



UNIVERSITY OF
BIRMINGHAM

**IDENTIFICATION OF VOLATILE ORGANIC
COMPOUNDS IN BREATH ASSOCIATED WITH
LIVER DISEASE AND THEIR POTENTIAL
APPLICATIONS FOR MEDICAL USE**

By

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Abstract

Proton Transfer Reaction Mass Spectrometry (PTR-MS) was applied to determine which volatile organic compounds in breath are associated with cirrhosis and hence diagnostically useful. A two-stage biomarker procedure was used. In the first-stage, alveolar breath samples of 31 cirrhotic patients and 30 controls were analysed and compared. In the second-stage, 12 of the patients had their breath analysed after liver transplant.

The first-stage study showed that seven volatiles were elevated in patients' breath compared to controls. Of these, limonene, methanol, 2-pentanone showed a statistically significant decrease post-transplant and hence can unequivocally be used as biomarkers for chronic liver disease. Limonene which is not produced in the body showed washout characteristics and the best diagnostic capability. These findings suggest that limonene, methanol and 2-pentanone are potential biomarkers for early-stage liver disease.

Limonene was detected in higher levels in patients with symptoms of hepatic encephalopathy (HE) in comparison to those with no symptoms. Limonene discriminates patients suffering from HE, but not methanol or 2-pentanone.

The elimination characteristics of post-operative isoflurane levels in breath of 5 patients were investigated. High concentrations of isoflurane remained in their breath for several weeks. This study raises the question about the effect of isoflurane in the neurocognitive function of patients after major surgery.

All of the three studies mentioned above have been published, the details of which are provided:

- R. Fernández del Río, M. E. O'Hara, A. Holt, P. Pemberton, T. Shah, T. Whitehouse, and C. A. Mayhew "Volatile Biomarkers in Breath Associated With Liver Cirrhosis-Comparisons of Pre- and Post-liver Transplant Breath Samples" *EBioMedicine* (2015) 1243-1250
- R. Fernández del Río, M. E. O'Hara, P. Pemberton, T. Whitehouse and C. A. Mayhew "Elimination characteristics of post-operative isoflurane levels in alveolar exhaled breath via PTR-MS analysis" *Journal of Breath Research* (2016) 10 046006
- M. E O'Hara, R. Fernández del Río, A. Holt, P. Pemberton, T Shah, T Whitehouse, and C.A. Mayhew "Limonene in exhaled breath is elevated in Hepatic Encephalopathy" *Journal of Breath Research* (2016) 10 046010

This thesis includes figures and text blocks from these three papers.

Dedication

En memoria de mi padre, Fernando Fernández Corcuera,

Tu gran fortaleza y valor es mi inspiración y guía en la vida.

To the memory of my father, Fernando Fernández Corcuera,

Your greatest strength and courage is my inspiration and guide
in my life.

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List of Abbreviations

ADO	average dipole orientation
AID	autoimmune liver disease
ALD	alcoholic liver disease
ALT	alanine aminotransferase
amu	atomic mass unit
AST	aspartate aminotransferase
AUROC	area under receiver operating characteristic
BMI	body mass index
CD	cryptogenic disease
CHE	covert hepatic encephalopathy
CHESS	clinical hepatic encephalopathy staging scale
CI	chemical ionization
CLD	chronic liver disease
cps	counts per second
DC	direct current
DT	drift tube
E/N	ratio of the reduced electric field strength, E , to total number density, N
ERV	expiratory reserve volume
FAIMS	field asymmetric ion mobility spectrometry
FP	false positive rate
FRC	functional residual capacity
FS	fibroscan
GB	gas –phase basicity
GC	gas chromatography
GC-FID	gas chromatography flame ionisation detection
GC-MS	gas chromatography mass spectrometry
GGT	gamma-glutamyl transferase
HBV	hepatitis B
HC	hollow cathode
HCC	hepatocellular cancer
HCV	hepatitis C
HE	hepatic encephalopathy
HESA	hepatic encephalopathy scoring algorithm
IC	inspiratory capacity
ICUs	intensive care units
IR	infrared spectroscopy

IRV	inspiratory reserve volume
ITU	intensive therapy unit
LFTs	liver function test
LQ	lower quartile
MHE	minimal hepatic encephalopathy
MID	multiple ion detection
MO-log	modified orientation log
NAFLD	non-alcoholic fatty liver disease
NASH	non-alcoholic steatohepatitis
Ncps	normalized counts per second
No-HCC	no hepatocellular cancer
OHE	overt hepatic encephalopathy
OPC	out-patient clinic
PA	proton affinity
PBC	primary biliary cirrhosis
ppbv	parts per billion by volume
ppmv	parts per million by volume
PSC	primary sclerosing cholangitis
PTR-MS	proton transfer reaction mass spectrometer
QMS	quadrupole mass spectrometer
RF	radio frequency
ROC	receiver operating characteristic
RV	residual volume
SDR	source drift region
SEM	secondary electron multiplier
SIFT-MS	selected ion flow tube mass spectrometry
STP	standard temperature and pressure
TAC	transplant assessment clinic
TV	tidal volume
Td	Townsend ($1 \text{ Td} = 10^{-17} \text{ V cm}^2$)
TE	transient elastography
TLC	total lung capacity
TP	true positive rate
TFA	trifluoroacetic acid
UKELD	United Kingdom model for end-stage liver disease
UQ	upper quartile
VC	vital capacity
VMR	volume mixing ratios
VOC	volatile organic compound

1 INTRODUCTION

The majority of clinical diagnosis focuses on the analysis of biological fluids such as serum and urine to collect information for disease prognosis or monitoring, allowing physicians to decide the best treatment for a patient. In recent years, with the fast improvement of new analytical techniques, such as Proton-Transfer-Reaction-Mass-Spectrometry (PTR-MS) there is a potential of non-invasive procedures to provide information about medical conditions. These have the benefit of reducing the inconvenience and stress caused on the patients by the invasive techniques and long periods of delays for a prognosis. Recently, the study of volatile organic compounds (VOCs) in breath has shown an increased clinical interest within the medical community. Breath contains VOCs that can be correlated with concentration levels of these compounds present in the human blood which are consequences of normal activity or due to pathological disorder [1] [2] [3] [4] [5]. Therefore, VOCs excreted via exhalation are representative of monitoring metabolic and pathological processes.

An analytical technology which has been used widely for the analysis of VOCs in breath as a fast, real-time and non-invasive diagnostic tool is Proton-Transfer-Reaction Mass-Spectrometry (PTR-MS) [6]. The collection of breath samples is painless for the patient and the method chosen can be easily replicate. Breath analysis is exclusive in the sense that it can provide continuous information about metabolic pathways and physiological processes that occur in the human body. Despite all these advantages, analysis of breath VOCs (and for that matter headspace analysis of serum, sputum, urine, and faeces) is still not used in the clinical practice [7]. One handicap is that there is a lack of a standardized [8] methodology for breath sampling and as a consequence there is a

huge variety of results with a very low confidence level. Another reason is that exhaled breath contains a diversity of volatiles with a large range of concentrations. Furthermore, despite over a decade of research, only few studies of volatiles compounds in breath have correlated a biomarker to a specific disease or habit [5] [9].

Arguably, too much reliance has been placed on multivariate statistics looking for correlations, which can lead to voodoo statistics, i.e. finding correlations when there are none. In addition to this, many compounds that have been put forward as biomarkers for a disease have either been incorrectly assigned or are not specific enough to be of use.

It is relevant to mention that although PTR-MS has rapidly established as an analytical tool for the analysis of VOCs in breath [10] [11], the technique is still used only for fundamental research at Universities, Technological Centres and University Hospitals. The huge amount of data generated for analysis and the expensive cost and maintenance of the instrument limit the technique, which is a main drawback for the development of the technique. The major outcome of the work presented in this thesis is to show for the first time how key volatiles in the complex chemical profile of breath have the potential for use as non-invasive probes in the diagnosis and monitoring of liver disease.

1.1 Thesis Outline

This thesis consists of seven chapters, each of them included in three main sections detailing (1) PTR-MS (2) methodology including breath samplings techniques and patient recruitment and (3) results and discussion. Included with the details of PTR-MS in Chapter 2 is an explanation of the theoretical aspects of the ion chemistry reactions involved. The section dealing with the methodology of breath sampling provided in Chapter 3, also briefly explains the theory of gas exchange and explains the different methods of sampling collection and drawbacks. Chapter 3 describes and explain the sampling protocol developed in this study for alveolar breath collection and the off-line measurements by PTR-MS. In this chapter is also involves the details and protocol of the patient recruitment.

In the third major section (Chapters 4-6), the results are presented. Chapters 4 and 5 deal with the liver disease studies. In these chapters, details of the two stages of the clinical trial procedure are explained. Comparisons are made between the concentrations of VOCs in exhaled breaths from patients versus controls and then the patients are used as their own controls. Biomarkers are detected in the alveolar breath of patients which are associated to the disease, although are not coming from the diseased liver. The chapter concludes with a discussion of the experimental results.

Chapter 6 describes the elimination characteristics of post-operative isoflurane levels in alveolar breath. Isoflurane anaesthetic agent was detected during the analysis of the breath profiles in patients who underwent liver transplantation, therefore taken the advantage of this fact, the washout characteristics of isoflurane anaesthetic were investigated.

