samples when compared to the NHLF samples, but only in collection 1 ($p < 0.0005$). Dodecane was metabolised by the cancer cells during the first week only and by the fibroblasts only during the second week. Therefore, the growing cancer cells consumed the hydrocarbon at a higher rate than the growing fibroblasts.

4-Methyloctane was produced by the cancer cells during both weeks of incubation and by the fibroblasts during the last week of incubation. Significant differences were found only between the A1 and N1 samples because the VOC was not detected in the N1 sample at all ($p = 0.0016$). No other differences were found, indicating that both cell lines produced 4-methyloctane at a similar rate.

The levels of 2,3,6,7-tetramethyloctane were found to be significantly higher for the A1 sample when compared to all the NHLF samples, as this VOC was detected only in the A1 sample ($p = 0.00064$).

2,3,5-Trimethylhexane was observed to be produced only in the second collection of the A549 cell samples and the VOC was not found at all in any of the NHLF samples. Therefore the levels of this VOC were observed to be significantly higher for the A2 sample when compared to all fibroblast samples in the ‘between-sample analysis’ ($p = 0.0011$). No differences were found between the A1 and the NHLF samples because the VOC was not detected in all the repetitions of the A1 cell sample.

The levels of 2,3,5-trimethyldecane were found to be significantly higher for the A1 sample when compared to the N1 and N2 samples ($p = 0.0014$), because the VOC was produced by the cancer cells during the first week of incubation and it was not found at all in the N1 and N2 samples. However, 2,3,5-trimethyldecane was detected in some, but not all, repetitions for the A2 and N3 samples and here the levels were not different to those of the A1 sample.

Aromatic hydrocarbons

A benzene derivative 1,1'-(1,2-cyclobutanediyl)-bis, cis-benzene was produced by both cell lines during the first two collections of the MT experiment. During the first week no differences in the levels of the compound were observed, suggesting that both cell lines were releasing the analyte at similar levels. However, the fibroblasts were found to produce the VOC at significantly higher levels than the cancer cells during the second week of cell culture ($p = 0.0049$). During the third week of incubation, the NHLF cells were not observed to produce the VOC, therefore no differences in the levels of the VOC between the A2 and N3 samples were detected.

Ethylbenzene was consumed by the cancer cells and fibroblasts during the first week of the experiment. The 'between-sample analysis' showed that the VOC was metabolised at a similar rate by both cell lines. Significantly lower levels, however, were found for the A1 samples when compared to the N3 sample ($p = 0.028$), which again may be due to different initial levels of the VOC between the two samples.

The levels of 1,3-di-*tert*-butyl-benzene were found to be significantly lower for the A1 sample when compared to the N1 sample. This indicates a higher rate of metabolism of 1,3-di-*tert*-butyl-benzene by the cancer cells, because both cell lines were observed to consume the VOC during the first week of incubation.

Alcohols

Benzyl alcohol was observed to be produced solely by the NHLF cells in the first week of incubation. This is mirrored by the results of the 'between-sample analysis', as levels of the compound were found to be significantly lower for both collections of the A549 samples when compared to the N1 sample ($p = 0.04$).

6-Dodecanol was consumed by the cancer cells and produced by the normal cells, therefore the 'between-sample analysis' showed that there were significantly lower levels of this VOC ($p < 0.002$) in the A549 samples in comparison to the fibroblast samples. Since the fibroblasts produced the alcohol only during the last two weeks of cultivation, its levels were not significantly different between the A2 and N1 samples. It is not clear, however, why the differences in the levels of the VOC were significant between the A1 and N1 samples as there were no differences found between the A1 and A2 samples. The p-value of 0.5636 in the comparison between the A2 and N1 samples could potentially be significant because of the conservative Bonferroni correction used in the Mann-Whitney U test. Therefore, the differences between the N1 sample, where the normal cells did not yet produce the VOC, and the A1 and A2 samples were the VOC was consumed, would be caused by metabolism of this VOC by the cancer cells.

2-Ethylhexanol was metabolised by the cancer cells for the whole period of their cultivation, while NHLF cells produced it during the first and second weeks of incubation. These results are consistent with the 'between-samples analysis' as significant differences for both A549 samples were detected, when compared to all fibroblast samples ($p < 0.0005$).

The alcohols detected at significantly higher levels in the A549 samples when compared to the fibroblast samples in all the collections were 3-heptanol and 1-phenylethanol ($p < 0.001$). The first alcohol was produced solely by the cancer cells. However, 1-phenylethanol was released by both cell lines, in the case of the fibroblasts, only during the third week of incubation. Therefore, these results indicate that 1-phenylethanol was produced at higher levels by the cancer cells.

The levels of 1-octanol were found to be significantly lower for the A1 sample when compared to the NHLF samples ($p < 0.001$), as the alcohol was metabolised by the cancer cells during the first week only.

Another alcohol, 1-nonanol was found to be consumed by both cell lines during both weeks for the A549 cells and during the last two weeks of incubation for the NHLF cells. As the 'between-sample analysis' detected lower levels of the alcohol in the A1 sample when compared to the fibroblast samples ($p < 0.03$), and in the A2 sample when compared to the N1 sample ($p = 0.00782$), this indicates a higher level of metabolism of 1-nonanol by the cancer cells.

Phenols

The levels of 2,4-di-*tert*-butyl-phenol were found to be significantly higher for the N3 sample when compared to both cancer cell samples ($p < 0.03$). This is consistent with the results of the 'between sample and control analysis', where the compound was found to be produced by the fibroblasts only during the third week of incubation. However, the differences of 2,4-di-*tert*-butyl-phenol levels appeared to be insignificant between the three NHLF collections, suggesting again the possibility of initial differences in the VOC levels between the used medium batches.

Both phenol and 2-nitrophenol were observed to be consumed by the A549 cells during the whole period of the MT experiment. However, significantly decreased concentrations in the cancer samples in comparison to all the NHLF samples were observed only for phenol ($p = 0.0041$). The differences in the levels of 2-nitrophenol were found to be significant but only between the A1 sample and all of the fibroblast samples ($p = 0.00041$). It is not clear why there was no difference between the A2 sample and the NHLF samples. 2-Nitrophenol is most likely another compound which demonstrates variable concentrations in different batches of cell culture medium.

Aldehydes

The levels of benzaldehyde were found to be significantly lower in the A1 samples when compared to all the NHLF samples ($p < 0.003$). When compared to pure medium controls, benzaldehyde was observed to be consumed by both cell lines in all collections. The 'between-sample analysis' indicates, therefore, that growing A549 cells metabolised benzaldehyde at a higher rate than both growing and confluent normal cells. Because the aldehyde was also one of the compounds metabolised at a higher rate during the first week in comparison to the second week of culture for the A549 cells ($p = 0.00041$), the differences in medians were not significant between the A2 sample and the NHLF samples.

Dodecanal was produced by the fibroblasts during the third week of incubation and consumed by the cancer cells during the second week of incubation. Consequently, the VOC was found at higher levels in the N3 sample when compared to the A2 sample ($p = 0.012$).

The levels of hexanal were found to be significantly lower for the A2 sample when compared to the N3 sample ($p < 0.003$). The aldehyde was consumed by the A549 cells in both collections and by the fibroblasts during the last two collections of the MT experiment. Therefore, hexanal was metabolised at a similar rate by the growing cancer cells and growing normal cells, but when both cell lines reached confluency, the aldehyde was metabolised by the A549 cells to a higher extent than their non-transformed counterparts.

The levels of heptanal and octanal were also found to be significantly lower for the A2 sample when compared to the N3 sample ($p < 0.005$). These aldehydes were observed to be metabolised by the cancer cells and the fibroblasts in collection 2 only. Therefore, the confluent A549 cells metabolised the VOCs at a similar rate to the growing fibroblasts. Because during the third week of incubation the normal cells stopped the metabolism of the aldehydes, the differences were found to be significant between the A2 and the N3 samples. Also heptanal and octanal may have different concentrations in different medium batches, as no differences were found between the A2 sample and the N1 sample where the NHLF cells have not started consuming these compounds yet.

2-Ethenyl-2-butenal was found to be consumed by both cell lines in all collections when compared to the pure medium as a control. The 'between-sample analysis' showed that the compound was present at significantly higher levels in the A549 samples when compared to the fibroblast samples ($p < 0.0005$), except for the A2 and N1 samples pair. The results suggest that this aldehyde was metabolised at a higher rate by the confluent fibroblasts than by the growing and confluent cancer cells. The levels of the VOC were found to be similar for the growing NHLF cell and the confluent A549 cells.

Ketones

Acetophenone, cyclohexanone and 2-tetradecanone were found to be consumed solely by the cancer cells in the MT experiment. Consequently, their levels were observed to be significantly decreased in the A549 samples of both collections, in comparison to all of the fibroblast samples in the 'between-sample comparison' ($p < 0.04$, $p < 0.0005$ and $p < 0.02$ respectively).

2,6-Di-*tert*-butyl-1,4-benzoquinone and 2-pentadecanone were found at increased levels in the A1 sample when compared to all of the NHLF samples ($p < 0.005$). No significant

differences were observed between the A2 sample and any of the NHLF samples for the first VOC. 2,6-di-*tert*-butyl-1,4-benzoquinone was produced by the A549 cells during the first week and by the fibroblasts during the second week of incubation. Therefore, the growing cancer cells produced it at higher rate than growing fibroblasts. 2-Pentadecanone was emitted from the A549 samples during the first week of cell culture, while the NHLF cells were observed to produce it continuously throughout the three weeks. The results of the 'between-sample analysis' suggest that 2-pentadecanone was produced at significantly higher levels by the growing A549 cells, in comparison to the growing and confluent fibroblasts. During the second week, the A549 cells were found to stop producing it. NHLF collections 2 and 3, therefore, showed significantly higher levels of 2-pentadecanone in comparison to the A2 sample. The lack of significant differences in the levels of 2-pentadecanone between the N1 sample (where the ketone was already produced) and the A2 sample (where the cells stopped producing it), might be again due to different initial amounts of 2-pentadecanone present in different batches of medium.

During the third week of cultivation the NHLF cells were found to produce geranyl acetone. However, its levels in the N3 sample were similar to the A1, N1 and N2 samples. The cancer cells were observed to consume the VOC during the second week. This was reflected in the significantly elevated levels of geranyl acetone found in the N3 sample when compared to the A2 sample ($p = 0.0057$).

Comparison of the trend levels within the same the cell line

Some analysed VOCs were found to be consumed or produced at significantly different rates between the collections of the same cell line. This may reflect different metabolic pathways working during the exponential growth of the cells and when they reach confluency (see Chapter 5.3.2 for further discussion).

Acetophenone, benzaldehyde, and dodecane were detected at significantly lower levels in the A1 sample when compared to the A2 sample ($p < 0.003$). For dodecane these results reflect the fact that it was found to be metabolised by the A549 cells during the first week of incubation only. Acetophenone and benzaldehyde were observed to be consumed throughout the whole period of cultivation, which indicates that the compounds were metabolised at a higher rate by the growing cancer cells than by the confluent cancer cells.

Ethylbenzene was observed at higher levels in the N3 sample when compared to the N1 sample. This is in agreement with the 'between sample and control analysis', where the VOC was observed to be consumed during the first week of incubation only. Heptanal and octanal were found at higher levels in the N3 sample when compared to the N2 sample, because they were both consumed by the fibroblasts in collection 2. Therefore, only growing fibroblasts were shown to consume ethylbenzene, heptanal and octanal. Because there were no differences found between the N1 and N2 samples for ethylbenzene and between the N1 and N2 samples for heptanal and octanal, these VOCs may have variable initial concentrations in different batches of medium or in different cell culture flasks.

An opposite trend, showing the production of VOCs at a higher rate by the growing cells in comparison to the confluent cells, was demonstrated for 3-heptanol and 1-phenylethanol by the A549 cells ($p = 0.00041$). The lower concentrations of 2,6-di-*tert*-butyl-1,4-benzoquinone and 2-pentadecanone in the A1 sample when compared to the A2 sample were due to the uptake of these VOCs by the growing cancer cells only.

Similarly, benzyl alcohol and dodecanoic acid isooctyl ester were produced only by the growing fibroblasts ($p < 0.04$). The levels of benzyl alcohol were observed to be significantly lower for the N3 sample when compared to the N2 sample ($p = 0.04$). Dodecanoic acid isooctyl ester was found to be elevated in the N1 and N2 samples, when compared to the N3 sample ($p < 0.008$). The Wilcoxon paired analysis showed, however, that the fibroblasts produced benzyl alcohol only during the first week of incubation and the ester during the second week of incubation. Therefore, the lack of any differences in the alcohol levels between the N1 and N2 samples and the observed differences in the ester levels between the N1 and N3 samples, were probably due to variable concentrations of the volatiles between different batches of RPMI 1640.

2-Ethylhexanol was found at higher levels in the A1 sample when compared to the A2 sample ($p = 0.00041$). As the VOC was shown to be consumed by this cell line in the 'between sample and control analysis', the 'between-sample analysis' indicates that the metabolism of this alcohol depends on the number of cells. It was consumed to a higher extent by the confluent cancer cells than by the growing cancer cells.

Dodecanal was produced exclusively by the confluent fibroblasts and therefore it was detected at higher levels in the N3 sample when compared to the N1 and N2 samples in the 'between-sample analysis' ($p = 0.04$).

The levels of pentanoic acid, 2,4-dimethyl-3-oxo-, methyl ester were found to be significantly increased in the A2 sample when compared to the A1 sample. However, because the VOC was not detected in the A1 sample at all, the observed difference is probably due to the initial concentration of the VOC in culture medium. It is not known whether the VOC would be consumed by the A549 cells in collection 1 if the ester was available to them.

Finally, the significantly lower concentrations of 2,4-dimethylheptane found in the N1 sample when compared to the N2 and N3 samples were probably the result of a combination of the production of the VOC by the fibroblasts during the second and third weeks of incubation, and the lack of the hydrocarbon in the N1 sample.

Chapter 4

The Thermal Desorption Experiment

Aims:

• To develop a thermal desorption method with the use of sorbent tubes for capturing and semi-quantification of VOCs present in the HS of the cell culture medium.

• To identify VOCs detected in the HS of the medium cultured with the A549 lung cancer cell line and with the BEAS-2B non-cancerous lung cell line, and in the HS of their pure medium controls.

• To determine precision of the TD method used.

• To compare the levels of the VOCs detected in the cell samples in the TD experiment between the A549 and the BEAS-2B cell lines, as well as between the collections of the same cell line.

• To compare the levels of the VOCs detected between water samples collected as "controls" after one and then two weeks of incubation.

4.1 Introduction

The technique of dynamic HS analysis was introduced in 1962 by Swinnerton *et al*. for the detection of volatiles dissolved in aqueous samples. Here the volatiles were stripped by an inert gas from the sample and transferred directly into a gas chromatograph. The technique was developed into P&T extraction by Bellar and Lichtenberg (1974). In P&T, the volatiles are carried with a carrier gas through a trap where the analytes are first retained. Next, the compounds are desorbed from the trap and introduced into the gas chromatograph for analysis. Since the development of the porous polymer Tenax in 1970 (Van Wijk), the method has became very popular for the analysis of VOCs in various scientific fields, such as food and alcoholic beverage analysis (Aznar and Arroyo, 2007; Heikes *et al.,* 1995; Vanderhaegen *et al.,* 2007), environmental monitoring of and assessment of human exposure to pollutants (Ashley *et al.,* 1992; Zoccolillo *et al.,* 2005), forensics (Ehara and Marumo, 1998), herbal medicine (Mazza and Cotrell, 1999), pharmaceutical quality control (Lakatos, 2008) and breath analysis and *in vitro* analysis for disease diagnosis (Filipiak *et al*., 2008; Phillips M. *et al*., 1997).

4.1.1 *TD sorbent tubes as an extraction technique*

A sorbent tube is filled with one or more types of adsorbent material such as porous polymers (e.g. Tenax®, PoraPak), active carbon and graphitised materials (e.g. Carbopack, Carbotrap®) or carbon molecular sieves (e.g. Carbosieve, Carboxen). When two or more adsorbents are used they are packed into the tube from the weakest to the strongest material, starting from the sampling inlet. The higher the surface area, the stronger the adsorbent material, although the strength of the material becomes more dependent on the size and shape of the pores for adsorbents with a surface area > 800 m^2 g^{-1} (Brown, 2013). The tubes require pre-conditioning at a certain temperature before the first sampling and after a longer period of non-use, to prevent the appearance of the interfering compounds in the chromatogram. The volatiles in the HS of the sample vial are transferred onto the trap with a stream of a carrier gas, or an aliquot of the HS air is sucked by a pump connected to the tube. Sampling should be always performed in the correct 'sampling flow' direction, as shown in Figure 4.1. This is because analytes with a lower BP will break through the weaker adsorbent but will be efficiently retained by the stronger adsorbent at the back (and easily released during desorption). The higher-boiling compounds should not be allowed

to reach the stronger adsorbent at the back (by placing a weaker one before it), as they will be too strongly adsorbed, or irreversibly adsorbed. With the use of a weaker adsorbent, they will be easily released during desorption (Brown and Shirey, 2001). During primary desorption the process is reversed.

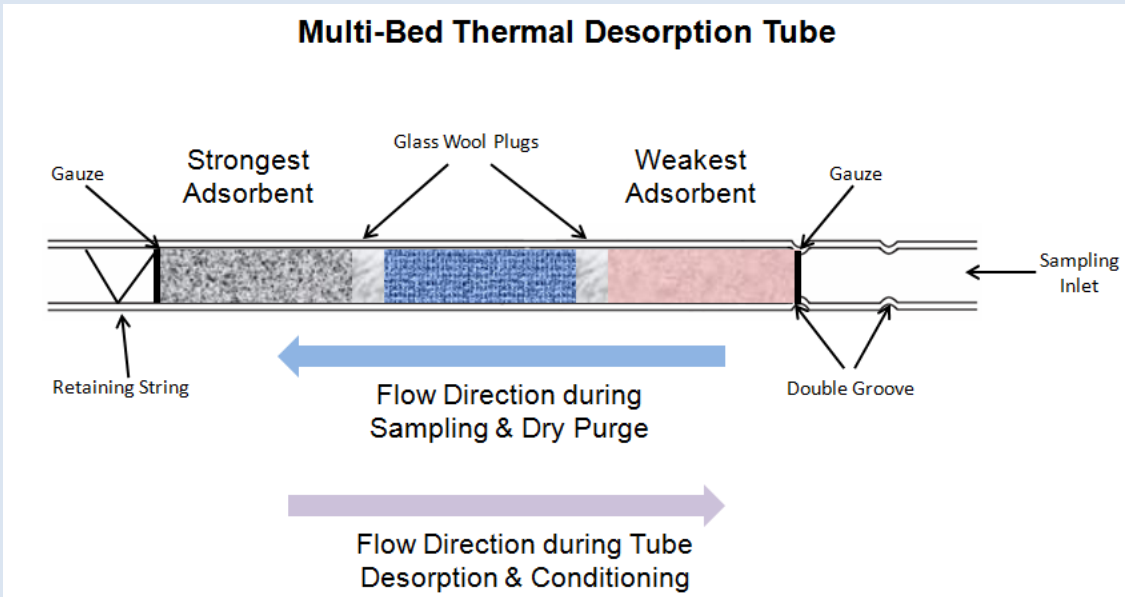

Figure 4.1 Diagram of multi-bed thermal desorption tube. Adsorbents are packed into the tube from the weakest to the strongest material, with glass wool plugs in between, starting from the sampling inlet (double grooved end). Sampling is performed in the sampling flow direction and primary desorption in the reverse direction (figure adapted from Brown J., 2013).

Once the tube is loaded onto the thermal desorber, the analysis starts by removing air and water so that analytes and sorbents are not subjected to oxidation at high temperatures and the water is not introduced into the GC. A flow of dry air or an inert gas at an ambient temperature are purged through the tube. Next, during primary desorption the tube is heated for a certain amount of time (3-5 min) at a particular temperature. The volatiles are desorbed from the tube and are transferred with a flow of the carrier gas onto the cold trap (Peltier trap). During primary desorption an inlet split might be applied so that only part of the sample is loaded onto the cold trap. The desorption flow of the tube is the reverse of the sampling flow, so that the smaller volatiles (lower BP) are removed first from the tube and retained at the front of the cold trap.

During secondary desorption, the cold trap is heated very rapidly to transfer the analysed compounds to the GC column. Here the outlet split flow might be adjusted for more concentrated samples (so that only part of the sample retained on the cold trap enters the GC column). Again, during secondary desorption the flow is reversed when compared to sampling flow, so that the lower MW compounds enter the GC column first. A diagram of primary and secondary desorption is shown in Figure 4.2.

As the TD experiment was simply a screening and un-targeted analysis, a multi-bed adsorbent tube was used to cover a greater range of potential analytes in terms of their MW, polarity and volatility.

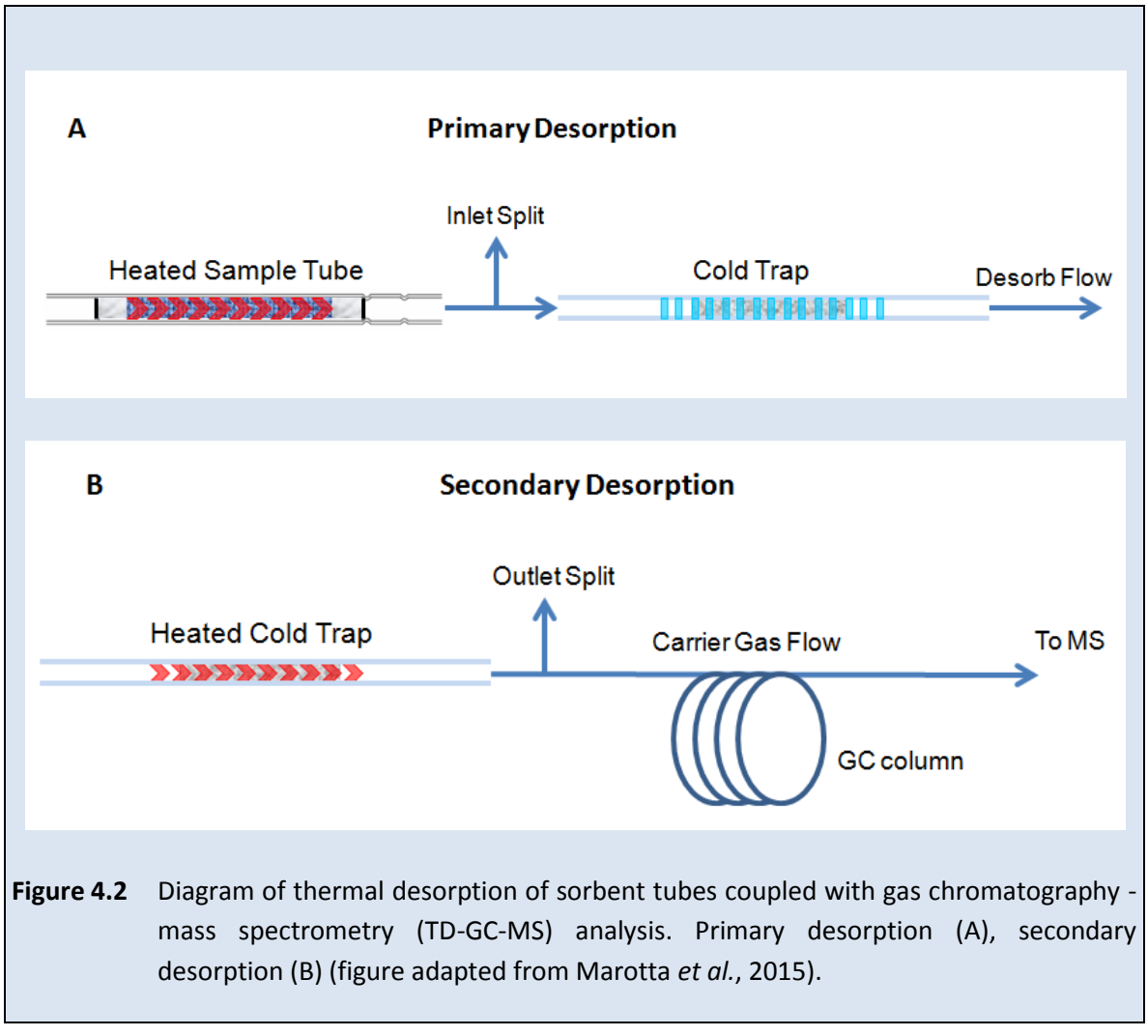

Figure 4.2 Diagram of thermal desorption of sorbent tubes coupled with gas chromatography - mass spectrometry (TD-GC-MS) analysis. Primary desorption (A), secondary desorption (B) (figure adapted from Marotta *et al.*, 2015).

4.1.2 *TD sorbent tubes versus other extraction techniques*

The comparison of TD sorbent tubes as a technique of extraction of VOCs from aqueous samples to three competitive extraction techniques i.e. MMSE, SPME and SBSE was discussed in chapter 3.1.2 (Table 3.1). Among these four techniques, only SPME and P&T onto sorbent tubes were used in studies where VOCs were collected from cell culture medium. However, loading the sample onto TD tubes with the use of an Easy-VOC™ pump has not been performed before in *in vitro* studies of VOCs. The Easy-VOC™ is a hand-held pump that allows for small volumes of sample air (50 - 500 ml) to be loaded directly onto TD tubes with no need for calibrated pumps. The HS sampling of the flask containing cell culture medium with the use of the Easy-VOC™ is shown in Figure 2.5.

4.2 TD sorbent tubes method development

In order to optimise the extraction of VOCs by TD tubes, several factors were varied. They included: the type of the multi-sorbent tubes used (Air Toxics™ or SVI™), the sample air volume (100 ml and 200 ml), the flow of the inlet split and the time of air flush.

The results of these studies are presented below. A comparison of the peak areas of ten selected VOCs from the TIC of RPMI 1640 medium (n = 3) was undertaken, in order to assess the impact of the sample air volume and the split ratio flows on VOC detection. The impact of the type of the TD tube used and time of air flush were assessed in singular experiments (n = 1). The method was not optimised on the cell medium incubated with cells, in order to exclude potential VOC variability arising from the presence of cells. A range of compounds from different areas of the chromatogram (i.e. VOCs with the low, medium or high RTs), with well separated peaks and with a relatively high intensity, was chosen.

The selected VOCs represented five chemical groups commonly detected in studies of VOCs *in vitro*: alcohols (2-ethylhexanol), aldehydes (benzaldehyde), ketones (acetophenone, 2-pentanone), hydrocarbons (heptane, decane) and aromatic hydrocarbons (benzene, ethylbenzene, styrene). 2-Methylfuran has not been previously detected in any *in vitro* study of VOCs. Identification of all the selected VOCs was based on spectral matching with the NIST library and was confirmed against the RTs of standards (data not shown). All VOCs (except 2-methylfuran) were detected in cell-free RPMI 1640 cell

culture medium in studies by others, but also in other types of cell culture media (Barash *et al.*, 2009, 2012; Davies *et al.*, 2014; Hanai *et al.*, 2012b; Filipiak *et al.*, 2008; 2010; Kwak *et al.*, 2013; Sponring *et al.*, 2009; Zimmermann *et al.*, 2007).

Similarly as in Chapter 3.2 the graphs presented in this chapter show peak areas (detector's response) for each of the selected compounds obtained with various experimental settings. Each bar represents a mean of peak areas (n = 3) for a particular analyte obtained from three samples. Error bars are marked on the graphs for each analyte and represent one SD of uncertainty. The differences in peak areas were compared with the use of the t-test (paired sample, 2-tailed) for two reasons. Firstly, because the data obtained for < 5% of VOCs in the TD method development failed the assumption of normality, this indicated that the results were approximately normally distributed (Shapiro-Wilk, $\alpha = 0.05$) (Appendix B, Tab. B3). Secondly, because the Wilcoxon signed-rank test (a non-parametric equivalent to paired t-test) requires at least 6 differences ($n \geq 6$). The differences in peak areas were considered significant when p value of the t-test < 0.05.

4.2.1 TD sorbent tube type

Two types of TD tubes were tested: Air Toxics™ and SVI™. Figure 4.3 shows two chromatograms of the extraction of the HS of the cell culture medium, performed with Air Toxics™ (A) and SVI™ (B) tubes. In general, the SVI™ tube allowed for the detection of compounds with a higher MW, while the Air Toxics™ tube adsorbed smaller MW compounds (< C11). VOCs eluting after undecane (RT: 18.54 min) were adsorbed at lower levels (for example nonanal, RT 18.69 min, dodecane, RT: 21.76 min, and benzothiazole, RT: 22.50 min), or not at all (for example decanal, RT: 21.50 min, tridecane, RT: 24.76 min, and diphenyl ether, RT: 26.90 min) with the use of the Air Toxics™ tube. However, some compounds with lower MW such as methylcyclohexane (RT: 4.05 min, MW: 98) and toluene (RT: 5.79 min, MW: 92) were adsorbed at higher levels when the Air Toxics™ tube was used. The observed results reflected the MW adsorption ranges of the tubes. SVI™ tubes contain four adsorbents (Carboxen and Carbotrap®) with the MW adsorption range of C2-C30, while Air Toxics™ tubes have an analyte volatility range of C2-C14. As the entire profile of VOCs that can be found in the

HS of the cell culture medium was of interest, the SVI^{TM} tubes were selected for the further analysis of VOCs in the TD experiment.

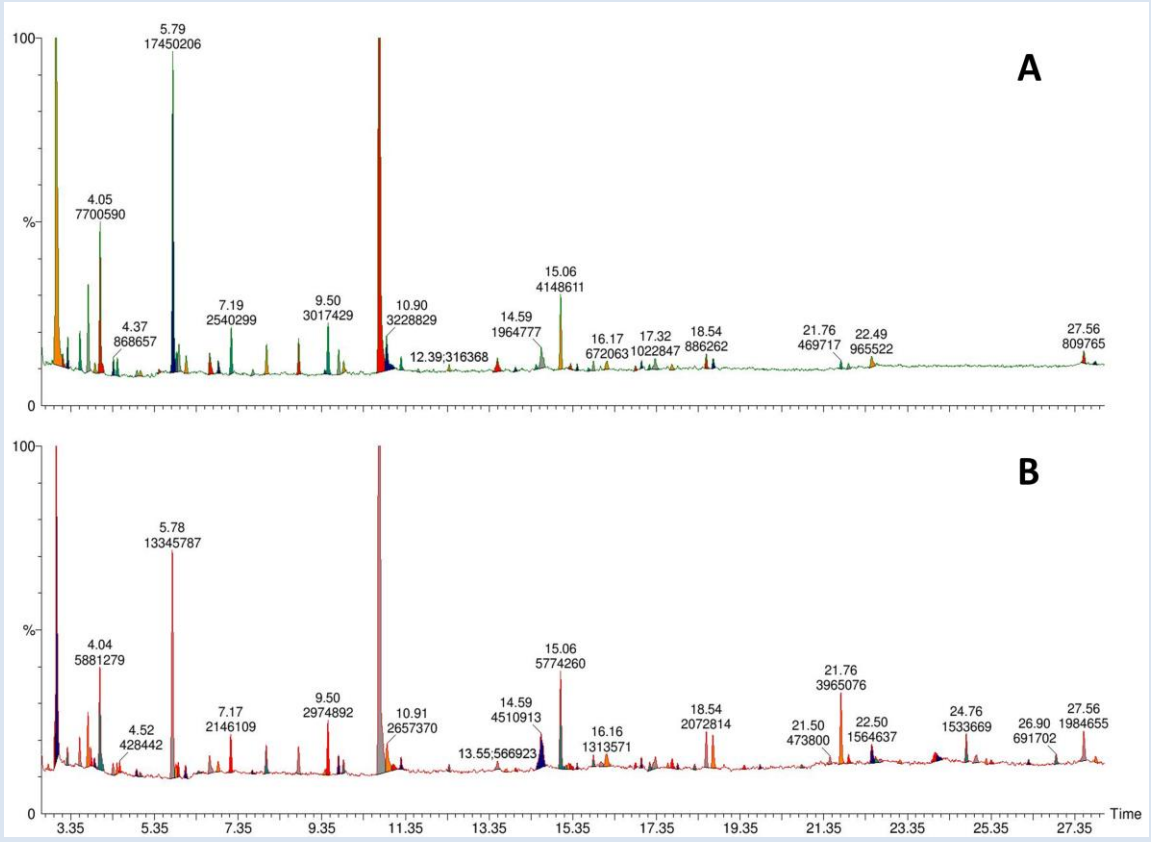

Figure 4.3 Total ion chromatograms of VOCs detected in the headspace of the RPMI 1640 cell culture medium incubated 7 days at 37°C with the use of thermal desorption of sorbent tubes coupled with GC-MS. (A) Extraction conducted with the use of Air Toxics^{TM} TD tube. (B) Extraction conducted with the use of Soil Vapour Intrusion (SVI^{TM}) TD tubes.

4.2.2 Volume of the extracted air

The use of an Easy-VOC^{TM} allows for sampling volumes in the range of 50 - 500 ml at 50 ml intervals (i.e. 50, 100, 150 ml). The volume of the ThermoFisher T-75 flask used is ~280 ml and this allowed for a maximum sampling HS volume of 200 ml. Therefore, the use of 100 and 200 ml volumes of the sampled HS was investigated. The results obtained for the 10 model compounds are shown in Figure 4.4. Most of the compounds (acetophenone, benzaldehyde, benzene, heptane, decane, 2-ethylhexanol and 2-pentanone) were extracted at approximately double amounts with the higher sample volume.

However, the sample volume did not have any significant effect on the extraction of ethylbenzene, 2-methyfuran and styrene. The volume experiment was performed without a dry air flush of the flasks and this might be a result of different residual amounts of these VOCs in different flasks (see Chapter 4.4.7 for further discussion).

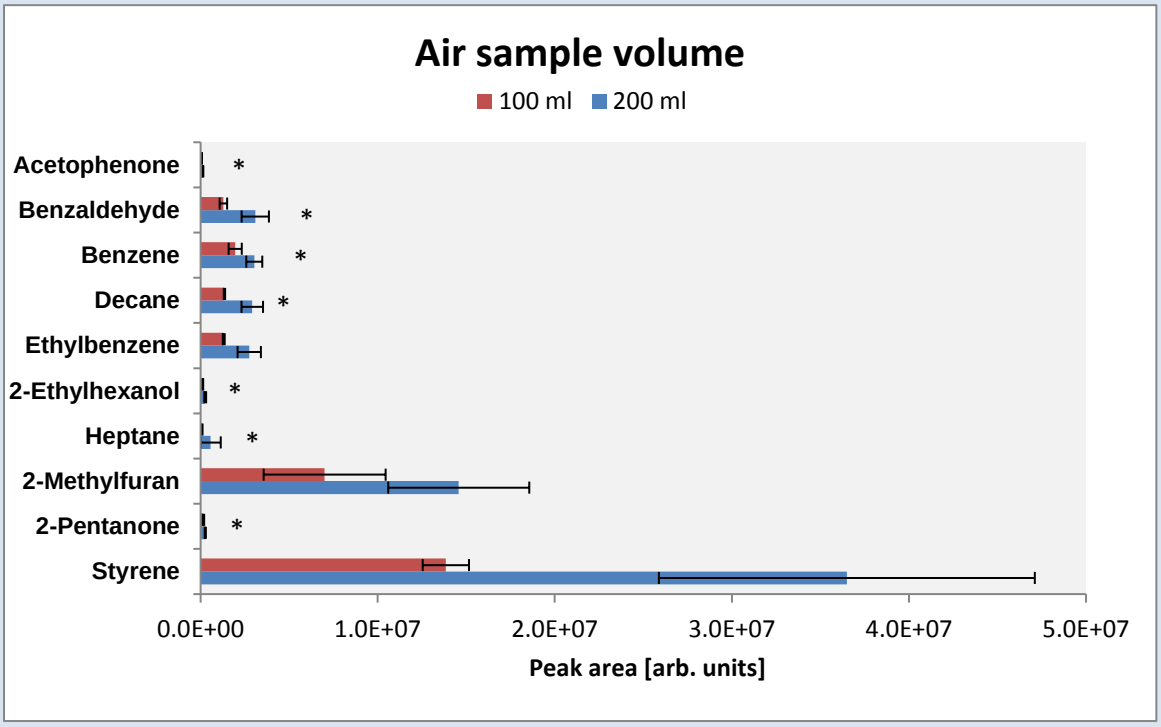

Figure 4.4 Influence of the air sample volume on the efficiency of thermal desorption of acetophenone, benzaldehyde, benzene, decane, ethylbenzene, 2-ethylhexanol, heptane, 2-methylfuran, 2-pentanone and styrene. The VOCs were detected in the headspace of RPMI 1640 complete cell culture medium incubated for 7 days at 37°C. Bars represent the means of peak areas for n = 3. Significant differences at $p < 0.05$ between the two tested volumes are marked with an asterix (paired samples t-test, 2-tailed).

4.2.3 Sample split ratio determination

During primary desorption an inlet split could be enabled, so that only a portion of the sample from the TD tube is transferred onto the cold trap. During secondary desorption an outlet split could be applied so that only a portion of the sample from the cold trap is transferred onto the GC column. The ability to have split points on the inlet and outlet of

the cold trap facilitates water management, as the amount of water entering the GC column is reduced. The percentage of the sample reaching the GC column (sample split ratio) can be calculated (Chapter 2.4.1, Carrier gas flow rates). The outlet split flow is recommended to be set at a minimum 10 ml min^{-1}. The inlet split flow during TD analysis was enabled, as very high water backgrounds were observed in the MS with inlet split off. This was also the case for inlet split flow set to 10 ml min^{-1}. Therefore, higher sample split ratios could not be investigated. Cell culture medium is a humid sample and high water trapping onto the TD tubes was most likely taking place. The effects of the sample split ratios are shown in Figure 4.5.

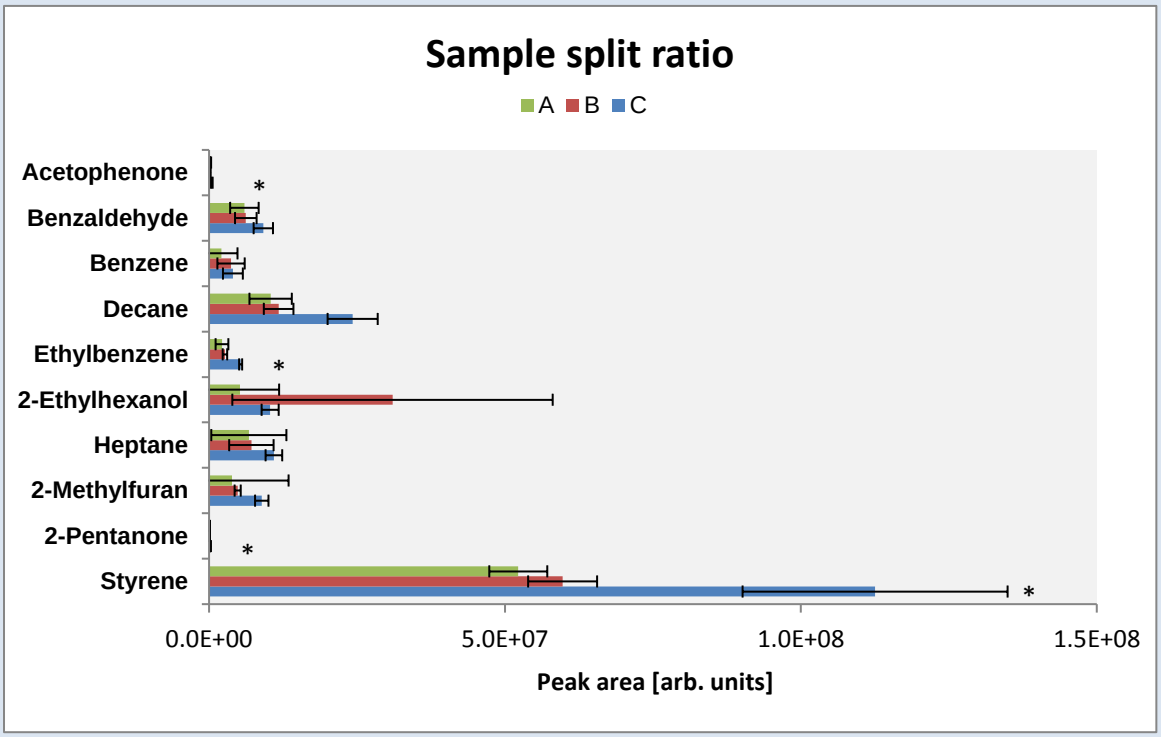

Figure 4.5 Influence of the sample split ratio used on the efficiency of thermal desorption of acetophenone, benzaldehyde, benzene, decane, ethylbenzene, 2-ethylhexanol, heptane, 2-methylfuran, 2-pentanone and styrene. The VOCs were detected in the headspace of RPMI 1640 complete cell culture medium incubated for 7 days at 37°C. A: inlet split 10 ml min^{-1} and outlet split 20 ml min^{-1}; B: inlet split 30 ml min^{-1} and outlet split 10 ml min^{-1}; C: inlet split 20 ml min^{-1} and outlet split 10 ml min^{-1}. Bars represent the means of peak areas for n = 3. Difference is significant between the bar with an asterix and the other tested split ratio at $p < 0.05$ (paired samples t-test, 2-tailed).

Three sample split ratios were investigated:

A - inlet split flow 10 ml min^{-1} and outlet split flow 20 ml min^{-1} (3.2% of the sample reaching the GC column).

B - inlet split flow 30 ml min^{-1} and outlet split flow 10 ml min^{-1} (3.6% of the sample reaching the GC column) and;

C - inlet split 20 ml min^{-1} and outlet split flow 10 ml min^{-1} (4.5% of the sample reaching the GC column);

As sampling cell culture medium is a trace analysis, sample split ratios < 3% were not investigated. Sample split ratios A and B allowed for the detection of the VOCs at similar levels. Sample split ratio C yielded significantly higher peak areas for acetophenone, ethylbenzene, styrene and 2-pentanone. However, it did not show significant differences when compared to the other two split ratios for the remaining compounds.

4.2.4 Air flush of cell culture flasks

The initial method development of the TD experiment involved the sampling of empty cell culture flasks. It was noted that freshly opened vessels had a relatively high amount of compounds present in the chromatogram, apparently originating from the flask. The amount and intensity of the compounds decreased visibly once the flask was left open at room temperature (data not shown). Because the flasks are sterile, it is not good practice to keep them open for a prolonged time, even in the cell culture cabinet. Therefore, to reduce the background levels of the VOCs originating from plastic, after seeding the cells, the cell culture flasks were flushed with dry air. Figure 4.6 shows that in this way the levels of the residual volatiles were significantly reduced. The dry air flush was performed for 20 min at the 90 ml min^{-1} flow.