A previous study found that isoflurane, which is hardly metabolised by the liver, is quickly eliminated from the body through breath, urine and sweat within days [12].

However, that study used healthy young males and isoflurane was administrated only for 30 minutes.

This thesis concludes with chapter 7 in which is included a further discussion with key conclusions and summary of findings, discussions on the limitations of the study, and proposals for future direction in breath analysis for the detection of liver disease.

1.2 Aims and Objectives

As mentioned above, one of the most crucial drawbacks in breath analysis is the lack of a standard protocol procedure to collect breath samples [13]. Unfortunately, the PTR-MS instrument used for this study was not placed at the Queen Elizabeth hospital where the patient recruitment takes place. Therefore, a main goal was to develop a reliable method to collect breath samples and to establish reproducible breath sampling protocol for the collection of breath samples for off-line measurements by PTR-MS. A standardized protocol will not avoid the intra-individual variations in breath. For this reason, and to avoid uncertainty in the measurements, four alveolar breath sample replicates were collected for each patient.

A major aim of research summarised in this thesis is to investigate the potential of PTR-MS for detecting and monitoring liver disease using non- invasive and real time breath sampling analysis techniques. The goal is to evaluate short and midterm changes of VOCs in the exhaled breath of patient after liver transplant and to raise the valuable diagnostic potential of breath analysis for clinical diagnose discuss the implications of the use of breath analysis for assessing liver function after liver transplant. The goal is to examine changes in the same volatiles during long periods of time and pre and after liver transplant.

Another aim of the work presented in this thesis is to measure VOCs in the breath of patients suffering with acute encephalopathy and examine which VOCs are elevated in the case of hepatic encephalopathy (HE) and which change over time in association with the severity of symptoms.

Finally, an investigation is presented which seeks to study the elimination profile of anaesthetics in the exhaled breath of patient's weeks after a major surgery and their implication to the cognitive function recovery of patients. Details are provided of which ions are diagnostically useful for the monitoring of isoflurane anaesthetic in alveolar exhaled breath. The goal of this investigation is to follow- up the changes in anaesthetic concentration following an operation.

2 PROTON TRANSFER REACTION MASS SPECTROMETRY

This chapter summarizes the technique of proton transfer reaction mass spectrometry and the basic chemical processes involved.

2.1 The PTR-MS

Proton transfer reaction mass spectrometry is a sensitive analytical technique for on-line real-time monitoring of volatile organic compounds (VOCs) in air with a minimal sample preparation. Physicists at the Institute for Ionic Physics at the Leopold-Frances University in Innsbruck first developed the technology in the mid-90s. The proton transfer reaction mass spectrometer uses gas phase hydronium ions as the reagent agents with limits of detections down to concentrations of part per billion (ppbv). New models of PTR-MS have detection limits down to part per trillion (pptv) [14]. This uses a quadrupole mass spectrometer for m/z selection. Figure 2.1 shows a photograph of the instrument used in this study, which is a first generation PTR-MS built by IONICON Analytik GmbH.



Figure 2.1 PTR-QUAD-MS Ionicon Analytik GmbH apparatus.

The spin-out company that resulted from their work, IONICON Analytik GmbH has become one of the leading manufacturers and suppliers of PTR-MS instruments. Figure 2.2 displays a top view photograph of the PTR-MS instrument.



Figure 2.2 Photograph of the top view of a PTR-QUAD-MS Ionicon Analytik GmbH apparatus.

The origin of the proton transfer reaction mass spectrometry comes from the investigation of the flowing afterglow technique for the study of ion-molecule reactions [15]. Chemical Ionization (CI) is a soft ionization technique developed by Munson and Field in the middle 1960s for the identification of trace organic compounds in complex chemical environments [16]. CI is a method based on gas phase ion/molecule chemistry and requires a transfer of charge, such as an electron or proton, between the reactants and the formation of the ions by chemical reactions. Owing to the low energies involved with CI fragmentation is often limited. This leads to a mass spectrum which is easier to interpret, than that obtained from conventional 70 eV electron impact mass spectrometry.

There are several types of ion/molecule chemistry. Some of the key ones are shown below:

- Proton transfer $M + BH^+ \rightarrow MH^+ + B$
- Anion abstraction $M + X^+ \rightarrow [M-A]^+ + AX$
- Charge transfer $M + X^+ \rightarrow M^+ + X$

CI methods implied the use of reagent ions that undergo exothermic ion molecule reactions with the neutral reactants. The reagent ion is chosen so that the ionisation reaction has a low exothermicity and therefore there is a low energy transferred to the product ion reducing the ion fragmentation.

For example, in the PTR-MS the chemical ionization mainly depends of the transfer of a proton from the protonated form to the neutral species. In PTR-MS the hydronium ion (protonated water H_3O^+) is used as a proton donor. The proton affinity (PA) of a neutral molecule is a determinant parameter in the proton transfer reactions.

In a protonation reaction defined at 298 K, PA is the negative of the enthalpy change for the addition of a proton (H^+). For example for a molecule M:



In PTR-MS, hydronium cations (H_3O^+) only transfer the proton when the gas molecule has a proton affinity higher than the water proton affinity (691 kJ mol^{-1} or 7.22 eV , where $96.5 \text{ kJ mol}^{-1} = 1 \text{ eV}$) [17]. The proton transfer reaction involving H_3O^+ is defined by the reaction:



In the gas phase, for exoergic reactions the process is spontaneous and fast with reaction rate coefficients being at the collisional limiting values. Importantly, for breath analysis (and many other applications), the common gases in air (N₂, O₂, CO₂, argon, etc.) have proton affinities (PAs) less than water. Table 2.1 shows the proton affinities of some volatile organic compounds including common constituents in air. Hence air (and breath) is functioning as carrier gas in the reaction chamber of the PTR-MS. Most volatile organic compounds have proton affinities in the range between 7 and 9 eV. Therefore, the proton transfer involving H₃O⁺ results in small enthalpy changes so that generally little fragmentation is involved.

Table 2.1 Proton affinities of some volatile organic compounds including common constituents in air. Taken from the compilation given in reference [18].

Molecule	Proton affinity (kJ/mol)
Oxygen	95
Nitrogen	118
Carbon monoxide	594
Carbon dioxide	540
Propane	626
<i>Water</i>	<i>691</i>
Methanol	754
Acetaldehyde	770
Ammonia	853
Pyridine	948
Limonene	875

The PTR-MS used in this study was a first generation Ionicon PTR-MS instrument with a serial number 01-13. Figure 2.3 shows a schematic representation of a PTR-MS apparatus used in the study [19] showing the different parts of the instrument in more detail.

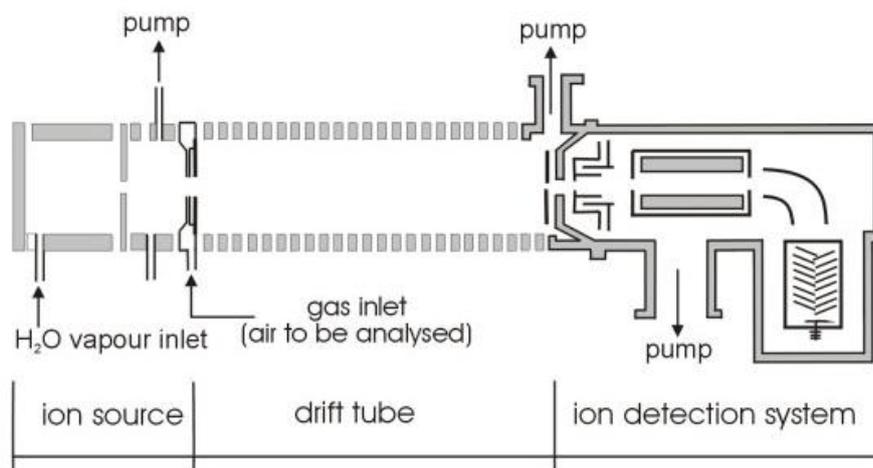


Figure 2.3 Schematic diagram of a PTR-MS apparatus [19]. Reprinted from International Journal of Mass Spectrometry and Ion Processes with the permission of Elsevier.

1. A reagent ion source - a hollow cathode discharge (HC) and a source drift region (SDR)
2. A drift tube - the reaction region (DT)
3. Detection - involving a mass to charge selection using a quadrupole mass spectrometer and secondary electron multiplier (QMS and SEM)

2.1.1 The ion source and source drift region

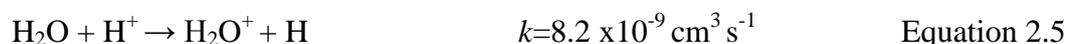
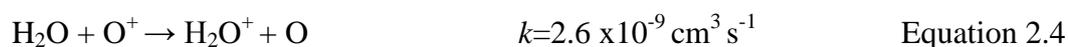
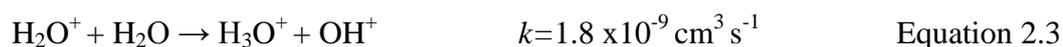
A detailed explanation of the ion source used in a PTR-MS instrument has been reported by Hansel et al. [19]. Researchers at the Institute of Ion Physics in Innsbruck have also published studies on comparable ion sources used in PTR-MS [20]. In PTR-MS instruments, the ion source is a DC hollow cathode discharge. H_3O^+ ions are produced from a plasma discharge in a region of water vapour. The hollow cathode operates at typically pressure of 1 millibar. The ion source is divided into two parts, a hollow cathode (HC) and a source ion drift region (SDR). The description of the ion source can be followed from the picture shown in figure 2.3.