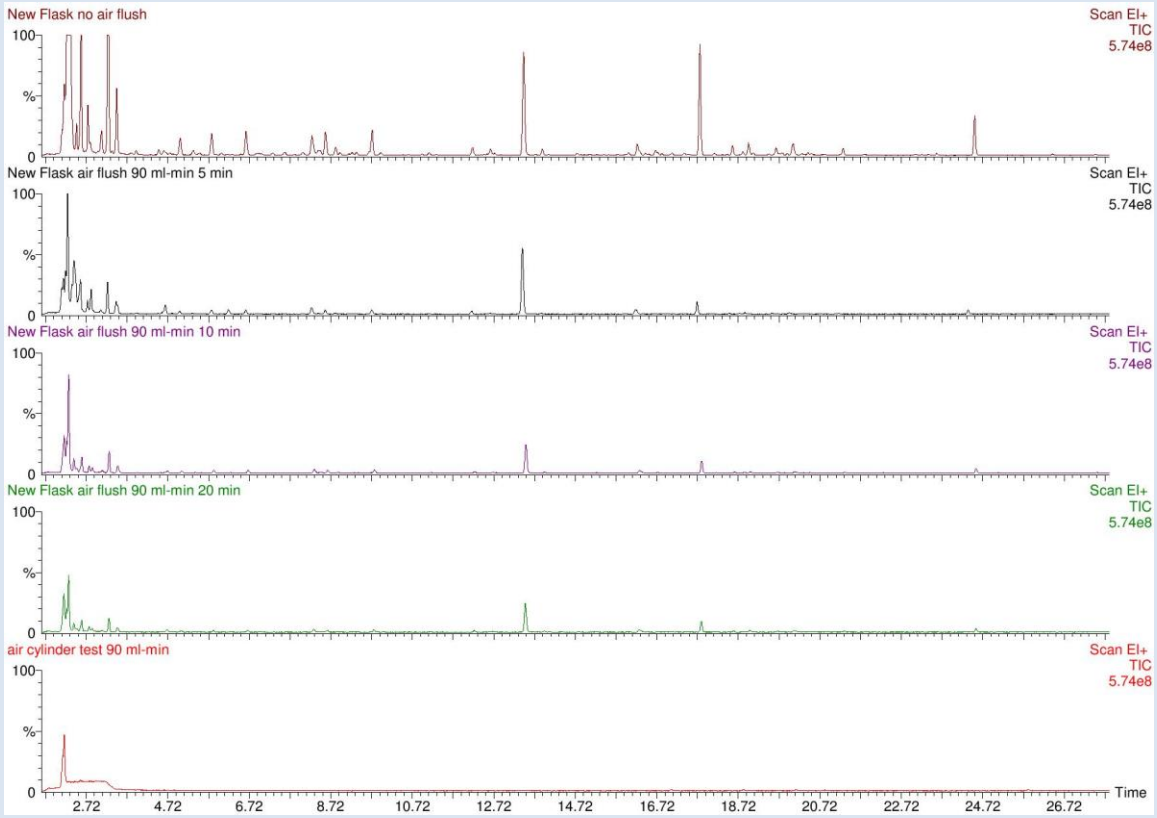

Figure 4.6 Total ion chromatograms of VOCs detected in the headspace of new empty cell culture T-75 flasks, both unflushed and flushed for 3 different periods of time, and in the air flowing from the cylinder, directly loaded onto the Thermal Desorption Tube. Sampling was performed on: a new flask with no air flush; a new flask air flushed for 5 min at 90 ml min^{-1}; a new flask air flushed for 10 min at 90 ml min^{-1}; a new flask air flushed for 20 min at 90 ml min^{-1}; dry air flowing from the cylinder. Extraction conducted with the use of Soil Vapor Intrusion (SVI™) TD tubes, air sample volume: 200 ml.

4.3 Cell culture conditions

4.3.1 Cell seeding density

Similar to the MT experiment (Chapter 3), the highest possible recommended seeding density was used for the A549 and BEAS-2B cell lines. The same volume of 30 ml was used in the TD experiment as in the MT experiment, as the seeding densities used were the highest recommended and the medium was not changed for a week. Therefore, a higher

amount of the medium is sufficient for supplying the essential nutrients and supplements to the cells for longer.

4.3.2 Time of incubation

The A549 and BEAS-2B cells were reaching confluency on the 6th day of cultivation. The HS of the flasks containing A549 and BEAS-2B growing cells were sampled after one week and then after two weeks of incubation. As confluent BEAS-2B cells demonstrated morphology of squamous cells, the second collection of the BEAS-2B cells was profiling VOCs of differentiated cells. The cell morphology of typical lung epithelial cells is predominantly cuboidal and polygonal. Differentiated squamous BEAS-2B cells have no well-defined cell borders and have a flat appearance (Zhao and Klimecki, 2015). Figure 2.1 shows growing BEAS-2B cells demonstrating a still epithelial-like morphology (C) and confluent BEAS-2B cells with a differentiated squamous appearance (D).

4.3.3 Cell culture medium

As discussed in chapter 3.3.3, different types of media are recommended for the cultivation of A549, NHLF and BEAS-2B cell lines. As different media types are likely to emit different VOCs, for a more direct comparison of the VOCs being released or consumed by the analysed cell lines, the same type of medium, RPMI 1640, was used for the cultivation of all the cell lines. It has been widely used in studies of VOCs for the culture of A549 and BEAS-2B cell lines by Barash *et al.* (2009, 2011), Brunner *et al.* (2010), Pyo *et al.* (2009) and Wang Y. *et al*., (2012).

4.3.4 Cell viability

Viability tests were performed on each of the sampled flasks after the period of incubation had finished. After two weeks of cultivation the average viability of the A549 cells was 97.1 ± 0.7 % (n = 9) and of the BEAS-2B cells the average viability was 97.7 ± 1.2 % (n = 9). The viabilty percentages for both cell lines indicated that the cell culture conditions were appropiate for the analysed cell lines and that the analysed VOCs were potentially coming from the living cells and were not due to the processes associating with cell death.

4.3.5 Internal standard concentration

1-Dimethoxyethane (DME) was selected as an ISTD for the analysis of VOCs from the cell culture medium in the TD experiment, as it did not co-elute with any VOC detected in medium during the TD method development, it is miscible in water, it was not found in the KEGG database as an intermediate or final metabolite produced by any living organism present in the database and finally its acute toxicity is relatively very low. The final concentrations of 4.0 mg l^{-1} and 2.0 mg l^{-1} for DME were tested. 2-F was investigated at the level of 1.0 ml l^{-1} and 2.0 mg l^{-1}. In the TD experiment only DME was used as an ISTD, because 2-F appeared to be consumed by the A549 cells.

4.4 *In vitro* TD-GC-MS analysis of VOCs

4.4.1 Optimised method for TD-GC-MS

The method that was developed after looking through the variety of effects of different factors on the adsorption and desorption of VOCs found in the RPMI 1640 culture medium with the use of TD sorbent tubes is shown in Figure 4.7. SVI^{TM} TD sorbent tubes were selected for this application. The volume of 200 ml of air sample was loaded onto the tube. Dry purge was set to 5 min. Primary desorption was carried out for 5 min at 330°C with a cold trap at 5°C temperature. The temperature of the cold trap during the secondary desorption was set to 300°C. Gas flows were set as follows: desorb flow to 20 ml min^{-1}, inlet split to 20 ml min^{-1} and outlet split to 10 ml min^{-1}.

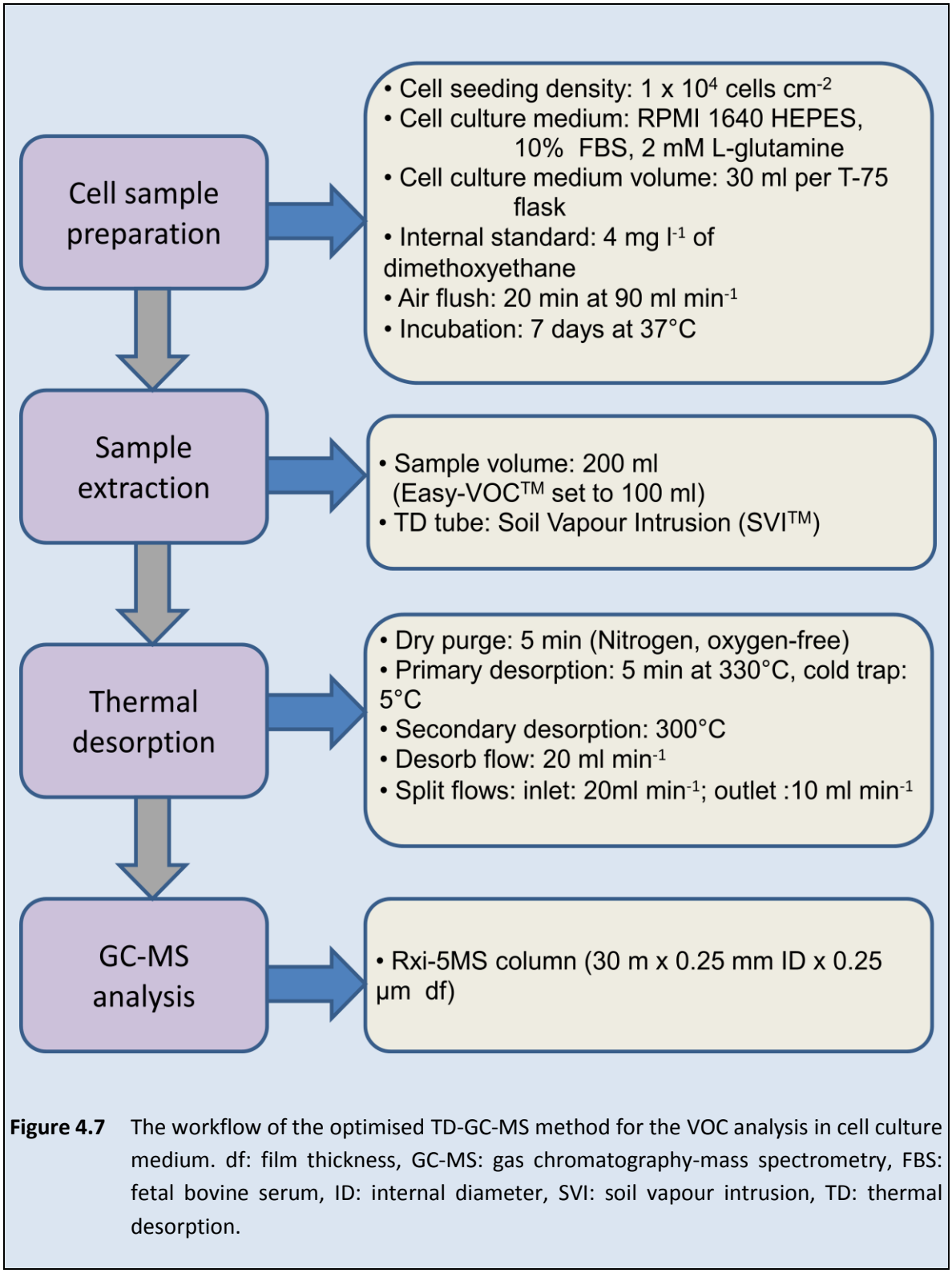

Figure 4.7 The workflow of the optimised TD-GC-MS method for the VOC analysis in cell culture medium. df: film thickness, GC-MS: gas chromatography-mass spectrometry, FBS: fetal bovine serum, ID: internal diameter, SVI: soil vapour intrusion, TD: thermal desorption.

4.4.2 Identification of the detected VOCs

In the TD experiment the two analysed cell lines, A549 and BEAS-2B, were cultivated for seven days. After one week of incubation the VOCs in the HS phase were collected onto the sorbent trap. Then the old medium was removed, a fresh medium added and the flasks were incubated for a further seven days. Here both cell lines were reaching confluence at the 6^{th} day of incubation. In this way, the HS from the flasks was collected from the proliferating cells (collection 1) and from the confluent cells (collection 2).

Figure 4.8 presents a chromatogram of the pure cell culture medium obtained in FS mode in the TD experiment. A typical TD chromatogram contained around 60 peaks. The peaks of all identified VOCs (except siloxanes) are numbered in the chromatogram and their identification is presented above it. Peaks of compounds such as acetone, acetophenone, DCM, 2,4-dimethylfuran, nonane, pentane, *tert*-butanol, d-limonene, 2-ethylhexanol, 4-methylundecane and 2,2,4-trimethylheptane were usually not visible in the FS mode. The RTs of these VOCs are marked with blue arrows.

All the VOCs (except siloxanes) that were detected in the HS of the cell culture medium and identified in the TD experiment are given in Table 4.1. Four compounds were identified as siloxanes and 44 VOCs were identified as members of other chemical groups. Thirty out of 44 VOCs were identified by not only the NIST library search, but also by comparison of their mass spectra and RTs against those of the reference standards. The VOCs identified by confirming their RTs against the RTs of chemical standards are noted in Table 4.1. The ions selected for peak integration (semi-quantification) are also shown. There were a few VOCs present in the chromatogram that were identified, however peak integration was not possible due to S/N < 3 in SIM. The table shows the analytes in order of their RTs. Closely eluting compounds and VOCs detected at S/N < 10 in the TIC were semi-quantified by the use of SIM mode. The MWs of the analytes were in the range of 44 - 135 Da and their BPs were between 18°C and 227°C.

The VOCs detected in the TD analysis belong to the following chemical groups: aliphatic, aromatic and halogenated hydrocarbons, alcohols, aldehydes, ketones, esters, furans and sulfides.

1: Acetaldehyde
2: Ethanol
3: Pentane
4: Acetone
5: tert-Butanol
6: Carbon disulfide
7: Dichloromethane
8: 2-Methylpentane
9: 3-Methylpentane
10: 2-Methylfuran
11: Ethyl acetate
12: Chloroform
13: Tetrahydrofuran
14: Dimethoxyethane (ISTD)
15: Benzene
16: 2- Methylbutanal
17: 2-Pentanone
18: 3-Methylhexane
19: Heptane
20: 2,4-Dimethylfuran
21: Methylcyclohexane
22: Toluene
23: 4-Methylheptane
24: 3-Methylheptane
25: Octane
26: 2,3,5-trimethylhexane
27: Ethylcyclohexane
28: 2,4-Dimethyl-1-heptene
29: Ethylbenzene
30: 4-Methyloctane
31: p-Xylene
32: Styrene
33: Nonane
34: Benzaldehyde
35: 2,2,4,6,6,-Pentamethylheptane
36: Decane
37: 5-Ethyl-2,2,3-trimethylheptane
38: 2-Ethylhexanol
39: d-Limonene
40: 4-Methylundecane
41: Acetophenone
42: Undecane
43: Dodecane
44: Benzothiazole

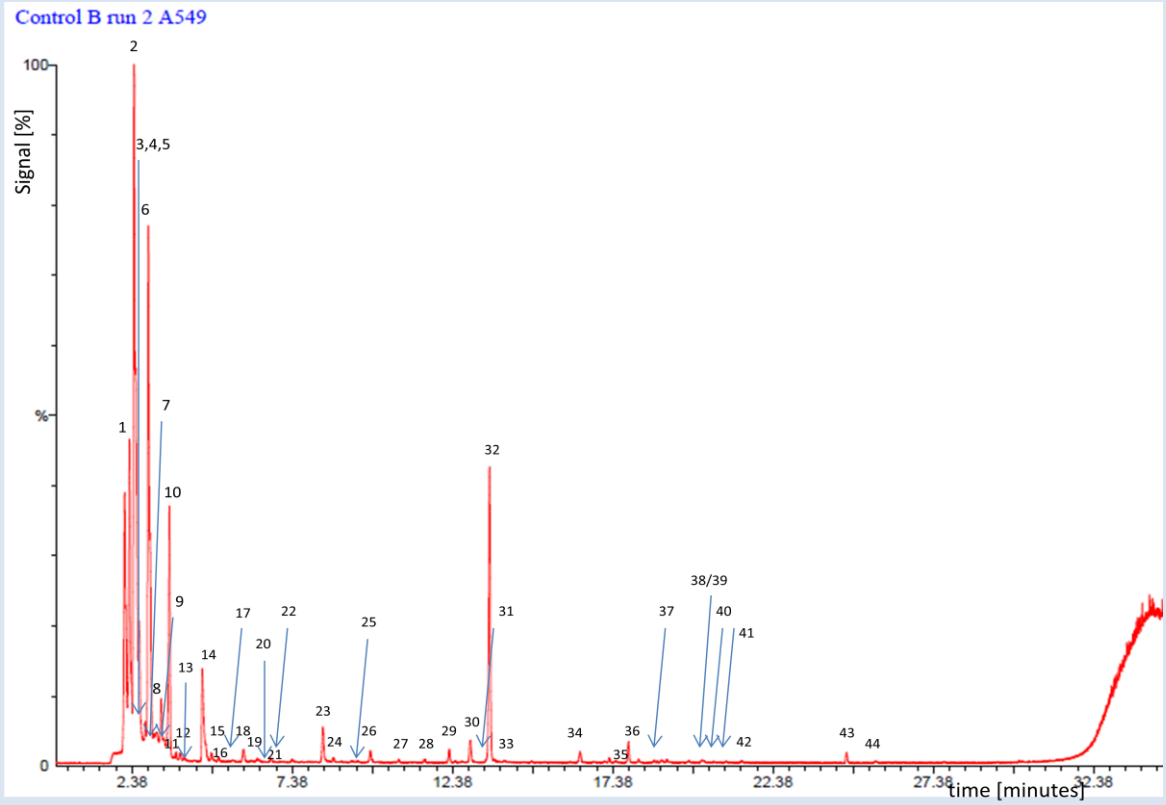

Figure 4.8 Total ion chromatogram (TIC) of the pure medium control. Arrows indicate compounds usually visible in TIC at S/N < 10.

NIST library search

The compounds identified in the TD experiment listed in Table 4.1 had NIST match factors > 600. The majority of the compounds detected in the TD experiment had a match factor > 850. What is more, the first three or four hits for these compounds were the same,

indicating a very good match. For most of the detected VOCs, the analyte reference standards were run to verify their identification. The VOCs for which the NIST library search yielded low match factors in the range of 600 - 700 demonstrated either S/N < 10 (nonane; d-limonene and methyl isobutyl ketone) or were co-eluting with other analytes (acetaldehyde; 2-ethylhexanol and tetrahydrofuran).

Top five hits of search NIST libraries for tentatively assigned peaks are given in Table 4.1. The compounds that were assigned tentatively, but with a high probability are marked in bold. These compounds were first on the hit list and also occupied 2^{nd} - 4^{th} positions on the list. These VOCs had relatively higher match factors in comparison to other best hits and/or the BPs of other best hits did not fit into the pattern. These compounds were: acetaldehyde, carbon disulfide, 2,3,5-trimethylhexane, 4-methyloctane, 2,2,4,6,6-pentamethylheptane and p-xylene. The possible alternative identification for these compounds is given below the name of the VOC. The compounds that were assigned tentatively, but with a lower probability, are underlined in Table 4.1. These compounds were first on the hit list, having the highest match factor, but they occupied first position only and the other best hits had a similar match factor value. These compounds were: 5-ethyl-2,2,3-trimethylheptane and 4-methylundecane. Similarly as in the MT experiment, peaks with similar match factors but with too high or too low BP can be disregarded as an alternative identification.

Co-eluting peaks

Some peaks were found to elute very closely to each other. This was the case for acetone and pentane; carbon disulfide, DCM and 2-methylpentane; CHF and EtAc; DME and benzene; 2-ethylhexanol and d-limonene. Co-eluting peaks can be identified by the selection of mutually exclusive ions present for each analyte, so they can be quantified. The ions monitored for the compounds detected in the TD experiments are presented in Table 4.1.

Table 4.1 Volatile organic compounds detected in the Thermal Desorption experiment listed in retention time order. Top five hits of search NIST libraries for tentatively assigned peaks are given. Number of top positions (out of 5) occupied by a particular hit (if > 1) is shown in the brackets after the factor match for tentatively assigned VOCs. BP: boiling point; CAS: chemical abstract service; FM: NIST library search factor match; ID: identification; ISTD: internal standard; LOD: limit of detection (S/N < 3); MW: molecular weight; NR: not resolved; RS: compound identified through the comparison of the retention time and mass spectrum with a reference standard; RT: retention time; QI: quantification ion; SM: smoothing applied for peak integration; TIC: total ion chromatogram; WB: water blank; ✓: compound also detected in a water "control" sample. **Bold:** compound that was assigned tentatively, but with a high probability i.e. it occupied the 1st and 2nd - 4th position on the hit list, had relatively higher factor match in comparison to other best hits and/or BP of other best hits did not fit into the pattern. Underlined: compound that was assigned tentatively, but with a lower probability i.e. it had the highest match factor but it occupied 1st position only, other hits had similar match factor and their BP fitted into the pattern.

No.	RT [min]	Volatile organic compound	WB	ID	FM	QI	CAS number	MW	BP [°C]
1	2.25	**Acetaldehyde** Formic acid, ethenyl ester Ethylene oxide		T	> 900(2) > 800 > 750(2)	NR	75-07-0 692-45-5 75-21-8	44 72 44	18 ± 3 44 ± 13 11 ± 0
2	2.40	Ethanol	✓	RS	> 940	45, 46	64-17-5	46	78 ± 0
3	2.60	Pentane	✓	RS	> 760	71, 72	109-66-0	72	36 ± 0
4	2.60	Acetone	✓	RS	> 870	36	67-64-1	58	56 ± 0
5	2.78	*tert*-Butanol	✓	RS	> 700	41, 59	75-65-0	74	83 ± 0
6	2.85	**Carbon disulfide** 4,4'-Diisothiocyanatostilbene-2,2'-disulfonic acid Glycine, N-(dithiocarboxy)-N-methyl-	✓	T	> 920(3) > 700 > 700	76, 78	75-15-0 53005-05-3 40520-03-4	76 454 165	46 ± 9 - 283 ± 42
7	2.90	Dichloromethane	✓	RS	> 750	74, 86	75-09-2	84	40 ± 0
8	3.10	Pentane, 2-methyl-	✓	RS	> 850	70, 71	107-83-5	86	60 ± 3
9	3.30	Pentane, 3-methyl-	✓	RS	> 850	57, 71	96-14-0	86	64 ± 1
10	3.55	Furan, 2-methyl-	✓	RS	> 900	81, 82	534-22-5	82	64 ± 1
11	3.74	Ethyl acetate	✓	RS	> 850	43, 88	141-78-6	88	77 ± 0
12	3.88	Chloroform	✓	RS	> 750	83, 85	67-66-3	119	61 ± 0.5
13	3.95	Tetrahydrofuran		RS	> 700	71, 72	109-99-9	72	66 ± 0
14	4.55	Dimethoxyethane		ISTD	-	45, 90	110-71-4	90	85 ± 1
15	4.70	Benzene	✓	RS	> 850	77, 78	71-43-2	78	80 ± 0

Table 4.1 Cont'd

No.	RT [min]	Volatile organic compound	WB	ID	FM	QI	CAS number	MW	BP [°C]
16	4.85	2-Methylbutanal		RS	> 850	41, 86 (SM)	96-17-3	86	95 ± 0
17	5.50	2-Pentanone	✓	RS	> 750	43, 86 (SM)	107-87-9	86	103 ± 3
18	5.05	**Hexane, 3-methyl-** Pentane, 2,3-dimethyl- Heptane	✓	T	> 750(3) > 700 > 700	< LOD	589-34-4 565-59-3 142-82-5	100 100 100	91 ± 7 89 ± 7 98 ± 1
19	5.85	Heptane	✓	RS	> 800	71, 100 (SM)	142-82-5	100	98 ± 1
20	6.35	2,4-Dimethylfuran	✓	RS	> 850	67, 96 (SM)	3710-43-8	96	95 ± 9
21	6.70	Methylcyclohexane	✓	RS	> 800	83, 98 (SM)	108-87-2	98	101 ± 0
22	8.30	Toluene	✓	RS	> 900	91, 92	108-88-3	92	111 ± 0
23	8.45	**Heptane, 4-methyl-** Hexane, 2,3-dimethyl- Heptane, 3-ethyl-4-methyl-	✓	T	> 750(3) > 700 > 700	71, 72 (SM)	589-53-7 584-94-1 52896-91-0	114 114 142	117 ± 7 115 ± 7 163 ± 7
24	8.65	**Heptane, 3-methyl-** Hexane, 3-ethyl Hexane, 2,4-dimethyl	✓	T	> 800(2) > 700 > 700(2)	84, 85 (SM)	589-81-1 619-99-8 589-43-5	114 114 114	119 ± 7 118 ± 7 109 ± 7
25	9.80	Octane	✓	RS	> 800	85, 114 (SM)	111-65-9	114	126 ± 3
26	10.70	**Hexane, 2,3,5-trimethyl-** Heptane, 2,4-dimethyl- Hexane, 3-ethyl- Octane, 4-methyl-	✓	T	> 820(2) > 700 > 800 > 800	84, 85 (SM)	1069-53-0 2213-23-2 619-99-8 2216-34-4	128 128 114 128	132 ± 7 134 ± 7 118 ± 7 142 ± 7
27	11.30	Ethylcyclohexane	✓	T	> 600(5)	< LOD	1678-91-7	112	129 ± 3
28	11.50	**Heptene, 2,4-dimethyl-, 1** " " " " 3-Heptene, 2,6-dimethyl-	✓	T	> 900 > 850(2) > 750(2)	TIC	19549-87-2 " " 2738-18-3	126 " 126	137 ± 7 " 135 ± 7
29	12.25	Ethylbenzene	✓	RS	> 900	91, 106	100-41-4	106	136 ± 3
30	12.50	**Octane, 4-methyl** " " " Hexane, 3-ethyl-	✓	T	> 900(1) > 850(2) > 800	43, 85 (SM)	2216-34-4 " " 619-99-8	128 " 114	142 ± 7 " 118 ± 7

Table 4.1 Cont'd

No.	RT [min]	Volatile organic compound	WB	ID	FM	QI	CAS number	MW	BP [°C]
Cont'd	Cont'd	Hexane, 2,3,5-trimethyl-	✓	T	> 750		1069-53-0	128	132 ± 7
31	12.70	**p-Xylene** o-Xylene	✓	T	> 820(4) > 800	< LOD	106-42-3 95-47-6	106 106	137-138 143-145
32	13.50	Styrene	✓	RS	> 950	TIC	100-42-5	104	145-146
33	14.05	Nonane	✓	RS	> 600	<LOD	111-84-2	128	151 ± 1
34	16.37	Benzaldehyde	✓	RS	> 880	TIC	100-52-7	106	178-179
35	17.50	**Heptane, 2,2,4,6,6-pentamethyl-** " " " " Heptane, 5-ethyl-2,2,3-trimethyl- Octane, 2,2,7,7-tetramethyl-	✓	T	> 800(1) > 700(2) > 700 > 650	57, 112 (SM)	13475-82-6 " " 62199-06-8 1071-31-4	170 " 170 170	177 ± 7 " 196 ± 7 185 ± 7
36	17.90	Decane	✓	RS	> 870	57, 142	124-18-5	142	172-174
37	18.65	Heptane, 5-ethyl-2,2,3-trimethyl- Nonane, 2,2,3-trimethyl- Heptane, 2,2,4,6,6-pentamethyl- Octane, 2,2,7,7-tetramethyl-	✓	T	> 800 > 770 > 700(2) >700	<LOD	62199-06-8 55499-04-2 13475-82-6 1071-31-4	170 170 170 170	195 ± 7 185 ± 7 177 ± 7 185 ± 7
38	18.90	2-Ethylhexanol	✓	RS	> 700	70, 83, 98, 112 (SM)	104-76-7	130	185 ± 0
39	18.95	d-Limonene	✓	RS	> 700	<LOD	5989-27-5	136	177-178
40	19.10	Undecane, 4-methyl- Dodecane, 2,7,10-trimethyl- Nonane, 3-methyl-5-propyl- Nonane, 5-(2-methylpropyl)- Hexadecane	✓	T	> 770 > 770 > 750 > 750 > 750	< LOD	2980-69-0 74645-98-0 31081-18-2 62185-53-9 544-76-3	170 212 184 184 226	209 ± 7 250 ± 7 213 ± 7 211 ± 7 287 ± 3
41	20.20	Acetophenone	✓	RS	> 750	105, 120 (SM)	98-86-2	120	202 ± 0
42	21.40	Undecane	✓	RS	> 780	43, 57, 71, 156 (SM)	1120-21-4	156	196 ± 0
43	24.70	Dodecane	✓	RS	> 830	57, 170 (SM)	112-40-3	170	216 ± 1
44	25.60	Benzothiazole	✓	RS	> 860	105, 138	95-16-9	135	227 ± 9

4.4.3 Blanks

Four types of blanks were used in the TD experiment:

(i) TD of a tube to be used for sampling to check for carry-overs, a blank TD tube was run between each experiment.

(ii) Empty cell culture flasks, sealed with Suba-Seals®, flushed with dry air and incubated for 7 days at 37°C.

(iii) Water samples containing 30 ml of distilled water and 4 mg l^{-1} of DME as an ISTD, sealed with Suba-Seals®, flushed with dry air, incubated for 7 days at 37°C and then sampled in the same way as cell culture medium samples (see Chapter 4.4.7 'Water experiment');

(iv) Air from the cell culture cabinet where the cell samples and controls were prepared.

Regarding the first type of blank, carry-overs of EtOH were observed for some of the analysed tubes during the TD experiment.

Empty cell culture flask blanks

Almost all of the detected VOCs in the TD experiment were also detected in empty flasks and water blanks (Tab. 4.1). For further discussion of the water experiment see Chapter 4.4.7.

The VOCs detected in freshly opened flasks which were not detected after the air flush and incubation of the flasks (either empty or with water) were: 2-methyl-butane (CAS: 78-78-4); methylcyclopentane (CAS: 96-37-7); 1,3-dimethyl-,cis-cyclohexane (CAS: 638-04-0); 1,4-dimethylcyclohexane (CAS: 589-90-2); 1,3,5-trimethylcyclohexane (CAS: 1839-63-0); 1,2,4-trimethylcyclohexane (CAS: 2234-75-5); propyl-cyclohexane (CAS: 1678-92-8); 2,6-dimethyloctane (CAS: 2051-30-1); 3,3-dimethylpentane (CAS: 562-49-2); hexane; 2-methylhexane (CAS: 591-76-4); 3-methylhexane (589-34-4); 3-ethylpentane (CAS: 617-78-7); 2-propenylidene-cyclobutene (CAS: 52097-85-5), 1,2-dimethyl-cis, cyclopentane (CAS: 1192-18-3); 3-methyl-2-hexene (CAS: 3683-22-5); 1,2,4-trimethylcyclopentane (CAS: 16883-48-0); 1,2,3-trimethylcyclopentane, (1α, 2α, 3β) (CAS: 15890-40-1); 5-methyl-1-heptene (CAS: 13151-04-7); 3,4-dimethylhexane (CAS: 583-48-2); 1,3-dimethyl, cis-cyclohexane (CAS: 591-21-9); 1,3-dimethyl, trans-

cyclohexane (CAS: 2207-03-6); 1,4-dimethylcyclohexane (CAS: 589-90-2); benzene(1-methylethyl); 2,6-dimethylundecane (CAS: 17301-23-4); 2-methyldecane (CAS: 6975-98-0); tetradecane (CAS: 629-59-4); 2-methyldodecane (CAS: 68551-19-9) and diphenyl ether (CAS: 101-84-8). This suggests that these compounds accumulated in the flasks during the production process, but they most likely did not originate from the flask itself.

Cell culture cabinet air blank samples

The VOCs always present in the chromatograms of the air in the cell culture cabinet samples (n = 6) were acetaldehyde, EtOH, toluene, and p-xylene. Acetal (1,1-diethoxyethane), acetic acid, 2-butanone, CHF, 1,1-diethoxyethane, d-limonene, EtAc, ethyl ether, ethylbenzene, 2-methylpentane and toluene were only found in some chromatograms of the air cell culture cabinet samples. The peak for EtOH was the most intensive peak in all the chromatograms (both blanks and samples), as it is used for the disinfection of any items introduced into the cabinet and of the cabinet itself. Acetaldehyde is an indoor contaminant, however its presence in the hood is probably from ultraviolet photo-degradation of thermoplastic polymers e.g. tip boxes or EtOH bottles left in the cabinet for UV sterilisation (Dornath, 2010). CHF, ethyl ether, EtAc, toluene, and xylene are common solvents used in chemistry laboratories. Their presence in the culture cabinet might be due to high concentrations of these VOCs in the lab air generally, which may vary on a daily basis depending on their use. Finally, all of the VOCs are common indoor contaminants originating from diverse chemical products such as paints, inks, waxes, sprays, adhesives and cleaning materials (Brown *et al*., 1994; Missia *et al*., 2010; Salthammer, 2014).

4.4.4 Precision

Table 4.2 presents the intra-batch precision (within the same sample; n = 3) and inter-batch precision (between the different samples; n = 9) expressed as % RSD calculated for each VOC analysed in each collection of the cell samples and their controls for the TD experiment. It must be noted that the intra-batch precision is not the intra-day precision, as desorption of the three 'tubes of one day' were not always performed on the same day. Also, the inter-batch precision is not the inter-day precision, as the latter should be determined by GC-MS analysis of the same sample run on the different days.

The intra-batch precision was obtained from three samples prepared on the same day, always with the use of the same batch of cell culture medium. The inter-batch precision was obtained from nine samples prepared on three different days using different batches of cell culture medium. Therefore, theoretically the inter-batch precision should be better than the inter-batch precision because of the lack of the variability originating from the medium batch, cell passage etc. However, for all analysed VOCs the intra-batch RSDs were similar or higher when compared to the inter-batch precision RSDs. This is a result of three 'samples of one day' instead of one as it was the case in the MT experiment (three injections of the same sample). Therefore, between the TD samples prepared on the same day there was still variability resulting from factors such as the flasks (and different initial concentrations of residual compounds), the Suba-Seals®, the cell seeding density and the variability in amount of the added ISTD. In addition, sensitivity of the MS detector could have varied between the days due to different water levels present in the system. Better inter-batch precision RSDs were the result of a greater sample size for the inter-batch precision as it produces statistically better results.

Relatively good intra- and inter-batch precision for most of the collections, < 30%, was yielded for acetone, benzaldehyde, benzene, decane, ethylbenzene, octane, styrene, tetrahydrofuran and toluene. Poor intra- and inter-batch precision, < 100%, obtained for acetophenone, benzothiazole, 2-ethylhexanol, methylcyclohexane, 2-methylbutanal, 2-pentanone and undecane were caused most likely by their low signal intensity (S/N ratio often < 10). For the remaining VOCs other factors had an impact on their poor precision. Even though the flush with dry air was applied to clean the flasks of the residual VOCs beforehand, the VOCs were still detected after a week of incubation. The levels of the released VOCs could be random for some compounds, affecting precision. Some of the VOCs were also found in the cell culture cabinet blanks so these VOCs could have been introduced during the sample preparation; and because there was no way to monitor how well the flush worked for each of the flasks, these compounds may also have had different initial levels between the flasks. Finally, some VOCs originating from Suba-Seals® might have been released at different intensity depending on the re-use time.

Table 4.2 Evaluation of the intra-batch and inter-batch precision, expressed as % relative standard deviation (RSD) of the thermal desorption - gas chromatography - mass spectrometry (TD-GC-MS) method used for the TD experiment. Col: collection; Intra: intra-batch precision RSD (%) for n= 3; inter-batch precision RSD (%) for n = 9; ND not detected.

Volatile organic compound	RSD (%)	A549 col 1		A549 col 2		BEAS-2B col 1		BEAS-2B col 2		Water	
		Sample	Control	Sample	Control	Sample	Control	Sample	Control	Col 1	Col 2
Acetone	Intra	20.5	22.5	27.5	4.1	7.9	12.2	11.3	11.7	37.2	30.0
	Inter	17.6	20.9	40.8	53.9	32.2	35.0	39.2	39.8	41.1	37.1
Acetophenone	Intra	40.7	57.7	66.6	31.9	28.1	17.6	ND	18.8	31.1	16.0
	Inter	49.4	58.7	74.9	69.9	31.4	21.0	ND	32.6	35.8	44.3
Benzaldehyde	Intra	ND	23.6	ND	24.0	ND	11.0	ND	23.8	22.8	37.0
	Inter	ND	21.9	ND	24.6	ND	12.6	ND	17.5	28.2	25.6
Benzene	Intra	14.9	16.3	16.1	31.1	11.1	11.0	43.0	12.1	15.7	28.2
	Inter	17.4	12.2	12.0	21.5	11.5	7.2	32.2	9.5	18.9	27.7
Benzothiazole	Intra	52.1	69.4	44.9	34.9	62.2	76.6	58.5	35.9	64.8	94.3
	Inter	45.9	61.6	36.6	27.7	55.8	79.4	64.1	42.7	47.5	49.9
Carbon disulfide	Intra	95.0	88.3	94.3	103.6	91.1	141.6	110.3	90.2	89.2	40.6
	Inter	75.8	81.3	70.9	75.8	71.3	96.7	87.5	74.7	112.7	72.1
Chloroform	Intra	99.6	41.5	53.1	43.0	111.2	115.0	70.7	77.9	28.1	147.8
	Inter	69.8	36.7	42.0	61.7	171.8	159.4	173.4	155.5	42.5	225.5
Decane	Intra	12.5	21.9	14.4	28.4	19.3	29.5	24.6	27.0	29.0	21.4
	Inter	20.7	19.3	17.8	33.0	15.6	22.4	14.4	21.4	32.4	16.5
Dichloromethane	Intra	14.3	13.9	24.5	24.5	42.0	25.6	11.1	25.7	145.5	62.6
	Inter	11.7	24.9	32.1	32.4	45.2	43.8	12.8	16.1	176.4	70.3
2,4-Dimethylfuran	Intra	47.7	145.2	160.2	103.4	77.6	104.0	70.3	54.0	110.3	50.2
	Inter	28.4	141.8	133.2	87.5	50.4	117.4	83.1	80.8	86.7	33.6
2,4-Dimethyl-1-heptene	Intra	72.7	49.0	39.3	65.0	47.5	77.6	21.7	24.1	66.2	64.6
	Inter	112.9	73.2	32.8	38.4	33.1	47.8	34.2	26.7	75.4	51.6
Dodecane	Intra	25.1	40.4	21.1	46.8	21.0	37.3	41.8	23.2	50.1	33.0
	Inter	19.8	54.4	13.6	52.1	20.5	24.6	40.6	26.6	40.6	35.7
Ethanol	Intra	75.7	108.8	73.3	53.7	47.2	63.3	17.0	43.2	76.8	76.6
	Inter	86.0	133.7	70.5	49.7	31.9	33.9	31.2	37.0	107.1	57.5

Table 4.2 Cont'd

Volatile organic compound	RSD (%)	A549 col 1		A549 col 2		BEAS-2B col 1		BEAS-2B col 2		Water	
		Sample	Control	Sample	Control	Sample	Control	Sample	Control	Col 1	Col 2
Ethyl acetate	Intra	12.9	4.7	ND	14.5	26.5	ND	21.9	ND	65.0	ND
	Inter	42.0	5.6	ND	14.8	31.7	ND	35.3	ND	40.7	ND
Ethylbenzene	Intra	17.8	17.7	15.7	13.7	15.5	11.4	14.7	17.4	14.2	23.1
	Inter	12.1	20.9	14.4	19.8	12.7	12.6	8.9	10.1	21.8	25.4
2-Ethylhexanol	Intra	26.1	15.1	20.5	38.9	34.9	28.3	28.3	27.0	20.1	ND
	Inter	23.6	15.1	25.4	20.5	41.4	45.7	30.7	39.1	16.8	ND
Heptane	Intra	34.3	21.0	33.9	49.8	51.0	35.3	38.7	17.6	18.1	61.0
	Inter	28.0	28.3	39.3	35.9	36.2	29.4	27.5	14.2	41.8	34.2
2-Methylbutanal	Intra	ND	32.4	ND	32.9	ND	29.0	ND	13.6	ND	ND
	Inter	ND	28.1	ND	27.9	ND	22.1	ND	17.5	ND	ND
Methylcyclohexane	Intra	50.0	18.4	47.4	51.5	54.9	29.7	40.9	35.0	30.4	52.5
	Inter	35.6	32.4	44.4	27.7	41.2	27.6	29.2	22.9	39.5	52.5
2-Methylfuran	Intra	31.8	23.4	42.6	59.4	22.3	31.9	51.1	44.2	64.9	37.7
	Inter	27.6	27.0	25.9	38.3	30.8	32.6	37.9	41.2	37.9	32.9
4-Methylheptane	Intra	57.3	40.3	18.8	69.7	66.3	28.9	21.3	20.9	54.5	33.1
	Inter	80.9	50.1	21.7	42.3	40.2	19.5	30.0	27.6	59.4	41.1
3-Methylheptane	Intra	44.0	24.0	54.9	85.0	54.4	34.9	10.3	26.6	17.1	30.6
	Inter	36.6	27.9	45.8	64.7	43.4	36.9	7.4	19.6	36.4	25.1
4-Methyloctane	Intra	68.9	63.8	26.9	105.3	51.1	96.1	33.2	34.8	62.7	44.2
	Inter	101.6	94.9	41.2	66.5	38.8	54.4	43.1	28.6	103.6	39.2
2-Methylpentane	Intra	83.3	24.9	98.8	54.6	104.9	98.5	31.3	118.3	45.5	32.1
	Inter	78.7	42.0	129.3	70.1	135.3	60.4	38.8	101.9	51.0	52.5
3-Methypentane	Intra	128.0	35.8	112.3	63.1	103.7	90.5	78.4	69.2	75.1	106.9
	Inter	98.8	56.6	90.7	63.7	73.1	90.2	65.8	47.0	52.1	54.8
Octane	Intra	14.5	16.5	26.7	40.1	18.2	22.3	9.4	16.1	29.3	21.6
	Inter	22.6	18.2	26.0	44.0	22.5	26.7	7.9	14.4	32.4	27.9

Table 4.2 Cont'd

Volatile organic compound	RSD (%)	A549 col 1		A549 col 2		BEAS-2B col 1		BEAS-2B col 2		Water	
		Sample	Control	Sample	Control	Sample	Control	Sample	Control	Col 1	Col 2
Pentane	Intra	99.9	45.7	18.8	48.9	108.7	93.9	78.9	88.8	58.8	34.7
	Inter	62.2	46.8	19.8	36.7	94.9	70.1	61.7	66.2	52.7	35.2
2-Pentanone	Intra	14.0	24.9	36.2	16.1	18.1	32.8	29.7	25.0	29.8	ND
	Inter	11.8	19.0	28.8	20.9	24.4	25.6	32.3	26.7	22.2	ND
2,2,4,6,6-pentamethylheptane	Intra	73.5	46.4	15.7	63.3	34.2	41.1	29.3	83.2	130.0	43.0
	Inter	69.1	67.9	35.7	42.7	52.5	38.2	21.8	94.5	78.5	54.2
Styrene	Intra	9.3	11.8	18.1	9.8	9.3	10.4	9.7	13.7	18.1	17.1
	Inter	8.5	16.1	12.0	13.3	6.7	8.7	7.8	9.2	24.4	18.9
Tetrahydrofuran	Intra	15.9	10.3	17.0	12.4	36.1	27.2	29.3	29.6	ND	ND
	Inter	15.7	6.4	18.8	15.9	154.7	27.2	84.5	24.1	ND	ND
2,3,5-Trimethylhexane	Intra	66.5	62.3	23.3	95.9	63.4	80.2	28.2	34.0	62.2	37.6
	Inter	87.1	87.7	34.2	61.1	44.2	45.9	34.3	37.3	94.9	44.7
Toluene	Intra	10.1	16.4	10.7	21.6	19.8	11.5	5.8	17.2	53.3	19.5
	Inter	10.2	19.0	11.6	17.8	15.1	9.1	8.1	12.7	59.4	24.0
tert-Butanol	Intra	39.9	16.4	25.5	13.2	42.4	41.6	6.8	11.3	ND	ND
	Inter	38.5	32.6	16.5	23.1	30.5	36.8	12.6	15.7	ND	ND
Undecane	Intra	47.7	56.1	30.0	59.5	18.7	57.4	32.2	62.7	56.1	35.1
	Inter	51.0	47.4	24.3	50.6	14.0	40.7	37.7	42.2	59.3	25.8

4.4.5 '*Between sample and control analysis*'

The data obtained for around 30% of VOCs in the TD experiment failed the assumption of normality (Shapiro-Wilk, $p < 0.05$) (Appendix B, Tab. B4). Therefore, for the comparison of VOCs present in the HS of the flask containing cell culture medium incubated with or without the cells, the Wilcoxon signed-rank test ($\alpha = 0.05$) was used. Tables 4.3 and 4.4 present the results of the Wilcoxon-signed rank test for the cell samples and their paired-controls for the TD experiment. Part A of Tables 4.3 and 4.4 shows the VOCs that were detected at significantly increased or decreased levels in the HS of the cell culture medium incubated with the A549 or BEAS-2B cells respectively, when compared to the HS of the pure medium controls ($p < 0.05$). Chemical structures of the VOCs found at altered levels in the TD experiment can be found in Appendix C2. Part B of Tables 4.3 and 4.4 shows the analytes that were found at similar levels for both cancer and normal cell samples and their controls. Both tables show the median peak area ratios for the sample and its control for each compound, as well as V statistics and p-values obtained with the use of the Wilcoxon-signed rank test.

A549 cell line

Five VOCs were found at increased levels and four at decreased levels in the A549 samples. After seven and 14 days of incubation, acetone and 2-pentanone were found to be significantly increased in the flasks incubated with the A549 cells, in comparison to the flasks with pure medium only ($V = 45$; $p = 0.003906$), while 2-ethylhexanol ($V = 45$; $p = 0.003906$), *tert*-butanol ($V = 40$; $p = 0.03906$) and 2,3,5-trimethylhexane ($V = 44$; $p = 0.02734$) were found to be increased only during the first week of incubation. Acetophenone, benzaldehyde and 2-methylbutanal ($V = 45$; $p = 0.003906$) were consumed by the cancer cells in collections 1 and 2 and octane was consumed only during the first week of incubation ($V = 5$; $p = 0.03906$).

BEAS-2B cell line

For the BEAS-2B cell line, four compounds were observed to be significantly elevated and six to be significantly lowered when compared to the medium controls.

Table 4.3 Comparison of the peak area ratios (peak area of an analyte/peak area of an internal standard) between the A549 samples (n = 9) and their pure medium controls (n = 9) for VOCs found in the thermal desorption (TD) experiment. NS: not significant; S: significant. **Part A**: VOCs for which the Wilcoxon-signed rank test detected the median difference between the pairs of observations not equal to zero at a level of significance α = 0.05. These VOCs were observed at increased or decreased levels in the A549 cell samples when compared to their pure medium controls. **Part B**: VOCs for which the Wilcoxon-signed rank test detected the median difference of the pairs of observations equal to zero at a level of significance α = 0.05. These VOCs were observed at similar levels in the A549 cell samples and their pure medium controls. Chemical structures of VOCs found at altered levels in the TD experiment can be found in Appendix C2.

Part A								
Volatile organic compound	**Sample Median**	**Control Median**	**V**	**p-value**	**Sample Median**	**Control Median**	**V**	**p-value**
	A549 collection 1				**A549 collection 2**			
Increased VOCs								
Acetone	5.319E-01	2.195E-01	45	0.003906	6.355E-01	2.072E-01	45	0.003906
2-Ethylhexanol	1.824E-02	9.378E-03	45	0.003906				NS
2-Pentanone	2.852E-02	1.278E-02	45	0.003906	2.336E-02	1.402E-02	45	0.003906
tert-Butanol	1.279E-01	1.142E-01	40	0.03906				NS
2,3,5-Trimethylhexane	2.385E-02	1.751E-02	44	0.02734				NS
Decreased VOCs								
Acetophenone	5.010E-03	1.333E-02	0	0.003906	2.242E-03	5.250E-03	0	0.003906
Benzaldehyde	0.000E+00	1.974E-01	0	0.003906	0.000E+00	1.033E-01	0	0.003906
2-Methylbutanal	0.000E+00	1.884E-02	0	0.003906	0.000E+00	1.617E-02	0	0.003906
Octane	1.382E-02	1.650E-02	5	0.03906				NS
Part B								
Benzene	9.792E-02	1.086E-01	17	0.5703	7.517E-02	6.652E-02	35	0.1641
Benzothiazole	3.213E-02	3.065E-02	17	0.5703	2.251E-02	1.829E-02	25	0.8203
Butane	3.034E-01	1.777E-01	31	0.3594	4.760E-02	3.717E-02	34	0.2031
Carbon disulfide	1.004E+01	1.237E+01	17	0.5703	1.202E+01	1.174E+01	22	1.000
Chloroform	1.773E-02	1.431E-02	29	0.4961	3.731E-02	2.864E-02	28	0.5703
Decane	3.696E-02	4.104E-02	14	0.3594	2.473E-02	2.242E-02	32	0.3008
Dichloromethane	5.343E-02	5.723E-02	7	0.07422	5.271E-02	5.572E-02	22	1.000

Table 4.3 Cont'd

Part B (cont'd)								
Volatile organic compound	**Sample Median**	**Control Median**	**V**	**p-value**	**Sample Median**	**Control Median**	**V**	**p-value**
	A549 collection 1				**A549 collection 2**			
2,4-Dimethylfuran	9.491E-03	1.042E-02	18	0.6523	8.734E-03	2.564E-02	24	0.9102
2,4-Dimethylheptene	1.669E-01	1.519E-01	37	0.09766	1.587E-01	1.391E-01	39	0.05469
Dodecane	1.101E-02	1.010E-02	20	0.8203	8.874E-03	7.525E-03	23	1
Ethanol	8.325E+00	6.527E+00	27	0.6523	3.536E+00	3.381E+00	26	0.7344
Ethyl acetate	2.307E-02	5.196E-02	6	05469	0.000E+00	0.000E+00	0	0.1003
Ethylbenzene	9.501E-02	9.633E-02	19	0.7344	5.612E-02	4.889E-02	33	0.25
2-Ethylhexanol				S	1.065E-02	1.232E-02	17	0.5703
Heptane	1.299E-02	1.450E-02	13	0.3008	5.017E-03	4.966E-03	23	1
Methylcyclohexane	1.107E-02	1.353E-02	18	0.6523	4.306E-03	0.000E+00	23	0.5286
2-Methylfuran	1.470E+00	1.326E+00	33	0.25	1.492E+00	1.314E+00	32	0.3008
2-Methylpentane	1.499E-02	2.298E-02	15	0.4258	4.027E-02	5.274E-02	9	0.1289
3-Methylpentane	1.130E-01	9.602E-02	31	0.3594	9.668E-02	9.030E-02	22	1
3-Methylhepatne	9.086E-03	9.556E-03	22	1	4.767E-03	3.327E-03	32	0.3008
4-Methylheptane	1.073E-02	1.354E-02	38	0.07422	1.232E-02	1.013E-02	35	0.1641
4-Methyloctane	2.664E-02	1.751E-02	39	0.05469	2.162E-02	2.001E-02	36	0.1289
Octane				S	9.073E-03	1.036E-02	30	0.4258
2,2,4,6,6-Pentamethylheptane	8.716E-03	1.123E-02	13	0.3008	6.570E-03	6.048E-03	25	0.8203
Pentane	3.001E-02	2.557E-02	27	0.6523	1.077E-02	9.034E-03	19	0.7344
Styrene	3.827E+00	3.973E+00	15	0.4258	2.446E+00	2.306E+00	36	0.1289
tert-Butanol				S	5.615E-02	5.585E-02	17	0.5703
Tetrahydrofuran	6.088E-03	6.109E-03	22	1	5.095E-03	4.776E-03	33	0.25
Toluene	3.380E-01	3.514E-01	16	0.4961	2.245E-01	2.109E-01	32	0.3008
Trichloromethane								
2,3,5-Trimethylhexane				S	2.335E-02	2.013E-02	36	0.1289
Undecane	1.180E-02	1.282E-02	26	0.7344	9.288E-03	8.908E-03	25	0.8203

Table 4.4 Comparison of the peak area ratios (peak area of an analyte/peak area of an internal standard) between the A549 samples (n = 9) and their pure medium controls (n = 9) for VOCs found in the thermal desorption (TD) experiment. NS: not significant; S: significant. **Part A**: VOCs for which the Wilcoxon-signed rank test detected the median difference between the pairs of observations not equal to zero at a level of significance α = 0.05. These VOCs were observed at increased or decreased levels in the A549 cell samples when compared to their pure medium controls. **Part B**: VOCs for which the Wilcoxon-signed rank test detected the median difference of the pairs of observations equal to zero at a level of significance α = 0.05. These VOCs were observed at similar levels in the A549 cell samples and their pure medium controls. Chemical structures of VOCs found at altered levels in the TD experiment can be found in Appendix C2.

Part A								
Volatile organic compound	**Sample Median**	**Control Median**	**V**	**p-value**	**Sample Median**	**Control Median**	**V**	**p-value**
	BEAS-2B collection 1				**BEAS-2B collection 2**			
Increased VOCs								
Acetone	4.582E-01	4.187E-01	44	0.007812	4.849E-01	4.335E-01	45	0.003906
Ethyl acetate	2.178E-02	0.000E+00	45	0.003906	2.129E-02	0.000E+00	45	0.003906
2-Ethylhexanol	2.327E-02	1.838E-02	43	0.01172	2.340E-02	1.641E-02	45	0.003906
2-Pentanone	1.737E-02	8.787E-03	45	0.003906	3.289E-02	9.516E-03	45	0.003906
Decreased VOCs								
Acetophenone				NS	0.000E+00	2.558E-03	0	0.003906
Benzaldehyde	0.000E+00	1.339E-01	0	0.003906	0.000E+00	8.689E-02	0	0.003906
2-Methylbutanal	0.000E+00	1.760E-02	0	0.003906	0.000E+00	1.268E-02	0	0.003906
Decane				NS	2.561E-02	2.749E-02	5	0.03906
2-Methylpenatne				NS	1.407E-02	2.828E-02	0	0.003906
Octane				NS	6.663E-03	7.910E-03	4	0.02734
Part B								
Acetophenone	2.751E-03	3.976E-03	10	0.1641				S
Benzene	1.080E-01	1.005E-01	32	0.3008	6.589E-02	6.221E-02	34	0.2031
Benzothiazole	1.613E-02	1.984E-02	19	0.7344	1.469E-02	1.401E-02	26	0.7344
Butane	1.000E-01	6.729E-02	30	0.4258	3.208E-02	3.227E-02	26	0.7344
Carbon disulfide	3.732E+00	1.989E+00	29	0.4961	1.812E+00	1.437E+00	34	0.2031

Table 4.4 Cont'd

Part B (cont'd)								
Volatile organic compound	**Sample Median**	**Control Median**	**V**	**p-value**	**Sample Median**	**Control Median**	**V**	**p-value**
	BEAS-2B Collection 1				**BEAS-2B Collection 2**			
Chloroform	2.222E-01	8.737E-02	20	0.8203	8.217E-02	5.141E-02	28	0.5703
Decane	3.921E-02	3.196E-02	35	0.1641				S
Dichloromethane	1.743E-02	2.436E-02	12	0.4412	3.130E-02	3.078E-02	26	0.7344
2,4-Dimethylfuran	9.842E-03	8.338E-03	13	0.5286	3.827E-02	3.402E-02	29	0.4961
2,4-Dimethylheptene	1.463E-01	1.371E-01	22	1	2.254E-01	1.684E-01	32	0.3008
Dodecane	1.051E-02	8.929E-03	31	0.3594	9.883E-03	1.423E-02	10	0.1641
Ethanol				S	4.637E+00	3.387E+00	34	0.2031
Ethylbenzene	7.411E-02	6.780E-02	36	0.1289	5.087E-02	5.323E-02	11	0.2031
Heptane	1.310E-02	9.365E-03	37	0.09766	3.918E-03	4.487E-03	20	0.8203
Methylcyclohexane	1.026E-02	8.826E-03	33	0.25	4.109E-03	4.487E-03	25	0.8203
2-Methylfuran	1.328E+00	1.282E+00	32	0.3008	1.784E+00	1.252E+00	31	0.3594
2-Methylpentane	3.013E-02	3.589E-02	26	0.7344				S
3-Methylpentane	2.365E-01	7.068E-02	32	0.3008	2.249E-02	3.614E-02	22	1
3-Methylhepatne	7.013E-03	5.930E-03	32	0.3008	3.961E-03	3.791E-03	30	0.4258
4-Methylheptane	1.308E-02	1.141E-02	29	0.4961	1.522E-02	1.390E-02	30	0.4258
4-Methyloctane	2.451E-02	2.784E-02	23	1	3.157E-02	3.192E-02	21	0.9102
Octane	1.244E-02	1.103E-02	34	0.2031				S
2,2,4,6,6-Pentamethylheptane	4.597E-03	3.551E-03	26	0.7344	2.501E-03	0.000E+00	12	0.7998
Pentane	1.684E-02	1.517E-02	27	0.6523	8.240E-03	7.460E-03	31	0.3594
Styrene	3.530E+00	3.241E+00	38	0.07422	2.472E+00	2.443E+00	19	0.7344
tert-Butanol	1.149E-01	6.561E-02	34	0.2031	4.660E-02	4.565E-02	20	0.8203
Tetrahydrofuran	0.000E+00	0.000E+00	3	1	3.194E-03	3.216E-03	11	1
Toluene	2.936E-01	2.655E-01	39	0.05469	1.898E-01	1.978E-01	17	0.5703
2,3,5-Trimethylhexane	2.191E-02	2.598E-02	34	0.9102	3.023E-02	2.426E-02	36	0.1289
Undecane	1.105E-02	8.642E-03	30	0.4258	1.740E-02	1.440E-02	22	1

Acetone, EtAc, 2-ethylhexanol and 2-pentanone were produced by the cells throughout the two weeks of cultivation (V = 44; p = 0.00781 for acetone collection 1; V = 43; p = 0.01172 for 2-ethylhexanol collection 1; V = 45; p = 45; p = 0.003906 for the rest of the VOCs). The uptake of benzaldehyde and 2-methylbutanal was observed during both collections (V = 0; p = 0.003906) while acetophenone, 2-methylpentane (V= 0; p = 0.003906), octane (V = 4; 0.02734) and decane (V = 5; p = 0.03906) were metabolised by the BEAS-2B cells during the second week of incubation only.

A549 versus BEAS-2B cell line

The release of acetone, 2-ethylhexanol and 2-pentanone and the uptake of acetophenone, benzaldehyde and 2-methylbutanal were observed in both cell lines. 2,3,5-Trimethylhexane and *tert*-butanol were found to be produced solely by the A549 cells. EtAc was a VOC produced only by the BEAS-2B cells. 2-Methylpentane and decane were found to be consumed exclusively by the non-transformed cells.

4.4.6 'Between-sample analysis'

Table 4.5 presents the results of the Kruskal-Wallis followed by Mann-Whitney U comparative analysis conducted for the standardised peak area ratios of the VOCs detected in the cell samples for the TD experiment. This is a comparison of the VOC levels between the A549 and the BEAS-2B cell lines, as well as between the collections of the same cell line. The analysis gives an extended view of the uptake and release of the VOCs by the studied cells. The paired Wilcoxon analysis compared the levels of the analytes in the samples to the pure medium. Here the VOC levels in the samples are discussed in relation to the other sample collections.

Part A of Table 4.5 groups eight VOCs for which differences in levels were found to be significant at a level of $p < 0.05$ when all the samples were compared. Then the pairwise sample comparisons using the Mann Whitney-U test were conducted. For two VOCs, octane and styrene, the Kruskal-Wallis analysis gave significant p-values, however, the follow up test did not detect any differences for the sample pairs. No differences in levels between all the samples were detected for 27 VOCs in the TD experiment. The compounds are listed in Part B of Table 4.5.

Table 4.5 Comparison of the VOC peak area ratios (peak area of an analyte/peak area of an internal standard) between the A549 cell samples (n = 9) and the BEAS-2B cell samples (n = 9), as well as between the collections of the same cell line for VOCs found in the TD experiment. **Part A**. VOCs for which the Kruskal-Wallis analysis found differences in peak area ratios when all the samples were compared, at the level of significance $\alpha = 0.05$ and then the Mann-Whitney U test was conducted to compare the peak area ratios between each of the analysed samples. **Part B**. VOCs for which the Kruskal-Wallis analysis found no significant differences in peak area ratios when all the samples were compared. A1: collection 1 of the A549 cells; A2: collection 2 of the A549 cells; B1: collection 1 of the BEAS-2B cells; B2: collection 2 of the BEAS-2B cells. Colour codes: green: the level of the VOC was found to be higher for the earlier collection of the same cell line; orange: the level of the VOC was found to be lower for the earlier collection of the same cell line; blue: the level of the VOC was found to be higher for the A549 cell line; purple: the level of the VOC was found to be lower for the A549 cell line.

Part A

Volatile organic compound	A1 vs A2	B1 vs B2	A1 vs B1	A1 vs B2	A2 vs B1	A2 vs B2	Kruskal-Wallis p-value
	Mann-Whitney U p-value						
Acetone	0.00740↓	1.00000	0.00025↑	0.00025↑	0.00025↑	0.00025↑	2.617e-06
Acetophenone	0.23990	0.01111↑	0.00099↓	0.06368	0.04665↓	1.00000	0.0003
Benzaldehyde	0.0017↓	0.0353↓	0.0030↓	0.0023↓	0.3752	1.0000	4.400e-05
Ethyl acetate	1.00000	1.00000	0.02394↓	0.00025↓	0.00215↓	0.00215↓	2.405e-05
2-Ethylhexanol	0.00025↑	1.00000	0.23990	0.18881	0.08515	0.00469↓	0.0003
2-Methylbutanal	0.6810	0.1125	1.0000	0.0047↓	1.0000	0.6810	0.0099
Octane	0.24	0.11	0.11	1.00	0.19	0.30	0.0180
Styrene	0.68	0.19	0.19	1.00	1.00	0.24	0.0324

Part B

Volatile organic compound	Kruskal-Wallis p-value
Benzene	0.6297
Benzothiazole	0.8756
Carbon disulfide	0.8538

Table 4.5 Cont'd

Part B (cont'd)	
Volatile organic compound	**Kruskal-Wallis p-value**
Chloroform	0.9821
Decane	0.0535
Dichloromethane	0.3879
2,4-Dimethyl-1-heptene	0.8042
2,4-Dimethylfuran	0.6891
Dodecane	0.4565
Ethanol	0.0974
Ethylbenzene	0.2200
Heptane	0.1191
Methylcyclohexane	0.3323
2-Methylfuran	0.9212
3-Methylheptane	0.5785
4-Methylheptane	0.8519
2-Methylpenatne	0.3302
3-Methylpentane	0.5127
4-Methyloctane	0.2857
2,2,4,6,6-Pentamethylheptane	0.7467
Pentane	0.7506
2-Pentanone	0.1752
tert-Butanol	0.0661
Tetrahydrofuran	0.6951
Toluene	0.1152
2,3,5-Trimethylhexane	0.5619
Undecane	0.9208

Comparison of level trends between the cell lines

The levels of acetone were found to be significantly higher for the A1 and A2 samples when compared to both B1 and B2 samples ($p = 0.00025$). The production of the ketone was observed for both cell lines during both weeks of incubation in the 'between sample and control analysis'. Therefore, acetone was produced at a higher rate by the cancer cells than by the normal cells throughout the whole period of incubation, regardless of their confluency.

Acetophenone was found at lower levels in the A1 and A2 samples when compared to the B1 sample ($p < 0.05$). As the VOC was shown to be consumed by both cell lines in the 'between sample and control analysis', the 'between-sample comparison' indicates that the metabolism of this ketone was performed at a higher rate by the growing and confluent cancer cells, when compared to the growing non-transformed cells. The levels of acetophenone metabolism 'levelled up' when both cell lines reached confluency, as no differences in the VOC levels were found between both the A1 and B2 samples and the A2 and B2 samples.

Significantly lower levels of benzaldehyde were observed for the A1 sample when compared to both BEAS-2B samples ($p < 0.003$). Therefore, as the aldehyde was shown to be consumed by both cell lines, the growing cancer cells metabolised it at a higher rate than both growing and confluent normal cells.

Also, significantly lower levels of EtAc were found for both the A1 and A2 samples when compared to the B1 and B2 samples ($p < 0.025$). These results are in agreement with the 'between sample and control analysis', because this VOC was found to be produced solely by the BEAS-2B cells in both weeks of incubation.

2-Ethylhexanol was observed to be produced by the A549 cells only in collection 1, while the BEAS-2B cells were observed to produce the VOC during both weeks of incubation. Therefore, the 'between sample analysis' showed that both cell lines emitted the alcohol at similar levels, as no differences were found between the A1 sample and the B1 and B2 samples. Lower levels of 2-ethylhexanol were found, however, for the A2 sample when compared to the B2 sample, as the VOC was not found to be produced by the cancer cells any more ($p = 000469$).

The levels of another aldehyde, 2-methylbutanal, were found to be significantly lower for the A1 sample when compared to the B2 sample ($p = 0.0047$). The VOC was observed to be metabolised by both cell lines during both weeks of incubation. This indicates that the growing A549 cells consumed it at a higher rate than the confluent BEAS-2B cells. The lack of any differences in 2-methylbutanal levels between the other sample pairs suggests that, similarly to the MT experiment, the concentration of this VOC is variable between the different batches of cell culture medium.

Comparison of VOC level trends within the same the cell line

The 'between sample analysis' also allowed for the comparison of the levels of VOCs between the two sample collections of the same cell line. The levels of acetone, benzaldehyde and 2-ethylhexanol were found to be significantly different between the two collections of the A549 cells ($p < 0.008$). Acetone was found to be produced at a higher rate by the confluent cancer cells than by the growing cancer cells. 2-Ethylhexanol was observed to be produced only by the growing A549 cells, while benzaldehyde was metabolised at a higher rate by the growing cancer cells than by the confluent cancer cells. The levels of acetophenone and benzaldehyde were observed to be significantly different for the two collections of the BEAS-2B cells ($p < 0.04$). The confluent BEAS-2B cells were found to consume acetophenone at a higher rate than the growing BEAS-2B cells. The opposite trend of the uptake of the VOC at a higher rate by the growing BEAS-2B cells was observed for benzaldehyde. The differences found may reflect differences in the metabolic pathways working during the exponential growth of the cells and when they reach confluency (see Chapter 5.3.2 for further discussion).

4.4.7 Residual VOCs from cell culture vessels

The initial TD experiments involved the sampling of empty cell culture flasks. It was noted that air samples from freshly opened vessels yielded a relatively high amount of compounds present in the chromatogram, apparently originating from the flask. Therefore, to reduce the background levels of VOCs originating from plastic, after seeding the cells, the cell culture flasks were flushed with dry air. Figure 4.6 shows that in this way the levels of the residual volatiles were significantly reduced. Later during the experiments, however, it appeared that the flasks continued to emit some of the residual and/or the PS radiolysis VOCs after flushing. Air flushed empty flasks and air flushed flasks containing

water yielded very similar chromatograms to the ones obtained from the cell samples and medium controls. This is shown in Figure 4.9, which presents the TICs of the water sample and cell-free medium sample. Because the VOCs were extracted after a week and then again after two weeks of incubation, a comparison between collections 1 and 2 of the water samples was conducted, in order to examine whether the levels of residual VOCs originating from the flasks changed during cell culture or remained constant. The findings are discussed below.

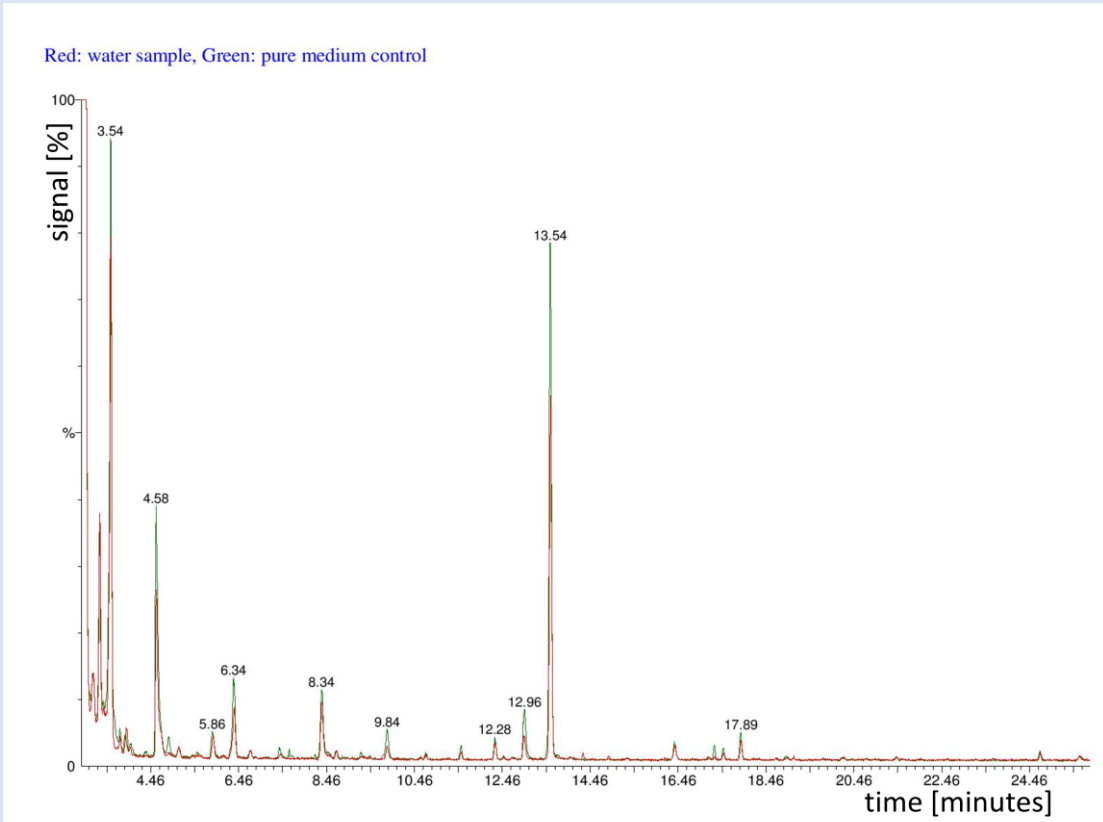

Figure 4.9 Total ion chromatograms of VOCs detected in the headspace of water sample (red) and pure medium control (green) with the use of thermal desorption of sorbent tube coupled with GC-MS. Both samples were incubated for 7 days at 37C. Extraction was conducted with the use of Soil Vapor Intrusion (SVI^{TM}) TD tubes, air sample volume: 200 ml, air-flushed.

‘Water experiment’

Table 4.6 presents the results of the Mann-Whitney U comparative analysis between collection 1 (n = 9) and collection 2 (n = 9) of the water samples. An important finding of the ‘water experiment’ was that only 2-methylbutanal and tetrahydrofuran were not detected in the empty flasks, nor the flasks containing water, suggesting that all the other compounds were released from the flasks through the process of ‘volatilisation’ of polystyrene (PS) and/or of Suba-Seals®.

The levels of nine VOCs were found to be significantly lower in the flasks sampled after two weeks of incubation when compared to the ones incubated for one week, namely acetophenone, benzene, EtAc, heptane, methylcyclohexane, octane, pentane and 2-pentanone ($p < 0.05$). 2-Ethylhexanol was not detected at all in the W2 sample. This suggests that the release of these VOCs from the flasks decreases with time of incubation. 2-Methylfuran, 2-methylpentane, and trichloromethane were found at significantly higher levels in the W2 samples when compared to the W1 samples. 2-Methylpentane and trichloromethane are possible environmental contaminants, as they were also detected in the blanks coming from the sampled air in the hood where the preparation of the samples and controls took place. Therefore, these compounds may have shown more random concentrations between days. Even though the flush with dry air was applied to clean the flasks of the residual VOCs before incubation, there was no way to monitor how well the flush worked for each of the flasks. A perfectly designed ‘water experiment’ would involve paired flasks used during both weeks. This could detect differences that were not visible here, due to the potential residual VOC variability between different flasks. 2-Methylfuran is a VOC most likely originating from the Suba-Seals® and therefore, its levels could differ between days, as the seals were reused during the TD experiment. Because no significant differences were found for the remaining VOCs, their release from the flasks appeared to be constant during the period of two weeks.

Table 4.6 Comparative analysis of the VOC peak area ratios (peak area of an analyte/peak area of an internal standard) between collection 1 of the water sample (W1, n = 9) and collection 2 of the water sample (W2, n = 9), conducted with the Mann-Whitney U test with the Bonferroni correction. Colour codes: yellow: VOCs not detected in the water samples (detected only in the cell samples and medium controls); grey: VOCs detected in the water samples which remained at constant levels during the two weeks of incubation; green↓: VOCs detected in the water samples found at lower levels in the W2 sample when compared to the W1 sample; blue↑: VOCs detected in water samples which were found at higher levels in the W2 sample when compared to the W1 sample; ●1: VOC detected only in collection 1.

Volatile organic compound	W1 vs W2 p-value	VOC	W1 vs W2 p-value	Volatile organic compound	W1 vs W2 p-value	Volatile organic compound	W1 vs W2 p-value
Acetone	0.34	2,4-Dimethylfuran	0.26	Methylcyclohexane	0.011↓	2-Pentanone	0.00058↓
Acetophenone	0.0027↓	2,4-Dimethyl-1-heptene	0.67	2-Methylfuran	0.0078↑	Pentane	0.013↓
Benzene	0.04↓	Dodecane	0.44	3-Methylheptane	0.077	Styrene	0.34
Benzaldehyde	0.16	Ethanol	0.16	4-Methylheptane	0.6	*tert*-Butanol	0.37
Carbon disulfide	0.8	Ethylbenzene	0.077	4-Methyloctane	0.39	Tetrahydrofuran	---
Trichloromethane	0.0012↑	Ethyl acetate	0.0053↓	2-Methylpentane	0.031↑	Toluene	0.19
Benzothiazole	0.11	2-Ethylhexanol	0.034●1	3-Methylpentane	0.86	2,3,5-Trimethylhexane	0.34
Decane	0.22	Heptane	0.024 ↓	Octane	0.031↓	Undecane	0.83
Dichloromethane	0.47	2-Methylbutanal	---	2,2,4,6,6-Pentamethylheptane	0.061		

Chapter 5

Discussion

Aims:

• To analyse differences in VOC patterns between the different cell lines and within the same cell line observed in the MT and TD experiments, in relation to cell culture conditions.

• To discuss the VOCs detected in the MT and TD experiments in terms of their potential origins from the cell culture flasks.

• To discuss the VOCs found at altered levels in the MT and TD experiments in relation to previous *in vitro*, *ex vivo* and *in vivo* studies of volatiles as potential biomarkers of disease.

• To establish the potential uses of the altered VOCs as biomarkers of lung cancer.

• To propose possible metabolic pathways for the production and/or metabolism of the VOCs detected at altered levels in the MT and TD experiments.

• To critically evaluate and compare the two techniques of VOC extraction used in the MT and TD experiments.

• To examine the inconsistency in the VOC patterns between the MT, TD and other *in vitro* studies.

• To examine the inconsistency in the VOC patterns between *in vitro* and *in vivo* studies of lung cancer.

5.1 Cell culture remarks

The A549 cell line was established in 1972 from the human alveolar epithelium and is a commonly used *in vitro* model of human bronchiolo-alveolar cell cancer (Foster *et al.*, 1998). This has been the most commonly studied cell line in studies of potential biomarkers of lung cancer and extraction techniques such as SPME, TD sorbent tubes and Ultra II SKC badge were applied for its analysis (Barash *et al.*, 2009; 2011; Filipiak *et al.*, 2010; Hanai *et al.* 2012b). As HS-MMSE and TD with an Easy-VOC^{TM} pump are new techniques for the extraction of VOCs emitted from cell culture medium, A549 cells were chosen so as to enable a more direct comparison between the VOCs detected with these techniques and the compounds linked with this cell line in previous studies. The BEAS-2B virus-transformed cell line, established from human bronchial epithelium, also has been analysed previously in studies of volatiles as a non-cancerous control (Brunner *et al.*, 2010; Yu J. *et al.*, 2009). The NHLFs have been studied for the first time in terms of emitted VOCs. Filipiak *et al*. (2010) have analysed fibroblasts derived from dermis in their study.

A general purpose medium, RPMI 1640 with HEPES was used in the MonoTrap and TD experiments. HEPES is a buffer stabilizing the pH in the range of 7.2 - 7.6 which is required for the cell culture at high cell densities when the pH otherwise would fall and for the sealed culture systems being in depletion of exogenous CO_2, which helps to regulate physiological pH (Freshney, 2005). The medium has been widely used in the literature for the culture of the A549 and BEAS-2B cell lines, in the studies of VOCs (Barash *et al.* 2009, 2011; Brunner *et al.* 2010; Pyo *et al.* 2009; Wang Y. *et al*., 2012).

High viability percentages > 90 % for all cell lines indicated that the cell culture conditions were appropriate for the analysed cell lines and that the analysed VOCs were potentially coming from the living cells and not due to the processes associated with cell death.

5.2 Residual VOCs from cell culture vessels

The air from freshly open empty cell culture flasks sampled in the TD experiment contained numerous VOCs accumulated during the production process. Dry air flush of

the flasks helped to substantially reduce their intensities. However, some of the VOCs were appearing again during the seven days of incubation. What is more almost all of the VOCs observed in the chromatogram of cell culture medium were also present in the chromatogram of empty flasks and flasks containing water in the TD experiment (Fig. 4.9). The water experiment aimed to establish whether the levels of these VOCs remains constant or changes during incubation period. The levels of some VOCs were shown to decrease, and of others remained constant (see Chapter 4.4.7). The VOCs detected in both MT and TD experiments are discussed below in terms of their potential origins from the flasks.

5.2.1 Polystyrene residual compounds and degradation products

Cell culture flasks used in the study were made of PS with a Nunclon™ Delta cell culture treated surface (a type of treatment that modifies the surface of hydrophobic PS to hydrophilic, in order to facilitate cell attachment), sterilised by ɤ-irradiation. Studies by Buchalla *et al.* (1999) and Buchalla (2000) showed that residual compounds found in PS (both irradiated and non-irradiated) are styrene; diphenylcyclobutane [1,1'-(1,2-cyclobutanediyl)bis-,trans-benzene and its cyclic analogue 1,1’-(1,2-cyclobutanediyl)bis-, cis-benzene]; toluene; ethylbenzene and 1,1′-(1,3-propanediyl)-bis-benzene. The main products of radiolysis are acetophenone; benzaldehyde; phenyl ethanol; phenyl acetaldehyde and phenol. There are also trace products of PS radiolysis such as benzene and aliphatic, often oxidised products, e.g. 1-dodecene, hexanal and acetic acid. Radiolysis was also investigated in Nunclon cell culture flasks by Buchalla (2000). A GC chromatogram obtained from a flask was typical, as expected, for irradiated PS. What is more, the time elapsed since irradiation was around 9.25 years for Nunclon cell culture flasks so the compounds can be still detected after such a long shelf-storage.

In the MT and TD experiments, most of the PS typical volatiles were detected, except phenyl acetaldehyde; 1,1’-(1,2-cyclobutanediyl)bis-,trans-benzene and 1-dodecene. However, phenyl acetaldehyde was not always present in the chromatograms of various PS samples studied by Buchalla *et al*. (2000) (e.g. it was absent in the TIC of PS Petri dishes). 1,1’-(1,2-Cyclobutanediyl)bis-,trans-benzene could co-elute with siloxane with an RT of 30.70 min, visible in the TIC as a peak between 2-pentadecanone and pentadecanal (peaks no. 57 and 58 respectively in Fig. 3.17), as the characteristic ion (104) of 1,1’-(1,2-cyclobutanediyl)bis-,trans-benzene was observed. On the other hand, the VOC could

actually co-elute with its isomer as both have the same BP (Tab. 3.3). Finally, 1-dodecene was a trace product of PS radiolysis in the study by Buchalla (2000) and therefore it may not have been detected in the TD experiment because of different conditions used in the study (GC column, sensitivity etc.).

Such potential residual and radiolysis compounds of PS as acetophenone; benzaldehyde; ethylbenzene and styrene were detected in both the MT and TD experiment. 1-Phenylethanol; hexanal; phenol and 1,1′-(1,3-propanediyl)-bis-,-cis-benzene were detected only in the MT experiment. These VOCs have relatively high BPs and therefore are probably not volatile enough at 37°C (the temperature of the sorbent tube extraction) to be detected in the HS phase in the TD experiment. Buchalla (2000) used a different method, utilising 160°C of dynamic TD, therefore these VOCs with higher BPs could be detected. The majority of the residual compounds and radiolysis products of PS found by Buchalla (2000), however, were dissolved in the cell culture medium, as they could be detected in the MT experiment. The exceptions were benzene and toluene which most likely eluted with the solvent peak (too low BP). They were detected in the TD experiment. It is not clear, however, why phenol and hexanal were not detected in the TD experiment. They are both polar VOCs and could be lost during the TD dry purge step (see Chapter 5.5 for further discussion).

Ethylbenzene and styrene, apart from the possible origin from PS, may also originate from the cell culture medium. Both VOCs were shown to migrate from polymer packaging material to food (Arvanitoyannis and Bosnea, 2004); therefore they could also potentially migrate into the medium, not only from the flask during cell culture, but also from the bottle before cell culture, during storage.

5.2.2 Degradation products of other polymers

Polymers and rubbers used commercially are complex materials. Manufacturing formulations always contain a number of additives that are used to give and/or enhance particular physical and chemical properties. These ingredients include adhesives, antioxidants, carbon black, extender oils, heat and light stabilizers, tackifying resins, cross-linking agents, antifatigue agents, pigments (masterbatches), plasticizers and others. Many of these additives are not sufficiently volatile for GC analysis; however, a range of alcohols, aldehydes, fatty acids and their esters, hydrocarbons, phthalate plasticizers,

phenols and low MW amines are (Hakkarainen and Karlsson, 2000). Therefore, degradation and/or radiolysis of polymers and rubbers may result in a complex mixture of VOCs that can be detected in polymer and rubber materials.

To add to the problematic presence of the residual VOCs of the culture flasks in the experiments performed, some volatiles may be also released from the open/vent type cap of the cell culture flask used in the MT experiment and from the Suba-Seal® in the TD experiment. The cap is made of high density polyethylene (HDPE) and masterbatch blue (Appendix D). Masterbatch is a mixture of dyes and/or additives encapsulated into a carrier resin which is then added to a polymer. The most generally used carrier resins are: low density polyethylene (LDPE), polypropylene (PP), PS and ethylene vinyl acetate (EVA) (Dyer, 1999). It is unknown however, which carrier resin was used in the caps of T-75 flasks. The seals are made of natural rubber (NR).

A typical chromatogram of HDPE is dominated by "even number" alkanes (octane, decane, etc.) and trace amounts of "odd number" alkanes (nonane, undecane, etc.) from C9 to C20. Other compounds include other hydrocarbons (C1-C9), 3-heptanone, 2-methyl-2-propanol, butanal and 2,6-di-*tert*-butyl-1,4-benzoquinone. Irradiation causes a dramatic decrease in the concentration of alkanes (Buchalla *et al.*, 1999). In the MT experiment, the peaks of "even number" alkanes (decane, dodecane, tetradecane, octadecane) were more intensive than the peaks of "odd number" alkanes (undecane and tridecane). This suggests that the straight alkanes detected in the MT experiment may originate from the cap. Moreover, 2,6-di-*tert*-butyl-1,4-benzoquinone was also one of the main VOCs present in the MT chromatogram, a compound typical for the radiolysis of HDPE (Buchalla, 2000).

Volatile products of pyrolysis of NR are xylene, toluene, benzene, isoprene, rubber dimer as well as some unidentified low MW hydrocarbons (C2-C10) (Naveau and Dieu, 1980; Seidelt *et al.*, 2006). Pyrolysis, however, is conducted at relatively high temperatures and involves rapid heating of the sample; for example, heating the sample from 40 to 500°C at a rate of 10°C/min (Mathew *et al.*, 2001). In thermal degradation studies solid rubber is stable, and below 200°C rubber volatility is negligible. This is also the case for PS, as no formation of volatiles occurs from PS below 300°C in thermal degradation studies (David, 1975). A range of techniques is used in thermal degradation studies of polymers such as thermogravometric analysis, differential scanning colorimetry, infrared spectroscopy and

also GC-MS. As GC-MS is used for methods based on polymer pyrolysis, high temperatures are applied (Crompton, 2010). No study is available regarding the volatility of any of the polymers of interest, at relatively low constant temperatures applied for prolonged periods and the MT and TD experiments both showed that some residual and radiolysis products of PS are emitted during incubation of the flask for a week at 37°C.

Compounds such as acetone; 2-methylfuran and benzothiazole were detected as degradation products of NR at 150°C (Gągol *et al*., 2015) and, therefore they may also be released by rubber seals. Carbon disulfide is another VOC of possible Suba-Seal® origin, as it is used as an additive for rubber in cold vulcanisation (Lay *et al*., 2000). The process is used to shape NR.

Other compounds detected in the MT experiment originating most likely from flasks are: 2,4-di-*tert*-butyl phenol; 2-ethenyl-2-butenal; cyclohexanone; dodecyl acrylate; pthalic acid, isobutyl nonyl ester and 2-tetradecanone. 2,4-Di-*tert*-butyl phenol is widely used as an antioxidant polymer additive in the plastic industry (Stoffers *et al*., 2004). 2-Ethenyl-2-butenal has been found to be a degradation product of PP (a common carrier material used in the masterbatch) and the cap is a probable source of its emission in the MT experiment (Liggat, 1999). On the other hand 2-tetradecanone may originate from LDPE, another common carrier material used in the masterbatch (Bravo and Hotchkiss, 1993). Dodecyl acrylate is an additive for polymers so may originate from PS but also is employed in inks and may originate from the masterbatch of the cap (BASF, 2015). Cyclohexanone may also originate from PS as it is used as thinner or solvent in the production of polymers and synthetic resins (Musser, 2000). Phthalic acid esters are common plasticisers (additives used to improve flexibility and durability of plastic) mainly in the industry of poly(vinylchloride) (PVC) but also of other plastics including PS (Jaworek and Czaplicka, 2013), therefore its origin in the cell culture medium may also be from the culture flask.

A number of PS and HDPE residual and radiolysis volatiles were shown to be consumed by the cells in the MT and TD experiments, namely acetophenone; benzaldehyde; decane; diisobutyl phthalate; 1,3-di-*tert*-butylbenzene; dodecane; ethylbenzene; hexanal; phenol and styrene. It highlights an important consideration for *in vitro* studies of volatiles as potential biomarkers of disease. How closely does the 'VOC environment' supplied to the cells *in vitro* mimic the *in vivo* state of the volatiles available to tumour cells? This is discussed further in Chapter 5.7.4.