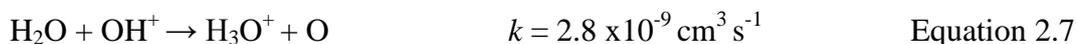
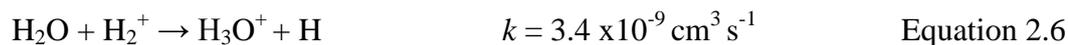
The hollow cathode consists in three main regions, cathode, cathode fall and negative glow. A plasma is produced by ionisation of water vapour between the anode and cathode. The discharge is a “negative glow” key region axial inside region of the hollow cathode. This region is a free-field cylindrical region, with a potential close to the anode potential. Between the negative glow and the cathode, a potential difference of hundreds of volts is created. This region is called cathode fall. In the cathode fall, electrons are accelerated up to several electron volts and collide with neutral molecules which are ionised by collisions with high-energy electrons to produce ions. In the negative glow region, neutral molecules undergo further collisions with the primary ions to form secondary and tertiary ions. These will be accelerated up to several electron volts and further ions collision occurs. This continuous effect produces a high-density plasma which is retained in the hollow cathode region. Two anode plates limit at either end the negative glow region in the HC. There is a small central orifice of 1.0 mm diameter in one these endplates [19].

The ions produced in the hollow cathode pass into the adjacent source drift region through the central orifice. The discharge current is maintained in the hollow cathode by a potentiometer set typically at 5 mA while the hollow cathode is in operational mode. In the PTR-MS the potentiometer can be regulated from 1-10 mA. The higher the discharge current is the higher the intensity ion current is produced and the more stable is the discharge and therefore the reagent ion signal. It is important to note that at higher discharge currents settings and with the continued use, the internal surface of the HC can become contaminated with compounds deposits affecting the production and stability of the reagent ions. Therefore, the HC ion source must be cleaned every 1-2 month to maintain a good instrument performance.

During operating conditions, a flow of high purity water vapour is introduced into the ion source and an electrical discharge will lead to the production of hydronium ions through a number of reactions. A mass flow controller maintains the water vapour flow rate. The flow rate can be set from 0 to 10 sccm (standard cubic centimetres per minute). In this study the flow rate was kept at 5 sccm. Electron impact ionization of water molecules lead to H_2O^+ or fragment ions such as O^+ , H^+ , H_2^+ and OH^+ .

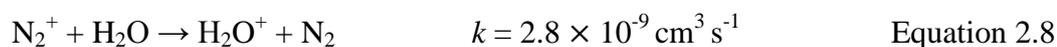
Parent and fragment ions produced in the glow discharge that have recombination energies greater than the ionisation potential of water will react with water, ultimately leading to H_3O^+ , equations 2.3-2.8.





where the k are the experimental reaction rate coefficients at 300 K. All the reactions are fast with the reaction rate coefficients being close to or at the collision value. The data above has been taken from the compilation from Ikezoe et al. published in 1987 [21].

The source drift region is a region placed between hollow cathode and the drift tube. Its purpose is to allow more time for the above reactions to take place and hence to maximise the reagent ion signal. It also serves to reduce protonated water clusters.

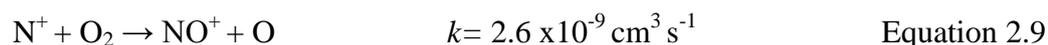


It is unpreventable that some gas from the drift tube flows back into the discharge region of a PTR-MS. This results in other types of reagent ions which do not react with water, such as O_2^+ , N_2^+ , NO^+ , being produced. N_2^+ is eliminated by charge exchange with water (Equation 2.8). N_2^+ lead to H_3O^+ formation via reaction of H_2O^+ with water.

The mechanism of O_2^+ elimination is more complicated. The ionization energy of the H_2O (12.6 eV) is higher than the recombination energy of O_2 which has a recombination energy of 12.1 eV compared with water which has an ionization energy of 12.6 eV. Therefore O_2^+ cannot exchange charge with neutral water. Hence O_2^+ is seen as

a low-level contaminant from the ion source and a low concentration of O_2^+ is always present in the DT.

NO^+ is another contaminant ion in PTR-MS that is generated by the reaction specified below:



The recombination energy of NO^+ (9.3 eV) is too low for it to be able to charge transfer with water.

Although present in the drift tube, it is possible to reduce their intensities by adjusting the PTR-MS operational parameters (gas flow, hollow cathode voltages) to minimise their concentrations. In this study, the abundance of NO^+ and O_2^+ was kept below 2% of the H_3O^+ ions. Consequently, a clean signal of reagent ion with a purity greater than 98% was produced [19].

2.1.1.1 Gas inlet air flow

In the PTR-MS the sample gas to analyse enters into the system via a needle valve. The needle valve controls the inlet flow going into the drift tube (DT). The pressure in the system is maintained by the pressure controller that can be set from 0- to 800 mbar using a specific software. Generally, the pressure controller is set to maintain a pressure of 2 mbar in the drift tube. In the back panel of the PTR-MS close to the inlet flow valve, there is an inlet called the ballast inlet. The goal of the ballast inlet is to supply a constant flow of dry air to the diaphragm vacuum pump. It is necessary to use this inlet ballast to avoid water condensation inside the diaphragm vacuum pump. The flow in the inlet

ballast is set at the specific value recommended in Ionicon's specifications manual for the instrument.

2.1.2 Drift tube

The drift tube is a cylindrical tube that contains a series of electrodes (stainless steel rings) equally spaced and separated by electrically insulated spacers (Teflon O-rings) to control the voltage to each electrode. A series of high impedance resistors are connected between the adjacent electrodes. These generate a homogeneous electric field that move ions along the drift tube when a voltage is set across the drift tube. The drift tube has a diameter of 3 cm and approximately 10 cm length. The drift tube can be heated up at to 100°C. Figure 2.4 shows a photograph of a drift tube in a PTR-instrument.

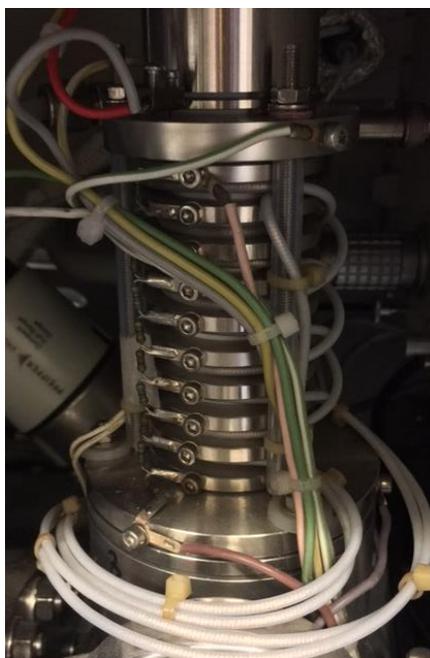
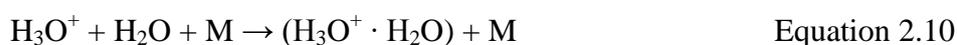


Figure 2.4 Photograph of a drift tube in a PTR-MS instrument.

The analyte is introduced through an inlet valve with air as the carrier gas into the drift tube. The reagent ions are guided into the drift tube and react with the analyte present in the air.

Given the presence of the H₂O in the air sample and from the hollow cathode, H₃O⁺ ions can associate with H₂O molecules through a three body process, forming hydrate clusters ions:



When monohydrate cluster ions are formed (H₃O⁺ · H₂O), the abundance of H₃O⁺ is reduced [17] [19]. Table 2.2 gives information on the proton affinities of neutral water and water clusters.

Table 2.2 Proton affinities of water and water clusters [21, 22].

Neutral Molecule	Proton Affinity (KJ/mol)	Protonated Molecule	<i>m/z</i>
H ₂ O	691	H ₃ O ⁺	19
(H ₂ O) ₂	833	H ₃ O ⁺ · H ₂ O	37
(H ₂ O) ₃	889	H ₃ O ⁺ · (H ₂ O) ₂	55
(H ₂ O) ₄	920	H ₃ O ⁺ · (H ₂ O) ₃	73

The electric field applied through the drift tube controls the energy delivered to ion-molecule collisions to reduce excessive water clusters and encourage the breakup of water cluster ions.

The operational conditions in the drift must be optimised to provide a balance between the amount of protonated water cluster ions formed and the energy for a soft ionisation to reduce the fragmentation of volatile organic compounds. The energy in an ion-molecule reaction depends on the electric field, E , and the total number density of gas, N , in the drift tube. E/N is a measure of the ions kinetic energy in the drift tube.