5.3 *In vitro* analysis of VOCs

Overall the two statistical tests conducted on the data from the MT and TD experiments, the Wilcoxon signed-rank test (used for the 'between sample and control analysis') and the Kruskal-Wallis followed by Mann-Whitney U test (used for the 'between-sample analysis'), provided a complete picture of the level trends of the analysed VOCs. The first test showed a general level trend of the VOCs in the presence of cells, while the second one gave additional information about the different rates of production or consumption of the VOCs between different cell lines and between growing and confluent cells of the same cell line. The two analyses were consistent with each other with some VOCs and, for example, when the paired test detected the production of a VOC only by the cancer cells, the level of this VOC was also found to be elevated in the cancer cell samples in comparison to the non-transformed cell samples, using the Mann-Whitney U test. For some VOCs however, the two tests yielded inconsistent results. For example, a VOC was found to be produced by the cancer cells but no differences were detected (where elevated levels would be expected) between the cancer cell samples and normal cell samples. Such phenomena were most likely caused by the different initial concentrations of the analyte in different cell culture flasks and/or in the different batches of cell culture medium. The results of the inter-batch precision for the MT and TD experiments confirm this, as for some VOCs it was relatively poor while for others it was good. However, there are other reasons for the poor intra-batch and inter-batch precision values obtained (see Chapters 3.4.3 and 4.4.4). The fact that non-parametric tests have less statistical power than parametric tests, and therefore are less likely to detect a true effect, may also contribute to the inconsistencies between the 'between sample and control' and 'between-sample' analyses (Worthy, 2015). On the other hand, using a parametric test on the non-normal distributed data could result in false positive results (De Winter, 2013). Moreover, in the Mann-Whitney U test a set of hypotheses were tested simultaneously (pairwise multiply testing). The probability of observing at least one significant result due to chance only increases with a number of multiply tests. To adjust the p-values according to the number of tests conducted, so that the probability of the observed significant results occurs due to chance only below a desired significance level (here < 0.05), the Bonferroni correction was applied. The Bonferroni correction is known to be slightly conservative i.e. it will tend to 'miss' significant differences (Sokal and Rohlf, 2012). Therefore, there might have been some significant differences not detected by the test for some VOCs (especially with

a p-value close to 0.05). As such, there is a need for further study to confirm some level trends of the VOCs analysed.

5.3.1 *Altered VOCs and comparison between the cell lines*

In the 'between sample and control analysis' there were seven VOCs observed to be produced solely by the A549 cells (cyclohexanol; 2,4-dimethyl-1-heptene; 4-methylundecane; 3-heptanol; 2,3,6,7-tetramethyloctane; 2,3,5-trimethylhexane and 2,3,5-trimethyldecane); 14 VOCs observed to be consumed solely by the cancer cells (acetophenone; cyclohexanone; dodecanal; 4-decanol; 6-dodecanol; 2,5-di-*tert*-butyl-phenol; 2-ethylhexanol; 1-octanol; phenol; 2-methoxy-diphenylmethane; 2-nitrophenol; pentanoic acid, 2,4-dimethyl-3-oxo-, methyl ester; tetradecane and 2-tetradecanone); 10 VOCs observed to be produced only by the fibroblasts (benzyl alcohol; 2,5-di-*tert*-butylphenol; 6-dodecanol; dodecanal; dodecanoic acid, isooctyl ester; 2-ethylhexanol; geranyl acetone; octadecanal; tetradecane and 2-tetradecanone). All the compounds found to be metabolised by the NHLF cells were also found to be metabolised by the A549 cells. Diisobutyl phthalate; ethylbenzene; heptanal; octanal; 1-nonanol and styrene were consumed by both cell lines at a similar rate and 4-methyloctane and 2,4-dimethylheptane were produced at a similar rate, as no significant differences were detected in the Kruskal-Wallis analysis. A higher rate of metabolism of VOCs, namely benzaldehyde; 1,3-di-*tert*-butyl-benzene; dodecane; hexanal and 1-nonanol was observed for the cancer cells while 2-ethenyl-2-butenal was consumed at a higher rate by the fibroblasts. The VOCs that were produced by both cell lines but were found to be produced at a higher rate by the A549 cells were 1-phenylethanol; 2,6-di-*tert*-butyl-1,4-benzoquinone and 2-pentadecanone. 1,1'-(1,2-Cyclobutanediyl)-bis, cis-benzene was observed to be produced by both A549 and NHLF cells, but the fibroblasts demonstrated a higher rate of its production than the cancer cells.

In the TD experiment, five VOCs were found to be produced and four to be metabolised by the A549 cells. The BEAS-2B cells were found to produce four VOCs and consume six. 2,3,5-Trimethylhexane and *tert*-butanol were produced exclusively by the cancer cells while EtAc was produced solely by the normal cells. Decane and 2-methylpentane were observed to be metabolised only by the BEAS-2B cells. All the VOCs found to be consumed by the cancer cells were also consumed by the non-transformed cells, however, acetophenone, benzaldehyde and 2-methylbutanal were consumed at higher levels by the

A549 cells in comparison to the BEAS-2B cells. 2-Ethylhexanol and 2-pentanone were released by both cell lines at a similar rate, while acetone was found to be produced at a higher rate by the A549 cells.

Rapid proliferation is a well-known characteristic of tumour models and cells *in vitro* (Cairns *et al.,* 2011). Therefore, it is not surprising that in the MT experiment, the cancer cells appeared to release or consume many of the analytes at a higher rate than the non-transformed cells. The fact that the fibroblasts were observed to produce or consume the same VOCs that were produced or consumed by the cancer cells later during cell culture is also a consequence of a more rapid proliferation of cancer cells. Fibroblasts have a longer doubling time when compared to A549 cells (32 ± 6 hours versus 22 hours) (ATCC, 2014a; Mio *et al*., 1992). BEAS-2B cells have a doubling time of ~22 hours which is similar to the doubling time of the A549 cell line of ~22 hours (ATCC, 2014a; Costa *et al*., 2010). As the cell lines were seeded at the same seeding density, there were fewer differences found in the VOC trends between the two cell lines.

In the MT experiment, there were seven VOCs produced and 14 VOCs consumed only by the A549 cells. Ten compounds were unique products of fibroblasts. In the TD experiment two analytes were observed to be consumed only by the A549 cells and one was produced and two metabolised by the BEAS-2B cells. This indicates that the tumour cells had different metabolic pathways active to the normal cells. Oncogenic research has revealed that tumour phenotypes are the result of thousands of mutations which affect many core signalling pathways (Parsons *et al*., 2008; CLCGP and NGM, 2013). Many of these pathways are an adaptation of cancer cells, supporting their growth and survival and some of them are absolutely necessary for carcinogenesis. Cancer cells are known to alter cellular metabolism to optimise cell proliferation via an increase in synthesis of macromolecules, rapid generation of ATP in order to maintain energy and maintenance of redox status of the cell (Cairns *et al*., 2011). Comparative proteomic analyses between A549 cells and MRC-5 lung fibroblasts, and between A549 and BEAS-2B cells have shown the overexpression of proteins related to stress, redox regulation of cells, glycolysis, hypoxia tolerance and allergic response in A549 cells when compared to normal cells (Chang *et al*., 2001; Martín-Bernabé *et al*., 2014; Rubporn *et al*., 2009). Many proteins have been found to be differentially expressed between the A549 and BEAS-2B cell lines such as alcohol dehydrogenases (ADHs), aldehyde dehydrogenases (ALDHs), aldo-keto reductases (AKRs) and cytochrome 450 (CYPs) superfamily

members (Courcot *et al*., 2012). Preferential expression of some of them was also displayed to be cancer tissue specific (Jelski and Szmitkowski, 2008; Marcato *et al.*, 2011). In fact, the number of either overexpressed or underexpressed genes has been found to be higher for A549 cells than BEAS-2B cells (Courcot *et al*., 2012). These alternations might be reflected in the very different VOC patterns demonstrated for the A549 and the normal lung cells in the MT and TD experiments.

5.3.2 *Comparison within the cell lines*

Some analysed VOCs were found to be consumed or produced at significantly different levels between collections of the same cell line. In the MT experiment dodecane was consumed, while 2,6-di-*tert*-butyl-1,4-benzoquinone and 2-pentadecanone were observed to be produced only by the growing A549 cells. 1-Phenylethanol and 3-heptanol were produced at a higher rate by the growing cancer cells than by the confluent cancer cells. Acetophenone and benzaldehyde were metabolised to a higher extent by the growing A549 cells than by the confluent A549 cells. 2-Ethylhexanol was consumed at a higher rate by the confluent cancer cells than by the growing cancer cells. Benzyl alcohol and dodecanoic acid isooctyl ester were produced while ethylbenzene, heptanal and octanal were found to be metabolised only by the growing fibroblasts. Finally, dodecanal was produced exclusively by the confluent fibroblasts.

In the TD experiment some changes were also observed in the VOC patterns, depending on the confluency of the cells. 2-Ethylhexanol was produced solely by the growing A549 cells. Acetone was produced at a higher rate by the confluent cancer cells, while benzaldehyde was metabolised at a higher rate by the growing cancer cells. Confluent BEAS-2B cells were observed to consume acetophenone at a higher rate than the growing BEAS-2B cells. Also the growing BEAS-2B cells consumed benzaldehyde at a higher rate than their confluent counterparts.

These results suggest different metabolic pathways active during both the exponential growth of the cells and when they reach confluency. When a cell line is reseeded, the growth of cells follows a standard pattern. Firstly, cells go through the lag phase of adaptation after subculture when cells do not proliferate. Next is the log phase when the number of cells exponentially increases. The log phase terminates after one or two population doublings after reaching confluency for non-transformed cells. Then, the cells

enter the plateau phase when a contact inhibition of proliferation occurs, due to cell-cell and cell-medium interactions, as well as cytoskeletal dynamics and reduced availability of nutrients. However, if the cell culture medium is replaced regularly, most tumour cultures will continue to proliferate (at a reduced rate) beyond confluency, growing in multilayers (Freshney, 2005). The A549 cells were observed to grow in multilayers after reaching confluency. Although the medium could not be changed regularly in order to make it comparable with the medium of the first collection, the cells continued to grow. The same was the case for the BEAS-2B cells which are an immortalised cell line by transformation with a virus and therefore their proliferative life span has been extended (Reddel *et al*., 1989).

It can be hypothesised that because the rate of proliferation in confluent A549 cells decreases, simultaneously the metabolic processes involved in the production or consumption of some VOCs are slowed down (or stopped). During the plateau phase there may also occur an increased synthesis of specialised proteins instead of structural proteins, as well as changes in content and charge of the cell surface (Freshney, 2005). Studies on A549 cells and other cell lines have demonstrated that the phenotype of cultured cells has an impact on the expression of cell adhesion proteins, cell sensitivity to UV light or nutrient deprivation, cell growth and cell-death related pathways (Castro *et al*., 2001; Singer *et al*., 2010; Zhou *et al*., 2015). These could have an impact on the utilisation of different metabolic pathways between the growing and confluent cells.

In an *in vitro* study of VOCs, Rutter *et al*. (2013) observed a similar phenomenon of a decrease of acetaldehyde production with increasing numbers of seeded Calu-1 cells. The group suggested that this might be due to increased cell death. The MT and TD experiment did not show, however, any significant death of the confluent cells. There have not been any other studies looking into the differences in VOC patterns between growing and confluent cells. More compounds were found to be consumed or produced at a higher rate by the growing cells than by the confluent cells. Since tumour cells proliferate rapidly *in vivo*, this could imply that the studies of confluent cells are probably of less value than of growing cells. Arguably however, studies on confluent cells reflect *in vivo* conditions more than those on sub-confluent cells because of the presence of cell-cell interactions and gap/tight junctions (Freshney, 2005). Since tumour cells *in vivo* proliferate at a slower rate than cancer cells *in vitro* and lung adenocarcinoma is a slower growing cancer than other types of lung cancer, a confluent culture might be a better model for VOC investigation

(Gazdar *et al*., 2010, Henschke *et al*., 2012). The use of confluent cells in the MT experiment also gave the possibility of a more direct comparison of the A549 and NHLF cell lines with such different proliferation rates.

5.4 Altered VOCs versus other studies

All the VOCs detected at increased or decreased levels in the cell samples when compared to the medium controls in the MT and TD experiment are discussed below, in relation to previous *in vitro* and *in vivo* studies of volatiles as potential biomarkers of disease. The analytes are discussed by chemical group. The status of each VOC as a potential biomarker of lung cancer is critically examined. Also possible metabolic pathways leading to the uptake and release of these VOCs by the A549, NHLF and BEAS-2B cell lines are proposed.

5.4.1 Aliphatic hydrocarbons

Aliphatic hydrocarbons were the most numerous group of the VOCs detected in both the MT experiment (16 identified) and the TD experiment (19 identified). Some of them were not semi-quantified because their peaks were not resolved or their signal intensity was too low (Tab. 3.3 and 4.1). The amounts of some of the straight hydrocarbons were not affected by the presence of cells (pentane; heptane; undecane; tridecane; pentadecane; heptadecane; nonadecane) and of some were changed (octane; decane; dodecane; tetradecane). Branched methylated hydrocarbons were mostly observed to be produced by the cells in the MT experiment (2,4-dimethylheptane; 2,4-dimethyl-1-heptene; 4-methyloctane; 4-methylundecane; 2,3,6,7-tetramethyloctane; 2,3,5-trimethyldecane and 2,3,5-trimethylhexane). In the TD experiment 2,3,5-trimethylhexane was found to be emitted by the cells and 2-methylpentane consumed, however, the levels of most of the branched methylated hydrocarbons were not changed in comparison to the medium controls (3-methylpentane; 3-methylheptane; 4-methylheptane; 2,4-dimethyl-1-heptene; 4-methyloctane; 2,2,4,6,6-pentamethyheptane). The only cyclic non-aromatic hydrocarbon, methylcyclohexane, was detected in the TD experiment and its levels were unchanged in the presence of cells.

Aliphatic hydrocarbons found at increased levels

The level of 2,4-dimethylheptane (CAS: 3074-71-3) was found to be significantly increased exclusively in the A549 samples of collection 1, when compared to the pure medium controls ($p = 0.0039$). 4-Methyloctane (CAS: 2216-34-4) was detected at significantly higher levels in relation to the control medium after one and two weeks of incubation of the A549 samples ($p = 0.0039$ and $p = 0.036$ respectively) and for collection 3 of the NHLF cells ($p = 0.03603$). 2,4-Dimethylheptane was not found either in the NHLF samples or in the A549 and BEAS-2B samples of the TD experiment. 4-Methyloctane was present in the HS of both cell lines analysed in the TD experiment. However, for the TD experiment the Wilcoxon-signed rank test did not show significant differences in peak area ratios between the A549 samples and the medium controls ($p = 0.05469$ and $p = 0.1289$ for collection 1 and 2 respectively), even though the levels of this compound were increased in most of the A549 samples when compared to their medium controls.

Neither of these methylated alkanes has been detected in A549 cells before. The compounds were previously detected in elevated levels in the HS of other lung cancer cell lines, Calu-1 (Filipiak *et al*., 2008) and NCI-H2087 (Sponring *et al.,* 2009). However, in the latter study it was not the case for all the experiments. Both analytes were also reported at increased levels in the urine of mice bearing A549 cell-induced tumours (Hanai *et al.,* 2012b). 4-Methyloctane was found to be elevated in the HS of the three mutated cell lines when compared to their parental HBEC cell line (Davies *et al*., 2014). 2,4-Dimethylheptane was observed in higher concentrations and 4-methyloctane exclusively, in the breath of lung cancer patients when compared to healthy controls (Peng *et al.,* 2009). 4-Dimethylheptane was one of the 22 breath biomarkers selected for the discriminant analysis between patients with lung cancer and without (Phillips M. *et al*., 1999a). 4-Methyloctane was included into a nine breath biomarkers set to distinguish lung cancer patients from healthy individuals in another study by this group (Phillips M. *et al*. 2003a). Both alkanes in a set of 20 VOCs were used as breath biomarkers to differentiate between not only lung cancer patients and healthy volunteers, but also between cancerous patients and persons suffering from COPD and asthma (Rudnicka *et al.,* 2015). Finally, 4-methyloctane was reported as one of the most abundant VOCs in the breath of healthy people (Phillips M. *et al.,* 2013). In general, the potential use of 4-methyloctane and 1,4-

dimethylheptane as biomarkers of lung cancer in the breath is very promising, but needs further study to verify their higher levels in the breath of lung cancer patients.

2,4-Dimethyl-1-heptene (CAS: 19549-87-2) is another hydrocarbon found in significantly elevated levels solely in collection 1 of the A549 cells in the MT experiment ($p = 0.01172$). The compound was not detected at all in collection 2 of the cancer cell samples and in collection 1 of the fibroblast samples. Collections 2 and 3 revealed the presence of this compound in both fibroblast samples and their controls, but without significant differences in the medians ($p = 0.4185$ and $p = 0.7893$ respectively). For the TD experiment 2,4-dimethyl-1-heptene was detected in all four set-ups, however no significant differences were observed between the cell samples and controls. The VOC was significantly increased in the HS of the A549, hFB and HBEC lung cell lines in the study by Filipiak *et al*. (2010). It was released at higher concentrations by five breast cancer cell lines in comparison to both control medium and a non-transformed breast cell line (Lavra *et al.,* 2015). Lower levels of 2,4-dimethyl-1-heptene were observed in A549 cells when compared to normal cells (WI-38 VA13 and OUS-11) by Hanai *et al.* (2012b). This study does not indicate however, whether the VOC was consumed or produced by the cancer cells. The levels of 2,4-dimethyl-1-heptene were observed to be lower in the breath of lung cancer patients when compared to healthy controls by Filipiak *et al.* (2014). The VOC was also reported as one of the most abundant volatiles in the breath of healthy non-smokers and therefore its higher levels in the breath of lung cancer patients as a potential biomarker needs further study (Phillips M. *et al.,* 2013).

Another branched hydrocarbon, 2,3,5-trimethylhexane (CAS: 1069-53-0), was observed in higher concentrations in the A549 samples in comparison to the pure medium for both the MT and TD experiments. However, this was only the case for collection 2 of the MT sample ($p = 0.0039$) and collection 1 of the TD experiment ($p = 0.027$). 2,3,5-Trimethylhexane is a potential biomarker of lung cancer, as no significant differences in the levels of the compound between the BEAS-2B cells and their medium controls were found. Also, NHLF cells were not observed to produce it. The VOC was reported in *in vitro* studies before, in the HS of the Calu-1 cell line (Sponring *et al*. 2009) but also in the HS of the non-cancer hFB cells (Filipiak *et al.*, 2010). Together with the previously described methylated alkanes, 2,4-dimethylheptane and 4-methyloctane and 17 other VOCs, it was used as a breath biomarker to differentiate between not only lung cancer

patients and healthy volunteers, but also between cancerous patients and persons suffering from COPD and asthma (Rudnicka *et al.*, 2015).

4-Methylundecane (CAS: 2980-69-0); 2,3,5-trimethyldecane (CAS: 1844-37-1) and 2,3,6,7-tetramethyloctane (CAS: 52670-34-5) were emitted by the growing A549 cell samples (collection 1) when compared to the control for the MT experiment. No significant differences in the levels of the branched hydrocarbons were observed for the growing and confluent fibroblasts and confluent cancer cells for the first two VOCs. 2,3,6,7-Tetramethyloctane was not detected in any other collection. 4-Methylundecane was also detected in the TD experiment; however, its levels were not quantified because of S/N ratio < 3. The three VOCs have not been previously reported in any *in vitro* studies. Regarding *in vivo* studies and cancer, 4-methylundecane was detected in the human exhaled breath and used as a discriminant compound (among 12 others) between colon cancer patients and healthy controls. However, it is not clear which trend this has shown i.e. whether its concentrations were higher in the breath of patients or of controls (Altomare *et al.*, 2013). 2,3,6,7-Tetramethyloctane was detected at higher levels in the breath of patients suffering from Parkinson's disease when compared to the breath of healthy individuals (Tisch *et al.*, 2013). Oxidative stress plays an important role in the pathogenesis of both Parkinson's disease and cancer, and the VOC might be generated during the process of lipid peroxidation induced by this oxidative stress (Filaire *et al.*, 2013; Tisch *et al.*, 2013). Therefore, 4-methylundecane and 2,3,6,7-tetramethyloctane could be candidate lung cancer biomarkers, however more data is needed to confirm this status, especially because the latter VOC had a possible alternative identification (Tab. 3.3). 2,3,5-Trimethyldecane has not been reported in breath or in any human biofluid yet and therefore its use as a potential biomarker of lung cancer is unlikely (De Lacy Costello *et al.*, 2014).

Aliphatic hydrocarbons found at decreased levels

Dodecane (CAS: 112-40-3) was found to be metabolised by the growing A549 cells (collection 1, $p = 0.003906$) and the growing NHLF cells (collection 2, $p = 0.03906$) in the MT experiment. However, the cancer cells were observed to consume the hydrocarbon at a higher rate than the fibroblasts. Although the VOC was also detected for the TD experiment, no differences in the levels of dodecane were found here between the cancer cells and their medium controls. This was also the case for the BEAS-2B cells. The

finding of the MT experiment is in agreement with the *in vivo* study of potential cancer biomarkers in breath by Peng *et al.* (2010). Peng and co-workers found levels of dodecane in the breath of healthy individuals were higher than in the breath of lung cancer patients, suggesting that tumour cells may metabolise it at a higher rate. The VOC (together with another five compounds) was therefore used by the group in the principal component analysis to distinguish cancer patients from healthy controls. However, Filipiak *et al.* (2014) observed an opposite trend (higher levels) for dodecane in the breath of lung cancer patients. Dodecane is one of the most prevalent VOCs in healthy human breath (Phillips M. *et al.*, 1999b; Van den Velde *et al.*, 2007; Phillips M. *et al*. 2013) and therefore lower amounts of dodecane may be the result of the presence of a tumour. More studies are required to confirm the status of dodecane as a potential lung cancer biomarker.

Tetradecane (CAS: 629-59-4) is another straight alkane that was found to be consumed by both the cancer cell lines (only during the second week, $p = 0.01172$) and the fibroblasts (only during the third week, $p = 0.02734$) for the MT experiment. There were no differences found in the levels of the emitted alkane between the cell lines. The level trend of tetradecane for the MT experiment is opposite, however, to other *in vitro*, *ex vivo* and *in vivo* studies of VOCs. The hydrocarbon was found to be released by the A549 cells in another study (Schallschmidt *et al.,* 2015a). Lung cancer tissues were observed to emit tetradecane in contrast to normal lung tissues (Wang Y. *et al.,* 2012). Tetradecane was also one of the proposed breath markers of breast cancer (Phillips M. *et al.,* 2010). Although it was found in the ventilated air from a cancerous lung during surgery, it was speculated that the VOC originated from the lipid peroxidation as result of reperfusion (Wang C. *et al.*, 2014). As tetradecane is one of the most abundant VOCs in the breath of a healthy individuals (Phillips M. *et al.,* 2013), and it is not a smoking or COPD-related compound (Gaida *et al.*, 2016), some new information regarding the metabolism of this alkane *in vivo* may yet be reported in future studies.

Significantly lower amounts of octane (CAS: 111-65-9) were detected in collection 1 of the A549 cell samples ($p = 0.03906$) and in collection 2 of the BEAS-2B cell samples ($p = 0.02734$) in the TD experiment, suggesting that the cancer cells metabolise this compound during the proliferation period and the BEAS-2B cells when they reach the confluent state. No significant differences in levels of octane, however, were observed between the cell lines. The hydrocarbon was found in decreased levels in the A549 samples in the study by Schallschmidt *et al.* (2015a) and at elevated levels, both in the A549 cells and in the

fibroblast cells by Filipiak *et al.* (2010). In other lung cancer cell lines Lu7466, Lu7387 and Calu-1 the levels of octane were similar to the concentrations in the pure medium controls (Filipiak *et al.,* 2008; Schallschmidt *et al.,* 2015a). Filipiak *et al.* (2014) also found higher concentrations of octane in the breath of cancer patients when compared to healthy subjects as well as in the HS of lung cancer tissue in comparison to non-cancer tissue controls. Octane is an abundant VOC in the breath of a healthy person (Libardoni *et al.,* 2006; Sanchez and Sacks, 2006; Van den Velde *et al.,* 2007). Higher concentrations of the VOC in the breath of lung cancer individuals when compared to healthy non-smokers and COPD patients (but not when compared to healthy smokers) were observed by Poli *et al.* (2005) and the VOC was included into the set of 13 biomarkers used for discriminant analysis. Octane was also proposed as a sensitive blood marker of liver cancer (Xue *et al.,* 2008). However, higher concentrations of octane in breath were linked to smoking (Buszewski *et al.,* 2009; Wallace and Pellizzari *et al*., 1987). Therefore, octane is unlikely to be a specific biomarker of lung cancer when found at higher levels. Lower concentrations of octane in breath in comparison to healthy controls have never been recorded. Such a finding would be needed to confirm octane found in lower levels as a potential marker of lung cancer.

2-Methylpentane (CAS: 107-83-5) was another aliphatic hydrocarbon consumed in the TD experiment exclusively by the BEAS-2B cells in collection 2 ($p = 0.003906$). The production of this VOC by cancer cell line NCI-H2087 was reported by Sponring *et al.* (2009). The VOC was observed at higher levels in breath of lung cancer patients (Poli *et al*., 2005; Rudnicka *et al*., 2011). Other studies did not find any significant differences in the levels of 2-methylpentane between the cancerous breath and healthy breath (Ligor M. *et al*., 2009; Ulanowska *et al*., 2011). More studies are required to determine whether the VOC has any use as a potential biomarker of lung cancer.

Biochemical background for the altered levels of aliphatic hydrocarbons

Oxidative stress is the main mechanism of production of hydrocarbons in the human body. Short straight chain alkanes and alkenes originate from the lipid peroxidation of PUFA by reactive oxygen species. It is known that the oxidation of ω-3 PUFA results in the formation of ethane, and ω-4 PUFA, ω-6 PUFA and ω-7 PUFA yields propane, pentane and hexane respectively in breath (De Zwart *et al.,* 1999). Other straight-chain hydrocarbons may also originate in this way but the exact mechanisms are not known

(Kneepkens *et al.,* 1994). Other sources of hydrocarbon formation *in vivo* are proteins and amino acids (hydrocarbons as free radical-induced oxidative degradation products) and bacterial flora (Kessler and Remmer, 1990; Kneepkens *et al.,* 1994). It is unclear how these other sources could possibly influence hydrocarbons present in exhaled breath. In the MT experiment some branched methylated hydrocarbons were produced by both the cancer and normal cells. At the moment the possible pathways of their formation are unknown. They are unlikely, however, to originate from lipid peroxidation of PUFA as there are no branched polyunsaturated fatty acids *in vivo* (Kneepkens *et al.,* 1994). Biosynthesis of long-chain hydrocarbons and branched methylated alkanes has been found and extensively studied in bacteria, plants and insects (Blomquist and Bagnères, 2010; Ladygina *et al.,* 2006; Leahy and Conwell, 1990; Nelson, 1978). However, an alternative pathway for the production of branched hydrocarbons and long-chained hydrocarbons in humans has not yet been reported despite their obvious association with many oxidative diseases, including lung cancer (Calenic *et al*., 2015; Phillips M. *et al*., 2003b; Phillips M. *et al*., 2004a).

Monooxygenases from the CYP superfamily have a significant role in the metabolism of hydrocarbons (via hydroxylation) in humans (Kneepkers *et al*., 1994). Over 50 CYP isoenzymes belonging to 18 families (CYP1, CYP2 etc.) have been described in humans. They have a broad affinity for various endogenous and exogenous compounds (Tomaszewski *et al.*, 2008). The A549 cells express numerous CYP enzymes from at least 12 different families (Castell *et al.*, 2005; Courcot *et al.,* 2012). Therefore, the A549 metabolism of octane, dodecane and tetradecane is most likely CYP-mediated. Some of the CYP enzymes expressed by BEAS-2B cells are different to the A549 cells (Courcot *et al.*, 2012). This might be the reason for differences in the consumption of decane between these cell lines. The differences in CYP expression probably also exist between the A549 cells and the fibroblasts, thereby differentiating the patterns of metabolised VOCs.

5.4.2 Aromatic hydrocarbons

Four aromatic hydrocarbons were identified in the MT experiment (1,3-di-*tert*-butyl benzene; 1,1'-(1,2-cyclobutanediyl)bis-,cis-benzene, styrene and p-xylene) and five in the TD experiment (benzene, ethylbenzene, styrene, toluene and p-xylene). The amounts of p-xylene in the MT experiment and benzene, styrene and toluene in the TD analysis were

not affected by the presence of cells. The peak areas of p-xylene were not quantified in the TD experiment because of S/N ratio < 3.

Aromatic hydrocarbons found at increased levels

The only aromatic hydrocarbon observed to be produced by cells in the MT experiment was 1,1'-(1,2-cyclobutanediyl)bis-,cis-benzene (CAS: 7694-30-6). Its levels were found to be significantly higher in the samples of both the A549 and NHLF cells, in comparison to their medium controls in collections 1 and 2 ($p = 0.039$, $p = 0.0039$, $p = 0.0391$, $p = 0.0039$ respectively) of the MT experiment. Its level was not elevated in collection 3 for the fibroblasts. Moreover, the cancer cells were found to emit the VOC at significantly higher levels than the normal cells during the second week of cell culture. The compound has not been reported in any *in vitro* study before. In *in vivo* studies of VOCs, 1,1'-(1,2-cyclobutanediyl)bis-,cis-benzene was one of the 30 biomarkers that were used in the multivariate model for the prediction of lung cancer employing VOCs in breath by Phillips M. *et al.* (2008). Because the MT experiment showed that 1,1'-(1,2-cyclobutanediyl)bis-,cis-benzene was produced by the A549 cells at a higher rate than by the NHLFs, the tumour cells, growing faster than the surrounding normal lung cells, may produce the VOC in higher concentrations that could be detected in breath. Interestingly, the VOC was found to induce cell proliferation in breast cancer cells *in vitro* via binding to the estrogen receptor (Ohyama *et al.,* 2001). Whether the compound is produced to enhance cell proliferation in cancer is only a speculation. However, all in all 1,1'-(1,2-cyclobutanediyl)bis-,cis-benzene could be a potential specific biomarker of lung cancer but more evidence is needed.

Aromatic hydrocarbons found at decreased levels

The levels of 1,3-di-*tert*-butylbenzene (CAS: 1014-60-4) were found to be significantly lower in the A549 cell samples in comparison to the control medium during the second week of cultivation in the MT experiment ($p = 0.03906$). It was also found in decreased levels in collection 1 of the NHLF cell samples ($p = 0.02734$), however, the A549 cells were observed to metabolise a VOC at a higher rate. The results for the cancer cells are consistent with another study of A549 cells where the VOC was found at lower levels when compared to normal cell controls during the first week of incubation (Hanai *et al.,* 2012b). However, the study by Barash *et al* (2012) showed the opposite trend for A459

cells and for the other NSCLC cell lines. 1,3-Di-*tert*-butylbenzene was one of three compounds proposed by Pyo *et al*. (2009) as a biomarker of apoptosis when the A549 cells were treated with Cisplatin. Therefore, in that study the VOC was also observed to be produced by A549 cells. There is every likelihood that the A549 cells in the MT experiment would start producing 1,3-di-*tert*-butylbenzene during apoptotic death.

However, the use of 1,3-di-*tert*-butylbenzene as a biomarker of cancer is unlikely in any scenario, regardless of its concentration trend. As a biomarker produced by lung cancer cells, 1,3-di-*tert*-butylbenzene would not be specific to lung cancer as it was also found in higher concentrations in breast cancer and normal breast cell samples when compared to pure medium controls (Lavra *et al.,* 2015). As a biomarker consumed by lung cancer cells, 1,3-di-*tert*-butylbenzene is probably not readily available to the tumour cells *in vivo* to be metabolised, as it has not been found in human breath or any other human bio-fluid except faeces (De Lacy Costello *et al.*, 2014). The presence of this VOC in the cell culture flasks most likely originates from FBS (Villard *et al.*, 2011) and/or the radiolysis of PS (Buchalla *et al.,* 1999) (see Chapter 5.2 for further discussion).

Ethylbenzene (CAS: 100-41-4) was detected in both the MT and TD experiments. However, its levels were observed to be significantly reduced for the cell samples when compared to the controls only in the MT analysis, and only for collection 1 of both the A549 and NHLF cells ($p = 0.003906$). No significant differences in the abundance of the VOC between the cell lines were present. The VOC was also consumed by A549 cells in the study by Schallschmidt *et al.* (2015a). Ethylbenzene is one of the most commonly occurring VOCs in human breath (occurrence > 75%, n = 1000). Studies *in vivo* reported mainly higher concentration trends for ethylbenzene in cancerous breath. Poli *et al.* (2005) found greater levels of ethylbenzene in the breath of lung cancer patients than in normal breath, but lower levels than in the COPD patients' breath. Higher concentrations of the VOC in lung cancer individuals were also reported by Ulanowska *et al.* (2011) and Buszewski *et al*. (2012b). However, some studies did not report any differences between cancerous breath and normal breath for ethylbenzene (Buszewski *et al*., 2012a; Peng *et al.*, 2009). Moreover, higher levels of ethylbenzene were linked to smoking (Buszewski *et al*., 2008; Filipiak *et al*., 2012; Wallace and Pellizzari, 1986). Therefore, more studies are needed to confirm the status of ethylbenzene as a specific biomarker of lung cancer.

Styrene (CAS: 100-42-5) was observed in lower levels in the samples of the growing A549 cells (collection 1) and of the confluent fibroblasts (collection 3) for the MT experiment. Although it was detected in the HS of all the sampled flasks for the TD experiment as well, no differences in median peak area ratios between the samples and controls and between the two cell lines were found (see Chapter 5.5 for further discussion). Different level trends of styrene in A549 cells were reported in the literature. In some studies it was found at lower levels when compared to normal cell samples (Hanai *et al*., 2012b), in others it was produced as higher levels were observed when compared to pure medium controls (Schallschmidt *et al*., 2015a; Barash *et al.,* 2012) and in some studies no differences were found between A549 cell samples and cell-free medium controls (Schallschmidt *et al.,* 2015a). Peled *et al.* (2013) observed an increase in styrene levels in the EGFR mutated cell line and wild-type cell lines, when compared to pure medium controls. Other human lung cancer cells (NSCLC and SCLC) were also observed to produce the VOC in comparison to the pure medium (Barash *et al.,* 2012) and normal cells (Chen X. *et al.,* 2007). Some other mammalian cells have been previously shown to metabolise styrene such as human skin cells cultured in monolayers (Acavedo *et al*., 2007) or mouse and rat isolated lung cells (Hynes *et al.,* 1999).

Styrene, similarly to ethylbenzene, is a widely distributed air pollutant in the environment (Mögel *et al.,* 2011), therefore it is also one of the most frequently occurring VOCs in the breath of a healthy person (occurrence >75%, n = 1000) (Needham *et al.,* 1995; Phillips M. *et al.,* 1999b; Phillips M. *et al.,* 2013; Van del Velde *et al.,* 2007). The VOC appeared in higher concentrations in the breath of lung cancer subjects when compared to the breath of healthy controls in the study by Peng *et al.* (2009) and it was used as a discriminant marker for the presence not only of lung cancer (Chen X. *et al.,* 2007; Phillips M. *et al.,* 1999a), but also of liver cancer (higher levels) (Qin *et al*., 2010). In another study, no differences were found in the levels of styrene between the lung cancer patients and healthy controls, but it was present in higher levels in the breath of COPD patients when compared to the two previous groups (Poli *et al*., 2005). Finally, styrene was identified as one of the VOCs arising in breath due to smoking (Buszewski *et al*., 2008; Filipiak *et al.,* 2012; Wallace and Pellizzari, 1986). Therefore, as the higher levels of styrene in the cell samples and in breath are not specific to lung cancer only, and the fact that styrene is related to smoking and that its concentration trends are inconsistent both in *in vitro* and *in vivo* studies, its use as a potential biomarker of lung cancer needs further evaluation.

Biochemical background for the altered levels of aromatic hydrocarbons

Similarly to aliphatic hydrocarbons the mechanism of production of aromatic hydrocarbons in the human body is currently not known so a potential metabolic pathway leading to the production of 1,1'-(1,2-cyclobutanediyl)bis-,cis-benzene could not be found in any living organism (KEGG, 2014). According to Yannai (2004) 1,1'-(1,2-cyclobutanediyl)bis-,cis-benzene is an endogenous compound but its biofunction is currently unknown. This would indicate, however, that *in vivo* benzene derivatives do not only originate from cigarette smoking.

There are three major pathways known for the metabolism of aromatic hydrocarbons *in vivo*: the CYP-mediated epoxidase hydrolase pathway, CYP-mediated peroxidise pathway and AKR pathway. The metabolism of aromatic hydrocarbons results in epoxides, phenols or catechols as metabolites. These compounds may be substrates for CYP themselves (Moorthy *et al*., 2015; Wolf, 1982). CYP1A1 is necessary for the detoxification of aromatic hydrocarbons (Moorthy *et al*., 2015). The enzyme has been shown to be expressed by A549 cells (Courcot *et al*., 2012). The metabolism of 1,3-di-*tert*-butylbenzene has not been described in any living organism but it may appear via these pathways.

The source of ethylbenzene and styrene in breath and biological fluids is exogenous. They enter the body mainly through inhalation via exposure to these VOCs from tobacco smoke and the exhaust from internal combustion engines (Needham *et al.,* 1995). Once in the body the detoxification mechanisms are induced to eliminate the compounds. However, they may also be accumulated in the body's fatty tissues and then slowly be released into breath (Hakim *et al*., 2012).

The pathways for styrene and ethylbenzene degradation in humans have been described previously. CYP-mediated oxidation of styrene leads to styrene oxide intermediate. The reaction takes place in the liver but also, to a lesser extent, in the lungs. Further hydrolysis of styrene oxide to styrene glycol (catalysed by microsomal epoxide hydrolase), and then oxidation of glycol (catalysed by ADHs and ALDHs), leads to the final products of styrene degradation: mandelic acid and phenylglyoxylic acid, the main metabolites of styrene found in urine (Rueff *et al*., 2009). The same compounds are the final metabolites in the degradation of ethylbenzene, however, via a different metabolic pathway leading though such intermediate compounds as 1-phenylethanol and possibly acetophenone

(Engström *et al*, 1984) (which are also found in urine as ethylbenzene metabolites). The pathway is CYP-initiated (Sams *et al.,* 2004). The observed degradation of styrene and ethylbenzene most probably occurred via these pathways. Interestingly, 1-phenylethanol appeared at elevated levels for the MT experiment which might be due to ethylbenzene metabolism.

The acute toxicity of ethylbenzene is low. However, a 3 hour exposure of leukaemia cells (HL-60) to a 0.36 mM (~ 35 ppm) dose of ethylbenzene (obtained IC_{20} concentration) resulted in an increased expression of the genes involved in immune response. The IC_{50} dose of 0.99 mM additionally caused the expression of genes involved in apoptosis (Sarma *et al.,* 2010). Styrene is slightly toxic to living organisms. Exposure of the A549 cells to a 1 mg m^{-3} (~ 0.2 ppm) dose of styrene for 20 hours or to a 10 mg m^{-3} (corresponding to ~ 0.02 ppm in cell culture medium) dose of styrene for 24 hours resulted in induced inflammatory reactions via the expression of such proteins as cycloxygenase-2 (COX-2) and monocyte chemoattractant protein-1 (MCP-1), which are proteins involved in inflammatory responses and oxidative stress. Interestingly, however, the VOCs had no effect on the viability and proliferation of the cells (Fischäder *et al.,* 2008; Mögel *et al.*, 2011).

The concentrations of styrene and ethylbenzene in the MT and TD experiments are not known, as quantification has not been conducted. The concentration of styrene in a cell-free RPMI 1640 medium quantified by Peled *et al.* (2013) who used PS culture dishes was 1.5 ± 0.8 ppm. It was the most abundant peak quantified by this group. Styrene was one of the most intensive peaks for the TIC of both the MT and TD experiments (Fig. 3.17, 4.8). Therefore, similar concentrations of styrene would be expected for the MT and TD experiments to those in Peled and co-workers' study. The exposure of the cells to a concentration of 1.5 ppm of styrene would be enough to induce detoxification mechanisms.

Ethylbenzene as a VOC *in vitro* has never been quantified by any group. In general, the concentrations of VOCs in studies where quantification was performed were found to be at medium or low ppb in various types of cell culture media incubated in either PS dishes or glass bottles (Davies *et al*., 2014; Mochalski *et al*., 2013b; 2014; 2015). Therefore, similar concentrations would be expected for the MT and TD experiments. The concentrations in sub or low-ppb could be enough to induce the metabolism of

ethylbenzene observed in the A549 and NHLFs cells as a mechanism of detoxification, especially given that the cells were exposed to the VOC for 7 days in contrast to 3 hours.

5.4.3 Alcohols

Alcohols were the second biggest group of VOCs detected in the MT and TD experiments. There were 11 alcohols identified in the MT analysis. Nine were found to be produced or consumed by the cells, namely benzyl alcohol; 2-ethylhexanol; heptanol; 3-heptanol; 1-nonanol; 1-octanol; 1-phenylethanol; 4-decanol and 6-dodecanol. The median peak area ratios of α-cumyl alcohol and cyclohexanol were similar in both the samples and controls. The TD analysis allowed for the identification of three alcohols: ethanol; 2-ethylhexanol and *tert*-butanol. Ethanol was excluded from the analysis because of the carry-overs and the levels of the other two alcohols appeared to be affected by the presence of cells.

Alcohols found at increased levels

1-Phenylethanol (CAS: 98-85-1) and 3-heptanol (CAS: 589-82-2) were alcohols found at significantly higher levels for both collections of the cancer cells in the MT experiment ($p = 0.0039$). 1-Phenylethanol was also observed in higher abundance in the fibroblast sample, but only after the third week of cell culture ($p = 0.0078$). The 'between-sample analysis' showed that 1-phenylethanol was produced at a higher level by the A549 cells when compared to the fibroblasts. 1-Phenylethanol was reported to be present at higher levels when compared to normal cells previously (Hanai *et al.*, 2012b). Melanoma cells were also shown to have greater relative amounts of this alcohol when compared to a control medium and non-transformed skin cells (Kwak *et al.*, 2013). 1-Phenylethanol is a main metabolite of ethylbenzene (Chan *et al.*, 1998). The levels of ethylbenzene were lower for both the A549 and NHLF cells in some collections of the MT experiment. 3-Heptanol is a main product of heptane metabolism (Perbellini *et al.*, 1986). The detection of heptane with the MT extraction was not possible because of the solvent peak; however, heptane was present in the HS of all the sampled flasks in the TD experiment. Therefore, the metabolism of ethylbenzene may be a possible pathway for 1-phenylethanol production and the metabolism of heptane for 3-heptanol production by the cells *in vitro*. The use of 1-phenylethanol and 3-heptanol as markers of lung cancer is questionable

however, as they have never been reported in human breath (De Lacy Costello *et al.*, 2014).

Benzyl alcohol (CAS: 100-51-6) was found to be produced by the fibroblasts ($p = 0.0078$) when compared to the medium control in the first week of the incubation for the MT experiment. The differences in medians were not significant for the remaining two collections. Interestingly, the A549 cells also emitted this alcohol in the first week, however the difference was beyond the chosen level of significance ($p = 0.0547$). Benzyl alcohol was found at lower or higher levels, depending on the week of cell culture, in A549 cell samples when compared to non-transformed cells by Hanai *et al.* (2012b). This alcohol was detected at higher levels in melanoma cells when compared to normal skin cells (there was no difference to the pure medium) (Kwak *et al.*, 2013). Benzyl alcohol was reported in the breath of healthy people and patients suffering from COPD and asthma, as well as in the EBC of healthy volunteers (Basanta *et al.*, 2012b; Peralbo-Molina *et al.*, 2015). It has never been used as a discriminant breath biomarker in any studies of cancer to date and its lower levels in cancerous breath when compared to normal breath would be in agreement with the results obtained for the MT experiment. However, benzyl alcohol may be related to a number of confounders. The VOC was applied to discriminate the breath of people suffering from asthma from healthy controls, with lower amounts present in the breath of the latter group (Ibrahim *et al.*, 2011). In addition, the VOC might be associated with sinus-related bacteria (Preti *et al.*, 2009). Finally, the VOC is an ingredient in many common cosmetic products and might be absorbed through the skin (Scognamiglio *et al.*, 2012). As such benzyl alcohol is rather unlikely to be a specific biomarker of lung cancer.