The reduced electric field (E/N) is referred to as the ratio of the electric field strength and the total number density of gas, N , in the drift tube, units of $V\text{ cm}^2$. The reduced electric field is more frequently defined in Townsend (Td), by the relation $1\text{ Td} = 10^{-17}\text{ V cm}^2$. For example, a voltage of 600 V applied across the drift tube provides an electric field of

$$E = \frac{V}{d} = \frac{600}{9.6} = 62.5\text{ V cm}^{-1} \quad \text{Equation 2.11}$$

The gas number density is the number of gas molecules per unit volume. Using the following equation, from the ideal gas equation [23]:

$$\frac{N_A}{V_M} \frac{273.15\text{ K}}{T_d} \frac{P_d}{101.325\text{ kPa}} \quad \text{Equation 2.12}$$

N_A is Avogadro's number ($6.022 \times 10^{23}\text{ mol}^{-1}$) and the molar volume is V_M ($22414\text{ cm}^3\text{ mol}^{-1}$) is the volume occupied by one mole of ideal gas at standard temperature (273.15 K) standard pressure, 1 atmosphere of pressure (101.325 kPa). P_d is the gas

pressure in the drift tube (kPa) and T_d is the operational temperature of the drift tube (Kelvin).

When operating the drift tube at a pressure of 2.06 mbar at a temperature of 318 K the total number of density is $4.69 \times 10^{16} \text{ cm}^{-3}$. Together this provides an E/N of 138 Td.

2.1.2.1 Determination of trace gas concentrations

One of the applications of PTR-MS is to determine without calibration absolute concentrations of a species M providing the reaction time t_d and the rate coefficient are known [10].

The reaction is a second-order reaction following this rate equation:

$$-\frac{d[\text{H}_3\text{O}^+]}{dt} = k[\text{H}_3\text{O}^+][M] \quad \text{Equation 2.13}$$

$[M]$ is a neutral gas and assuming that $[M] \gg [\text{H}_3\text{O}^+]$, then $[M]$ is constant. The equation 2.13 can be integrated and the reaction follows a pseudo first order kinetics.

$$[\text{H}_3\text{O}^+]_t = [\text{H}_3\text{O}^+]_0 e^{-k[M]t}, \quad \text{Equation 2.14}$$

where t is the reaction time, the time it takes for the hydronium ion to travel to the end of the drift tube. The concentration of $[\text{H}_3\text{O}^+]$ can be related to the concentration of $[\text{MH}^+]$ by

$$[\text{MH}^+]_t = [\text{H}_3\text{O}^+]_0 - [\text{H}_3\text{O}^+]_t \quad \text{Equation 2.15}$$

Rearranging both equations a and b the $[\text{MH}^+]_t$ is given by

$$[\text{MH}^+]_t = [\text{H}_3\text{O}^+]_0 (1 - e^{-k[\text{M}]t}) \quad \text{Equation 2.16}$$

Firstly, the condition can be simplified assuming that only a low concentration of $[\text{H}_3\text{O}^+]$ is consumed by the reaction then $[\text{H}_3\text{O}^+]_0 \approx [\text{H}_3\text{O}^+]_t$ that is $[\text{MH}^+] \ll [\text{H}_3\text{O}^+]$ therefore, the product ion density $[\text{MH}^+]$ can be described:

$$\frac{[\text{MH}^+]_t}{[\text{H}_3\text{O}^+]_t} = (1 - e^{-k[\text{M}]t}) \quad \text{Equation 2.17}$$

Secondly, $k[\text{M}]t \ll 1$ assuming that VOC are not greater than part per million level.

The concentration of the gas M can be calculated from the ratio of the product ion density and the hydronium ion density by:

$$\frac{[\text{MH}^+]}{[\text{H}_3\text{O}^+]} = k[\text{M}]t \quad \text{Equation 2.18}$$

The concentration of the gas M can be calculated from the ratio of the product ion density and the hydronium ion density

2.1.2.2 Determination of the reaction time in the drift tube.

In the drift tube the reaction time (t_d) can be determined by calculating the drift velocity from the ion mobility. The ion drift velocity (v_d) is given by:

$$v_d = \mu E \quad \text{Equation 2.19}$$

Where μ is the ion mobility ($\text{cm}^2 \text{V}^{-1} \text{s}^{-1}$). Often a reduced mobility is used, μ_0 , which is given by:

$$\mu_0 = \frac{N}{N_0} \mu \quad \text{Equation 2.20}$$

N is defined as the number of density of the carrier gas at experimental condition and N_0 is the carrier gas number density at standard temperature and pressure (STP).

The drift velocity is expressed in terms of the reduced mobility, carrier gas density at STP and the electric reduced field by:

$$v_d = N_0 \mu_0 \frac{E}{N} \quad \text{Equation 2.21}$$

The value for the reduced mobility coefficient for H_3O^+ is $2.76 \text{ cm}^2 \text{V}^{-1} \text{s}^{-1}$ [24].

Under operating conditions the velocity of the ions in the drift tube is

$$v_d = 1.02 \times 10^5 \text{ cm s}^{-1}$$

The drift tube length is 9.6 cm and gives a value of drift time of reaction of reaction time $t_d = 0.09 \text{ ms}$. The reaction time in the drift tube can be calculated by:

$$t_d = \frac{L}{\mu_0 N_0} \frac{N}{E} \quad \text{Equation 2.22}$$

2.1.2.3 Collisional Reaction Rate Coefficient

The reaction rate coefficient is an important parameter of ion-molecule reaction and is defined in terms of reactants (consumed) and products (formed). The reaction rate

measures the speed at which a reaction takes place. Chemical reactions have to be efficient

in order to obtain adequate products. An efficient reaction takes place at or close to the collisional rate. Exoergic proton transfer reactions are close to the collisional limiting values. For example, for the reaction:



The reaction rate coefficient is defined by

$$\frac{d[R^+]}{dt} = -k [R^+][M], \quad \text{Equation 2.23}$$

where k is the rate of reaction (cm^3s^{-1}). The collisional rate coefficient is the upper limit to the reaction rate coefficient. In the literature are calculated many of the reactions rate coefficients [21] for proton transfer reactions.

Different theoretical studies have been proposed to determine the rate coefficient of exothermic ion-molecule reactions. One of the models developed for non-polar molecules, was proposed by Langevin over a century ago. The Langevin rate coefficient is classically calculated assuming that the reaction occurs upon every collision [24]. The principle is based on the idea that the ion induces a dipole in the non-polar neutral molecule.

The Langevin rate constant is independent of the relative velocity and the temperature. The collisional rate constant or the Langevin non-polar constant (k_L) is described by:

$$k_L = \sqrt{\frac{\pi\alpha q^2}{\mu\epsilon_0}} \quad \text{Equation 2.24}$$

Here α is the polarizability, μ is the reduced mass and ϵ_0 the free space permittivity.

This method has been used as a standard for the calculation of ion molecule and ion-atom collisions. This approach does not work well for the interaction of polar molecule and ion because it omits interactions between permanent electric dipole moment and positive charge and of an underestimation of the reaction rate coefficient.

The locked dipole approximation takes into account the effect of the reaction between an ion and a polar molecule. However, this model tends to overestimate rate coefficients by deleting a range of dipole orientations.

A more accurate calculation is that by Su and Bowers who developed an average dipole orientation (ADO) theory. The ADO rate coefficient is described by:

$$k_{ADO} = \sqrt{\frac{\pi\alpha q^2}{\mu\epsilon_0}} + \frac{C\mu_D q}{\epsilon_0} \sqrt{\frac{A}{2\pi\mu k_B T}} \quad \text{Equation 2.25}$$

k_B is the Boltzman constant and μ_D is the dipole moment. The first part of the equation is the Langevin contribution shown in equation 2.24 whereas the second part of the equation takes into account the ion-permanent dipole. A comparison of the rate coefficient calculated by ADO theory and experimental values of rates coefficients, the result is that ADO calculations tend to underestimate the rate coefficients by 10-20% [25]. The reason is because k_{ADO} does not take into account the dipole moment of a charged reagent.

2.1.3 Detection system

The detection system in the PTR-MS used in this study consist of a quadrupole mass spectrometer (QMS) which filters the ions according to their m/z (mass/charge ratio), coupled to a secondary electron multiplier (SEM) which has the task of detecting the presence of the ions passed by the mass filter. The SEM multiplier operated in pulse counting detection.

The quadrupole consists of a set of four metal horizontal, cylindrical parallel rods positioned equidistant from a central axis. They are arranged as shown in figure 2.5. Adjacent rods are electrically connected together and a radio frequency voltage (RF) with a DC offset voltage is applied so that a quadrupole electric field can be created [26].

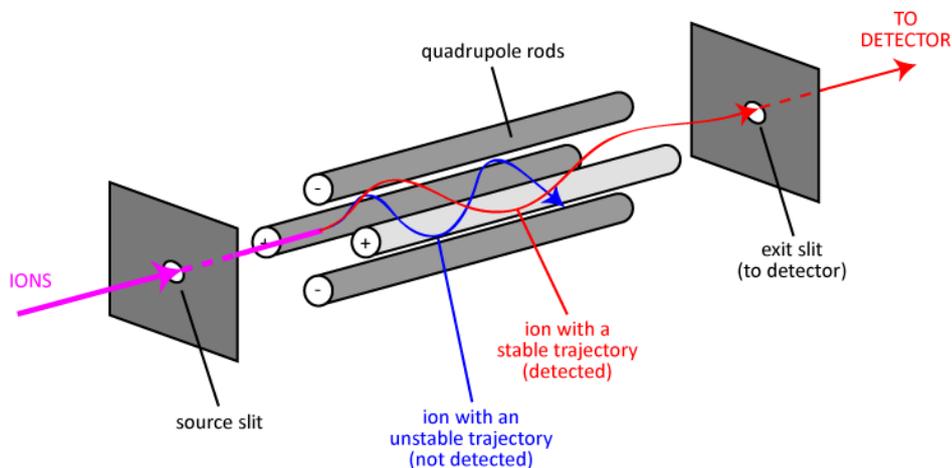


Figure 2.5 Schematic diagram of a quadrupole mass spectrometer [26]. Figure used with permission from Dr Paul Gates, University of Bristol, United Kingdom.