Cyclohexanol (CAS: 108-93-0) was a VOC observed to be produced solely by the A549 cells during the second week of incubation ($p = 0.003906$). The alcohol was observed to be produced by A549 and Lu7387 cell lines (but not by Lu7466 cells) although with the use of some sampling methods, no changes were observed in its levels for A549 cells (Schallschmidt *et al.*, 2015a). Cyclohexanol was reported in the urine and blood of individuals exposed to cyclohexanone (Mráz *et al.*, 1999; Sakata *et al.*, 1989), however it has never been observed in breath (De Lacy Costello *et al.*, 2014). Therefore, its production by the A549 cells in the MT experiment might be due to the metabolism of cyclohexanone, a VOC most likely originating from PS (Musser, 2000). In such a

scenario, cyclohexanol is unlikely to serve as a biomarker of lung cancer. However, if found in breath in future studies its use as a lung cancer biomarker is promising.

Alcohols found at decreased levels

1-Heptanol (CAS: 111-70-6) was found in significantly decreased levels in the A549 samples when compared to the medium controls during the first week of the MT experiment. However, it was not detected at all in any other collection of the MT experiment (neither in the samples nor in the controls). Therefore, it is unknown whether the NHLF cells would consume the compound as well if it was available to them. In relation to other *in vitro* studies of cancer, the VOC was observed to be produced by colon cancer cells in comparison to a pure medium control (Zimmerman *et al*., 2007). The alcohol was found in the EBC and breath of healthy individuals, which is promising if the VOC in lower levels in lung cancer breath is a biomarker of the disease (Hryniuk and Ross, 2010; Hubbard *et al*., 2009; Pleil *et al*., 2008). 1-Heptanol is known to be produced by gut bacteria (Ewen *et al*., 2005; Garner *et al*., 2007) and as such it can be transported to the lungs and exhaled in breath (Ulanowska *et al*., 2011b). Therefore, further study is needed to verify the status of this VOC as a potential biomarker of lung cancer.

2-Ethylhexanol (CAS: 104-76-7) was detected in all collections of both experiments but with different level trends. In the experiment employing MTs, it was found in significantly decreased levels in the A549 cell samples when compared to medium controls ($p = 0.0039$ for both collections). In contrast, the NHLF cells appeared to produce the alcohol but only in the second week of the culture ($p = 0.0078$) and the levels of the VOC were similar for the samples and controls in the two other weeks. The BEAS-2B cells also generated 2-ethylhexanol into the HS ($p = 0.0117$ for collection 1 and $p = 0.0039$ for collection 2). Finally, the TD experiment showed an opposite trend for the A549 cells, as in the first collection the cells were observed to produce the VOC ($p = 0.0039$). The same contradictory results regarding this VOC exist in the literature. Therefore, for the A549 cell line, 2-ethylhexanol was not only shown to be produced (Barash *et al.,* 2012) or consumed (Hanai *et al.,* 2012b; Schallschmidt *et al.,* 2015a), but furthermore no differences were found between the cells and pure medium (Schallschmidt *et al*., 2015a). Other *in vitro* studies also reported its production by lung cancer cells (Barash *et al.,* 2012; Sponring *et al.,* 2009), metabolism by lung cancer cells (Filipiak *et al*., 2010) or no differences in the concentrations of the alcohol between the medium and breast cancer

cells (Lavra *et al.*, 2015). It also was shown to be emitted in higher levels by two breast cancer cell lines when compared to normal breast cell lines (Huang Y. *et al.*, 2016). In fact, Sponring *et al.* (2009) observed that NCI-H2087 cells produced 2-ethylhexanol with low passage numbers and the VOC could not be detected with high passage numbers. The status of this VOC is not any clearer in the *in vivo* or *ex vivo* studies of lung cancer. 2-Ethylhexanol was reported as one of the VOCs found only in the exhaled breath of lung cancer patients (n = 40, occurrence >85%), and was not detected in healthy, non-smoking subjects (n = 56) (Peng *et al.*, 2009). Its levels were also elevated in the urine of lung cancer patients (n = 20) in comparison to healthy individuals (n=20) (Hanai *et al.*, 2012a). In other studies, however, the VOC was reported as one of the most abundant VOCs in the breath of healthy non-smokers (n = 34, occurrence > 90%) (Martin *et al.*, 2010; Phillips M. *et al.*, 2013). The *ex vivo* analysis of pleural effusions from lung cancer individuals showed significantly higher levels of 2-ethylhexanol than the effusions of patients without lung cancer. The VOC was proposed as a biomarker of lung cancer to differentiate between cancerous and benign effusions (Liu H. *et al.*, 2014). The possible reasons for such contradictory results will be discussed later in this chapter.

Another two alcohols, 1-octanol (CAS: 111-87-5) and 4-decanol (CAS: 2051-31-2) were significantly decreased in the A549 samples of the MT experiment, but only in the first collection ($p = 0.0039$ and $p = 0.01953$ respectively). 1-Octanol was reported in the HS of two colon cancer cell lines but no significant differences in relation to medium controls were observed. However, a normal colon cell line consumed 1-octanol in the same study (Zimmermann *et al.*, 2007). 1-Octanol was reported to be present in urine, faeces and skin emanations from healthy humans, however, not in breath (De Lacy Costello, *et al.*, 2014; Garner *et al.*, 2007; Yamazaki *et al.*, 2010). 4-Decanol has never been reported in the literature as a volatile in any body fluid of a healthy or diseased person (De Lacy Costello, *et al.*, 2014). Therefore, the use of both alcohols as potential specific biomarkers of lung cancer is unlikely.

There were different patterns observed for 6-dodecanol (CAS: 6836-38-0) between the A549 and NHLF cells. It was significantly consumed by the cancer cells in both collections ($p = 0.0039$ and $p = 0.0078$ respectively) while the fibroblasts appeared to produce it during the last week of incubation ($p = 0.0039$). 6-Dodecanol, similarly to 4-decanol, has never been recorded in the literature regarding VOC studies in humans and, therefore, its use as a biomarker is doubtful (De Lacy Costello *et al.*, 2014).

1-Nonanol (CAS: 143-08-8) was present at significantly lower levels in both collections for the cancer cells ($p = 0.00391$) and for the last two weeks for the normal cells ($p = 0.0039$) in the MT experiment. Higher levels of the alcohol were found in the HS of gastric cancer MGC-803 cells and of non-transformed GES-1 gastric cells, when compared to medium controls. In addition, the MGC-803 cell line appeared to produce it at a higher rate than the GES-1 cells (Zhang Y., 2014). The VOC was previously observed only in human faeces, never in breath studies (De Lacy Costello *et al.,* 2014; Garner *et al.*, 2007). Therefore, it is rather unlikely that 1-nonanol is a specific biomarker of lung cancer.

The simplest tertiary alcohol, *tert*-butanol (CAS: 75-65-0) was found to be produced by the A549 cells in collection 1 of the TD experiment ($p = 0.03906$). The A549 cells apparently stopped producing the VOC or started metabolising it in the second week. This result is consistent with the study by Hanai *et al.* (2012b) who reported an increase of this alcohol in their samples, in comparison with normal cell samples, similarly only after a week of cell culture (no difference for weeks 2 and 3). No differences in the levels of *tert*-butanol between A549 cells and cell medium controls were observed by Schallschmidt *et al.* (2015a; 2015b). On the other hand, another adenocarcinoma cell line, Lu7466, was found to consume the VOC in the same studies. The alcohol was also released by HBEC cells (Filipiak *et al.*, 2010). The VOC has been reported previously in human breath but only as an exposure marker to methyl-*tert*-butyl-ether, a potential carcinogen present in motor vehicle exhaust (Pleil, 2009). However, because *tert*-butanol is also a typical hospital contaminant (Hakim *et al*., 2011) and was found to be produced by oral bacteria species (Khalid *et al*., 2013b), at the moment the use of *tert*-butanol as a potential biomarker of lung cancer is questionable.

Biochemical background of the altered levels of alcohols

In vivo the alcohols are the products of CYP-mediated hydroxylation of hydrocarbons (Ortiz de Montellano, 2010). Therefore, the higher levels of 2-ethylhexanol; 1-phenylethanol and 3-heptanol found in the cell samples are most likely to be the result of such reactions. *tert*-Butanol is a one of the main metabolites of methy-*tert*-butyl-ether which was observed in cell culture medium (DMEM and RPMI 1640) by others (Filipiak *et al.*, 2010; Sponring *et al.*, 2010). It is possible that the TD experiment did not allow for the detection of this VOC, however, it was present in the flasks and metabolised by the

cancer cells into *tert*-butanol. This is supported by the fact that methyl-*tert*-butyl-ether metabolism is mediated mainly by CYP2A6 and CYP2E1 in humans (Phillips, S. *et al.*, 2008) and CYP2E1 was found to be expressed by A549 cells (Hukkanen *et al.*, 2000; Sheets *et al.*, 2004). Cyclohexanol is a metabolite of the first step in cyclohexanone metabolism in humans, a reduction catalysed by ADH (Mráz *et al.*, 1999). Cyclohexanol was found to be produced and cyclohexanone consumed by the A549 cells in the MT experiment. *In vivo*, cyclohexanol is subsequently oxidised to 1,2- and 1,4-cyclohexanediol, final products that can both be detected in urine. However, the presence of EtOH was shown to alter this pathway, which was reflected in the increase of cyclohexanol metabolites in urine. This was probably due to EtOH inhibition of CYPs, the superfamily of enzymes most likely to be responsible for the oxidation of cyclohexanol to -diols (Espinosa-Aguirre *et al.*, 1997; Mráz *et al.*, 1999). EtOH was abundant in the cell culture flasks as it was the most intensive peak in the TIC in the TD experiment, which supports this theory.

The degradation of alcohols *in vivo* occurs either via ADH or CYP-catalysed reactions (mainly happening in the liver) leading to aldehyde products. ADHs constitute a family of enzymes with a wide substrate specificity which break down toxic alcohols, and are implicated in many physiological processes such as the metabolism of lipid peroxidation products steroids, biogenic amines, ω-hydroxyfatty acids (Yin *et al*., 2003). There are five classes (I - V) of human ADH divided according to their subunit and their physiochemical properties (Agarwal, 2001). Many alcohols (primary, secondary, tertiary, cyclic, hemiacetal) can be oxidized by ADH in humans (Von Wartburg, 1964; Hakim *et al*., 2012). For example 1-octanol, benzyl alcohol and heptanol were reported in literature as substrates for ADH (Jelski *et al.*, 2004; Smith, M. *et al.* 1972; Wagner *et al.*, 1983). CYPs (CYP2E1 and CYP3A4) are also involved in the metabolism (oxidation) of alcohols in humans (Vieira *et al.*, 1996; Tomaszewski *et al.*, 2008). The expression of ADH has been previously reported in lung fibroblasts (Buehler *et al*., 1982). Such members of the ADH superfamily as ADH3, ADH5 and ADHFE1 and both CYP enzymes have been previously reported in A549 cells (Castell *et al*., 2005; Courcot *et al.*, 2012; Speit *et al.*, 2010) and ADH4, ADH1B, ADHFE1 and CYP2E1 in BEAS-2B cells (Courcot *et al.*, 2012; Sheets *et al.* 2004).

5.4.4 Aldehydes

In the MT experiment 14 aldehydes were detected and the levels of six were found to be significantly reduced in comparison to the pure culture medium (benzaldehyde; 2-ethenyl-2-butenal; hexanal; heptanal; octanal and tetradecanal). Dodecanal and octadecanal were the only aldehydes found to be produced in the experiment (exclusively by the fibroblasts). The levels of nonanal; decanal; tridecanal; pentadecanal; hexadecanal and heptadecanal were not altered by the presence of the cells. These aldehydes were contaminants originating from the MT or the sampling vials as they were also detected in water blanks in the MT experiment (Tab. 3.3). The TD experiment allowed for the detection of two aldehydes (benzaldehyde and 2-methylbutanal) and the concentrations of both were observed to be significantly decreased in both A549 and BEAS-2B cells in comparison with the controls.

Aldehydes found at increased levels

NHLF cells were observed to produce dodecanal (CAS: 112-54-9) during the last week of cell culture ($p = 0.01172$) and octadecanal during the first week of cell culture ($p = 0.03906$). Both aldehydes have not been previously reported in any *in vitro* studies of VOCs produced or consumed by cells. In *in vivo* studies of cancer, dodecanal was detected in the urine of bladder cancer patients and no presence of the VOC was observed in normal urine (Jobu *et al.*, 2012). It was found to be present in the saliva and sweat of healthy people (Al-Kateb *et al.*, 2013; Penn *et al.*, 2007; Soini *et al.*, 2010). Dodecanal was one of the characteristic VOCs present in the breath of healthy children in contrast to the breath of children with allergic asthma (Caldeira *et al.*, 2012). Its higher concentration in breath was also linked to a gluten-free diet (Baranska *et al.*, 2013). Octadecanal has been reported in human feaces but never in the breath or other biofluids (De Lacy Costello *et al*., 2014). As both dodecanal and octadecanal were only produced by the fibroblasts, and cancer cells neither consumed them nor metabolised them, the VOCs are unlikely to be of any use as biomarkers of lung cancer.

Aldehydes found at decreased levels

In the MT experiment hexanal (CAS: 66-25-1), heptanal (CAS: 111-71-7) and octanal (CAS: 124-13-0) were metabolised both by the cancer cells and the fibroblasts. Hexanal

was consumed throughout the whole period of cell culture by the A549 cells ($p = 0.003906$) and during the second and third weeks by the NHLF cells ($p = 0.003906$). Heptanal and octanal were found in significantly lower concentrations only for collection 2 for both cell lines (heptanal: $p = 0.003906$ and octanal: $p = 0.007812$ for the cancer cells and $p = 0.003906$ for the fibroblasts). However, the concentrations of hexanal were found to be significantly different between the A2 and N3 samples ($p = 0.0029$). As hexanal was consumed by both cell lines in these samples, it was metabolised at a higher rate by the confluent cancer cells than by the confluent fibroblasts. These results are in agreement with other *in vitro* studies in which these aldehydes were detected. Hexanal was observed to be consumed by a number of cancer and non-transformed cell lines: A549 (Brunner *et al.,* 2010; Schallschmidt *et al.,* 2015a), Calu-1 (Sponring *et al.,* 2009), NCI-H1666 (Sponring *et al.,* 2010), HepG2 (Mochalski *et al.,* 2013b), HUVEC (Mochalski *et al.,* 2015), HBEC (Filipiak *et al.,* 2010) and RPE (Brunner *et al.,* 2010). Heptanal was found in lower levels in A549, BEAS-2B (Brunner *et al.,* 2010), and Lu7466 and Lu7387 cells (Schallschmidt *et al.,* 2015a). Finally, octanal was observed to be metabolised by normal lung cells HBEC (Filipiak *et al.,* 2010) and HUVEC (Mochalski *et al.,* 2015).

Some *in vivo* studies are in consensus with the *in vitro* findings of the MT experiment for the three aldehydes. Lower levels of octanal were observed in the breath of lung cancer patients when compared to healthy controls by Filipiak *et al*. (2014). Heptanal was found in the majority of the EBC samples of healthy controls and was not found in most of the EBC samples from lung cancer patients (Xu *et al.*, 2014). The urine concentrations of hexanal in colon cancer, lymphoma and leukaemia patients were lower than their levels in normal urine (Silva *et al.,* 2011). On the other hand, no significant differences in levels of hexanal were found in the breath of lung cancer patients in comparison to non-smoking controls, however, its levels were significantly lower in the breath of smokers when compared to the two previous groups in a study by Kischkel *et al.* (2010). The increased concentration trends for hexanal and/or heptanal were usually reported in breath samples of lung cancer individuals (Deng *et al*., 2004a; Filipiak *et al*., 2014; Fuchs *et al.,* 2010; Phillips M. *et al.,* 1999a; Poli *et al.,* 2010; Rudnicka *et al*., 2015) with no smoking influence on their level. In addition, the concentrations of hexanal and heptanal were observed to be increased in lung cancer blood (Deng *et al.,* 2004a; 2004b; Lilli *et al*., 2010). The comparison between breath and blood levels of the two aldehydes suggested that hexanal and heptanal in breath originate from blood (Deng *et al.*, 2004a). Therefore,

they are most likely endogenous VOCs. Elevated levels of hexanal, heptanal and octanal were found in the breath of women suffering from breast cancer in comparison to healthy subjects (Li J. *et al.,* 2014). As such, these VOCs are likely to be of limited use as biomarkers of lung cancer at present, and further study of these compounds is required in terms of their transition mechanisms and biochemical origin *in vivo* (See Chapter 5.7 for further discussion).

Benzaldehyde is an analyte that was found to be metabolised by the A549, NHLF and BEAS-2B cells in all collections of both the MT and TD experiments ($p = 0.03906$ for all). The 'between-sample analyses' have shown, however, that the proliferating cancer cells were consuming it at a higher rate than the growing fibroblasts in the MT experiment; and both proliferating and confluent A549 cells were metabolising it more than their BEAS-2B counterparts in the TD experiment.

Another study of A549 cells showed an opposite trend for benzaldehyde, as the VOC was released by this cell line (Barash *et al.,* 2012). On the other hand, a number of cell lines of other cancers and non-transformed cells were observed to metabolise benzaldehyde. These were: HeLa of cervix cancer (Nozoe *et al.,* 2015), two colon cancer and one normal colon cell line (Zimmerman *et al*., 2007), three skin cancer cell lines (Kwak *et al.,* 2013), HepG2 (Mochalski *et al.*, 2013b), lung fibroblasts (Filipiak *et al.,* 2010), five breast cancer and one control breast cell line (Lavra *et al.,* 2015); HUVEC (Mochalski *et al.,* 2015) and primary HDFs (Acavedo *et al.,* 2007; 2010). Interestingly, a study investigating volatile patterns of lung cancer-specific genetic mutations showed a correlation between benzaldehyde consumption and KRAS mutation. Benzaldehyde was not found in the HS of cells carrying the KRAS mutation (A549 among them) when compared to pure medium (Peled *et al.,* 2013). Another study by the same group found benzaldehyde at lower concentrations in bronchial cells carrying the KRAS mutation, TP53 knockdown, or both alterations in relation to the parental HBEC-3KT cells (Davies *et al*., 2014). Similarly, the TD experiment showed a total depletion of benzaldehyde in the HS of the A549 cells, while in the HS of the BEAS-2B cells it was reduced. In the MT experiment benzaldehyde was detected in the cell samples but here a different matrix (HS of the heated medium) was analysed. Therefore, the increased metabolism of benzaldehyde by the A549 cells in the TD and MT experiments is most likely due to the KRAS mutation carried by the cells.

Nevertheless, similarly as for C6-C8 aldehydes, *in vivo* studies give contradictory results for benzaldehyde as a VOC linked to lung cancer. Its higher amounts in the breath of lung cancer patients when compared to normal breath were found by Bajtarevic *et al.,* (2009) and Ligor M. *et al.* (2009). In another study benzaldehyde was detected in the HS of lung cancer and normal lung tissue as well as in the breath of lung cancer patients and normal individuals, however, no significant differences between the groups for this VOC were found (Filipiak *et al*., 2014). No significant differences for benzaldehyde blood concentrations of lung cancer patients and normal subjects were found by Deng *et al.* (2004a). Benzaldehyde is commonly present in the breath of a healthy person (Kwak *et al.*, 2014; Martin *et al*., 2010; Phillips, M. *et al.,* 1999b; Van den Velde *et al.,* 2007;) and therefore lowered levels of the VOC in cancerous breath are theoretically possible. More studies are required to confirm the concentration trend (if any) for benzaldehyde as a potential biomarker of lung cancer. It would be especially interesting to conduct *ex vivo* and *in vivo* experiments involving patients with and without the KRAS mutation in order to investigate whether the correlation between lower amounts of benzaldehyde and the mutation exists outside of the *in vitro* environment.

2-Ethenyl-2-butenal (CAS: 20521-42-0) was consumed by the A549 cells and NHLFs throughout the whole period of cell culture ($p = 0.003906$). Interestingly, this is the only VOC found to be metabolised at a higher level by the fibroblasts (in the second and third weeks of incubation) than by the cancer cells. This is the first time the aldehyde is reported to be a part of the volatile background of the cell culture medium. It is an aroma volatile found in food (Iwaoka *et al*., 1994; Lee J. *et al*., 2003) but it has not been found in any human biofluid (De Lacy Costello *et al*., 2014). It has been found to be a degradation product of PP and this is its probable origin in the cell culture flask (Liggat, 1999). Therefore, it has little potential as a useful biomarker in cancer studies.

Tetradecanal (CAS: 124-25-4) was observed at significantly lower levels in the cancer cell samples during the first week of incubation ($p = 0.0306$) and in the fibroblast samples during the second week of incubation ($p = 0.007812$) when compared to their medium controls. The VOC was observed also in lower levels in HeLa cells when compared to the pure medium (Nozoe *et al*., 2015). Because tetradecanal was reported in human breath as a VOC related with ventilator-associated pneumonia (Schnabel *et al*., 2015), further study is required to assess the use of the VOC as a potential biomarker of lung cancer.

2-Methylbutanal (CAS: 96-17-3) is another VOC that was consumed by both the A549 and BEAS-2B cells in the TD experiment ($p = 0.003906$). The differences in peak area ratios were also found to be significant between the A1 and B2 samples, suggesting a possible higher metabolism of 2-methylbutanal by the cancer cells. However, the differences were not significant between the confluent cancer and confluent BEAS-2B cells. These results are in agreement with other *in vitro* studies of lung cancer as 2-methylbutanal was found to be metabolised by A549 cells (Schallschmidt *et al.*, 2015b), another lung cancer cell line NCI-H2087 (Sponring *et al*., 2009) and by HDFs (Filipiak *et al.*, 2010). HUVEC also consumed the VOC (Mochalski *et al*., 2015). Filipiak *et al.* (2014) reported the VOC in the breath of both healthy non-smokers and lung cancer patients, but no significant differences between the groups were found for this VOC. However, 2-methylbutanal is not common in the breath of healthy individual and, therefore, its use as a biomarker that is at lower levels in lung cancer breath is unlikely.

Biochemical background for altered levels of aldehydes

Only two aldehydes in the MT experiment were found to be emitted by the cells (none in the TD experiment): dodecanal and octadecanal (by the fibroblasts). It is not known which metabolic pathway was involved in their production. Human endogenous aldehydes are formed during various physiological processes such as lipid peroxidation, carbohydrate and amino acid metabolism, CYP and amine oxidases-catalysed metabolic activation (Marcato *et al*., 2011; O'Brien *et al.*, 2005). Besides endogenous aldehydes, other contributors to the *in vivo* aldehydes burden are inhaled aldehydes (some of them are common air pollutants), aldehyde products of alcohol metabolism, aldehydes in ingested food and drugs and aldehydes related to tobacco smoking (inhaled tobacco aldehydes and aldehydes as by-products of tobacco metabolism (Clark and Bunch, 1997; Hakim *et al*., 2012; Miyake and Shibamoto, 1995; O'Brien *et al*., 2005; Schultheiss *et al.*, 2000).

Six aldehydes were observed to be metabolised in the MT experiment and two in the TD experiment. Aldehydes are metabolised *in vivo* by oxidation to acids and/or reduction to alcohols. Oxidation in humans usually involves ALDHs or NAD^+ (nicotinamide adenine dinucleotide) which oxidise a broad range of endogenous and exogenous aldehydes to their equivalent carboxylic acids. The human enzymes responsible for the reduction of aldehydes are the ADH superfamily, AKR superfamily and short-chain

dehydrogenases/reductases superfamily (SDR). ADHs are also able to oxidise aldehydes (O'Brien *et al.,* 2005).

ADHs, have been shown to be expressed in A549, BEAS-2B and lung fibroblast cells (Buehler *et al*., 1982; Courtos *et al*., 2012). The expression of ALDH1, ALDH2 and ALDH3 members of the ALDH superfamily in the A549 and BEAS-2B cell lines has also been reported in the literature (Courtos *et al.,* 2012; Rubporn *et al*., 2009; Speit *et al.,* 2010). However, different members of the enzyme families were found to be expressed in A549 and BEAS-2B cells with ALDH1A1 and ALDH3A1 not being expressed by the latter cells at all (Moreb *et al*., 2007). This may result in a higher rate of benzaldehyde and 2-methylbutanal consumption demonstrated by the A549 cells in comparison to the BEAS-2B cells in the TD experiment. ALDH1 has been observed to be either underexpressed in MCR-5 lung fibroblasts or not expressed at all in CCL-210 lung fibroblasts when compared to A549 cells (Fromigué *et al.,* 2003; Rubporn *et al*., 2009). What is more, in general ALDHs displayed preferential expression in cancer cells in comparison to non-transformed cells (Ma S. *et al.,* 2008) and ADH activity was shown to be higher in cancer tissues than in healthy tissues (Jelski and Szmitkowski, 2008). These differences could explain the higher rate of the metabolism of hexanal and benzaldehyde by the cancer cells in the MT experiment. Hexanal, heptanal, octanal and benzaldehyde were observed as specific substrates for human ALDHs (Klyosov, 1996). Benzaldehyde has been shown as a substrate for human AKR1A1 (Petrash and Srivastava, 1982). The enzyme (as well as a few other AKRs) was shown to be expressed by the A549 cells and BEAS-2B cells and therefore the metabolism of this aldehyde may also partly occur via this pathway (Courcot *et al*., 2012). Similar mechanisms for the metabolism of aldehydes may exist for lung fibroblasts as well, however, there is no information in the literature about this.

5.4.5 Ketones

There were seven ketones identified in the MT and three in the TD experiment. In the MT analysis six ketones displayed altered amounts in comparison to the medium controls: acetophenone, cyclohexanone, geranyl acetone and 2-tetradecanone were consumed by the A549 cells only. Geranyl acetone and 2-tetradecanone were produced by the fibroblasts. 2-Pentadecanone and 2,6-di-*tert*-butyl-1,4-benzoquinone were found to be emitted by both

the A549 and NHLF cells. In the TD experiment acetone and 2-pentanone were found to be emitted, while acetophenone was metabolised by the A549 and BEAS-2B cells.

Ketones found at increased levels

Acetone (CAS: 67-64-1) and 2-pentanone (CAS: 107-87-9) were found in significantly higher levels, in comparison to the pure medium, in the HS of the A549 ($p = 0.0039$) and BEAS-2B samples in the TD experiment (collection 1 $p = 0.0078$, collection 2 $p = 0.0039$). The comparative analysis of the median peak area ratios between the samples showed, however, that the cancer cells produced significantly more acetone in both collections than the normal cells. There were no differences found in the amounts of 2-pentanone emitted between the cell lines.

The A549 cell line in other studies was observed to release acetone or consume it (Filipak *et al.* 2010; Schallschmidt *et al*., 2015a). The production of acetone was previously also attributed to HBEC, VGP and tracheobronchial epithelial (TBE) cells (Filipak *et al.* 2010; Kwak *et al.*, 2013; Schivo *et al.*, 2014). It was also a discriminant compound (found in higher amounts) used to differentiate two mutated cell lines from a parental HBEC cells (Davies *et al.*, 2014). 2-Pentanone was reported to be significantly increased in A549 samples in comparison to normal cells and pure medium by Hanai *et al.* (2012b) and to normal medium only by Filipiak *et al.* (2010). It was also shown to be emitted by HDF, HPEC and HepG2 cells (Filipiak *et al.*, 2010; Mochalski *et al.*, 2013b). Lower amounts (for acetone) or no differences in amounts (depending on the sampling method) between the cell samples and medium controls for both ketones, however, were found in another study of the VOCs emitted by A549 cells (Schallschmidt *et al.*, 2015a).

Acetone and 2-pentanone are two of the most abundant compounds present in the breath, blood and urine of a healthy person (Mochalski *et al.*, 2012; 2013a; Phillips, M. *et al.*, 2013; Van den Velde *et al.*, 2007; Zlatkis *et al.*, 1973). The studies *in vivo* showed inconsistent results regarding both ketones in terms of their concentration trends in breath and biofluids of lung cancer patients and healthy people. For example some studies reported higher concentrations of these VOCs in the breath of lung cancer patients when compared to healthy controls (Kischkel *et al.*, 2010; Ulanowska *et al.*, 2011; Buszewski *et al.*, 2012a; 2012b; Rudnicka *et al*., 2015). Bajtarevic *et al.* (2009) observed the opposite situation for acetone and Filipiak *et al*. (2014) did not detect any significant differences for

2-pentanone, neither in breath nor in the HS of lung tissues, in comparison to control samples from healthy subjects. A study of acetone in blood did not reveal any differences in acetone concentration between lung cancer and normal blood either (Li N. *et al*., 2005). The reasons for these contradictory observations might be that both ketones are probably not specific markers of lung cancer.

Acetone is one of the three ketone bodies (along with acetoacetate and 3-beta-hydroxybutyrate) which are produced in the liver during lipolysis as an alternative energy source during shortages of glucose. During prolonged fasting or in a diabetic organism it is also produced during ketogenic amino acid catabolism (such as leucine) (Laffel, 1999; Ochs, 2011). The levels of protein metabolism, and therefore acetone, are increased in cachexia. This is a process of body wasting associated with various chronic diseases including cancer. Cachexia is usually associated with cancer in its final stages, and therefore acetone production too is increased (Datta, 2004). Thus, differing acetone levels dependent on the stage of a cancer might be one of the reasons for the different results obtained in different *in vivo* studies. Acetone is a well characterised VOC in human breath with determined typical levels in healthy volunteers of different body weight, age and genders (Manolis *et al*., 1983; Schwarz *et al*., 2009; Turner, 2013). However, its use as a cancer biomarker could be problematic, as its levels may also be influenced by diet, excessive exercising and/or fasting (Španěl *et al*., 2011; Turner, 2013). The physiological reason behind this is that fasting people or people who exercise a lot have low concentrations of glucose and increased concentrations of ketone bodies in their blood. The ketone bodies are formed in the breakdown of lipids as a response to an energy shortage. Acetone will appear in the breath as it is more volatile than the other two ketone bodies (Turner, 2013). One of the solutions would be measuring glucose levels in blood whilst testing for acetone in breath. Such a study was performed on patients after fasting and then after the consumption of glucose. As expected, acetone was found at higher levels in the breath after fasting and decreased after glucose intake (Turner *et al*., 2008). Similarly as for acetone, breath concentrations of 2-pentanone were also observed to be higher than at rest after excessive exercising and were linked to fasting and liver cirrhosis (King *et al.*, 2010; Statheropoulos *et al.*, 2006; Van den Velde *et al.*, 2008). However, the endogenous origin of this ketone is still disputed. All in all, more studies are required to assess the usefulness of the two ketones as specific lung cancer biomarkers.

2-Pentadacenone (CAS: 2345-28-0) was detected in higher concentrations for collection 1 of the cancer cell samples ($p = 0.0039$) and for all collections of the fibroblasts ($p = 0.0039$), and the levels of the produced ketone were higher for the A549 cells in comparison to the NHLF cells (in collection 1). A study by Y. Wang *et al.* (2012) showed that 2-pentadecanone was a characteristic compound not only for *in vitro* cultured lung cancer cells (A549, NCI-H446 and SK-MES1) but also for lung cancer tissue specimens ($n = 18$). Moreover, in the same study the ketone was also found as one of the breath VOCs discriminating between lung cancer patients ($n = 85$), patients with benign lung diseases ($n = 70$) and healthy controls ($n = 88$). Although the MT experiment shows that 2-pentadecanone is also produced by the fibroblasts, the differences in concentrations of the ketone between the A549 and NHLF cells were found to be significant (for the first collection). 2-Pentadecanone was produced at significantly higher levels by the growing cancer cells in comparison to the growing non-cancerous cells. In addition, the ketone was also found at increased levels, in comparison to the culture medium, in HeLa cells and SW480 cells (Nozoe *et al.*, 2015; Zimmermann *et al*., 2007). Altogether this indicates that 2-pentadecanone is a candidate biomarker of cancer, however, because it was also observed to be produced by cervical and colon cancer cells, it might not be specific to lung cancer.

Another ketone 2,6-di-*tert*-butyl-1,4-benzoquinone (CAS: 719-22-2) was found to be emitted by the proliferating A549 cells (collection 1) and the proliferating NHLF cells (collection 2) in the MT experiment. However, the cancer cells produced it at higher rate than the fibroblasts. The VOC has been reported previously in *in vitro* studies of the A549 cell line by Pyo *et al.* (2008). The ketone was suggested here as another biomarker of apoptosis as it was released in higher amounts by Cisplatin-treated cells than by control cells and its concentration was Cisplatin-concentration dependent. It is not clear whether Pyo *et al.* detected any differences between untreated cells and pure medium (with or without Cisplatin), or whether they did not detect the VOC at all in the two types of media. The medium used by Pyo *et al.* was the same as for the MT experiment, RPMI 1640. The MT experiment, however, showed that 2,6-di-*tert*-butyl-1,4-benzoquinone was present in all the cell-free RPMI 1640 medium controls. It also showed that 2,6-di-*tert*-butyl-1,4-benzoquinone was produced only by the growing cancer cells. Confluent cancer cells did not release it. Nevertheless, it is possible that the A549 cells would start emitting

2,6-di-*tert*-butyl-1,4-benzoquinone again upon reaching the apoptotic state. This matter needs further investigation.

Regarding *in vivo* studies of volatiles; 2,6-di-*tert*-butyl-1,4-benzoquinone was one of the most frequently occurring VOCs in the breath of healthy individuals in studies by M. Phillips *et al.* (1999b) and Van den Velde *et al.* (2007) (occurrence 100%, n = 50 and 92.5 %, n = 40 respectively). A later study by M. Phillips' group included 2,6-di-*tert*-butyl-1,4-benzoquinone in a set of 16 VOCs discriminating the breath of lung cancer patients from the breath of healthy subjects (Phillips, M. *et al.,* 2007b). However, raised concentrations of the VOC were also linked to asthma breath in comparison to healthy controls (Ibrahim *et al.,* 2011). It also was found at higher levels in urine of renal cell carcinoma patients when compared to healthy controls (Wang D. *et al.,* 2016). Therefore, the ketone might be a candidate biomarker for general pulmonary diseases/and or cancer, but it may not be specific to lung cancer. This requires further study.

Ketones found at lower levels

Geranyl acetone (CAS: 3796-70-1) was found at significantly lower levels in collection 2 of the A549 cells ($p = 0.03461$) and at significantly higher levels in collection 3 of the NHLF cells ($p = 0.003906$). Therefore, lower levels of the ketone in breath could be indicative of tumour presence. Geranyl acetone was observed in human sweat (Meijerink *et al.,* 2000). Although it is a common indoor air VOC, it has never been reported in human breath (De Lacy Costello *et al*., 2014; Weschler and Nazaroff, 2012). Also it has not been observed in any *in vitro* studies of VOCs and, therefore, its use as a biomarker of lung cancer is unlikely.

The reduced concentrations of acetophenone (CAS: 98-86-2) in the A549 cell samples were significant in both the MT and the TD experiments for collections 1 and 2 ($p = 0.0039$). The BEAS-2B cells were also found to consume the ketone but only during the second week of incubation ($p = 0.0039$). The median peak area ratios were significantly lower, however, for the growing A549 cells than for the growing BEAS-2B cells. This indicates that A549 metabolised acetophenone to greater extent during the first week of the experiment.

Acetophenone (CAS: 98-86-2) is a commonly occurring VOC in studies of lung cancer, however, again its status as a potential biomarker of lung cancer is not clear. The results of

the MT and TD experiments are in agreement with a study by Barash *et al.* (2012) who also found A549 cells (as well as 17 other lung cancer cell lines) to consume the ketone when compared to medium controls. Acetophenone was consumed by A549 cells during the first week of incubation in a study by Hanai *et al.* (2012b) and then produced in weeks 2 and 3. No differences were found in the concentrations of acetophenone for A549 and Lu7466 cells when compared to their medium controls in the study by Schallschmidt *et al.* (2015). Such a difference was found for Lu7387 cells in the same study. These cells appeared to consume acetophenone. An opposite trend was observed for skin cancer and normal skin cells (Kwak *et al.*, 2013).

Acetophenone has been one of the breath biomarkers used for differentiation between lung cancer patients and healthy controls. It was found to be present only in the breath of lung cancer individuals (Bajtarevic *et al.*, 2009). In other studies, however, it was detected in the breath of normal healthy subjects (Kwak *et al*., 2014; Van den Velde *et al.*, 2007) (n = 40, occurrence 95%). In another study comparing VOCs between the breath of lung cancer patients and of normal controls acetophenone was found at similar levels in the two studied groups (Preti *et al.*, 1988). The ketone was also associated with breast cancer, although its breath level trends were not given (Li J. *et al*., 2013; Phillips M., *et al.*, 2006). Lower levels of acetophenone in breath of lung cancer patients would be in agreement with the MT and TD experiments. As it is present in normal human breath, its lower levels related to lung tumour presence may yet be reported and possibly used as biomarker of the disease. Therefore, further validation of the ketone as a potential lung cancer biomarker is needed.

2-Tetradecanone (CAS: 2345-27-9) was observed for the first time in *in vitro* studies of VOCs in cancer. It was detected at lower levels in the two collections of the A549 cells ($p = 0.007812$) and at higher levels in collection 3 of the fibroblasts ($p = 0.007812$), when compared to pure medium controls for the MT experiment. Although it was consumed by the A549 cells and produced by the fibroblasts, its use as a lung cancer biomarker is unlikely. The VOC has never been detected in human breath, even though it was found in the saliva and sweat of healthy individuals, so it is present in the body (Penn *et al.*, 2007; Soini *et al.*, 2010). It is not readily available in air, but can however be ingested with food. Its origin in the cell culture flask is most likely due to the thermal degradation of PE (Bravo and Hotchkiss, 1993).

Cyclohexanone (CAS: 108-94-1) was observed to be metabolised exclusively by the A549 cells in both collections of the MT experiment ($p = 0.0039$). The VOC had different level trends in the study by Hanai *et al.* (2012b) for this cell line as it was observed at higher levels in the first week, lower levels during the second week and then at higher levels again in the last week of incubation when compared to normal cells. Cyclohexanone was found in lower concentrations in the HS of three mutated lung cell lines, in comparison to their parental HBEC cell line (Davies *et al.*, 2014).

Exhaled concentrations of cyclohexanone also had different trends in different studies. There were no differences observed in cyclohexanone levels between three groups (lung cancer patients, smokers and healthy controls) studied by Kischkel *et al.* (2010). In contrast, *ex vivo* analysis of pleural effusions from lung cancer patients showed significantly higher concentrations of cyclohexanone than effusions of patients without lung cancer. The VOC was proposed as a biomarker of lung cancer to differentiate between cancerous and benign effusions (Liu H. *et al.*, 2014). In addition, tentatively assigned cyclohexanone was a single VOC discriminating between COPD patients (with or without lung cancer) from healthy individuals (Westhoff *et al.*, 2010). Finally, the study by Kischkel *et al.* (2012) indicated that cyclohexanone can be mistakenly taken as a biomarker of lung cancer. Although it was observed to be present in higher contractions in a diseased lung than in a healthy lung during a resection of the lung, the group showed that the ketone originated from tubing attached to a device. Nevertheless, cyclohexanone is a common industrial solvent and indoor air pollutant and one of the most frequently found VOCs in normal human breath (Musser, 2000; Van den Velde *et al.*, 2007; Verhoeff *et al*., 1988). Therefore, the potential metabolism of the ketone is possible *in vivo*. More studies are required to resolve the status of cyclohexanone as a cancer biomarker. For the MT experiment cyclohexanone may originate from PS (Musser, 2000).

Biochemical background for altered levels of ketones

Ketones are produced via oxidation of alcohols, with the reduction of NAD^+ to NADH catalysed by ADH. Therefore, 2-pentanone, 2-pentadecanone, 2-teradecanone and 2,6-di-*tert*-butyl-1,4-benzoquinone are most likely produced by the cells *in vitro* via this mechanism. However, no entries for 2,6-di-*tert*-butyl-1,4-benzoquinone; 2-pentadecanone and 2-tetradecanone were found in the KEGG database and therefore no pathways involving these ketones are known in any living organism at the moment.

Acetone *in vivo* is formed either by non-enzymatic or acetoacetate decarboxylase-catalysed decarboxylation of acetoacetate. The enzyme is induced by starvation and inhibited by acetone. Acetoacetate arises in mammals due to lipolysis or degradation of amino acids. Furthermore, small amounts of acetone can be produced via another pathway i.e. the dehydrogenation of IPA by ADH (Kalapos, 2003). Similarly, 2-pentanone could derive from the oxidation of pentanol. Both alcohols were found as substrates of human ADH (Diltow *et al.*, 1984). Acetone is produced during glucose metabolism and the Warburg effect is a well-characterised metabolic phenotype observed in cancer cells. This is a shift from ATP production via oxidative phosphorylation to ATP production via glycolysis (Cairns *et al*., 2011). Because in the TD experiment, acetone was produced not only by the A549 cells but also by the BEAS-2B cells, it is possible that both cell lines released acetone due to low-nutrient conditions. A study by C. Wu *et al*. (2013) has shown that nutrient deprivation (lack of serum and amino acids) can induce the Warburg effect, which delays cell apoptosis not only in HeLa cells but also in non-transformed cells (although this was dependent on the cell specificity and the reasons are not known). Nutrient deprivation is (along with hypoxia) a characteristic feature of solid tumours and as such its role in cell metabolism has not yet been entirely explained. Studies of VOCs in controlled starvation conditions could be of great value in this field.

Metabolism of some ketones occurs via their reduction by NADH and ADH to form primary and secondary alcohols (O'Brein *et al.*, 2005). Similarly to aldehydes, ketones are reduced by the ADH, AKR and SDR superfamilies of enzymes to form their corresponding alcohol. However, the possible pathways for the metabolism of geranyl acetone and 2-teradecanone are not known (no entries in the KEGG database). The pathway for cyclohexanone metabolism in humans is known. The main urinary metabolites of the ketone are 1,2- and 1,4-cyclohexanediol. The first step is the reduction of cyclohexanone to cyclohexanol catalysed by ADH. Cyclohexanol was found to be produced by the A549 cells in the MT experiment so it might be a product of this pathway. The enzymes responsible for the subsequent oxidation of cyclohexanol to -diols have not yet been identified. In all likelihood, however, they belong to the CYP enzymes family (Espinosa-Aguirre *et al.*, 1997; Mráz *et al.*, 1999). The pathway for acetophenone metabolism in humans is not known. However, acetophenone is a substrate for rat AKR1C1 (with 1-phenylethanol as a product) (Uwai *et al.*, 2008). The enzyme was shown to be expressed at higher levels in A549 cells than in BEAS-2B cells (Courcot *et al.*,

2012) which suggests that a similar pathway for acetophenone metabolism may be also utilised by human cells *in vitro* and explain why cancer cells metabolised the ketone at higher rate than the BEAS-2B cells.

5.4.6 Phenols

Three phenols were detected in the MT experiment (phenol, 2-nitrophenol and 2,5-di-*tert*-butylphenol) and none in the TD experiment. All these VOCs were observed to be consumed by the A549 cells, whilst the levels of the latter compound were found to be increased in the NHLF sample of collection 3.

Phenols found at increased or decreased levels

The NHLF cells were observed to produce 2,4-di-*tert*-butyl phenol (CAS: 96-76-4) during the last week of cultivation ($p = 0.003906$) in contrast to the A549 cells that consumed the VOC only in the last week of incubation. In *in vitro* studies of VOCs, 2,4-di-*tert*-butyl phenol was reported only by Hanai *et al*. (2012b). The VOC here was consumed by the A549 cells during the first week of cell culture and then found at higher levels when compared to normal cells during the next two weeks. 2,4-Di-*tert*-butyl phenol was detected in the urine of healthy people (Zhang S. *et al.*, 2012), however, concentrations *in vivo* may arise due to food, pharmaceutical products, cosmetics and occupational exposure (Chen M.L. *et al*., 2005; Pouech *et al.*, 2015). The compound is widely used as an antioxidant polymer additive in the plastic industry (Stoffers *et al*., 2004); therefore, its presence in the A549 samples and medium controls most likely originates from the cell culture flasks. As it has never been detected in breath, the use of lower levels of the phenol as a marker of lung cancer is rather unlikely.

Both phenol (CAS: 108-95-2) and 2-nitrophenol (CAS: 88-75-5) were observed in significantly lower amounts during the two weeks of incubation in the A549 cell samples ($p = 0.0039$ and $p = 0.01953$ for 2-nitrophenol collection 2) when compared to the controls and to the fibroblast samples. In addition, there was a complete depletion of 2-F in the HS of the flasks containing cancer cells in the TD experiment. 2-F could not be therefore used as an ISTD. Phenol was also entirely consumed by Caco-2 cells in comparison to medium controls (Baranska *et al*., 2015). Other studies *in vitro* for A549 cells, however, have found phenol at higher levels in this cell line when compared to pure medium or normal

control cells (Hanai *et al.,* 2012b; Schallschmidt *et al.,* 2015a). Melanoma and TBE cells were also observed to emit this VOC (Kwak *et al.,* 2013; Schivo *et al.,* 2014). In some studies, medium without phenol red was used and here phenol presence was not reported (Sponring *et al.,* 2009, 2010; Filipiak *et al*., 2010). 2-Nitrophenol is observed for the first time in *in vitro* studies of VOCs in cancer.

Phenol *in vivo* is known as a contaminant originating from Tedlar bags used for breath collection, appearing in significant quantities that vary greatly between the bags (Steeghs *et al.,* 2007). Unless an effective method for cleaning Tedlar bags or a different collection method is used, phenol cannot be included in the analysis (Caldeira *et al.,* 2011). Examples of alternative breath samplers are RTubes®, Bio-VOC® and Teflon bulbs®. With the use of alternative collection systems, phenol was found to be one of the typical VOCs in the breath of healthy individuals (Kleeblatt *et al.,* 2014; Kwak *et al*., 2014; Martin *et al.*, 2010; Van den Velde *et al.,* 2007). On-line measurements of human breath with single photon ionisation time-of-flight mass spectrometry (SPI-TOF-MS) also detected phenol in the breath gas of healthy people (Mühlberger *et al.,* 2005). Finally, Kumar *et al.* (2013), with the use of Nalophan bags, found higher levels of phenol in the breath of people suffering from esophago-gastric cancer in comparison to healthy controls, potentially due to a rise in protein catabolism, up-regulation of tyrosine metabolism and/or changes in microbial flora. Phenol may yet appear as a VOC being metabolised by lung tumour cells in *vivo*. 2-Nitrophenol has never been reported in any *in vitro* and *in vivo* studies of VOCs and, therefore, its use as a biomarker of lung cancer is unlikely (De Lacy Costello *et al.*, 2014; Filipiak *et al*., 2016).

Biochemical background for altered levels of phenols

Endogenous phenol can arise from the metabolism of aromatic amino acids, benzene, aromatic methyl and ethyl esters (Kumar *et al.,* 2013; Timbreell, 2008). Phenol is also present in food and, therefore, may be ingested as an exogenous VOC (Bravo L., 1998). 2,4-Di-*tert*-butyl phenol is produced by some bacteria as an antifungal agent (Dharni *et al*., 2014) but its production by human cells has never been reported before. Therefore, the metabolic pathway leading to the production of 2,4-di-*tert*-butyl phenol by the NHLF cells is unknown.

The metabolism of phenol may occur in humans in a number of ways: methylation (phenol-O-methyltransferase), conjugation with sulfate (sulfotransferase) or glucoronic acid (glucuronosyl transferase) (Timbrell, 2008). Phenol occurs as a metabolic product of animals and humans in the breath and biofluids, both as phenol itself as well as in conjugated forms of glucuronide and sulfate. Another route of its metabolism is CYP-mediated hydroxylation of phenol with the formation of catechol and hydroquinone (Snyder *et al*., 1993; Timbrell, 2008). The metabolic pathway for 2-nitrophenol degradation in humans is not known.

Phenolic antioxidants were observed exerting toxicity towards lung cells. A plant extract of 2,4-di-*tert*-butyl phenol displayed strong cytotoxicity against A549 cells in a study by Malek *et al*. (2009). The metabolism of the phenol by the A549 cells observed in the MT experiment might therefore be the result of a detoxification mechanism. 2,4-Di-*tert*-butyl phenol is widely used as an antioxidant polymer additive in the plastic industry and therefore most likely originated from PS and/or HDPE, in both the cancer cell samples and the controls for the MT experiment (Stoffers *et al*., 2004).

5.4.7 Esters

Six esters were detected in the MT experiment. The levels of dodecanoic acid isooctyl ester; pentanoic acid, 2,4-dimethyl-3-oxo-, methyl ester (alternative ID: 1-butoxypentane); propanoic acid, 2-methyl-, 1-(1,1-dimethyl)-2-methyl-1,3-propanediyl ester and pthalic acid, isobutyl nonyl ester were altered by the cells. The levels of propanoic acid, 2-methy-(2,2-dimethy-1-(2-hydroxy-1-methylethyl) propyl ester and propanoic acid, 2-methyl-, 3-hydroxy-2,4,4-trimethylpentyl ester remained the same during the two weeks of cell cultivation. The A549 cells were found to metabolise diisobutyl phthalate and pentanoic acid, 2, 4-dimethyl-3-oxo, methyl ester during the second week of cultivation. The NHLF cells were observed to produce propanoic acid, 2-methyl-,1-(1,1-dimethyl)-2-methyl-1,3-propanediyl ester (collection 1 and 2) and dodecanoic acid isooctyl ester (collection 2) and to consume diisobutyl phthalate (collection 3). Ethyl acetate was the only ester found in the TD experiment, produced solely by the BEAS-2B cells.

Esters found at increased levels

Two esters were found at significantly higher levels in the NHLF cell samples when compared to the medium controls: propanoic acid, 2-methyl-, 1-(1,1-dimethyl)-2-methyl-1,3-propanediyl ester (collection 1: $p = 0.03903$; collection 2: $p = 0.007812$) and dodecanoic acid isooctyl ester (collection 3: $p = 0.0117$).

Propanoic acid, 2-methyl-, 1-(1,1-dimethyl)-2-methyl-1,3-propanediyl ester (CAS: 74381-40-1) and its alternative ID pentanoic acid, 2,2,4-trimethyl-3-carboxyisopropyl, isobutyl ester (no CAS) have never been reported in *in vitro* studies of VOCs before. However, the first VOC was included in the sets of VOCs discriminating the breath of lung cancer patients from the breath of healthy controls in two studies by M. Phillips *et al.* (2007b, 2008). The second VOC was found at higher levels in pleural effusions of lung cancer patients in comparison to benign controls (Huang Z. *et al.*, 2016). The MT experiment questions, therefore, the use of these VOCs as a potential lung cancer biomarker present in higher levels in cancerous breath as it was found to be produced only by the normal cells.

Dodecanoic acid isooctyl ester (CAS: 84713-06-4) has not yet been reported in any *in vitro* or *in vivo* study of VOCs. Therefore, its use as a potential biomarker of lung cancer is very unlikely, especially that it was observed to be emitted only by the NHLF cells.

Ethyl acetate (CAS: 141-78-6) was found in significantly elevated levels solely in the HS of the BEAS-2B cells in both collections in the TD experiment ($p = 0.003906$). The levels of the ester remained unchanged in the A549 cell samples when compared to their medium controls. However, the emission of ethyl acetate by A549 cells has been observed before (Schallschmidt *et al.*, 2015a; 2015b). Another cell line reported to produce ethyl acetate was HUVEC (Mochalski *et al.*, 2015). Ethyl acetate is another common VOC present in the breath of a healthy person (Buszewski *et al.*, 2009; Ligor T. *et al.*, 2008; Van den Velde *et al.*, 2007). It also was detected in the blood of healthy individuals (Mochalski *et al.*, 2013a). It was found at higher levels in the breath of lung cancer patients when compared to smoking and non-smoking controls (Buszewski *et al.*, 2012a). However, no differences in concentration between the breath of lung cancer patients and healthy volunteers were observed in another study by the same group (Rudnicka *et al.*, 2011). Ethyl acetate also appeared only in the breath of healthy volunteers, in contrast to the breath of gastric cancer patients (Ligor T. *et al.*, 2007). For the TD experiment only non-transformed cells produced ethyl acetate, therefore were the VOC to be a potential

biomarker of lung cancer, it would be expected to be found at lower levels in the breath of lung cancer patients. However, no supporting studies of such a trend exists, leading to the conclusion that ethyl acetate is unlikely to be a potential biomarker of lung cancer.

Esters found at decreased levels

In this project pentanoic acid, 2, 4-dimethyl-3-oxo, methyl ester (CAS: 55107-14-7) as well as its alternative identification, 1-buroxypentane (CAS: 18636-66-3), were reported for the first time in studies of VOCs as potential biomarkers of disease. Therefore, their use as potential biomarkers of lung cancer is not likely.

Diisobutyl phthalate (CAS: 84-69-5) was another ester observed to be consumed by the cells in the MT experiment. Its levels were found to be significantly lower in the A549 cell samples of collection 2 ($p = 0.007812$) and in the NHLF cell samples of collection 3 ($p = 0.03906$), when compared to the pure medium controls. The 'between-sample analysis' showed that the ester was consumed at similar levels by both cell lines. Phthalic acid esters are common plasticisers, therefore its origin in the cell culture medium may be from the culture flask (Jaworek and Czaplicka, 2013). Diisobutyl phthalate has never been found in breath or reported in any *in vitro* studies of VOCs. Consequently, the ester is very unlikely to be of any use as a lung cancer biomarker.

Biochemical background for the altered levels of esters

Esters *in vivo* can be metabolised by hydrolysis, the reaction catalysed by carboxyesterases and amidases with different substrate specificities (Timbrell, 2008). Therefore, pentanoic acid, 2, 4-dimethyl-3-oxo, methyl ester and diisobutyl phthalate were most likely metabolised in this way. Ethyl acetate is known to be rapidly hydrolysed *in vitro* and *in vivo* by various esterases with the final products of ethanol and acetic acid, as many studies on animal tissues and in animals *in vivo* have shown (Gallaher and Loomis 1975, Dahl *et al.* 1987, MAK 2012). However, in the TD experiment the BEAS-2B cells released ethyl acetate. Mochalski *et al*. (2013b) suggested esterification of acetic acid with ethanol as a mechanism of ethyl acetate formation *in vitro*. Ethanol would be oxidised to acetic acid (reaction catalysed by ADH and ALDH) with the subsequent esterification of the acid product. Ethanol was readily available to the cells in the TD experiment which supports this route of ethyl acetate production. The pathways of the esterfication leading to

dodecanoic acid isooctyl ester and propanoic acid, 2-methyl-, 1-(1,1-dimethyl)-2-methyl-1,3-propanediyl ester are unknown.

5.4.8 Ethers

Two aliphatic ethers (2-butoxyethanol and dodecyl acrylate) and one aromatic ether (2-methoxy-diphenylmethane) were detected in the MT experiment. 2-Butoxyethanol could not be quantified because of the presence of an unknown co-eluting peak. 2-Methoxy-diphenyl methane was observed to be consumed by the cells. The levels of dodecyl acrylate were not affected by the presence of the cells. For the TD experiment no ethers were found.

Ethers found at decreased levels

The presence of cells only altered the level of one ether, 2-methoxy-diphenyl methane (CAS: 883-90-9) (alternative ID: 3-methoxy-diphenyl methane). The ether was metabolised by the A549 cells in the MT experiment. Neither of these ethers has been ever reported in any *in vitro* or *in vivo* studies of VOCs. Therefore, their use as a potential biomarker of lung cancer is not likely.

Biochemical background for the altered levels of ethers

Aromatic methyl and ethyl ethers may be metabolised via o-dealkylation to give the phenol and corresponding aldehyde. The preferred route for ethers with longer alkyl chains is via ω-1-hydroxylation, as they are less readily O-dealkylated (Timbrell, 2008). CYPs were also shown to play a crucial role in the metabolism of methyl-*tert*-butyl ether and other gasoline ethers (Hong *et al*., 1999). The pathway for metabolism of 2-methoxy-diphenyl methane is not known. It may occur via o-dealkylation or ω-1-hydroxylation as it is a C_{14} aromatic ether.

5.4.9 Furans

Furans were detected only in the TD experiment and these were tetrahydrofuran; 2-methylfuran and 2,4-dimethylfuran. Their levels were not affected by the presence of

cells. 2-Methylfuran and 2,4-dimethylfuran were most likely emitted from the rubber Suba-Seals® (see Chapter 5.2.3).

5.4.10 Sulfides

Carbon disulfide was the only sulfide detected. It was one of the most intensive peaks present in the chromatogram of the TD experiment. Its levels were not affected by the presence of the cells. Carbon disulfide most likely originated from the Suba-Seals® (see Chapter 5.2.3).

5.4.11 Thiazoles

One member of this chemical group, benzothiazole, was detected in the TD experiment. Its levels in the HS were similar for the cell samples and medium controls. Again, it probably originates from the rubber Suba-Seals® (see Chapter 5.2.3).

5.5 MMSE versus TD sorbent tubes extraction

The use of MMSE allowed for the identification of 69 VOCs while the extraction of volatiles on the sorbent tubes yielded 44 analytes. Aliphatic hydrocarbons were the most numerous chemical group in both experiments. Aromatic hydrocarbons, alcohols, aldehydes, ketones and esters were represented by more compounds in the MT experiment than in the TD experiment. Ethers and phenols were detected only in the MT experiment, while furans and singular members of sulfides and thiazoles were observed solely in the TD experiment. Thirteen VOCs were common to both experiments, mainly aliphatic hydrocarbons, and these were 2,3,5-trimethylhexane; 4-methyloctane; 2,4-dimethyl-heptane; 2,4-dimethyl-1-heptene; benzaldehyde; decane; p-xylene; ethylbenzene; styrene; 2-ethylhexanol; acetophenone; dodecane and undecane. The statistical analysis for the two experiments gave the same results in terms of level trends for the A549 cells in the case of 2,3,5-trimethylhexane; benzaldehyde and acetophenone. The TD experiment did not detect any differences between the cancer cell samples and controls for 4-methyloctane; 2,4-dimethylheptane; 2,4-dimethyl-1-heptene; dodecane; styrene and ethylbenzene, while the MT experiment showed that the cells produced the methylated hydrocarbons and

consumed styrene; dodecane and ethylbenzene. Finally, 2-ethylhexanol was found to be consumed in the MT analysis and produced in the TD experiment by the A549 cells. There are a number of potential reasons explaining why the two techniques yielded different results in terms of the number of detected VOCs, the represented chemical groups and level trends. They are discussed below.

5.5.1 Experiment design

The MMSE and extraction with TD sorbent tubes yielded analytes with different BP ranges. An important difference between the two techniques is the presence of a solvent peak in the MT experiment introducing so called 'solvent delay' into the analysis, which was set to 5.30 minutes. The peaks eluting in the TD experiment could be monitored straight from the start of the sample introduction onto the column. Therefore, for the TD experiment, VOCs with low BPs could be detected (although not resolved), such as acetaldehyde (18°C). On the other hand, 41 VOCs with BP > 200°C were identified in the MT chromatogram, in contrast to only four such compounds present in the TD chromatogram. The reason for this difference lies in the experiment design, as two different sample matrices were analysed in the two experiments. For the MT experiment, VOCs were extracted from the HS of the culture medium collected from the cell culture flask and transferred into the MT vial. Therefore, the VOCs detected here were a result of the accumulation of analytes in the culture medium phase throughout the week. For the TD experiment, the HS above the culture medium in the culture flask was loaded onto the sorbent traps. The analytes detected in this experiment were due to the accumulation of VOCs in the HS phase during the week. Essentially, the culture medium phase and the HS phase could be expected to differ in both the content and levels of the VOCs; the HS containing non-polar and more volatile analytes and the medium containing more polar and less volatile compounds. This difference is dependent on the distribution constants of the VOCs between the cell culture medium and the HS at 37°C. The temperature of extraction applied to the collected culture medium phase in the MT experiment was 60°C, therefore, at this temperature a different equilibrium was achieved and higher BP compounds could travel into the HS and onto the trap. In addition, the MT experiment allowed for matrix modifications such as the addition of salt and pH adjustment. Salt addition could result in further travelling of less volatile analytes into the HS. Another change to the sampled matrix that was done only in the MT experiment was the

adjustment of the pH of the A549 cell samples, which were slightly more acidic when compared to controls (Tab. 2.2). Such an adjustment was not possible for the TD experiment. This might be the reason why no differences in peak areas were found for the four hydrocarbons, between the samples and controls in the TD experiment.

5.5.2 Type of adsorbent

An obvious difference between the two techniques is the different type of trapping sorbent exploited. MT is made of silica with graphitised carbon, while SVI™ TD tubes combine four types of sorbents (graphitised carbon and molecular sieve sorbents; detailed sorbent types are not known as the patent for these tubes is pending). They have different loading capacities (the maximum weight of material that is retained on the adsorbent in mg of analyte per g of sorbent) and different average surface areas (Tab. 3.1). Theoretically SVI™ sorbent tubes have a broader VOC molecular range for the collection of gases than DCC18. The SVI™ TD tube allows for the detection of VOCs in the C2 - C30 range. The MT experiment has shown the ability of the MTs to detect analytes in the range of C6 - C20 with DCM as a solvent. Moreover, surface area volumes are usually higher for the sorbent tubes than for MTs, therefore offering more reaction sites for the analytes (Tab. 3.1). However, sorbent tubes have shown a smaller range of detected chemical groups and an almost complete absence of some. For example, aldehydes and alcohols were very abundant in the MT experiment but only two alcohols and two aldehydes were detected in the TD experiment. In contrast, aliphatic hydrocarbons were abundant in both experiments suggesting that a loss of polar compounds might have occurred during the sampling with TD tubes.

5.5.3 Loss of polar compounds

The first step of the TD analysis is a trapping stage when the VOCs are loaded onto the sorbent tube with the use of an Easy-VOC™ pump. On the surface of the sorbents, competitive reactions of adsorption-desorption between water and analyte molecules occur. During the first minutes of the P&T technique, in humid samples, a large proportion of the adsorption sites in the sorbent are occupied by water, regardless of the affinity of the adsorbent to water. After some time the trap will be saturated with water and then the water molecules no longer retained will carry away polar compounds (Canac-

Arteaga *et al*., 1999). Sampling with the use of an Easy-VOC™ pump takes 30 s per 100 ml of a sample volume. It is not known what proportion of the sample vapours are retained on the sorbent during such a short sampling time. Water not retained can be easily purged away during a dry purge step.

In the TD experiment a dry purge of the sorbent tube was set to 5 minutes (ambient temperature). During this step, water and residual air are eliminated from the adsorbents (via loss through the vent) but may also cause desorption of other volatiles. Previous studies showed that polar compounds may be lost with the increased time of a dry purge (Canac-Arteaga *et al*., 1999; Lee K. *et al*., 2001). Polar compounds could be lost during this stage of the TD analysis, especially given that this step was not optimised during the TD method development. However, problems with water management during the initial experiments did not allow for testing shorter purge times. The inlet split facilitates TD analysis of humid samples by the reduction of the amount of water entering the cold trap. Even though inlet split and outlet split were in 'on mode' for the TD analysis, there was still a high water background observed on the MS instrument. High water backgrounds can cause transformations of VOCs in the water matrix, shifts of RTs (which were observed during the TD experiment) and can cause problems during MS detection such as an increase in noise level (and a decrease in sensitivity), peak broadening and finally deterioration of the column stationary phase and MS detector (Canac-Arteaga *et al*., 1999; Helmig and Vierling, 1995; Ras *et al.,* 2009). On the other hand, the same studies have shown better recovery with a longer period of dry purge for some VOCs, and the amounts of other VOCs adsorbed were the same regardless of the dry purge time (Canac-Arteaga *et al*., 1999; Lee K. *et al*., 2001). In addition, a study by Gawłowski *et al*. (2000) showed that longer purge times of 20 min and even 30 min did not cause any loss of polar compounds, suggesting that the opposite phenomenon may have taken place during the TD experiment i.e. the loss of polar VOCs because of too short a purge time. Taking into account that water may decrease the sensitivity of the GC-MS analysis, it might be that longer purge times would improve the recovery of polar compounds in the TD experiment. Potentially, the use of Tenax TA™ tubes could help with water management. Tenax adsorbent has a low affinity to water (in comparison to Carboxen® and Carbosieve® sorbents) and therefore it could be potentially used with the inlet split turned off, resulting in higher amounts of the sample being loaded onto the column, thereby potentially increasing

sensitivity (Brown J., 2013). These TD tubes were designed for the analysis of VOCs in the range of C7-C26, so they cover a wide range of compounds.

A portion of the sample desorbed during the primary desorption step was lost due to the use of an enabled inlet split (only 4.5% of the original sample desorbed from the TD tube was loaded onto the GC column). However, testing the TD analysis with the inlet split closed resulted in even higher water backgrounds; therefore the inlet split was set to 20 ml min^{-1}. The outlet split for the low-bore GC capillary columns must be set in this type of instrument to a minimum of 10 ml min^{-1} (see Chapter 2.4.1 and 4.2.3).

It is also possible that the HS phase concentrations of polar analytes were low, because polar compounds tend to dissolve in the water phase and 200 ml of collected HS volume was not sufficient for the detection of lower abundant compounds. The MT experiment allowed for a 2 hour trapping of VOCs coming from the heated culture medium. Here the compound molecules travel from the sample into the HS and from the HS onto the MT. Once the analyte molecules are trapped onto the disk, more of the analyte molecules travel into the HS. This occurs until equilibrium is reached because there are no more adsorption sites available. It must be noted that MTs have a low affinity to water and even if water molecules initially react with the adsorption sites on the trap, they are probably quickly replaced by the analyte molecules. An EasyVOC^{TM} pump is a very quick sampling process. As mentioned before, it is not known what proportion of the sample vapours is actually retained on the sorbents with a use of the pump. Filipiak *et al*. (2010) have detected more polar compounds (such as hexanal, octanal, 3-methylbutanal, 2-methyl-2-butenal, methacrolein and 2-butanone) in the HS of cultured cells with the use of TD sorbent tubes for extraction. The group also used a 200 ml air sample volume, however, first of all, it was loaded on the sorbent tube at a slow, controlled flow (30 ml min^{-1}) and secondly, the sampled air was diluted with dry air to reduce the humidity of sample. Therefore, the recovery of VOCs present in the HS of the cell culture flasks in the TD experiment could potentially be greatly improved if HS samples were loaded onto the sorbent tubes with the use of P&T or static headspace techniques.

5.5.4 Precision

Both techniques, MMSE-GC-MS and TD-GC-MS, appeared to be suitable in differentiating between the patterns of VOCs of the cell medium samples and their cell-

free medium controls, however with different precision. The TD experiment showed poorer inter-batch precision than the MT experiment (the intra-batch precision could not be directly compared). This was the result of the differences in VOC initial concentrations between the flasks and/or the batch of cell culture medium. For some VOCs, however, the poorer precision obtained in the TD experiment could have been caused by less accurate and less precise ISTD addition. In the MT experiment the ISTD was added into the collected sample placed in a volumetric flask. In the TD experiment the ISTD was added a week before collection into the sample placed in a cell culture flask. Here an appropriate volume of the sample was achieved with the use of serological pipettes, losing accuracy and precision. This could also have an impact on the detection of fewer differences in VOC levels between the sample and its control than for the MT experiment. However, preparation of a TD sample in a volumetric flask would introduce an additional step of risking contamination and autoclaved volumetric flasks posed problems with drying. The precision of the TD analysis could be improved by incorporating the ISTD addition accessory for the thermal desorber which automatically loads ISTD into clean TD tubes just before desorption.

5.5.5 Sensitivity of a method

Finally, the two extraction techniques used in the MT and TD experiments were coupled to two different analytical instruments with different sensitivities. The amounts of ISTDs added to the samples yielded peak areas in the midrange of the levels obtained for the analytes in both experiments. For the MT experiment 2-F and 2-B were added to make final concentrations of 0.25 mg l^{-1} and 0.50 mg l^{-1} respectively, while in the TD experiment DME and 2-F were added to make final concentrations of 4 mg l^{-1} and 2 mg l^{-1} respectively. If the added amounts of ISTDs were close to the amounts of VOCs in the samples there was ~1 order of magnitude difference in the levels of the detected compounds between the two techniques. In trace analysis, the compounds with low concentrations may have been below the detection level of the analytical technique. Some polar compounds, which in the MT experiment were shown to be less intense than non-polar analytes, could be not detected for this reason, rather than as a result of any loss during the TD analysis.

Schallschmidt *et al.* (2015a) also analysed different sample matrices in their study:

(i) *in situ* sampling (SPME fiber inside a cell culture flask with the cells);
(ii) liquid sample (HS of the collected cell culture medium transferred to a vial for SPME sampling);
(iii) gas sample (HS of the cell culture flask removed with a syringe and then sampled in a vial with SPME).

As the group used the same sampling technique, they were able to compare the results between the three set ups more directly. Similarly as for the MT and TD experiment, the group observed that signal intensities for less volatile compounds were decreased in the gas sample while the analysis of the liquid sample (and sampling *in situ*) allowed for a detection of a larger number of VOCs. For most of the VOCs the level trends were altered in the same direction with the use of one or two sampling matrices and remained not changed with the use of the third matrix. For example, ethylbenzene was observed to be consumed in the *in situ* and the liquid samples and no differences were observed for the gas sample. Similarly, for the MT experiment (liquid sample) ethylbenzene was found to be metabolised by the cells and for the TD experiment (gas sample) no differences were detected in the levels of the VOC between the samples and controls. Interestingly, Schallschmidt *et al*. found opposite level trends for decane between the three sampling methods. Decane was observed at increased levels in the gas sample, at reduced levels in the liquid sample, and no differences were found *in situ*. Similarly, 2-ethylhexanol was observed at decreased levels for the MT experiment (liquid sample) and increased levels for the TD experiment (gas sample) for the A549 cells. Schallschmidt *et al*. proposed that the cells 'relocated' decane from the liquid phase into the HS but neither produced it, nor consumed it. If the opposite trends observed for 2-ethylhexanol for the MT and TD experiments were due to 'relocation' by the A549 cells, interestingly, fibroblasts did not show such an ability. However, the mechanism of such 'relocation' is currently unknown. Nevertheless, the MT and TD experiments show that using different sampling matrices gives a complementary picture of the VOC trends. Some potentially wrong conclusions about level trends can be avoided by the analysis of different matrices simultaneously.

5.6 Different VOC patterns between different *in vitro* studies of the A549 cell line

It is common in *in vitro* studies of VOCs as potential biomarkers of cancer to find differences in VOC patterns and level trends not only between different cell lines of the same cancer, but also within the same cell line. Level trends of such VOCs as acetone; acetophenone; benzaldehyde; cyclohexanone; 2,4-dimethyl-1-heptene; 1,3-di-*tert*-butylbenzene; 2-ethylhexanol; octane; 2-pentanone; phenol; styrene and tetradecane were observed to be different for the A549 cell line between the MT and TD experiments and between other *in vitro* studies. The possible reasons behind this are associated with:

(i) the different extraction and analytical techniques used, which has an impact on such factors as the analysis of different matrices and the applied methodology;
(ii) the different cell culture conditions such as the use of different cell culture medium, different periods of cell cultivation and different seeding density, differences in the cell passage number cultivated, the different cell culture vessels used and different controls used, and finally;
(iii) the different statistical test applied.

As different studies of VOCs produced or consumed by the A549 cell line performed to date differ in almost all these factors, differences between the patterns of the detected VOCs are to be expected.

5.6.1 Extraction and analytical technique

Differences in the extraction technique used in different studies are an obvious potential reason for the different patterns observed for the same VOC between studies. Different extraction techniques have different sensitivity, require a different study design and may be coupled to different analytical instruments (with a different sensitivity again). As discussed in the previous chapter, MMSE and TD sorbent tubes have different requirements as an extraction technique, such as the use of a solvent, the temperature of extraction or the selection of a sampled matrix. It also has been discussed that certain modifications to the sample matrix are possible when working with a liquid phase of a sample, while these modifications cannot be utilised in the sampling of the HS phase. This can have a direct impact on the different level trends of the same VOC between the two

techniques applied. The MT and TD experiments were conducted with the use of a different brand of GC-MS which have different sensitivities.

Different extraction and analytical techniques used in *in vitro* studies of VOCs are presented in Table 1.1. Most of the studies investigating the A549 cell line were performed with the use of SPME coupled with GC-MS (Hanai *et al*., 2012b; Pyo *et al*., 2009; Yu J. *et al*., 2009; Schallschmidt *et al*., 2015; Wang Y. *et al*., 2012). Barash *et al*. (2009; 2012) used Ultra II SKC badges for the adsorption of VOCs from the cell culture medium and then applied SPME at a higher temperature of 270°C to extract the VOCs from the badge. Other groups used P&T coupled to GC-MS (Filipiak *et al*. 2008, 2010; Sponring *et al*., 2009, 2010). Finally, a different analytical technique, PTR-MS, was also used by Brunner *et al*. (2010) for the on-line and off-line analysis of VOCs emitted from A549 cells. Such a range of different techniques applied results in different matrices being sampled, different achievable sensitivities and different methodologies which all may have an impact on the range and patterns of the VOCs detected.

Analysis of different matrices

The MT and TD experiments have shown that sampling different matrices may have an impact on the range and trend levels of the VOCs detected. The main matrices analysed to study the VOCs generated by cancer cells are:

(i) the HS of the cell-free culture medium of a target cell and
(ii) the HS of the medium still containing the cells (Tab. 1.1).

The HS of cell lysate (pre-concentrated supernatant of the lysed cells) could potentially be used as another matrix in such studies (Kato *et al*., 2003). As mentioned before, there are some substantial differences in terms of the extraction procedure details for the main two matrices. First of all, the analysis of culture medium with cells takes place at 37°C, while the analysis of cell-free medium may employ a higher temperature. Also, the efficiency of the analysis of cell-free medium samples can be improved by the addition of salts or by a change of pH, while such changes are not possible when cells are present. On the other hand, the analysis of medium with cells ensures that no VOCs are lost and no environmental contaminants are introduced during storage or transfer of the sample to a sampling dish. Sampling both types of matrix has its pros and cons and the best approach to screening for potential volatile biomarkers of lung cancer is using both.

5.6.2 Cell culture study design

There are many important factors that may result in the contradictory results obtained in different *in vitro* studies related to cell culture study design, such as incubation period and chosen cell seeding density, the type of cell culture medium used, the senescence of the cultured cells and the type of culture vessel used.

Incubation period and cell seeding density

Two important factors that may have a huge impact on the different VOC patterns observed in the same cell line between different *in vitro* studies are the chosen period of cell cultivation and hence cell seeding density. Both the MT and TD experiments showed that differences in level trends of a particular VOC are common between growing and confluent cells. Some of these VOCs, namely acetone; acetophenone; benzaldehyde and 2-ethylhexanol are the compounds found to have different trends for the A549 cell line by others, being the result of different metabolic pathways being active.

In order to analyse VOCs as potential cancer biomarkers in cell culture medium, different times of cell cultivation were applied by others. Hanai *et al.* (2012b) collected culture medium from dishes cultivated with A549 cells for one, two and three weeks until reaching confluency. Y. Wang *et al.* (2012) aquired samples after eight days of A549 culture when the cells reached confluency. Although the cell seeding density is not given for both studies, it is probably correct to state that both studies were examining only growing A549 cells, however the rates at which the cells reached confluency were very different. Barash *et al.* (2009; 2011) cultured the A549 cell line for 68 h with a seeding density of 3.6×10^4 cells cm^{-2} until reaching 95% confluency, a period roughly 2.5 times shorter and with a cell density three times higher than the conditions used in the MT and TD experiments. Again the study examined growing cancer cells. Filipiak *et al.* (2010) sampled the HS of a cell fermenter after 21 hours of A549 cell cultivation (2.5×10^5, 7.5×10^5 and 1×10^6 cells ml^{-1}). Schallschmidt *et al.* (2015a, 2015b) started new A549 cultures at 3×10^4 cells cm^{-2} and conducted sampling after 24 hours of incubation. Such short incubation times in both studies may have resulted in the sampling of cells during the lag phase, whilst they were still recovering from sub-culture and therefore not proliferating at a high rate. A lag phase measured for the A549 cell line by Iloki-Assanga *et al.* (2013) was ~50 hours. Moreover, the recommended seeding density for the A549 cell line is

between 2 x 10^3 and 1 x 10^4 cells cm^{-2} and higher seeding densities used in other studies could potentially result in changed patterns of VOCs, due to a higher level of cellular stress. In conclusion, collection 1 of both the MT and TD experiments may only be directly comparable in terms of cell confluency and time of incubation to the studies of Hanai *et al*. (2012b) and Y. Wang *et al*. (2012). The analysis of VOCs produced or consumed by the A549 cell line after the second week of incubation cannot be directly compared to any of the previous *in vitro* studies, as this was the first investigation of VOCs in confluent cells.

Cell culture medium

The use of a different cell culture medium, as well as a different supplementation of it, may have an impact on the different patterns of the same VOC found between different *in vitro* experiments. Some nutrients that are available to cells in one type of culture medium may not be present in another type of culture medium. Therefore, even the same cell line can potentially switch different metabolic pathways on or off in response to the presence, absence, abundance or scarcity of a particular nutrient. A study by Hartmann *et al*. (2008) has shown that VOC levels in colon cancer cell lines were affected by the use of a substitute for FBS, SErEx™ as well as serum-free medium. In the MT and TD experiments RPMI 1640 was used for the cultivation of all cell lines to make the study more comparable. It has been used for the culture of the A549 and BEAS-2B cell lines in the studies of VOCs by Barash *et al.* (2009; 2011), Brunner *et al.* (2010), Pyo *et al.* (2009) and Y. Wang *et al*. (2012). Others used DMEM high-glucose, DMEM-F12 or Ham's F12 cell culture medium (Filipiak *et al*., 2010; Hanai *et al*., 2012b; Schallschmidt *et al*., 2015a, 2015b). Filipiak *et al*. (2010) used 5% FBS in order to reduce a high background level of detected VOCs.

Cell culture vessel

The use of different cell culture vessels might be one of the main reasons for the differences in some VOC patterns observed in different studies of the same cell line. Different types of culture vessel were used for A549 cells such as 100 mm dishes (made of PS) (Barash *et al.*, 2012; Hanai *et al.*, 2012b), the Bellco® cell culture fermenter system (a bottle made of polyethylene terephthalate glycol-modified, LDPE/EVA and PP; and carriers for the cells growth from irradiated PS) (Sponring *et al*., 2009; Filipiak *et al.*,

2008; 2010), and T-75 flasks (Brunner *et al.*, 2010; Yu J. *et al*., 2009) or T-150 flasks with special vent filter caps (made of PE) (Schallschmidt *et al.*, 2015a; 2015b). It is surprising that the problem of residual compounds originating from plastic culture dishes is not often mentioned in the literature regarding *in vitro* analysis of volatiles. Zimmerman *et al.* (2007) noticed higher amounts of compounds in the chromatogram obtained from cells grown in PS flasks than from cells cultured in glass flasks. The group therefore chose to extract the VOCs using the glass flasks to bypass the problem. A major drawback of such a study design is that the glass has to be pre-treated with poly-D-lysine to facilitate cell attachment to the glass. This is an additional step in method development, as it requires testing for the appropriate concentration of Poly-D lysine to achieve the desired attachment for the cell density required. The use of poly-D-lysine may also introduce variability via potential differences in cell density. In addition, the initial TD experiments involved sampling of the 250 ml conical flasks. The differences between the glass flask and the PS flask in the range of present contaminants were not very obvious (however, partly because Suba-Seals® were used in both settings) (data not shown). To avoid the problem of contaminant compounds released from plastic during extraction, Kwak *et al.* (2013) collected the culture medium from above the cells cultured in a plastic flask and sampled it in a glass vial. The MT experiment showed, however, that the residual and radiolysis compounds of plastic dissolve in medium, so are present when sampling in another dish as well. Schallschmidt *et al*. (2015a) observed the same phenomenon. The group also detected some alkanes and aromatic compounds in new culture flasks, which were observed in the chromatogram of the collected cell culture medium sampled in a glass vial.

Control used

To assign the VOC level trend, most *in vitro* studies used cell-free medium incubated simultaneously with the cell medium using the same conditions as a control. However, some results obtained in some studies of the A549 cell line cannot be directly compared with one another, as the levels of VOCs were compared to the levels observed in normal-cell medium, instead of the cell-free culture medium. Therefore, VOC level trends in some cases cannot be assigned. For example, Hanai *et al*. (2012b) found benzaldehyde to be at higher levels in A549 samples when compared to normal cell samples. However, it is not known whether the cancer cells produced it, as this difference could be caused by the use

of a different batch of medium for the different cells lines. It is not known whether the three cell lines used in this study were cultured simultaneously using the same batch of culture medium or not. What is more, this group has observed pattern change between the three weeks of incubation for some VOCs, which again might have been caused by the use of different batches of medium, rather than by the presence of cells.

Passage number

The study of Sponring *et al.* (2009) showed the possibility of a change of the released volatile metabolites with increasing passage number. In their study, 2-ethylhexanol was increased in the cancer cell samples with low passage numbers and it was not detected in the cell samples with high passage numbers. For the TD and MT experiments, 2-ethylhexanol was observed to have different level trends, however, as the A549 passage numbers grown were in the range of 10 - 14 in both experiments, the reasons for the different level trends lie in the different experimental design and/or the 'relocation' phenomenon. Different passage numbers of the A549 cell line were almost certainly used in different studies of VOCs as biomarkers (although no publication mentions the passage number used in the study) and this can have a potential impact on the detected VOCs. Primary and senescent HDFs were shown to differ in VOC patterns by Acavedo *et al*. (2010). Early and late passages were shown to influence metabolism, cell proliferation and cell membrane transport characteristics of cells *in vitro* in many studies (Chantret *et al*., 1994; Hughes *et al*., 2007). It is known that cancer cell lines display a greater genetic instability than tumour cells *in vivo*, especially with long term cultivation, through the accumulation of multiple mutations. This may result in the selective growth of initial cell line subpopulations characterised by being less differentiated. However, this could easily be reduced by the correct cell culture techniques (Gazdar *et al*., 2010).

5.6.3 Statistical analysis

Another important factor impacting on the different level trends of the same VOC between studies is the choice of applied statistical test. As mentioned before, parametric methods such as the t-test applied to non-normal distributed data may result in false positive significant differences (De Winter, 2013). On the other hand, the use of non-parametric methods may result in false negative differences (Worthy, 2015). Higher levels of 4-

methyloctane and 2,4-dimethyl-1-heptene were observed in the MT experiment. No significant differences for these VOCs were found in the TD experiment at a significance level $p < 0.05$. However, the p-values of $p = 0.0549$ for both 2,4-dimethyl-1-heptene (in collection 2) and 4-methyloctane (in collection 1) were close enough to 0.05 to argue that the A549 cells were producing the VOCs.

Other studies of cells *in vitro* used mainly non-parametric analysis to test group medians such as Wilcoxon/Kruskal-Wallis (Barash *et al.* 2012; Davies *et al*., 2014); Kruskal-Wallis only (Filipiak *et al*., 2010); Wilcoxon signed-rank test (Mochalski *et al*., 2013b; 2015) or Mann-Whitney U test (He *et al*., 2014; Huang Y. *et al*., 2016). Some, however, used parametric tests such as multivariate analysis of variance (MANOVA) without (Lavra *et al*., 2015) or with the Bonferroni correction (Kwak *et al*., 2013), one-way ANOVA (Schallschmidt *et al*., 2012a, 2012b) or t-test (Hanai *et al*., 2012b).

5.7 Different VOC patterns between *in vitro* and *in vivo* studies

Chapter 5.4 discussed in detail the possible use of the VOCs found at altered levels in the cell samples for the MT and TD experiments as biomarkers of lung cancer. There were differences observed in the level trends between different *in vitro* studies for the same cell line. There were also different level trends found between different cell lines of the same cancer, showing that they have different metabolic pathways active. Genetic and phenotypic differences and the fact that each cell line is representative of only a small part of a primary tumour are the main reason for this. However, the contradictions in the concentration trends were most obvious between *in vitro* and *in vivo* studies of VOCs in cancer.

The poor correlation between *in vivo* and *in vitro* studies may arise from:

(i) the complex transition mechanisms of the VOCs produced by tumour cells in the body and found in breath or biofluids;
(ii) exogenous VOCs being included in the predictive models of cancer;
(iii) different extraction and detection techniques used in different studies;
(iv) different experimental design;
(v) the relatively lower number of *in vitro* studies performed to date, in comparison to VOC studies of breath samples and biofluids and;

(vi) problems with *in vitro* culture as a model of *in vivo* VOC presence in breath and biofluids.

In addition, studies which show that the VOC patterns do not change after tumour removal imply that some VOCs may be biomarkers of the risk of cancer developing, rather than being indicative of the presence of a tumour (see Section 1.4.1 for further discussion).

5.7.1 Transition mechanisms

While cells *in vitro* release VOCs into the HS of the cell culture flask and consume VOCs emitted from the cell culture medium and/or from the cell culture vessel, VOCs emitted or metabolised by cells *in vivo* have more complex transition mechanisms. VOCs *in vivo* are present in different body fluids and their distribution in the organism depends on various physiological properties. Fat:blood ($\lambda_{f:b}$) and blood:air ($\lambda_{b:a}$) partition coefficients are the two main physiological parameters that impact on the fate of VOCs *in vivo*. These coefficients might be measured experimentally or theoretically predicted for each VOC, in order to estimate the equilibrium concentrations of the VOC between body fluids. A high ($\lambda_{f:b}$) results in the storage of the VOC in the body fat compartments and lipophilic cell membranes, while a low $\lambda_{f:b}$ leads to the release of the VOC into the blood stream. VOCs with high $\lambda_{b:a}$ tend to be exchanged in the airways (mainly polar compounds), while VOCs with low $\lambda_{b:a}$ only in alveoli (mainly non-polar compounds). The alveolar clearance mechanisms of VOCs have an influence on their final partition and exhalation (Broza *et al*., 2015). Even if some VOCs are present at similar concentrations in exhaled breath, their concentrations in blood and fat tissues could vary by as much as 12 and 8 orders of magnitude respectively. This suggests that different VOCs have different concentrations in different storage tissues and that their synthesis and metabolism rates differ. Modelling and simulation of the fate of VOCs *in vivo* will help greatly towards gathering knowledge about their metabolic pathways and exhalation kinetics (Amann *et al*., 2014).