A sequence of DC and RF voltages applied to the rods determine the pathway of the ions passing through the quadrupole field. The geometry of the quadrupole and the X, Y directions are defined in the figure 2.6 [27].

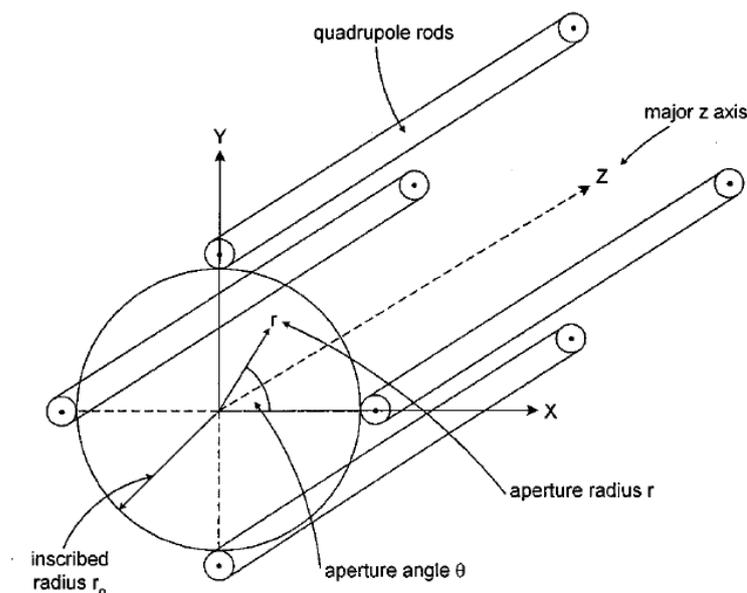


Figure 2.6 Schematic diagram of the geometry of a quadrupole mass filter defined in the x, y directions. Reprinted from Journal of Vacuum Science & Technology with the permission of AIP Publishing [27].

For given values of DC and RF voltage applied to the rods is defined an oscillating electric field that only ions of a specific m/z ratio are transmitted and recorded leading to a mass spectrum. The other ions with different m/z will be lost on the rods of the quadrupole.

The model of the quadrupole in the PTR-MS is a Balzers QMS-422. This model of quadrupole spectrometer has limited mass range, typically unit mass resolution (a low mass resolving power), and with at operating mass range up to 500 m/z .

One of the drawbacks of the Balzers QMS-422 is that unambiguous identification of compounds is difficult based only on m/z values. However, additional analytical techniques, such as gas chromatography can be used to for pre-separation.

The SEM provides an electrical pulse for each incident ion. As the SEM ages, it loses sensitivity, and this has to be periodically checked. The operating voltage is determined by gradually increasing the voltage and observing the point at which the instrument response counts per second (cps) plateaus. The value of plateau has to be chosen to operate at the shoulder of the sigmoid. If the voltage is set too low than the sensitivity will be reduced whereas for a voltage too high the signal to noise ratio deteriorates owing to dark currents causing a poor quality signal. Figure 2.7 shows a picture of a secondary electron multiplier used in a PTR-MS instrument.

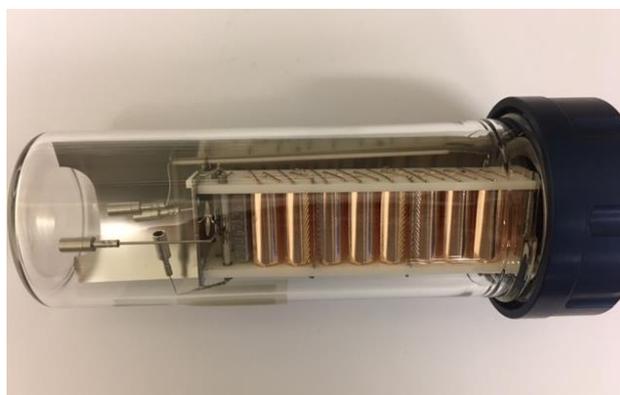


Figure 2.7 Photograph of a new secondary electron multiplier. The dynodes are visible between the white ceramic layers.

Furthermore operating at high voltage leads to a reduction in the lifetime of the SEM. It is recommended by the supplier to operate the instrument at a voltage that gives for every 100 V increase in the SEM potential a 10% increase at m/z 21.

If the cps incident on the SEM are more than a million, the output signal loses linearity. Furthermore, a high number of ions will accelerate the damage to the detector.

At m/z 19 the hydronium signal saturates the detector. As an alternative, the count rate of the ^{18}O isotope of H_3O^+ is measured and indirectly calculated the m/z 19 signal. The relative abundance of ^{16}O is 99.8% and for ^{18}O is 0.2%. The m/z 19 count rate is calculated by multiplying by 500 the count rate signal at m/z 21. Changes in the operating conditions result in changes in the H_3O^+ signal intensity. Furthermore, the signal intensity is dependent on E/N. Therefore, it is common to normalise the intensity of the signal to the number of the reagent ion H_3O^+ .

2.1.3.1 Reagent ion and normalization

To ensure that data acquired during measurements is comparable, normalisation of the product ion intensity signals to reagent ion signal is required. In this way it is possible to make comparisons of the measurements under different modes or conditions. In the PTR-MS instrument, H_3O^+ signal can be different from one day to another day or it can also change over the course of a day.

The following equation was used for the normalization:

$$ncps = 50 \times 10^6 \frac{(MH^+)}{(H_3O^+)} \quad \text{Equation 2.26}$$

The data acquired during this study was normalised to the H_3O^+ signal of 50 million counts. In the equation 2.26,

$\frac{(MH^+)}{(H_3O^+)}$ is the ratio of the product ion density and the hydronium ion density.

In the DT, the hydronium ion is the dominant reagent ion. When the analyte gas is humid, there are significant concentrations of the monohydrate water cluster in the sample gas which may also react by proton transfer with the analyte. When proton transfer from the protonated water cluster can occur then the intensity of the product ion signal should be normalized to the hydronium H_3O^+ and monohydrate water cluster $H_3O^+ \cdot H_2O$.

Several studies have shown that the VOCs concentration using PTR-MS has a dependency on the water vapour content in the samples gas [28]. De Gouw and Warneke reported that the normalization has to be considered for water clustering, the signal H_3O^+ and $H_3O^+ \cdot H_2O$ may not be constant and varies with the humidity [29]. The intensity of the signal H_3O^+ changes in the HC and the water content in the samples often is different. Therefore the impact of the humidity has to take into account during proton transfer reaction [29].

The following equation was suggested [29] where X is a factor which is specific for each compound and account for the reaction rate of the VOC with the monohydrate cluster ion and the transmission efficiency of the monohydrate water cluster ion.

$$\frac{MH^+}{H_3O^+} \rightarrow \frac{MH^+}{H_3O^+ + X \times (H_3O^+ (H_2O))} \quad \text{Equation 2. 27}$$

The factor X depends of the compound measured and reproduce the difference in efficiency for the detection of H_3O^+ and $H_3O^+ \cdot H_2O$ ions in the QMS and the rate coefficient different for H_3O^+ and $H_3O^+ \cdot H_2O$ in the proton transfer reactions [29]. Values of $X \approx 1$ imply H_3O^+ normalized signals independent of the humidity. Aromatics compounds do not react at all with the $H_3O^+ \cdot H_2O$ their X values are approximately to zero. For other VOCs $X \approx 0.5$ [29].

2.1.4 Advantages of PTR-MS

The application of PTR-MS technology has several advantages compared to other types of mass spectrometers. These are the major ones

- (i) Detection of low concentrations of volatiles due to the high sensitivity (down to part per billion by volume (ppbv)) with no gas sample pre-concentration.
- (ii) Minimal fragmentation of the volatiles compounds analysed; during soft ionization process a small amount of energy is transferred as a result very little fragmentation are obtained. The advantage is that the spectra are easier to interpret compared to other ionization methods.
- (iii) Provide real-time measurements and quantification of volatile organic compounds with low response times.

- (iv) Minimal sample preparation, headspace of volatiles are measured.
- (v) The sensitivity of the measurements is similar for detection of volatiles with different m/z .
- (vi) No reaction with the main constituents in air (N_2 , O_2 , Ar, CO_2 , etc.) due to the lower proton affinity compared to water.
- (vii) PTR-MS is a transportable instrument. The device can be used in different places or locations.