5.7.2 Exogenous VOCs as potential biomarkers of lung cancer

Some argue that breath analysis should include exclusively endogenous compounds, as only they may reflect potential pathophysiological changes of endogenous metabolic processes that arise due to tumour presence (Kwak and Preti *et al*., 2013). *In vitro* studies help towards this approach by providing information on the endogenous or exogenous origin of VOCs. For example, studies by M. Phillips *et al.* (1999b; 2003c) were criticised by Mitsui and Konodo (2003) for suggesting that branched methylated hydrocarbons are markers of oxidative stress, as there is 'no available data to support the contention that methylated alkanes derive not from environmental contamination but from endogenous lipid peroxidation'. Some *in vitro* analyses of branched hydrocarbons, however, including the MT and TD experiments, constitute such data. They indicate that these compounds may be produced by human cells (although it is not known via which metabolic processes) and therefore their presence in breath might be of endogenous origin.

It is thought by some that exogenous VOCs cannot be reliable biomarkers, as their levels may be affected by factors such as their variable concentrations present in the environment and therefore, the levels of the VOCs in breath will depend on the exposure to and accumulation of exogenous VOCs in body tissues (Kwak and Preti, 2013). Arguably, exogenous VOCs should be used as breath cancer biomarkers. Exposure to certain VOCs may increase the risk of cancer, therefore their potential higher level of storage in body tissues and then release at higher concentrations in breath, may be characteristic of tumour presence. Many exogenous VOCs are present in the human breath. Metabolism of inhaled or ingested exogenous VOCs might be impaired or enhanced by the presence of a tumour, leading to either higher or lower concentrations in cancerous breath when compared to normal breath. As long as a biochemical pathway of the metabolism of a VOC and the relationship between its metabolism and the disease is well understood, exogenous compounds could be used as biomarkers. Large population studies could be applied to measure the concentration ranges of VOCs commonly present in normal human breath. Lower or higher levels in the breath of lung cancer patients could then be used as a biomarker. However, defining normal ranges of VOCs in breath requires evaluation of thousands of healthy breath samples performed on many research sites (Solga and Risby, 2013). Before this happens, the standardisation of breath collection methods and the analytical technique used must occur.

5.7.3 Breath analysis standardisation

Effective standardisation of the sampling and analysis of breath is the main challenge in breath research and its absence is probably the main reason for inconsistent results in this field, not only between *in vitro* and *in vivo* studies but also between different *in vivo* studies. The main reasons behind this are:

(i) the diversity of exhaled VOCs because of inter- and intra-individual variability of exhaled VOCs;
(ii) different sampling and analytical techniques employed;
(iii) the diverse goals of different studies, and;
(iv) potential unidentified confounders (Herbig and Beauchamp, 2014).

Inter- and intra-individual variability of exhaled VOCs

Breath is a complex mixture of chemically diverse VOCs. It is important to note that over 3000 VOCs were detected in a single study (n = 50 subjects) and 200 VOCs were observed on average in a single sample by M. Phillips *et al*. (1999b). The number of analytes common to all subjects was relatively low (27 VOCs with occurrence = 100%). The remaining VOCs were mostly detected only once, reflecting the individuality of VOC patterns in humans. Therefore, only ~1% of exhaled breath is likely to contain lung cancer specific VOCs. What is more, most of these VOCs are also present in healthy breath, but in altered concentrations. Therefore, there is a huge amount of inter-individual variability depending on many factors such as previous or present exposure to the compounds of interest, compounds associated with smoking, the medical history of the patient, their food consumption, gender, age etc. (Di Francesco *et al.*, 2005; Kischkel *et al.*, 2010; Miekisch *et al*., 2012) (see Chapter 1.4). Moreover, intra-individual variability must be also taken into account in breath research when formulating future recommended practices. Studies have shown variations in VOC concentrations in the same individual depending on the day of sampling and on the sampling methodology (Boshier *et al*., 2010; Bunkowski *et al*., 2010).

Different sampling and analytical techniques employed

Differences in sampling procedures have been shown to have an impact on results in VOC breath analysis by Miekisch *et al*. (2008) (alveolar versus mixed expiratory sampling) and Boshier *et al*. (2010) (on-line versus off-line sampling). A single breath sampling procedure would allow for the direct comparison of results obtained with the use of various techniques. However, this is not feasible in reality, as it has to address a broad range of issues that may vary depending on the study goal, such as on-line versus off-line measurement, the rest period before sampling, breathing manoeuvre, selection of breath phase, inspired compound concentration and alveolar gradients (Herbig and Beauchamp, 2014). In order to distinguish between endogenous VOCs and exogenous volatiles originating from the ambient air, correction for background concentrations of VOCs in inspired air is mandatory. However, there are different approaches to overcoming this issue that need to be agreed upon (Miekisch *et al*., 2004). Because they are stored in different tissues *in vivo*, different VOCs can be expected to have different exhalation kinetics. The release of a VOC from the tissue in which it is stored is dependent on the blood flowing through this tissue during sampling. Therefore, different breath collection protocols may lead to different concentrations of the compounds in breath, due to the application of different breathing manoeuvres or periods of resting before sampling etc. (Amann *et al*., 2014).

A variety of different analytical techniques have been used to date in *in vivo* studies of VOCs as potential biomarkers of lung cancer, namely gas chromatography - mass spectrometry (GC-MS), selected ion flow tube - mass spectrometry (SIFT-MS), proton transfer reaction - mass spectrometry (PTR-MS), ion mobility spectrometry (IMS), laser absorption spectroscopy (LAS) and e-noses (see Chapter 1.2.2). It is difficult to directly compare the results obtained with the use of such diverse tools. They all result in different sample preparation, leading to potentially different results being obtained between studies.

Although a single breath sampling procedure might be difficult to introduce, because of the diversity of the sampling and analytical tools as well as the differing goals of various studies in the field of VOCs a potential biomarkers of disease, it will be necessary to do so if it is ever to reach the clinic. An initial framework considering the individual stages of breath sampling and analysis has been proposed as first step towards standardisation (Herbig and Beauchamp, 2014).

Diverse goals of different studies

Breath research can be divided into three main areas:

(i) exploratory (screening) research: the discovery of new VOCs of possible relevance to a healthy or a diseased state;
(ii) focused (targeted) research: the comprehensive evaluation of a single VOC or a combination of VOCs with a well understood biochemistry, and;
(iii) breath condensate: the analysis of non-volatile and volatile compounds present in the water vapour condensation of exhaled breath.

These areas employ different breath collection procedures, sampling techniques and analytical instruments, representing a diversity of challenges (Risby and Solga, 2006).

Potential unidentified confounders

No single VOC biomarker has been so far found to discriminate between lung cancerous breath and normal breath. The potential diagnostic use of breath analysis relies on differences in VOC concentrations between healthy and diseased states, rather than on unique biomarkers of disease. Therefore, data processing and interpretation is crucial in this field. A major challenge in data interpretation is identification of confounders. These can be split into three groups: variable confounders, so-called 'voodoo' confounders and statistical misconceptions in the study design (Miekisch *et al*., 2012).

Confounding variables display a real statistical relationship to the disease and a potential VOC biomarker. This may result in the inaccurate conclusion that the disease and VOC are causally correlated. Examples of confounding variables are the factors leading to inter- and intra-individual variability. Breath sampling and analytical techniques themselves may also result in potential confounders such as contaminants originating from Tedlar bags, artefacts created during storage of breath VOCs on Tenax tubes, bleeding contaminants from TD tubes etc. (Kang and Thomas, 2016; Kwak *et al*., 2014; Miekisch *et al*., 2012;). 'Voodoo' confounders are statistically significant correlations occurring coincidentally in the analysis of too many variables. The chances of finding a significant effect increases with the number of measured variables. A cross-validation on a different dataset, the performance of estimations of random correlations (minimising type II errors), and/or the application of methods to control overall type-I errors (false positive)

helps to reduce the incidence of 'voodoo' confounders (Miekisch *et al*., 2012; Phillips M. *et al*., 2010). Finally, statistical misconceptions in study design are connected with the selection of an appropriate control and sample size (Miekisch *et al*., 2012).

Similarly to *in vitro* studies, the studies *in vivo* used different statistical methods making it difficult or impossible to compare or extrapolate the results between studies. Either statistical hypothesis tests are used to test whether a single VOC is related to a disease (e.g. ANOVA followed by Student-Newman-Keuls, paired t-test, Kruskal-Wallis and Mann-Whitney U), or multivariate analysis is employed to determine the many variables discriminating between groups (e.g. decision tree, discriminant analysis, PCA, factor analysis) (Bajtarevic *et al*., 2009, Kischkel *et al*., 2012, Phillips M. *et al*., 1999a, Preti, 1988, Ulanowska *et al*., 2011; Wang C. *et al*., 2014). Because VOC concentrations in breath may depend on a variety of confounders, the method used to normalise data must be chosen carefully. A study by Kischkel *et al*. (2010) showed that the use of different statistical algorithms and data normalisation methods resulted in changes in statistical information. Finally, methods to control overall Type-I (false positive) errors by correction for multiple comparisons (e.g. the Bonferroni, Šidák or Tukey's corrections) should be used.

5.7.4 Cell culture in vitro as a lung cancer in vivo model

Some of the VOCs shown to be produced or consumed by the A549 cells in the MT and TD experiments were observed to have opposite concentration trends in cancerous breath (or in other biofluids) when compared to normal breath in some of the studies. Kalluri *et al.* (2014) postulated that the overlap between VOCs found in the exhaled breath of lung cancer patients and compounds produced by lung cancer cells *in vitro* (approximately one-quarter being common to both matrices) is not sufficient at the moment for *in vitro* culture to be a good model for the VOCs present in exhaled breath. The authors propose that this could be due to cell cultivation in hyperoxic conditions (at atmospheric oxygen concentration levels), emphasising this as a potential limitation of the *in vitro* studies performed to date. Tumours have been shown to grow in hypoxic (oxygen depleted) or anoxic (oxygen absent) conditions in contrast to normal tissues (Vaupel *et al*., 2004). Cellular oxidative stress would lead to the production of different VOCs by cells in comparison to hyperoxic cell culture conditions. Studies comparing the patterns of VOCs

present in the HS of cells cultured in hyperoxic and hypoxic conditions are needed to address this potential limitation of the *in vitro* approach.

However, another issue related to cell culture conditions could also result in differences in the VOCs present in the HS of cell culture and samples taken from a patient. Standard 2D cell culture conditions may have a great impact on the cell metabolic behaviour, thereby losing accuracy when looking for biomarkers when compared to 3D culture that better mimics the growth of the tumour (Rutter *et al*., 2013). It is known that *in vitro* cell culture may differ from the original tissue or organ significantly in many biological responses, such as receptor and transcriptional expression, cellular migration and apoptosis (Haycock, 2011). For example, in a study investigating the gene expression profiles of A549 cells grown in a 4D model and in a monolayer, as well as several genes being differentially expressed (involved in extracellular matrix functioning, polarity and cell fate and development), significant differences in proliferation rates and cell death were also observed (Mishra *et al*., 2014). Patterns of VOCs were shown to be different between conventional 2D cultures and 3D encapsulated cultures of HDFs in a study by Acavedo *et al*. (2007). Also, the production of acetaldehyde was affected by the type of cell culture (2D versus 3D scaffolds) in a study by Rutter *et al*. (2013). Exposure to BTEX compounds also showed that 2D A549 cell cultures are more sensitive than 3D A549 spheroids (Liu F. *et al*., 2013). Although 3D cell/tissue culture is certainly the future for *in vitro* studies, it is still an evolving area requiring further studies for its optimisation. Challenges involve vascularisation of the tumour models and co-culture with other closely related cells that are present in tumours to more closely mimic tumours *in vivo*. It also has been shown that different cell lines do not grow well when cultured using a certain method which needs more research (Breslin and O'Driscoli, 2013). In addition, the use of different 3D cell culture technologies may potentially also introduce inconsistency into the VOC patterns observed. Finally, even with the use of the same 3D technique, the reproduction of exact 3D structures from experiment to experiment may be difficult to achieve (Liu F. *et al*., 2013).

Another limitation of cell cultures which can introduce differences in VOC patterns between *in vitro* and *in vivo* studies is the lack of interactions with stroma cells and other non-malignant cells surrounding tumours. Tumours are complex systems and their microenvironment plays a major role in carcinogenesis. The stroma mediates signals between epithelial cells and neighbouring fibroblasts. The co-culture of A549 cells with

normal fibroblasts resulted in the altered expression of genes coding cell growth, survival and angiogenic factors, transcriptor regulators, enzymatic activity and transmembrane receptors (Fromigué *et al*., 2003).

The TD experiment showing that the majority of analytes were emitted from the cell culture flask raised the question of how closely the 'VOC burden' supplied to the cells *in vitro* mimics the *in vivo* state of the volatiles available to tumour cells. It is likely that it does not mimic it at all. There is a minimal chance that a given combination of VOCs present in a culture flask/dish is similar to the VOCs 'supplied' to tumour cells *in vivo*. And it is the combination of VOCs (and nutrients) available to cells that dictate which metabolic pathways are used. Cells *in vitro* and *in vivo* both constantly adapt to the changing environment of available nutrients. Therefore, it is very unlikely that exactly the same metabolic pathways are turned on for the cultured cells and tumour cells *in vivo*. The resulting different concentration trends for some VOCs are the consequence of different environments. For example, ethylbenzene and styrene were reported to be present at higher levels in the breath of cancer patients when compared to healthy controls (Buszewski *et al*. 2012b, Chen X. *et al.,* 2007, Peng *et al.,* 2009, Phillips M. *et al.,* 1999a, Poli *et al.,* 2005 and Ulanowska *et al.,* 2011). The consumption of ethylbenzene and styrene demonstrated by both cancer and fibroblast cells in the MT experiment may be the result of detoxification mechanisms stimulated by the presence of these VOCs in the cell culture flask. Therefore, it appears that *in vitro* the opposite biochemical processes involving these VOCs occur to *in vivo*. However, there are many basic cellular processes which will be occurring via the same metabolic pathways both *in vitro* and *in vivo*. A direct way of finding out whether the availability of a particular VOC results in its metabolism by tumour cells is an *in vitro* culture.

Chapter 6

Conclusions and Future Directions

The present study aimed at the detection, identification and semi-quantification of VOCs released or consumed by the A549 cell line, and at the comparison of the A549 VOC level trends to the trends of the NHLF or BEAS-2B cell lines. For this purpose, methods of HS-MMSE using MT disk and TD sorbent tube extraction with an Easy-VOC™ pump as a sample loading tool, both coupled with GC-MS, were developed, optimised and applied. Both techniques have never been used in *in vitro* studies of VOCs as potential biomarkers of cancer, and both were proven to be suitable in differentiating not only between the patterns of VOCs of the cell medium samples and their cell-free medium controls, but also between the cell lines and between the growing and confluent cells of the same cell line. However, some improvements in the methods of both techniques should be introduced in future studies. For the HS-MMSE-GC-MS method, a higher injector port temperature should be tested in order to possibly gain better precision in semi-quantitation of analytes with a higher BP. In addition, blanks of an MT disk without sample extraction and water samples (prepared as for the TD experiment) could be introduced to further investigate the origin of the detected VOCs. In TD experiments, longer and shorter dry purge periods as well as higher volumes of the HS sample (e.g. two samples combined or the use of a larger cell culture vessel) could potentially improve sensitivity and the range of detected compounds. Finally, both methods require validation of intra-day and inter-day precision.

For both the MT and TD experiments, numerous VOCs originating from the cell culture flasks were observed. The ‘water experiment’, investigating for the first time the residual and radiolysis compounds of the T-75 cell culture flasks, showed that the levels of some of these VOCs decrease with longer incubation, while the levels of others remain constant. Relatively high intensities of these VOCs may have an impact on cell metabolism, growth and behaviour and new methods of cell culture should be investigated. Different caps could potentially be applied to seal cell culture flasks (instead of Suba-Seals®) and/or a different type of cell culture vessel used, in order to decrease the background levels of contaminant VOCs.

In the MT experiment, seven VOCs were produced and 14 VOCs metabolised only by the cancer cells (Wilcoxon signed-rank test, $p < 0.05$). Among the released compounds were mainly methylated hydrocarbons (2,4-dimethyl-1-heptene; 4-methylundecane; 2,3,6,7-tetramethyloctane; 2,3,5-trimethylhexane and 2,3,5-trimethyldecane) and alcohols (cyclohexanol and 3-heptanol). The metabolised analytes were alcohols (4-decanol; 6-dodecanol; 2-ethylhexanol; 1-octanol), aldehydes (dodecanal; tetradecanal), ketones (acetophenone; cyclohexanone; 2-tetradecanone) phenols (phenol and 2-nitrophenol); an ether (2-methoxydiphenylmethane), an ester (pentanoic acid, 2,4-dimethyl-3-oxo-, methyl ester) and a hydrocarbon (tetradecane). The NHLF cells were observed to also produce unique VOCs belonging to different chemical groups, such as alcohols (benzyl alcohol; 6-dodecanol; 2-ethylhexanol); aldehydes (dodecanal; octadecanal); an ester (dodecyl acid isooctyl ester), a hydrocarbon (tetradecane), ketones (geranyl acetone and 2-tetradecanone) and a phenol (2,5-di-*tert*-butylphenol) (Wilcoxon signed-rank test, $p < 0.05$).

2,4-Dimethylheptane; 1-phenylethanol; 2,6-di-*tert*-butyl-1,4-benzoquinone and 2-pentadecanone were produced by both the A549 and NHLF cells but were found to be produced at a higher rate by the A549 cells. Compounds such as 1,1'-(1,2-cyclobutanediyl)-bis, cis-benzene were observed to be produced by both cell lines, but the fibroblasts demonstrated a higher rate of their production than the cancer cells. A higher rate of metabolism of VOCs such as benzaldehyde; 1,3-di-*tert*-butyl-benzene; hexanal and 1-nonanol was observed for the cancer cells, while 2-ethenyl-2-butenal was consumed at a higher rate by the fibroblasts (Mann-Whitney U, $p < 0.05$).

For the TD experiment, 2,3,5-trimethylhexane and *tert*-butanol were produced exclusively by the A549 cells, while ethyl acetate solely by the BEAS-2B cells. Decane and 2-methylpentane were observed to be metabolised only by the BEAS-2B cells (Wilcoxon signed-rank test, $p < 0.05$). All the VOCs found to be consumed by the cancer cells were also consumed by the non-transformed cells, however, acetophenone, benzaldehyde and 2-methylbutanal were consumed at higher levels by the A549 cells in comparison to the BEAS-2B cells. 2-Ethylhexanol and 2-pentanone were released by both cell lines at a similar rate, while acetone was found to be produced at a higher rate by the A549 cells (Mann-Whitney U, $p < 0.05$).

Of these VOCs, the production of 2,4-dimethylheptane; 4-methyloctane and 1,1'-(1,2-cyclobutanediyl)bis-,cis-benzene is particularly interesting from the perspective of VOCs as potential biomarkers of lung cancer, as they were found previously in cancerous breath in higher concentrations, when compared to normal breath by more than one group (Peng *et al*., 2009; Phillips M. *et al*., 1999a, 2003a, 2008; Rudnicka *et al.*, 2015). 2,3,5-Trimethylhexane might be also of potential interest, as it was reported at higher levels in A549 cells and other lung cancer cells and in cancerous breath (Sponring *et al*., 2009; Filipiak *et al*., 2010; Rudnicka *et al.*,2015). 2-Pentadecanone and 2,6-di-*tert*-butyl-1,4-benzoquinone might also be candidate biomarkers but their specificity towards lung cancer needs further investigation. A VOC found to be metabolised by the A549 cell line which might be of potential interest is dodecane, as it was found to be present at lower levels in the breath of cancer patients (Peng *et al*., 2010). The metabolism of benzaldehyde might potentially be a marker of the KRAS mutation (Davies *et al*., 2014; Peled *et al*., 2013). 1-Phenylethanol has never been reported in breath, but its higher levels for cancer cells were observed in other *in vitro* studies of VOCs (Hanai *et al*., 2012b; Kwak *et al*., 2013). Similarly, cyclohexanol could be of potential use if found in breath in future studies at it has been found at higher levels in lung cancer cell lines by others (Schallschmidt *et al*., 2015a). More studies are needed to confirm these VOCs as potentially useful as biomarkers of lung cancer.

This project has investigated the patterns of the VOCs *in vitro* between growing and confluent cells for the first time. Some analysed VOCs were found to be consumed or produced at significantly differing levels between collections of the same cell line or exclusively by one collection. In the MT experiment dodecane was consumed, while 2,6-di-*tert*-butyl-1,4-benzoquinone and 2-pentadecanone were observed to be produced, only by the growing A549 cells. 1-Phenylethanol and 3-heptanol were produced at a higher rate by the growing cancer cells than by the confluent cancer cells. Acetophenone and benzaldehyde were metabolised to a higher extent by the growing A549 cells than by the confluent A549 cells. 2-Ethylhexanol was consumed at a higher rate by the confluent cancer cells than by the growing cancer cells. Benzyl alcohol and dodecanoic acid isooctyl ester were produced while ethylbenzene, heptanal and octanal were found to be metabolised only by the growing fibroblasts. Finally, dodecanal was produced exclusively by the confluent fibroblasts (Mann-Whitney U, $p < 0.05$).

For the TD experiment some changes were also observed in VOC patterns, depending on the confluency of the cells. 2-Ethylhexanol was produced solely by the growing A549 cells. Acetone was produced at a higher rate by the confluent cancer cells, while benzaldehyde was metabolised at a higher rate by the growing cancer cells. Confluent BEAS-2B cells were observed to consume acetophenone at a higher rate than the growing BEAS-2B cells. Also, the growing BEAS-2B cells consumed benzaldehyde at a higher rate than their confluent counterparts (Mann-Whitney U, $p < 0.05$).

These results suggest that different metabolic pathways are active during both the exponential growth of the cells and when they reached confluency. Arguably, studies on confluent cells better reflect *in vivo* conditions than those on sub-confluent cells, because of the presence of cell:cell interactions and gap:tight junctions.

The synthesis and metabolism of most of the detected VOCs (alcohols, aldehydes, ketones, benzene derivatives) can be attributed to the activity of such enzymes as CYP, ALDH and ADH which were shown to be expressed by the A549, BEAS-2B and lung fibroblast cells previously (Buehler *et al*., 1982; Courcot *et al*., 2012; Fromigué *et al*., 2003; Rubporn *et al*., 2009). The biochemical pathways for the production of methylated branched hydrocarbons need to be established. Detailed knowledge about metabolic pathways of the VOCs detected *in vitro* would greatly assist the investigation of the fate and behaviour of VOCs *in vivo*, and therefore aid their use as potential biomarkers of cancer. From this perspective, one of the possible future *in vitro* approaches could involve C13 labelled precursors being supplied to cells and the investigation of compounds with increased C13 content (Oldiges *et al.*, 2007).

The use of the majority of the VOCs detected at altered levels in this project as potential biomarkers of lung cancer is questionable, and need further study as their level trends were often opposite to the trends found in other *in vitro* and *in vivo* studies of VOCs. The differences between the different *in vitro* studies are associated with different experimental design in terms of extraction technique, analytical technique, statistical analysis and cell culture conditions. Different cell culture medium and supplementation, cell passage number, applied control, cell culture vessel, seeding density and period of cultivation may have had an impact on the obtained differences.

The poor correlation between *in vivo* and *in vitro* studies of VOCs as potential biomarkers of cancer is associated with different experimental design (extraction technique, analytical

technique, statistical analysis etc.), but may also arise from complex transition mechanisms of VOCs produced by tumour cells in the body and found in breath or biofluids, from exogenous VOCs being included in the predictive models of cancer and/or from problems with *in vitro* culture as a model of *in vivo* VOC presence in breath and biofluids. In addition, there is a generally relatively lower number of *in vitro* studies performed to date, in comparison to VOC studies of breath samples and biofluids. More *in vitro* studies may bring more correlations.

One of the underestimated problems relating to the *in vitro* analysis of VOCs is the influence of the environment in which the cells are cultivated on the VOC patterns detected. This environment includes the type of cell culture medium, the type of culture dish, hypoxia or hyperoxia conditions of the culture, 2-D or 3-D cultures etc. This means that the cells consume a compound from the medium/culture flask, metabolise it and then release VOC by-products of this metabolism into the HS/cell medium. Such products can be biomarkers of adaptations in metabolism between cancer and normal cells, in response to the particular conditions, rather than being biomarkers of disease. *In vivo,* the same compounds may simply not be available to the tumour cells and consequently the same products would not be present in breath. Moreover, it is not known what impact some VOCs emitted from PS (or other polymers used in cell culture) may have on cultured cells. For example, the observed metabolism of styrene and ethylbenzene in the MT experiment might be a mechanism of detoxification as a response to low but constant styrene and ethylbenzene concentrations.

Nevertheless, studies over the past 25 years have revealed that there are thousands of cancer-related mutations, many of them involved in core signalling pathways and carcinogenesis processes. They are the result of an adaptation of tumour cell metabolism, supporting their growth and survival. Some of these metabolic changes are obligatory for tumorgenesis and therefore they are distinctive features of cancer. These alternations are surely reflected in different VOC patterns produced or metabolised by cancer cells *in vitro*. The MT and TD *in vitro* analyses as well as other *in vitro* and *in vivo* studies of volatiles as potential biomarkers of lung cancer show that compounds common to all matrices exist, regardless of the potential limitations of the *in vivo* and *in vitro* approaches.

Researchers take different approaches when looking for potential biomarkers of cancer. The first decision is whether to use an *in vivo* or *in vitro* system for study. The aim is to

apply differential VOCs of cancer to a device that will enable the detection of cancer in a patient with 100% certainty, ideally noninvasively (patient-friendly), as the less invasive a procedure is, the cheaper and more simple it will be to conduct. Whether it is breath, blood, urine, or any other sample coming from the patient, at this stage none of these matrices is ideal for looking for potential volatile biomarkers. The main reason is the uncertain origin of the detected VOCs, as their patterns may depend not only on the presence of the disease, but also on a long list of other variables such as genetic and environmental factors, age, gender, and so forth.

Therefore, it seems obvious to complement *in vivo* studies with an investigation of the VOC profiles produced by tumours at the microcellular level, where an explanation of the presence of a compound in the chromatogram is more straightforward. Studies on cells are of great informative value about the biochemistry of tumours and are valuable tools in advancing effective cancer diagnosis, regardless of some uncertainties arising. Interestingly however, VOC fingerprints were shown to be different between not only cancer cells and non-transformed cells but also between the cell lines of different types of cancer and cell lines of the same type of cancer. The latter phenomenon might be a result of various mutations carried by different cell lines, variability in expressed receptors, and different proliferation rates and metastatic potential of the cell lines, as all of these variables have been shown to have differentiating VOC patterns. This may aid not only an early detection of cancer but also differentiation between cancer stages, treatment monitoring and treatment choice.

Without doubt, more studies are needed for the comparison of VOCs produced by tumour cells to the ones found in breath or biofluids, as well as to compare VOC patterns generated by many cell lines and primary tumour samples, in order to profile as many cell lines as possible, so that an attempt can be made to find the common VOCs for particular types of cancer. Ideally, research should be directed to comparing VOC patterns in the HS of primary cancer cells or tissues of one particular patient with the compounds detected in breath, urine, and/or blood of the same patient. On the other hand, multiple cell-lines and many samples from different patients must be studied to reflect the natural diversity of lung cancer tumours.

Studies of the “scent of cancer” are elegant in the simplicity of the idea; however there are still limitations in applying this idea clinically, regardless of the technique used. At

present, it cannot be stated with any certainty that a particular VOC is a biomarker of cancer. The analysis of breath and other matrices in the investigation of the potential biomarkers of cancer is still in its infancy. Evidently, large-scale screening studies are first required in order to describe the normal profiles of VOCs in all the matrices being studied. Knowledge of the VOC concentration ranges for a normal, non-diseased state and validation studies using larger populations in relation to all forms of cancer, will further evaluate the promising results of existing studies of these diseases. And here surely the path to the use of VOCs as "smellprints" of cancer in the clinic lies in using information gleaned from a variety of different approaches in complement.

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Appendix A

Principles of Gas Chromatography – Mass Spectrometry

Chromatography is a method for the separation of the components of a mixture. It is based on the different affinities of the different sample components for the stationary phase and the mobile phase of the chromatographic method. Gas chromatography (GC) is a common type of chromatography used in analytical chemistry for the analysis of compounds that can be vaporised without decomposition [1]. In GC, the mobile phase is a carrier gas (usually an inert gas). The most commonly used inert gas in GC is helium (over 90% of instruments), however, the use of hydrogen provides improved separations [2]. On the other hand, hydrogen can react with the sample, converting it into another substance and there is a risk of explosion with its use [2,3]. The stationary phase is a thin layer of solid polymer or liquid on an inert solid support, inside a piece of glass, fused silica or stainless steel tubing called a GC column [4]. There are two types of GC depending on the type of equilibration process which is dependent on the type of the stationary phase. In adsorption chromatography (gas-solid chromatography), the stationary phase is solid, while in partition chromatography (gas-liquid chromatography) the stationary phase is liquid [1].

The gas chromatograph is an instrument used to perform gas chromatography. A diagram of gas chromatography conjugated to mass spectrometry (MS) is shown in Figure A1. The sample is injected into the column through a rubber septum. The injector port, oven and detector are set to high temperatures so that the vapour pressure of the sample is at least 10 torr. The vaporised sample is transported with a carrier gas through the column contained in the temperature regulated oven. The compounds coming off the column are automatically detected in the various types of detector.

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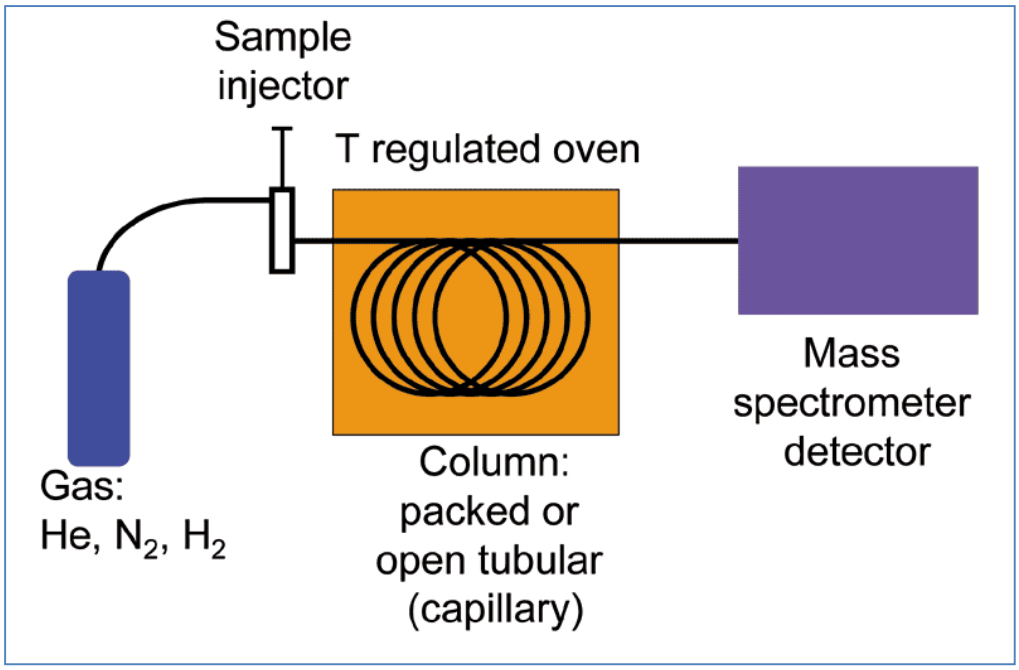


Figure A1 Diagram of gas chromatography - mass spectrometry [5].

There are two types of GC column: packed and capillary (open tubular). Packed columns contain an inert, finely divided, solid support material which is coated with liquid stationary phase. They are usually 1.5 - 10 m in length and 2 - 4 mm in ID. Capillary columns can be 5 - 120 m long (30 m is a standard length) and have an ID of a few tenths of a millimetre. There are two main types of capillary column: wall-coated open tubular (WCOT) or support-coated open tubular (SCOT). WCOT columns are capillary tubes whose walls are coated with liquid stationary phase. The inner wall of the SCOT column is lined with a thin layer of support material (such as diatomaceous earth), onto which the stationary phase has been bonded. WCOT columns are generally more efficient than SCOT columns and both types of capillary column are more efficient than packed columns [4]

The separation of compounds depends on the dimensions of the column (length, ID and film thickness) as well as the phase properties (e.g. 5% phenyl polysiloxane). Due to differences in chemical properties, different compounds will be retained to a greater or lesser extent by the stationary phase when travelling through the column and will elute from the column at different times (this is called the retention time). Each component of

[5] Murray, K. (2006) 'Gas chromatography mass spectrometry schematic', on the *Wikimedia Commons* website <https://commons.wikimedia.org/wiki/File:Gcms_schematic.gif> [accessed: 01/08/2016]

the sample ideally produces a specific peak that is recorded electronically as a chromatogram. The size of the peaks depends on the amount of the corresponding compounds in the analysed sample. The peak is measured from the baseline to the top of the peak [3]. The comparison of retention times gives GC its analytical usefulness, as a particular compound at given GC conditions will always elute at the same time. However, the appearance of a compound peak in the chromatogram at a particular RT does not guarantee the presence of that particular compound. This depends on the complexity of the sample mixture. In order to enable more confident compound identification a range of detectors can be used such as FID, TCD and MS.

Mass spectrometry is an analytical technique for the identification of both high and low MW compounds. A mass spectrometer consists of three main parts: the ion source, the mass analyser and the detector. First the ion source ionises compound molecules. Next, the mass analyser separates the ions according to their m/z ratio. The ions are then collected by the detector and a plot of the ion signal as a function of the m/z ratio is produced, called a mass spectrum. Mass spectra are used for the determination of the elemental or isotopic composition of a sample and the masses of analyte molecules, and for elucidation of the chemical structures of the analytes. The electron ionisation (EI) and chemical ionisation (CI) ion sources as well as the mass analyser and detector require a vacuum [1].

EI is the most frequently used method for the ionisation of chemical species. In this method, a gaseous sample is ionised by an electron beam emitted from a heated wire filament according to the following reaction [1]:

$$\mathbf{M + e^- \rightarrow M^{+\bullet} + 2e^-}$$

M is the parent molecule
e^- is the electron from the heated filament
$M^{+\bullet}$ is the molecular ion
$2e^-$ is the electron from the filament and the electron removed from the parental molecule

As a result of a collision between the parent molecule (M) and a high-energy (70 eV) electron from the filament (e^-), an electron from the parent molecule is removed and the parent molecule is converted into a positive ion with an odd number of electrons (molecular ion $M^{+\bullet}$). This molecular ion is a non-dissociative result of EI (Fig. 3) and it

has the same mass as the neutral compound molecule, because the loss of the electron is negligible in relation to mass spectrometer resolution. Because the molecular ion has absorbed an excess of energy during the ionisation process, it may break into fragments (the primary product ion and the neutral fragment/fragments or the free radical). This is a dissociative result of EI (Fig. 3). Each primary product ion can undergo fragmentation into secondary product ions and so on. The ions which appear in the mass spectrum are called fragment ions. Under particular EI conditions, each compound has its own characteristic fragmentation fingerprint. Because of this fragmentation, EI is called a hard ionisation technique. It has its advantages and disadvantages. Fragmentation patterns help in establishing the chemical structure of an analyte, however, an excessive fragmentation may result in the absence of a molecular ion in the mass spectra (hindering compound identification) [6].

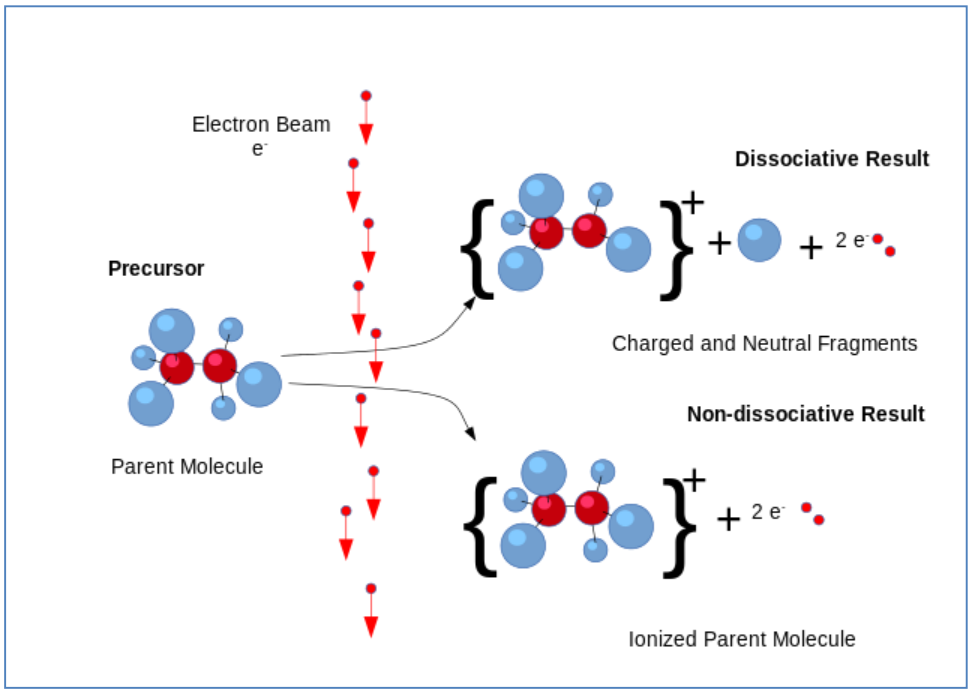


Figure A2 Diagram of electron ionisation (EI) [7].

Ions generated in the ion source are separated according to their m/z ratio, typically by being accelerated in the mass analyser. In this project, quadrupole mass spectrometers (Fig. A3 and A4) were used with an electron multiplier as a detector. Quadrupole mass

[6] Robinson, J.W., Skelly Frame, E.M. and Frame, G.M. (2014) 'Undergraduate instrumental analysis', 7th edition, Boca Raton: CRC Press

[7] Mason, M. (2015) 'Diagram of electron ionisation' on the *Wikimedia Commons* website <https://commons.wikimedia.org/wiki/File:Electron_Ionization.svg> [accessed: 01/08/2016]

analysers are ideally suited for GC analysis as they are compact and relatively cheap. This type of mass analyser is made up of four parallel cylindrical rods. The rods create a radiofrequency (RF) field, filtering the ions. Only ions with a particular m/z ratio will go through the field taking a 'stable path' and will reach the detector. Other ions will collide with the rods. The operator can scan for a range of m/z ions via continuous changes in the applied RF voltage [1].

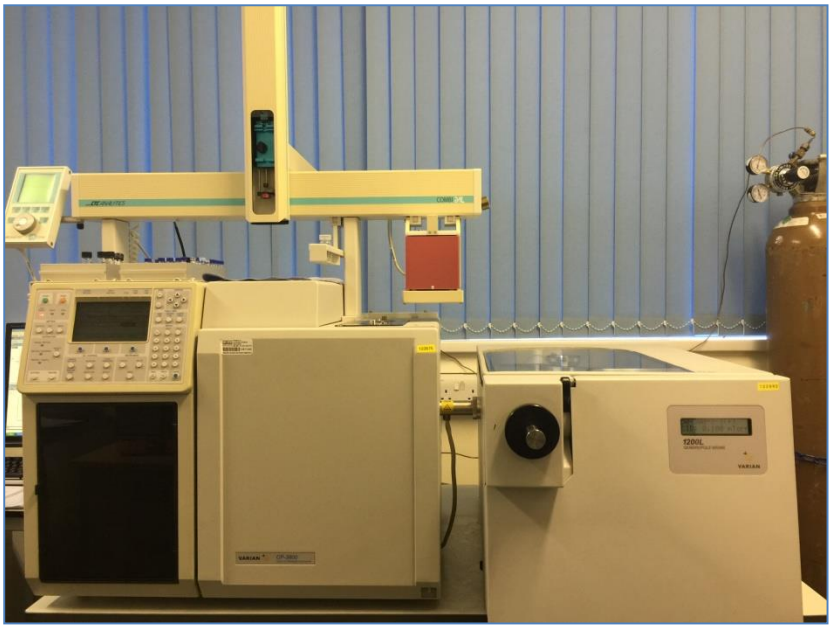


Figure A3 Varian CP-3800 gas chromatograph and 1200 MS/MS mass spectrometer.

Figure A4 PerkinElmer Turbomatrix 300 thermal desorber, Clarus 800 mass spectrometer and Clarus 5800 gas chromatograph (from left to right).

Appendix B

Results of the Shapiro-Wilk test of normality

Table B1 Results of Shapiro-Wilk test of normality with Lilliefors significance correction for the MonoTrap method development data.

Table B2 Results of Shapiro-Wilk test of normality with Lilliefors significance correction for the MonoTrap experiment data.

Table B3 Results of Shapiro-Wilk test of normality with Lilliefors significance correction for the Thermal desorption method development data.

Table B4 Results of Shapiro-Wilk test of normality with Lilliefors significance correction for the Thermal Desorption experiment data.

Table B1 Results of Shapiro-Wilk test of normality with Lilliefors significance correction for the MonoTrap method development data. White: the Sig. value was $p > 0.05$; therefore the data was normal; Green: Sig. value was $p < 0.05$; therefore the data significantly deviated from a normal distribution; Grey: compound not detected. The number of degrees of freedom was df = 3. ACN: acetonitrile; CHF: chlororform; DCM: dichloromethane; EtAc: ethyl acetate; EtOH: ethanol; Sig.: significance value; Temp.: temperature.