2.2 PTR-MS Data Analysis

2.2.1 PTR-MS and the recording of data

The quadrupole is controlled using the software for data acquisition supplied by Balzers QMS-422. The software consists of different controller programs for the control of the PTR-MS system. The software allows the use of different settings controlling water flows and gas inlet flows, voltages in the drift tube and data recording and visualisation as well.

The PTR-MS can be used in two different ways for ion detection; Mass Scan and Multiple Ion Detection mode (MID). A mass scan, is generally used when the ion products of the reactions are unknown. When the PTR-MS instrument is run in the MID mode, the user can monitor individual peaks of selected m/z values as a function of time so that rapid changes (time resolution 100 ms) in concentrations can be recorded.

An important parameter for mass scan and MID modes is the dwell time. The dwell time characterizes the time spent for the quadrupole for the data acquisition on a particular m/z . In mass scan mode the dwell time can be chosen from 0.5 ms up to 60 s per channel. The setting used for the data acquisition in this study was found for an

optimal data acquisition of 0.5 s. The dwell time can be used to improve signal to noise. For instance, volatiles at high concentrations (e.g. greater than 100s of ppbv) do not need a long integration time whereas volatiles at low concentrations (several ppbv) need longer integration times (seconds). When measuring low concentrations and the product ions are known for a given compound the dwell time can be increased significantly over that possible with mass scan mode by using a MID mode, thereby improving the counting statistics.

In the PTR-MS instrument used in this investigation, data extraction and analysis of the spectra (mass scans and MID) was not an automatized procedure. Every cycle generated was converted to an ascii file through the Balzers Quadstar 422 software package and then copied to an excel spreadsheet.

2.2.1.1 Mass scan mode

In this selected mode the mass scan generates a complete spectrum showing ion counts per second versus m/z . In the mass scan mode the instrument records all the intensities between two selected m/z values in the software. Figure 2.8 shows an example of a typical mass spectrum generated in the mass scan mode of a breath sample over the mass range from m/z 30-75.

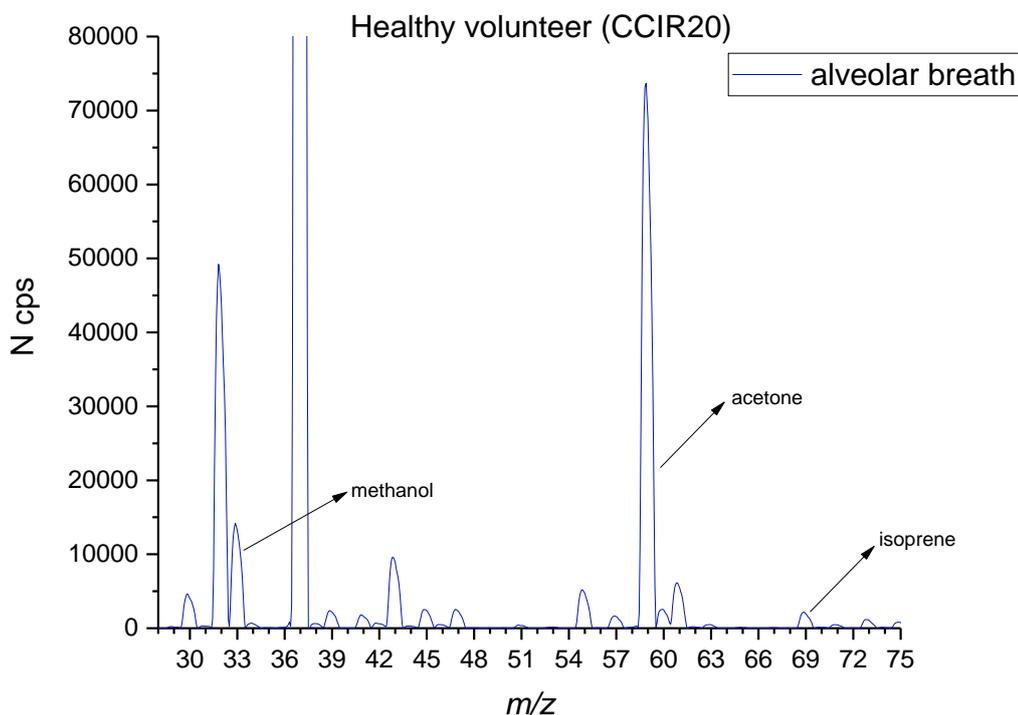


Figure 2.8 Mass scan spectrum of an alveolar exhaled breath sample from a healthy volunteer. Key volatiles present in the breath are identified.

In the software settings, the mass scan mode does not allow the user to choose individual mass dwell times instead a specific dwell time is required for the complete cycle. A scan cycle is finished when the whole range of m/z values chosen has been scanned over a specified dwell time for each point.

2.2.1.2 Multiple ion detection (MID)

By way of illustration, figure 2.9 shows a typical MID spectrum for an on-line real-time breath sampling for which ions at m/z 33 (protonated methanol), m/z 59 (protonated acetone) and m/z 69 (protonated isoprene) were monitored. MID generates a file with the specific masses measured. This mode is often used to measure concentration of specific ions compounds as well as monitoring changes on VOCs over time.

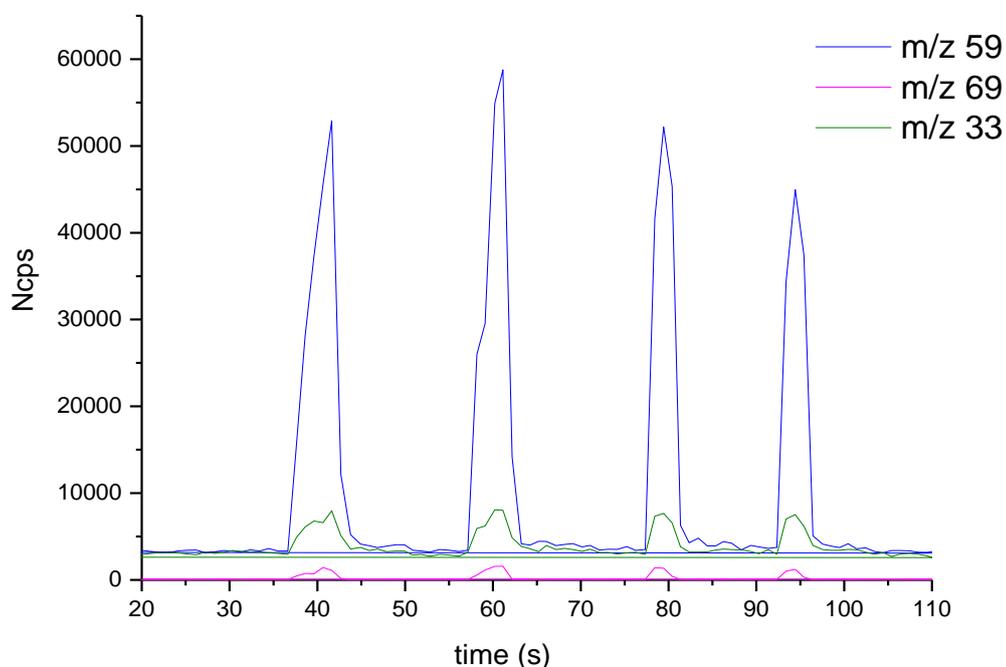


Figure 2.9 MID spectrum of alveolar breath sample.

2.2.2 Statistical analysis

For each measurement three scans were averaged and added to one sum spectrum. The following approach was applied to all data presented in this study. For the comparison study between patients and healthy volunteers, the appropriate analysis tool was selected.

Choosing the statistical test is essential depending on the data generated. IBM SPSS version 22 software was used for all statistical analysis. The distribution of the data was assessed using Shapiro-Wilks test which found that the data was not normally distributed. A non-parametric statistical test based on the result obtained was chosen with Shapiro-Wilks test. Mann-Whitney U-tests determined which m/z values differed between the patients and controls. A Wilcoxon signed rank test was used to determine which volatile concentrations differed between pre- and post-transplant breath samples. To compare blood chemistry values with breath volatiles Kendall's tau-b correlation coefficients were measured.

Finally, in order to know how reliable is the study for diagnostic test evaluation of volatiles Receiver Operating Characteristic (ROC) curves were used to graphically plot the true positive rate (TP), sensitivity in function of the false positive rate (FP) (100-Specificity) [30]. The area under the ROC curve measures how well the volatiles can distinguish between two diagnostic groups (in this case patients versus healthy volunteers).

2.2.3 Calibration

The PTR-MS instrument was regularly checked to measure the sensitivity using limonene as the calibration gas standard. The calibrations were performed at the same pressure and at a constant temperature set during the breath samples measurements from patients and controls described in chapter 4 and 5. During the calibration the DT voltage was 600 V, the pressure in the DT was 2.07 ± 0.01 mbar at a temperature of $45 \pm 1^\circ\text{C}$. The PTR-MS was calibrated using a KINTEK gas standard generator and permeation tubes. The emission rate of the permeation tube was calculated by measuring the rate of weight loss. The permeation tube was placed inside the KINTEK unit and limonene vapour was emitted through the tube wall when the gas standard generator was at constant temperature. The sensitivity obtained was 700 cps/ppb. In general, a good agreement was found between the gravimetrically method and the calculated method using the collisional reaction rate coefficients.

3 BREATH ANALYSIS FOR CLINICAL DIAGNOSIS AND MONITORING

3.1 Introduction

The potential of breath as a diagnostic medium for prognosis of a disease has been reported since the time of Hippocrates (460–370 BC). He described a fecal smell, which is a sign of a liver failure called fetor hepaticus, in his studies on volatile organic compounds in breath. Modern breath analysis took place many centuries later when in the early 70s, Linus Pauling analysed an extensive number of volatiles in exhaled breath and in urine vapour by using gas chromatography (GC) [31].

In recent years, the analysis of blood, urine and other body fluids have been established as routine analysis in clinical settings. However, breath analysis is now attracting great interest clinically owing to its potential as non-invasive and faster diagnostic method for disease monitoring and diagnosis [23]. Breath analysis can also be used as a technique to provide information about the metabolic processes occurring in organs or places in the body. Alterations in the metabolic pathways due to abnormal metabolism or a disease can lead to changes of concentrations in the breath VOCs or new ones being produced related to a disease.

The analysis of exhaled breath has many advantages [32]. For example, breath analysis has a low risk of infection and breath is a biological sample that does not need to be stored and there is no need to be concerned about safe disposal compared with the blood and urine analysis samples. A technology that has been applied in the medical

setting in the last 20 years for breath analysis is Proton Transfer Reaction Mass Spectrometry.

This technique has been used in research for the on-line monitoring of VOCs in variety of areas from the analysis and the detection of anaesthetics in exhaled breath [4, 33] [34], to skin volatiles [35] and the monitoring of microbial volatile metabolites [36].

However, it is important to highlight that breath sampling and data analysis are not trivial. There are many pitfalls such a confounding variables that can lead to erroneous results and conclusions [37].

3.2 Human Respiratory System Physiology

The main function of the human respiratory system is to provide the cells of the human body with a continuous flow of oxygen and to remove carbon dioxide. The respiratory system consists of two sections, the upper respiratory tract or upper airway and the lower airways.

The upper respiratory tract includes the parts of the respiratory system above the glottis or vocal cords. The upper respiratory tract (upper airways) consists of nasal and oral cavities, pharynx and larynx (voice box). The lower respiratory tract (lower airways) consists of the trachea (wind pipe). The trachea divides into the two primary bronchi, bronchioles and the lungs. Figure 3.1 shows schematically the structure of the human respiratory system and the alveoli in the lungs [38]. The bronchial tree includes the bronchi, terminal bronchioles, alveolar sac and alveoli. The bronchial tree allows the air pass from the trachea to the lungs.

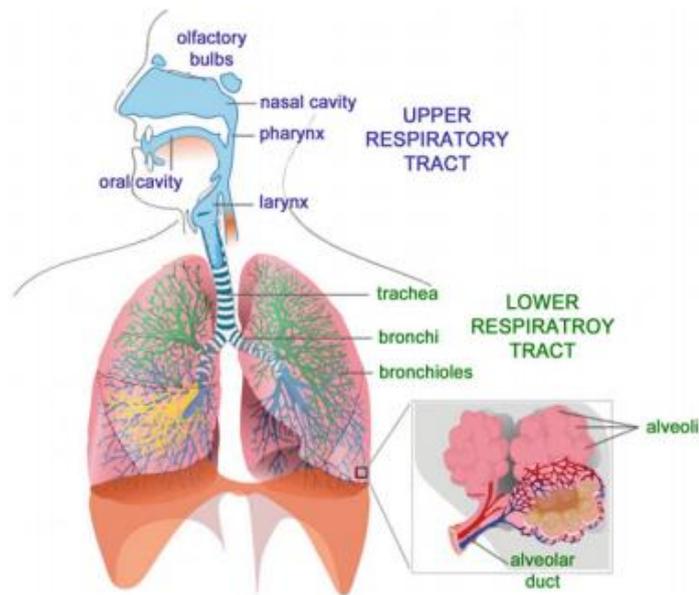


Figure 3.1 Human respiratory system and the structure of the alveoli in the lungs [38]. Reprinted from Computational Fluid and Particle Dynamics in the Human Respiratory System with the permission of Springer International Publishing.

3.2.1 Gas exchange

The primary function of the human respiratory system is the gas exchange. Gas exchange takes place in the capillaries of each alveolus in the lungs and occurs through diffusion by a concentration gradient. The oxygen is taken from the air and diffuses in the alveoli into the bloodstream and waste carbon dioxide is removed in the lungs via air sacs (alveoli) at the end of the bronchial tubes.

The composition of O_2 and CO_2 contained in the breath changes during the process of breathing due to gas exchange, for instance inhaled air contains 21% oxygen and 0.04% CO_2 in contrast exhaled breath contains 16% and approximately 4% CO_2 .

3.2.2 Lung capacities and volumes

An adult male has a lung capacity of approximately 6 litres; the actual lung capacity is determined by factors such as age, gender, weight, height and ethnicity. During normal and relaxed breathing, only a small volume of this capacity is used and is on average 500 ml of air. This volume of air breathed during normal inhalation and exhalation is the tidal volume. Figure 3.2 shows a schematic diagram of the different lung volumes and capacities [38].

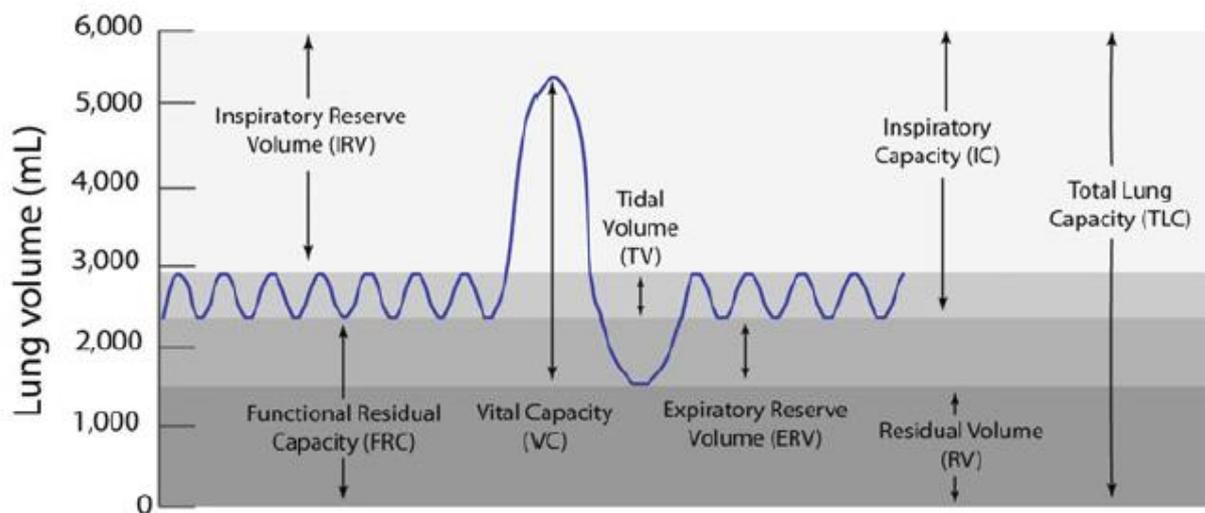


Figure 3.2 Lung volumes and capacities. Reprinted from Computational Fluid and Particle Dynamics in the Human Respiratory System with the permission of Springer International Publishing [38].

The respiratory lung capacities can be define into four different volumes:

- Tidal volume (TV) (previously described)
- Expiratory reserve volume (ERV)
- Residual volume (RV)
- Inspiratory reserve volume (IRV)

The inspiratory capacity (IC) is the maximum volume of air that can be held in the lungs and inspired in a full inhalation. The vital capacity (VC) is described as the maximum volume of air that is forcibly expired from the lungs after a fully inhalation. The VC can be calculated by the sum of the expiratory reserve volume (ERV), inspiratory reserve volume (IRV) and tidal volume (TV) [39].

The expiratory reserve volume (ERV) is the additional volume of air that can be exhaled after a normal exhalation of a tidal volume of air. The additional volume of extra air that can be inhaled in a normal expiration of a tidal volume of air is called the inspiratory reserve volume (IRV).

The residual volume is describes as the volume of air that still remains in the lungs after a maximal exhalation. The maximum volume of air that can hold in the lungs is defined as the functional residual capacity (FRC). Total lung capacity (TLC) is the total volume of air that can hold in the lungs which is approximately 6000 ml of air in healthy adult males.

3.3 The Origin and Composition of Breath

According to the origin of the volatile organic compound exhaled in the breath can be divided into two well differentiated groups, exogenous or endogenous [40]. Endogenous compounds are produced in the human body and provide information about metabolic pathways and different pathologies and diseases. Endogenous compounds can contain inorganic gases such as nitric oxide and carbon monoxide, or volatile organic compounds such, methanol, isoprene and acetone [41].

If the origin of the VOCs is due to an exposure of pollutants from the environment, diet intake, drugs or absorbed through the skin, then they are referred to as exogenous compounds.