Volatile organic compound	Statistic & Sig.	Disk type		Mode			Time				Temp.
		DCC18	DSC18	Floating	Stirring	Static	60 min	90 min	120 min	150 min	45°C
Acetophenone	Statistic	.802		.999	.958	.777	.878	.999	.954	.990	.802
	Sig.	.118		.954	.605	.060	.319	.931	.588	.806	.119
Benzaldehyde	Statistic	.856		.805	.988	.916	.855	.993	.855	.998	.904
	Sig.	.256		.125	.786	.439	.253	.843	.253	.915	.398
Cyclohexanol	Statistic	.973	.941	.947	.969	.755	.800	.842	.970	.923	.752
	Sig.	.687	.532	.554	.661	.011	.114	.220	.668	.463	.005
2-Ethylhexanol	Statistic	.998	.1.000	.904	.917	.981	.838	.852	.980	.829	.912
	Sig.	.908	.982	.398	.441	.736	.209	.246	.727	.185	.424
Heptanal	Statistic	.996	.923	1.000	.876	.970	.812	.941	.976	.916	.974
	Sig.	.881	.463	.996	.312	.667	.144	.533	.702	.439	.693
Nonanal	Statistic	.802	.951	.764	.840	.822	.964	.984	.938	.942	.830
	Sig.	.120	.573	.031	.214	.168	.637	.756	.520	.537	.189
1-Octanol	Statistic	.773	.967	.970	.786	.785	.942	.957	.890	.902	
	Sig.	.051	.653	.669	.081	.079	.537	.600	.354	.391	
Styrene	Statistic	.797	.842	.927	.954	.951	.842	.940	.839	.878	.944
	Sig.	.108	.220	.477	.589	.576	.220	.526	.213	.317	.545
Volatile organic compound	**Statistic & Sig.**	**Temp.**		**Salt**			**pH**			**Solvent type**	
		60°C	**75°C**	**0%**	**15%**	**30%**	**3.0**	**7.5**	**10.0**	**DCM**	**CHF**
Acetophenone	Statistic	.996	.897	.817	1.000	.987	.938	.791	.997	.999	.964
	Sig.	.882	.377	.156	1.000	.780	.521	.092	.904	.956	.637
Benzaldehyde	Statistic	.828	.844	.998	.812	.980	1.000	.997	.897	.969	.792
	Sig.	.183	.225	.923	.144	.726	.959	.896	.375	.664	.094
Cyclohexanol	Statistic	.822	.998	.855	.945	.784	.995	.997	.795	.837	.932
	Sig.	.169	.906	.253	.546	.077	.868	.902	.103	.206	.497

Table B1 Cont'd

Volatile organic compound	Statistic & Sig.	Temp.		Salt			pH			Solvent type	
		60°C	75°C	0%	15%	30%	3.0	7.5	10.0	DCM	CHF
2-Ethylhexanol	Statistic	.995	1.000	.964	.842	.989	.757	.997	.993	.972	.999
	Sig.	.870	.959	.637	.220	.797	.016	.900	.839	.679	.931
Heptanal	Statistic	.773	.938	.907	.893	.791	.949	.974	.999	.787	.824
	Sig.	.052	.520	.407	.363	.093	.567	.691	.928	.085	.174
Nonanal	Statistic	.862	.993	.997	.778	.998	.875	.845	.949	.991	.845
	Sig.	.274	.843	.891	.064	.911	.311	.226	.567	.822	.227
1-Octanol	Statistic	.841	.972	.923	.891	.993	.935	.995	.850	.851	.913
	Sig.	.216	.681	.463	.356	.843	.508	.863	.241	.244	.427
Styrene	Statistic	.815	.929	.987	.793	.901	1.000	.964	1.000	.866	.878
	Sig.	.150	.483	.780	.098	.388	.985	.637	.962	.286	.317
Volatile organic compound	**Statistic & Sig.**	**Solvent type**					**Solvent volume**				**Sample volume**
		ACN	**EtOH**	**Hexane**	**EtAc**	**IPA**	**70 µl***	**100 µl**	**130 µl**	**160µl**	**15 ml**
Acetophenone	Statistic		.998		.986			.997			.855
	Sig.		.911		.776			.892			.255
Benzaldehyde	Statistic		.999		.998			.891	.896	.895	.842
	Sig.		.929		.921			.357	.372	.368	.220
Cyclohexanol	Statistic	.999	.929	.754	.992	.923		.807	.810	.886	.972
	Sig.	.955	.485	.010	.831	.463		.132	.138	.341	.679
2-Ethylhexanol	Statistic	.969	1.000	.931	.788	.952		.989	.898	.901	.855
	Sig.	.663	1.000	.492	.086	.577		.798	.795	.389	.253
Heptanal	Statistic	.876	.991	.954	.977	.787		.993	.832	.905	.987
	Sig.	.312	.823	.587	.712	.084		.843	.194	.401	.780
Nonanal	Statistic	.883	.993	1.000	.915			.941	.993	.751	.964
	Sig.	.333	.835	1.000	.433			.531	.843	.002	.637
1-Octanol	Statistic			.981	.999			.969	.845	.888	.939
	Sig.			.739	.927			.663	.228	.348	.525

Table B1 Cont'd

Volatile organic compound	Statistic & Sig.	Solvent type					Solvent volume				Sample volume
		ACN	EtOH	Hexane	EtAc	IPA	70 µl*	100 µl	130 µl	160µl	15 ml
Styrene	Statistic	.899	.772	.869	.907	.787		.842	1.000	.959	1.000
	Sig.	.383	.050	.294	.407	.085		.220	1.000	.612	.962
Volatile organic compound	**Statistic & Sig.**	**Sample volume**									
		20 ml	**25 ml**	**30 ml**							
Acetophenone	Statistic	.982	.976	.769							
	Sig.	.744	.705	.042							
Benzaldehyde	Statistic	.951	.998	.983							
	Sig.	.576	.918	.747							
Cyclohexanol	Statistic	.970	.987	.987							
	Sig.	.669	.780	.780							
2-Ethylhexanol	Statistic	.900	1.000	.923							
	Sig.	.385	.958	.463							
Heptanal	Statistic	.951	.946	.828							
	Sig.	.573	.551	.183							
Nonanal	Statistic	.831	1.000	.998							
	Sig.	.191	.969	.915							
1-Octanol	Statistic	.818	1.000	.754							
	Sig.	.157	.984	.008							
Styrene	Statistic	.997	.998	.980							
	Sig.	.896	.905	.726							

Table B2 Results of Shapiro-Wilk test of normality with Lilliefors significance correction for the MonoTrap experiment data. Col: collection; df: degrees of freedom; Sig.: significance value; White: the Sig. value was $p > 0.05$; therefore the data was normal; Green: Sig. value was $p < 0.05$; therefore the data significantly deviated from a normal distribution; Grey: compound not detected. Blue: The number of degrees of freedom was df = 9 unless otherwise stated.

Volatile organic compound	Statistic & Sig.	A549 Col 1		A549 Col 2		NHLF Col 1		NHLF Col 2		NHLF Col 3	
		Sample	Control	Sample	Control	Sample	Control	Sample	Control	Sample	Control
Hexanal	Statistic	.832	.762	.815	.850	.957	.874	.933	.902	.875	.893
	Sig.	.047	.007	.031	.074	.765	.135	.511	.265	.140	.216
Hexane, 2,3,5-trimethyl	Statistic	.885	.958	.670							
	Sig.	.177	.773	.001							
Heptane, 2,4-dimethyl	Statistic	.840	.816	.658	.673			.929	.911	.841	.983
	Sig.	.058	.031	.000	.001			.470	.324	.060	.977
Heptene, 2,4-dimethyl, 1-	Statistic	.839	.878	.633	.652			.778	.672	.666	.681
	Sig.	.056	.149	.000	.000			.011	.001	.001	.001
Heptane, 2,3-dimethyl	Statistic	.825	.849	.624	.677						
	Sig.	.040	.072	.000	.001						
Octane, 4-methyl	Statistic	.832	.845	.660	.747			.818	.753	.888	.852
	Sig.	.047	.065	.000	.005			.033	.006	.192	.078
p-Xylene	Statistic	.896	.807	.888	.879	.902	.877	.944	.236	.770	.888
	Sig.	.228	.025	.190	.155	.265	.147	.623	.049	.009	.191
Ethylbenzene	Statistic	.877	.821	.803	.934	.897	.939	.939	.892	.947	.974
	Sig.	.144	.036	0.22	.525	.233	.575	.568	.209	.660	.927
Cyclohexanol	Statistic	.854	.817	.521	.860	.969	.807	.953	.896	.910	.942
	Sig.	.083	.032	.000	.095	.889	.025	.728	.229	.314	.599
Styrene	Statistic	.920	.891	.903	.885	.966	.940	.971	.844	.862	.866
	Sig.	.388	.206	.270	.177	.857	.577	.901	.065	.102	.110
2-Ethenyl-2-butenal	Statistic		.932		.936	.773	.794	.921	.813	.654	.881
	Sig.		.504		.543	.010	.017	.398	.029	.000	.160
Cyclohexanone	Statistic		.815		.842	.883	.935	.885	.763	.850	.981
	Sig.		.030		.060	.168	.534	.179	.008	.074	.969

Table B2 Cont'd

Volatile organic compound	Statistic & Sig	A549 Col 1		A549 Col 2		NHLF Col 1		NHLF Col 2		NHLF Col 3	
		Sample	Control	Sample	Control	Sample	Control	Sample	Control	Sample	Control
3-Heptanol	Statistic	.848	.910	.690	.687	.963	.855	.905	.928	.982	.925
	Sig.	.071	.318	.001	.001	.832	.084	.279	.460	.975	.435
Heptanal	Statistic	.731	.924	.932	.975	.926	.844	.924	.851	.903	.907
	Sig.	0.003	.427	.501	.930	.444	.065	.428	.077	.268	.294
Benzaldehyde	Statistic	.888	.754	.883	.821	.827	.940	.839	.953	.869	.765
	Sig.	.190	.006	.168	.035	.042	.585	.056	.728	.195	.008
Pentanoic acid, 2,4-dimethyl-3-oxo-, methyl ester	Statistic				.869	.757	.859	.884	.768	.842	.855
	Sig.				.119	.006	.095	.172	.009	.061	.085
2-Ethylhexanol	Statistic	.910	.873	.672	.886	.983	.919	.928	.880	.955	.890
	Sig.	.317	.133	.001	.180	.977	.380	.461	.155	.746	.200
1-Heptanol	Statistic	.885	.928								
	Sig.	.176	.458								
Phenol	Statistic	.891	.855	.930	.981	.781	.860	.939	.924	.834	.787
	Sig.	.207	.085	.486	.970	.013	.096	.573	.424	.049	.014
Octanal	Statistic	.860	.957	.799	.857	.815	.765	.932	.858	.927	.963
	Sig.	.095	.763	.020	.089	.030	.008	.502	.092	.449	.833
Decane	Statistic	.750	.868	.807	.891	.965	.928	.954	.909	.910	.948
	Sig.	.005	.118	.025	.207	.852	.460	.738	.309	.319	.665
Benzyl alcohol	Statistic	.981	.950	.973	.826	.678	.700	.769	.740	.877	.955
	Sig.	.970	.686	.923	.040	.001	.001	.009	.004	.145	.742
Phenyl ethanol	Statistic	.896	.912	.893	.903	.853	.839	.884	.931	.929	.900
	Sig.	.231	.330	.214	.273	.081	.056	.173	.495	.473	.252
Acetophenone	Statistic	.953	.963	.746	.925	.634	.862	.902	.919	.955	.860
	Sig.	.721	.826	.005	.431	.000	.101	.261	.382	.742	.096
1-Octanol	Statistic	.891	.785	.763	.940	.841	.951	.886	.971	.896	.928
	Sig.	.203	.014	.008	.584	.059	.701	.182	.900	.231	.462

Table B2 Cont'd

Volatile organic compound	Statistic & Sig	A549 Col 1		A549 Col 2		NHLF Col 1		NHLF Col 2		NHLF Col 3	
		Sample	Control	Sample	Control	Sample	Control	Sample	Control	Sample	Control
Decane, 2,4,6-trimethyl	Statistic	.778	.661								
	Sig.	.011	.000								
α-Cumyl alcohol	Statistic	.855	.880	.941	.868	.759	.854	.918	.955	.874	.870
	Sig.	.084	.156	.587	.117	.007	.082	.374	.749	.137	.122
Undecane	Statistic	.978	.885	.731	.940	.947	.936	.883	.813	.897	.894
	Sig.	.954	.176	.003	.586	.662	.543	.168	.029	.235	.220
Nonanal	Statistic	.777	.880	.817	.809	.694	.735	.850	.750	.689	.786
	Sig.	.011	.156	.032	.026	.001	.004	.075	.005	.001	.014
Undecane,4-methyl	Statistic	.813	.913	.637	.925	.763	.844	.845	.840	.952	.902
	Sig.	.029	.335	.000	.437	.008	.063	.066	.057	.712	.267
2-Nitrophenol	Statistic	.907	.896	.625	.942	.924	.945	.941	.949	.784	.914
	Sig.	.297	.229	.000	.604	.428	.637	.588	.679	.014	.343
1-Nonanol	Statistic	.119	.953	.862	.853	.928	.965	.827	.950	.931	.901
	Sig.	.306	.725	.101	.080	.461	.850	.042	.693	.490	.257
4-Decanol	Statistic	.973	.839	.872	.935	.912	.928	.905	.961	.970	.960
	Sig.	.919	.056	.128	.528	.333	.462	.285	.809	.897	.797
Dodecane	Statistic	.721	.786	.922	.891	.866	.910	.970	.931	.860	.933
	Sig.	.003	.014	.410	.207	.113	.318	.894	.493	.095	.508
Decanal	Statistic	.848	.943	.726	.796	.868	.739	.846	.887	.887	.858
	Sig.	.071	.609	.003	.018	.117	.004	.068	.186	.187	.090
Benzene, 1,3-di-*tert*-butyl	Statistic	.969	.934	.956	.930	.958	.930	.883	.887	.964	.876
	Sig.	.883	.517	.754	.479	.778	.483	.167	.185	.843	.141
Tridecane	Statistic	.685	.963	.669	.897	.969	.958	.851	.949	.920	.941
	Sig.	.001	.825	.001	.232	.886	.778	.076	.676	.390	.588
Decane, 2,3,5-trimethyl	Statistic	.741	.643	.631	.672					.676	.690
	Sig.	.004	.000	.000	.001					.001	.001

Table B2 Cont'd

Volatile organic compound	Statistic & Sig	A549 Col 1		A549 Col 2		NHLF Col 1		NHLF Col 2		NHLF Col 3	
		Sample	Control	Sample	Control	Sample	Control	Sample	Control	Sample	Control
Propanoic acid, 2-methyl- 2,2-dimethyl-1-(2-hydroxy-1-methylethyl)-, propyl ester	Statistic	.816	.962	.664	.960	.807	.893	.911	.850	.935	.904
	Sig.	.031	.819	.001	.796	.025	.214	.325	.074	.530	.273
Propanoic acid, 2-methyl-, 3-hydroxy-2,4,4-trimethylpentyl ester	Statistic	.814	.942	.894	.881	.800	.953	.973	.953	.924	.769
	Sig.	.029	.598	.218	.161	.021	.727	.920	.722	.424	.009
6-Dodecanol	Statistic	.847	.721	.811	.954	.989	.954	.926	.969	.939	.908
	Sig.	.068	.003	.028	.734	.995	.738	.444	.890	.573	.302
Tetradecane	Statistic	.920	.873	.800	.929	.958	.953	.963	.958	.886	.870
	Sig.	.393	.134	.020	.470	.776	.718	.827	.776	.182	.122
Dodecanal	Statistic	.859	.882	.801	.897	.895	.961	.866	.868	.846	.958
	Sig.	.093	.165	.021	.234	.224	.806	.111	.118	.067	.782
Geranyl acetone	Statistic	.911	.721	.875	.779	.941	.876	.969	.910	.800	.946
	Sig.	.321	.003	.138	0.12	.595	.142	.888	.319	.021	.646
2,6-Di-*tert*-butyl-1,4-benzoquinone	Statistic	.922	.945	.888	.743	.759	.922	.852	.896	.914	.828
	Sig.	.405	.640	.191	.005	.007	.407	.078	.231	.346	.042
Tridecanal	Statistic	.958	.995	.901	.954	.903	.941	.927	.905	.841	.856
	Sig.	.775	1.000	.255	.739	.270	.588	.456	.282	.059	.087
2,4-Di-*tert*-butylphenol	Statistic	.911	.863	.775	.899	.861	.853	.710	.698	.773	.737
	Sig.	.326	.104	.011	.247	.098	.081	.002	.001	.010	.004
2-Tetradecanone	Statistic	.843	.983	.980	.739	.908	.856	.870	.937	.999	.949
	Sig.	.062	.977	.001	.004	.302	.088	.123	.556	.1.000	.680
Propanoic acid, 2-methyl-, 1-(1,1-dimethyl)-2-methyl-1,3-propanediyl ester	Statistic	.805	.684	.544	.894	.641	.819	.803	.909	.843	.918
	Sig.	.023	.001	.000	.221	.000	.033	.022	.306	.062	.377
Tetradecanal	Statistic	.957	.903	.896	.950	.900	.878	.910	.901	.809	.880
	Sig.	.764	.270	.230	.692	.253	.151	.315	.257	.026	.156

Table B2 Cont'd

Volatile organic compound	Statistic & Sig.	A549 Col 1		A549 Col 2		NHLF Col 1		NHLF Col 2		NHLF Col 3	
		Sample	Control	Sample	Control	Sample	Control	Sample	Control	Sample	Control
2-Methoxy-diphenylmethane	Statistic	.872	.819	.757	.833	.882	.836		.844	.843	.953
	Sig.	.129	.033	.007	.048	.164	.052		.064	.062	.727
Dodecyl acrylate	Statistic	.756	.693	.848	.829	.723	.781	.841	.886	.827	.935
	Sig.	.006	.001	.072	.044	.004	.018	.059	.180	.075	.595
	df					8	8	8	8	7	7
2-Pentadecanone	Statistic	.833	.943	.743	.815	.894	.903	.920	.966	.891	.948
	Sig.	.049	.614	.004	.030	.221	.273	.394	.855	.203	.665
Pentadecanal	Statistic	.902	.929	.836	.966	.942	.321	.922	.916	.915	.920
	Sig.	.265	.475	.052	.862	.603	.401	.410	.357	.352	.392
Benzene, 1,1'-(1,2-cyclobutanediyl)bis-, cis-	Statistic	.923	.887	.952	.986	.786	.905	.934	.881	.771	.896
	Sig.	.418	.185	.711	.989	.014	.284	.522	.161	.010	.228
Hexadecanal	Statistic	.877	.877	.826	.821	.772	.867	.725	.813	.749	.700
	Sig.	.144	.147	.041	.035	.010	.114	.003	.029	.005	.001
Phthalic acid, isobutyl nonyl ester	Statistic	.809	.888	.748	.919	.798	.892	.766	.844	.729	.789
	Sig.	.026	.188	.005	.386	.039	.286	.008	.064	.003	.015
	df					7	7				
Heptadecanal	Statistic	.963	.871	.908	.885	.867	.949	.944	.869	.919	.912
	Sig.	.825	.127	.304	.176	.114	.675	.624	.120	.387	.330
2-Nonadecanone	Statistic	.972	.843	.928	.907	.807	.881	.832	.869	.914	.867
	Sig.	.909	.062	.465	.294	.024	.161	.062	.149	.345	.115
	df							8	8		
Octadecanal	Statistic	.903	.918	.928	.849	.886	.878	.782	.704	.701	
	Sig.	.269	.373	.465	.074	.180	.151	.018	.003	.001	
	df							8	8		
Dodecanoic acid, isooctyl ester	Statistic	.793	.866			.797	.787	.909	.925		
	Sig.	.017	.112			.019	.014	.308	.434		

Table B3 Results of Shapiro-Wilk test of normality with Lilliefors significance correction for the Thermal desorption method development data. White: the Sig. value was $p > 0.05$; therefore the data was normal; Green: Sig. value was $p < 0.05$; therefore the data significantly deviated from a normal; Grey: compound not detected. The number of degrees of freedom was df = 3 for all data.

Volatile organic compound	Statistic & Sig.	Sample volume		Split ratio		
		100 ml	200 ml	A	B	C
Acetophenone	Statistic	1.000	.986	.942	.985	.991
	Sig.	.973	.771	.534	.768	.814
Benzaldehyde	Statistic	.768	.896	.987	.783	.943
	Sig.	.041	.374	.784	.073	.539
Benzene	Statistic	.992	.965	.829	.801	.986
	Sig.	.833	.641	.186	.116	.775
Decane	Statistic	.884	.959	.873	.797	.997
	Sig.	.337	.612	.303	.107	.896
Ethylbenzene	Statistic	.951	.982	.787	.949	1.000
	Sig.	.572	.744	.085	.565	1.000
2-Ethylhexanol	Statistic	.880	.936	.873	.825	.997
	Sig.	.325	.510	.304	.176	.891
Heptane	Statistic	.946	.793	.938	.974	.872
	Sig.	.551	.097	.521	.693	.302
2-Pentanone	Statistic	.777	.841	.948	.904	.984
	Sig.	.060	.216	.563	.399	.754
2-Methylfuran	Statistic	.990	.844	.768	.999	.792
	Sig.	.807	.225	.041	.950	.095
Styrene	Statistic	.850	.901	.999	.898	.839
	Sig.	.240	.388	.938	.381	.211

Table B4 Results of Shapiro-Wilk test of normality with Lilliefors significance correction for the Thermal Desorption experiment data. Col: collection; df: degrees of freedom; Sig.: significance value; White: the Sig. value was $p > 0.05$; therefore the data was normal; Green: Sig. value was $p < 0.05$; therefore the data significantly deviated from a normal distribution; Grey: compound not detected. The number of degrees of freedom was df = 9 for all data.

Volatile organic compound	Statistic & Sig.	A549 col 1		A549 col 2		BEAS-2B col 1		BEAS-2B col 2		Water	
		Sample	Control	Sample	Control	Sample	Control	Sample	Control	col 1	col 2
Acetone	Statistic	.898	.860	.832	.707	.813	.814	.765	.696	.911	.887
	Sig.	.241	.097	.047	.002	.029	.029	.008	.001	.321	.185
Acetophenone	Statistic	.887	.715	.835	.872	.851	.945		.928	.933	.924
	Sig.	.185	.002	.050	.129	.075	.632		.459	.510	.424
Benzaldehyde	Statistic		.851		.889		.941		.847	.929	.944
	Sig.		.076		.195		.592		.069	.472	.621
Benzene	Statistic	.822	.932	.916	.626	.877	.861	.499	.860	.951	.947
	Sig.	.037	.501	.364	.000	.147	.099	.000	.095	.699	.661
Benzothiazole	Statistic	.858	.875	.972	.935	.614	.617	.710	.865	.929	.963
	Sig.	.091	.141	.913	.528	.000	.000	.002	.109	.472	.829
Carbon disulfide	Statistic	.873	.894	.940	.874	.920	.854	.862	.929	.836	.852
	Sig.	.132	.217	.578	.134	.391	.083	.101	.469	.053	.078
Decane	Statistic	.935	.865	.940	.763	.946	.789	.935	.821	.925	.846
	Sig.	.529	.108	.582	.008	.648	.015	.532	.035	.434	.068
Dichloromethane	Statistic	.953	.893	.902	.889	.958	.941	.948	.952	.491	.767
	Sig.	.719	.215	.264	.197	.778	.595	.669	.710	.000	.008
2,4-Dimethylfuran	Statistic	.902	.567	.516	.889	.852	.674	.838	.891	.825	.909
	Sig.	.265	.000	.000	.194	.078	.001	.055	.206	.039	.310
2,4-Dimethyl-1-heptene	Statistic	.776	.875	.906	.925	.910	.755	.981	.854	.785	.949
	Sig.	.011	.139	.289	.438	.313	.006	.968	.083	.014	.679
Dodecane	Statistic	.901	.849	.933	.834	.927	.957	.823	.936	.969	.923
	Sig.	.259	.072	.515	.050	.455	.765	.037	.544	.887	.416

Table B4 Cont'd

Volatile organic compound	Statistic & Sig.	A549 col 1		A549 col 2		BEAS-2B col 1		BEAS-2B col 2		Water	
		Sample	Control	Sample	Control	Sample	Control	Sample	Control	Col 1	Col 2
Ethyl acetate	Statistic	.923	.684		.527	.922		.854		.798	
	Sig.	.418	.001		.000	.409		.082		.020	
Ethylbenzene	Statistic	.912	.909	.901	.868	.968	.912	.859	.982	.956	.912
	Sig.	.330	.306	.256	116	.874	.332	.093	.973	.755	.332
2-Ethylhexanol	Statistic	.880	.865	.952	.902	.909	.796	.854	.949	.743	
	Sig.	.157	.110	.709	.263	.310	.019	.082	.677	.004	
Heptane	Statistic	.926	.869	.738	.942	.916	.819	.830	.955	.931	.924
	Sig.	.444	.120	.004	.598	.363	.033	.045	.741	.489	.425
2-Methylbutanal	Statistic		.875		.968		.965		.918		
	Sig.		.139		.881		.848		.375		
Methylcyclohexane	Statistic	.820	.932	.826	.773	.815	.929	.941	.947	.961	.692
	Sig.	.034	.496	.040	.010	.030	.467	.594	.655	.804	.001
2-Methylfuran	Statistic	.900	.948	.958	.939	.851	.681	.971	.941	.945	.958
	Sig.	.253	.663	.777	.571	.077	.001	.906	.589	.632	.782
4-Methylheptane	Statistic	.857	.969	.878	.906	.922	.989	.991	.923	.837	.948
	Sig.	.089	.888	.150	.287	.409	.994	.997	.420	.053	.673
3-Methylheptane	Sig.	.738	.941	.886	.881	.835	.851	.945	.929	.837	.869
	Statistic	.004	.590	.182	.159	.050	.077	.630	.476	.053	.119
4-Methyloctane	Sig.	.769	.781	.850	.781	.917	.862	.884	.902	.648	.984
	Statistic	.009	.012	.074	.012	.365	.101	.173	.266	.000	.982
2-Methylpentane	Sig.	.676	.872	.655	.892	.640	.930	.911	.552	.903	.857
	Statistic	.001	.128	.000	.209	.000	.477	.323	.000	.271	.089
3-Methypentane	Sig.	.753	.902	.806	.939	.849	.719	.781	.952	.847	.893
	Statistic	.006	.265	.024	.576	.072	.002	.012	.711	.069	.213
Octane	Sig.	.909	.886	.946	.975	.893	.920	.904	.963	.855	.921
	Sig.	.311	.183	.646	.934	.212	.396	.273	.832	.084	.398

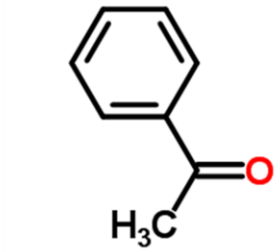
Table B4 Cont'd

Volatile organic compound	Statistic & Sig.	A549 col 1		A549 col 2		BEAS-2B col 1		BEAS-2B col 2		Water	
		Sample	Control	Sample	Control	Sample	Control	Sample	Control	Col 1	Col 2
Pentane	Statistic	.863	.888	.890	.877	.744	.875	.747	.923	.933	.901
	Sig.	.103	.190	.200	.146	.005	.141	.005	.419	.506	.259
2-Pentanone	Statistic	.921	.824	.811	.975	.933	.920	.919	.934	.837	
	Sig.	.403	.038	.027	.935	.511	.392	.380	.524	.054	
2,2,4,6,6-pentamethylheptane	Statistic	.893	.962	.90	.976	.924	.915	.830	.638	.918	.838
	Sig.	.216	.820	.271	.939	.422	.356	.044	.000	.374	.055
Styrene	Statistic	.890	.895	.869	.790	.907	.855	.976	.905	.966	.880
	Sig.	.198	.224	.120	.016	.293	.085	.941	.284	.857	.157
Tetrahydrofuran	Statistic	.927	.816	.857	.940	.685	.681	.890	.865		
	Statistic	.457	.031	.090	.581	.001	.001	.201	.109		
Chloroform	Sig.	.629	.787	.955	.932	.666	.670	.651	.680	.929	.468
	Statistic	.000	.014	.741	.498	.001	.001	.000	.001	.471	.000
2,3,5-Trimethylhexane	Sig.	.842	.820	.950	.826	.941	.863	.912	.922	.640	.987
	Statistic	.060	.034	.687	.041	.589	.102	.332	.412	.000	.991
Toluene	Sig.	.932	.953	.906	.703	.852	.851	.918	.911	.696	.989
	Statistic	.502	.725	.290	.002	.078	.077	.379	.323	.001	.994
tert-Butanol	Sig.	.946	.803	.971	.940	.812	.800	.968	.927		
	Statistic	.642	.022	.906	.579	.028	.020	.873	.456		
Undecane	Sig.	.845	.786	.847	.654	.948	.830	.909	.823	.886	.898
	Statistic	.066	.014	.069	.000	.668	.044	.312	.038	.181	.240

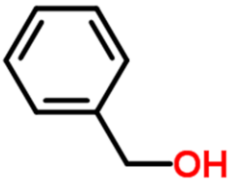
Appendix C

Chemical structures of VOCs found at altered levels in the MT and TD experiments

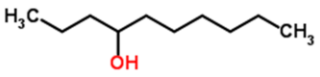
C.1 VOCs found at altered levels in the MT experiment



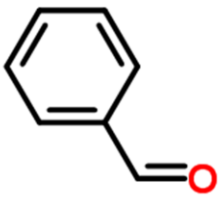
Acetophenone
CAS: 98-86-2
MW: 120
Molecular formula: C_8H_8O



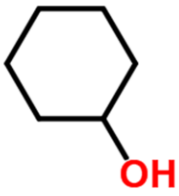
Benzyl alcohol
CAS: 100-51-6
MW: 108
Molecular formula: C_7H_8O



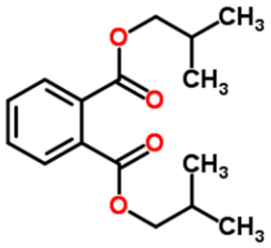
4-Decanol
CAS: 2051-31-2
MW: 158
Molecular formula: $C_{10}H_{22}$



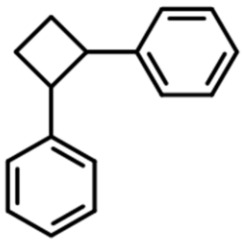
Benzaldehyde
CAS: 100-52-7
MW: 120
Molecular formula: C_7H_6O



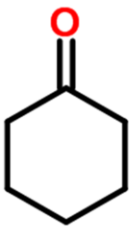
Cyclohexanol
CAS: 108-93-0
MW: 100
Molecular formula: $C_6H_{12}O$



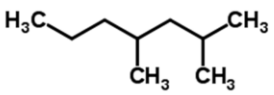
Diisobutyl phthalate
CAS: 84-69-5
MW: 278
Molecular formula: $C_{16}H_{22}O_4$



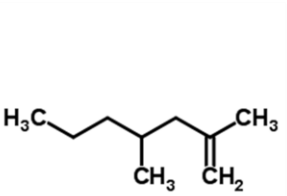
Benzene, 1,1'-(1,2-cyclobutanediyl)bis-, cis-
CAS: 7694-30-6
MW: 208
Molecular formula: $C_{16}H_{16}$



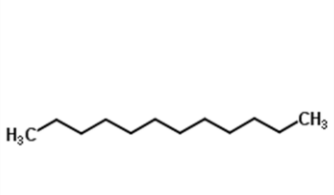
Cyclohexanone
CAS: 108-94-1
MW: 98
Molecular formula: $C_6H_{10}O$



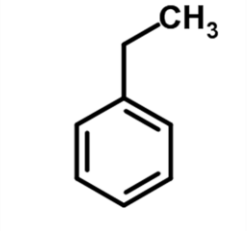
2,4-Dimethylheptane
CAS: 2213-23-2
MW: 128
Molecular formula: C_9H_{20}



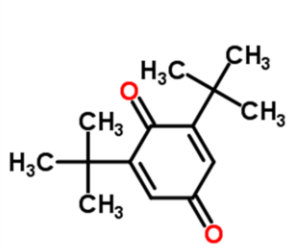
2,4-Dimethyl-1-heptene
CAS: 19549-87-2
MW: 128
Molecular formula: C_9H_{18}



Dodecane
CAS: 112-40-3
MW: 170
Molecular formula: $C_{12}H_{23}$

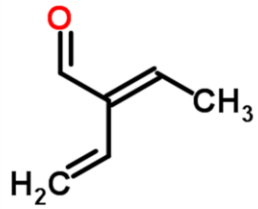


Ethylbenzene
CAS: 100-41-4
MW: 106
Molecular formula: C_8H_{10}

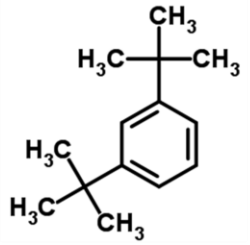


2,6-Di-*tert*-butyl-1,4-benzoquinone
CAS: 719-22-2
MW: 220
Molecular formula: $C_{14}H_{20}O_2$

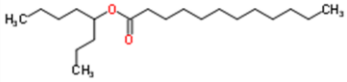
Dodecanal
CAS: 112-54-9
MW: 184
Molecular formula: $C_{12}H_{24}O$



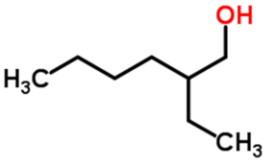
2-Ethenyl-2-butenal
CAS: 20521-42-0
MW: 96
Molecular formula: C_6H_8O



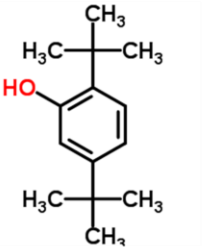
1,3-Di-*tert*-butylbenzene
CAS: 1014-60-4
MW: 190
Molecular formula: $C_{14}H_{22}$



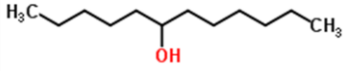
Dodecanoic acid , isooctyl ester
CAS: 84713-06-4
MW: 312
Molecular formula: $C_{20}H_{40}O_2$



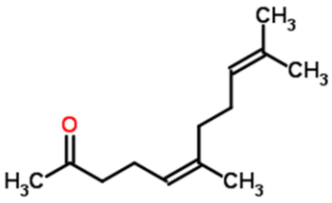
2-Ethylhexanol
CAS: 104-76-7
MW: 130
Molecular formula: $C_8H_{18}O$



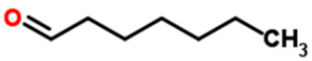
2,5-Di-tert-butylphenol
CAS: 5875-45-6
MW: 206
Molecular formula: $C_{14}H_{22}$



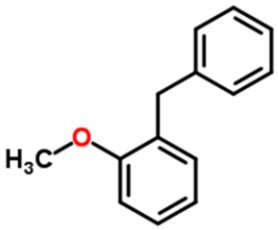
6-Dodecanol
CAS: 6836-38-0
MW: 186
Molecular formula: $C_{12}H_{26}$



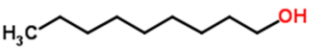
Geranyl acetone
CAS: 3796-70-1
MW: 194
Molecular formula: $C_{13}H_{22}O$



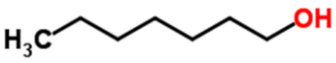
Heptanal
CAS: 111-71-7
MW: 114
Molecular formula: $C_7H_{14}O$



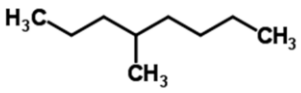
2-Methoxy-diphenylmethane
CAS: 883-90-9
MW: 198
Molecular formula: $C_{14}H_{14}$



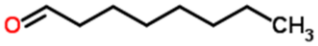
1-Nonanol
CAS: 143-08-8
MW: 144
Molecular formula: $C_9H_{20}O$



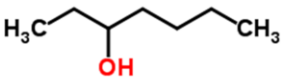
Heptanol
CAS: 111-70-6
MW: 116
Molecular formula: $C_7H_{16}O$



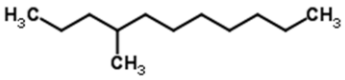
4-Methyloctane
CAS: 2216-34-4
MW: 128
Molecular formula: C_9H_{20}



Octanal
CAS: 124-13-0
MW: 128
Molecular formula: $C_8H_{16}O$

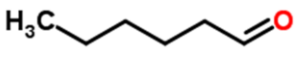


3-Heptanol
CAS: 589-82-2
MW: 116
Molecular formula: $C_7H_{16}O$

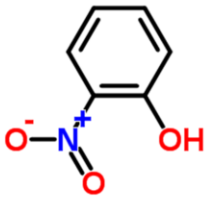


4-Methylundecane
CAS: 2980-69-0
MW: 170
Molecular formula: $C_{12}H_{26}$

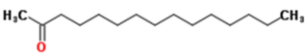
1-Octanol
CAS: 111-87-5
MW: 130
Molecular formula: $C_8H_{16}O$



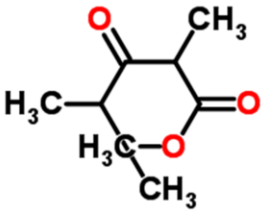
Hexanal
CAS: 66-25-1
MW: 100
Molecular formula: $C_6H_{12}O$



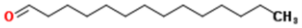
2-Nitrophenol
CAS: 88-75-5
MW: 139
Molecular formula: $C_6H_5NO_3$



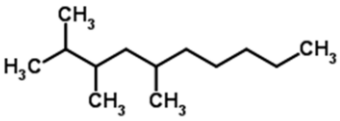
2-Pentadecanone
CAS: 2345-28-0
MW: 226
Molecular formula: $C_{15}H_{30}O$



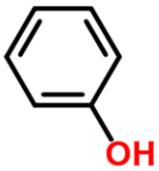
Pentanoic acid, 2,4-dimethyl-3-oxo-, methyl ester
CAS: 59742-51-7
MW: 158
Molecular formula: $C_8H_{14}O_3$



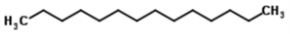
Tetradecanal
CAS: 124-25-4
MW: 212
Molecular formula: $C_{14}H_{28}O$



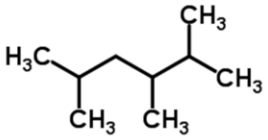
2,3,5-Trimethyldecane
CAS: 18344-37-1
MW: 184
Molecular formula: $C_{13}H_{28}$



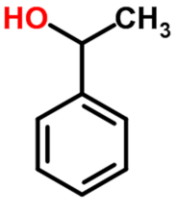
Phenol
CAS: 108-95-2
MW: 94
Molecular formula: C_6H_6O



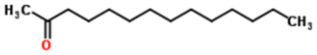
Tetradecane
CAS: 629-59-4
MW: 198
Molecular formula: $C_{14}H_{30}$



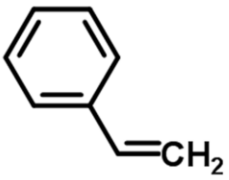
2,3,5-Trimethylhexane
CAS: 1069-53-0
MW: 128
Molecular formula: C_9H_{20}



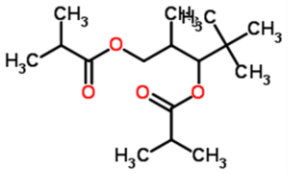
1-Phenylethanol
CAS: 98-85-1
MW: 122
Molecular formula: $C_8H_{10}O$



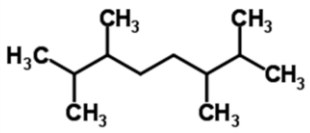
2-Tetradecanone
CAS: 2345-27-9
MW: 212
Molecular formula: $C_{14}H_{28}O$



Styrene
CAS: 100-42-5
MW: 104
Molecular formula: C_8H_8

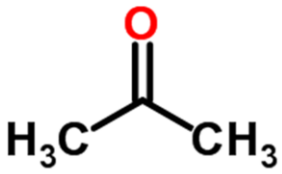


Propanoic acid,2-methyl-, 1-(1,1-dimethyl)-2-methyl-1,3-propanediyl ester
CAS: 74381-40-1
MW: 286
Molecular formula: $C_{16}H_{30}O_4$

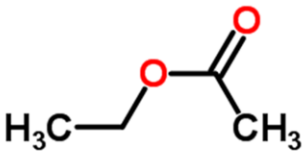


2,3,6,7-Tetramethyloctane
CAS: 52670-34-5
MW: 170
Molecular formula: $C_{12}H_{26}$

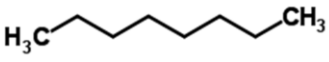
C.2 VOCs found at altered levels in the TD experiment



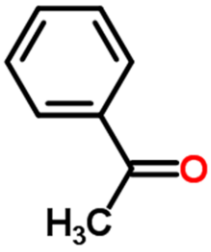
Acetone
CAS: 67-64-1
MW: 58
Molecular formula: C_3H_6O



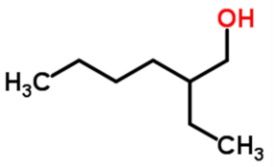
Ethyl acetate
CAS: 141-78-6
MW: 88
Molecular formula: C_4H_8O



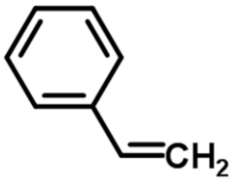
Octane
CAS: 111-65-9
MW: 114
Molecular formula: C_8H_{18}



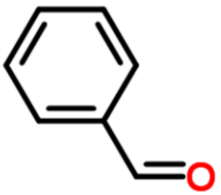
Acetophenone
CAS: 98-86-2
MW: 120
Molecular formula: C_8H_8O



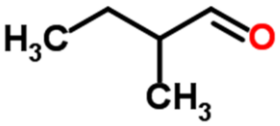
2-Ethylhexanol
CAS: 104-76-7
MW: 130
Molecular formula: $C_8H_{18}O$



Styrene
CAS: 100-42-5
MW: 104
Molecular formula: C_8H_8



Benzaldehyde
CAS: 100-52-7
MW: 120
Molecular formula: C_7H_6O



2-Methylbutanal
CAS: 96-17-3
MW: 86
Molecular formula: $C_5H_{10}O$

Appendix D

Raw materials of T-75 flask

ThermoFisher SCIENTIFIC

The world leader
in serving science

Product Statement

Products:	Nunc EasYFlasks Nunclon
Raw materials:	Polystyrene, PS (65927)
	High Density Polyethylene HDPE (43847)
	Masterbatch, blue (48452)
	Gore Filter (35688) [1]

Thermo Fisher Scientific hereby confirms that the above-mentioned products have been designed and are manufactured from selected materials.

Inspection point of the products:
Visual inspection according to specification
Test of function according to specification

The material has successfully passed the following test:

- *Biological evaluation of medical devices – ISO 10993-12, Sample preparation and reference materials, 2008*
- *Biological evaluation of medical devices – ISO 10993-18, Chemical characterization of materials, 2005*
- *USP <661> Physicochemical Tests – Plastics*
- *SOP PM 061, version # 03, „Recording of GC/MS fingerprints of medical devices and packaging materials"* and have passed the tests according to the product intended use.
- *USP <88> "Biological Reactivity Tests, In Vivo" class VI:* 50°C (7 days implant).
- *Biological evaluation of medical devices – ISO 10993-5, tests for in vitro cytotoxicity.* The cell line used is L-929 (mouse connective tissue)
- *USP <87> "Biological Reactivity Tests, In Vitro, Elution test"*

Cell culture validation tests:
Monolayer formation of two different cell lines and one primary cell culture. The cell lines are L-929 Connective tissue from mouse and Diploid human lung, and the primary cell culture is chicken embryo.

Cloning efficiency:
Specially selected cell line with high sensitivity to toxic components is used: V-79 Chinese hamster lung cell.

Non-pyrogenic:
The products are tested according to the principles of the Limulus Amoebocyte Lysate (LAL) test and certified non-pyrogenic with a documented endotoxin level of less than 20 endotoxin units/device (0.5 endotoxin units/ml), as stated in the United States Pharmacopoeia, Bacterial Endotoxin Test Criteria.

[1] Only for filter caps

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www.nuncbrand.com

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Sterility and bioburden:
Sterility is obtained through irradiation (gamma irradiation) according to ISO 11137-2 (sterilization of health care products – requirements for validation and routine control – radiation sterilization).
Consecutive audit of sterility is performed every 3 month.
Sequential inspection of the particle level is made in the production environment.

The expiry date is manufacturing date plus 5 (five) years.

Quality assurance of the products are performed in accordance with the requirements of the Quality Management System ISO 9001:2008 "Quality management system – Requirements" and ISO 13485:2003, "Medical devices – Quality management systems – System requirements for regulatory purposes".

Roskilde Site

Signature: _____ Date: 2013-10-23
Louise Wohlfahrt, QA Engineer (YYYY-MM-DD)