Exhaled breath contains a variety of gases such as oxygen, nitrogen, carbon dioxide, inert gases, water vapour and small concentration of gas traces in variable concentrations [42]. These volatile compounds are detected in human breath at typically parts per billion (ppbv) and part per million (ppmv) by volume. For example, a previous research has measured breath acetone and isoprene levels ranging from 515–2335 ppbv and 308–702, respectively [3]. Compounds such as isoprene and acetone can be correlated with metabolic processes in the human body. Table 3.1 shows a list of typical molecules measured in human exhaled breath, adapted from references [7] [43] and [44]. Ketones bodies, such acetone is associated to lipolysis and glucose metabolism. Isoprene in exhaled breath is a by-product of cholesterol biosynthesis. High levels of volatile sulphur compounds have been detected in patients with chronic liver disease with respect to healthy volunteers [5, 42, 45].

Table 3.1 Details of volatiles in breath, concentration range and metabolic process involved [7] [43] [44].

Compound	Range	Metabolic process
Acetaldehyde	ppb	Lipid peroxidation/ Ethanol metabolism
Acetone	ppb-ppm	Fat catabolism
Dimethyl sulphide	ppb	Liver dysfunction/ Oral bacteria
2- butanone 3- butanone	ppb	Liver dysfunction/ Fatty acid oxidation
Methanol	ppb	Liver dysfunction/ Pectin degradation/ Gut flora
Ammonia	ppb	Protein metabolism
Isoprene	ppb	Cholesterol biosynthesis
1-pentane	ppb	Lipid peroxidation
Hydrogen sulphide	ppb	Liver function/ Protein metabolism of bacteria
Ethanol	ppb	Gut bacteria/ Drinking

3.3.1 Breath volatiles for medical diagnosis

To date, the use of breath volatiles for medical diagnosis has been met with limited success. Confounding factors, such as volatiles present in the environment, contamination in the sampling procedures, poor sampling methods and influence of food intake, have meant that there is a great deal of uncertainty in volatile discovery [46] [47]. Problems of bias and false findings in biomarker discovery research have been widely reviewed [48, 49].

Most of the studies in breath analysis have been mainly focused on the determination of concentration of volatile organic compounds such as isoprene, acetone, ethanol, methanol since they are contained in a significant range in the human breath [3, 50]. The production of acetone in breath is related to the insulin and blood sugar and fat metabolism. When the glucose levels are low in the blood, the body produces ketones (acetone) from adipose tissue as a source of energy. The triglycerides (TG) are major constituents in the body fat, which can be break down into one glycerol molecule and 3 fatty acid molecules by the hormone lipase (HSL). If the glucose levels are low, the fatty acid levels in the blood increase and the catabolic process called beta-oxidation takes places. In the process the TG are broken down and generate Acetyl-CoA. When the concentration of Acetyl-CoA rises, it triggers ketogenesis. During the ketogenesis acetoacetate is formed first and the converted into two types of ketones: beta-hydroxybutyrate and acetone [51].

Acetone levels rise in patients suffering from diabetes because of the influence of glucose metabolism. Previous studies have shown that the concentration of acetone rise when the volunteer is on a low-carbohydrate diet or are fasting [52] and in adults with alcoholism. The concentration of acetone in breath has been proposed to predict ketosis and can be used as a tool to understand metabolic pathways of the diet, fat loss or to predict diabetes. However, due to the large variability in acetone levels in exhaled breath through the day in individuals and other caused that can influence acetone levels such as health status, diet, and age, the monitored acetone level cannot be used with confidence.

Another common endogenous breath volatile which has been the focus of attention is isoprene. Isoprene is linked to be part of the cholesterol biosynthesis, although its biological pathway of biosynthesis and origin is still unclear. Therefore,

analysis of isoprene levels in exhaled breath proves to be particularly valuable as a method to detect lipid digestion, diagnose cholesterol or lipid disorders. It is important to mention that due to its variability with movement, isoprene can only be used as non-invasive marker under cardiac output controlled conditions and only if heart rate is maintained constant during the breath sampling.

For instance, isoprene concentration increases during exercise in part because the heart rate has a direct impact on its concentration increased the blood flow. Isoprene is excreted from the muscles and exhaled in the breath during exercise due to pulmonary gas exchange process occurring in the body tissues. It is known from previous studies that isoprene concentration can increase by a factor of ~3-4 within about one minute during exercise [53].

A number of recent studies on breath analysis for the diagnosis of lung cancer have been based on the analysis of breath samples by GC-MS techniques. A large combination of VOCs that either increase or decrease in concentration if cancer is present has been identified [54]. Feinberg and co-workers investigated the volatile profile before and after an oral glucose test in patients suffering from lung cancer. The findings suggested that the oral glucose tolerance test has low effect on the tumour cells metabolism [55]. A cross-sectional study, carried out by Phillips et al. proposed that 22 VOCs are found in patients with lung cancer [2].

In order to support the study of volatiles in breath and understand better the metabolism and their physiological processes involved, several studies have focus on the investigation of volatiles emission from growth medium [55]. Feinberg and co-workers investigate the volatile profile of lung cancer cells by PTR-MS. They found that lung

cells had a distinct sub-population depending on the activity of the mitochondrial cells which can be discriminated by a different volatile profile [56].

In particular, O'Hara et. al investigated the profile of volatiles of bacterial cultures. The finding suggested that there were unique molecular marker for the bacterial culture which was independent of the culture growth [36].

3.4 Methods of Alveolar Breath Sampling collection

Lack of standardization and sampling procedures has led to differing results and conclusions. Although a number of groups have suggested a standardized practise in breath analysis, it is still not being commonly used and so it has not been established in the breath community [57]. A problem is in part associated with the fact that no one method may be suitable, depending on the disease and volatiles.

Following an established method for collecting breath samples is crucial to have reproducible results. The difficulty in breath collection is that exhaled breath is not an homogenous sample. For this reason, breath should be sampled under specific protocol and controlled sampling procedures [58]. The challenge in breath sampling is the separation and collection of the alveolar breath from the dead space (volume of breath contained in the upper airways). The aim in breath analysis for biomarker discovery is to be able to determine when the alveolar phase is reached. For this CO₂ levels could be monitored during breath sampling.

For PTR-MS, and especially where breath to breath analysis is being done in real-time it is possible to monitor for instance acetone levels to ascertain when the alveolar

phase has been reached. However, this is limited because the PTR-MS can not be brought to a patient.

The sampling of VOCs in breath can be undertaken following two different methods. In the off-line analysis, the sample is collected in bags, canisters, syringes or using pre-concentration methods such as needle traps. In contrast, for on-line breath gas sampling, patients are able to breath directly into the instrument and the sample is analysed in real time [59]. In this way, certain problems due to sample decomposition and contamination are avoided.

3.4.1 Use of capnography for alveolar breath sampling

Capnography is a technique that gives us an excellent picture of the respiratory process and can be used to improve gas-sampling methods. Capnography provides a wave display of the carbon dioxide CO_2 during the respiratory cycle. In medicine is used during anaesthesia and intensive care as a non-invasive analysis providing and recording CO_2 partial pressure measurements in exhaled breath over time.

It is important to note that a capnograph will display an erratic waveform when there are air leaks in the airways system, tubing obstructions, disconnections and CO_2 recording apparatus fails to function normally [60]. For instance, the shape of CO_2 waveform is monitored in order to detect any malfunction in the system. The capnogram displays a uniform and stable shape when CO_2 measurements are acceptable. Whereas a capnograph shows an irregular and anomalous shape of CO_2 waveform when there is a dilution of expired CO_2 in the system.

The end of exhalation, called end-tidal CO₂, (Et-CO₂), is correlated with the alveolar phase in exhaled breath. Et- CO₂ represents the maximum concentration of CO₂ or partial pressure expressed as a percentage or in units of mmHg. The Et- CO₂ is measured by a sensor located between the patient airway and the capnograph.

The technology is based on infrared spectroscopy (IR). CO₂ absorbs in the IR part of the electromagnetic spectrum at very specific wavenumbers (usually the line at 2350 cm⁻¹ is used) [61]. Detecting these energy absorptions, using an appropriate photo detector sensitive in this spectral region, allows CO₂ concentrations in a gas sample to be easily determined.

The capnograph monitor displays the CO₂ waveform which is called capnogram, showing the relationship of CO₂ concentration versus time. The normal values are 5% to 6% CO₂ and 35-45 mmHg [62].

Figure 3.3 shows a normal capnogram and the phases in the expiratory and inspiratory cycle. The first phase point A to B, represents the end of inspiration. The phase from B to C represents the beginning of expiration, exhalation of CO₂ free gas from the anatomical and apparatus dead space. This is the first step in exhalation. From point C to D the volunteer begins to exhale additional CO₂ from the connecting airways and alveoli of the lungs. This phase represents air from the alveoli and CO₂ remains relatively constant because mainly alveolar gas is exhaled and is defined as the alveolar plateau. The Et-CO₂ is measured at the end of the plateau (D) correspond with the highest concentration of CO₂. Once the majority of air is exhaled from the airways, the inhalation begins and the increasing slope of the waveform will begin to slow down (D-E). Oxygen fills the airways and CO₂ levels decreases back to zero.

Uncontrolled breath sampling has been shown to be unreliable. For the correct analysis of exhaled breath it is crucial to monitor and collect only the alveolar phase of the volunteer breath. Previous investigations have determined the impact of sampling methods on the analysis of VOCs in exhaled breath. A higher concentration of volatile organic compounds in alveolar breath samples is found compared to mixed exhaled breath samples [63].

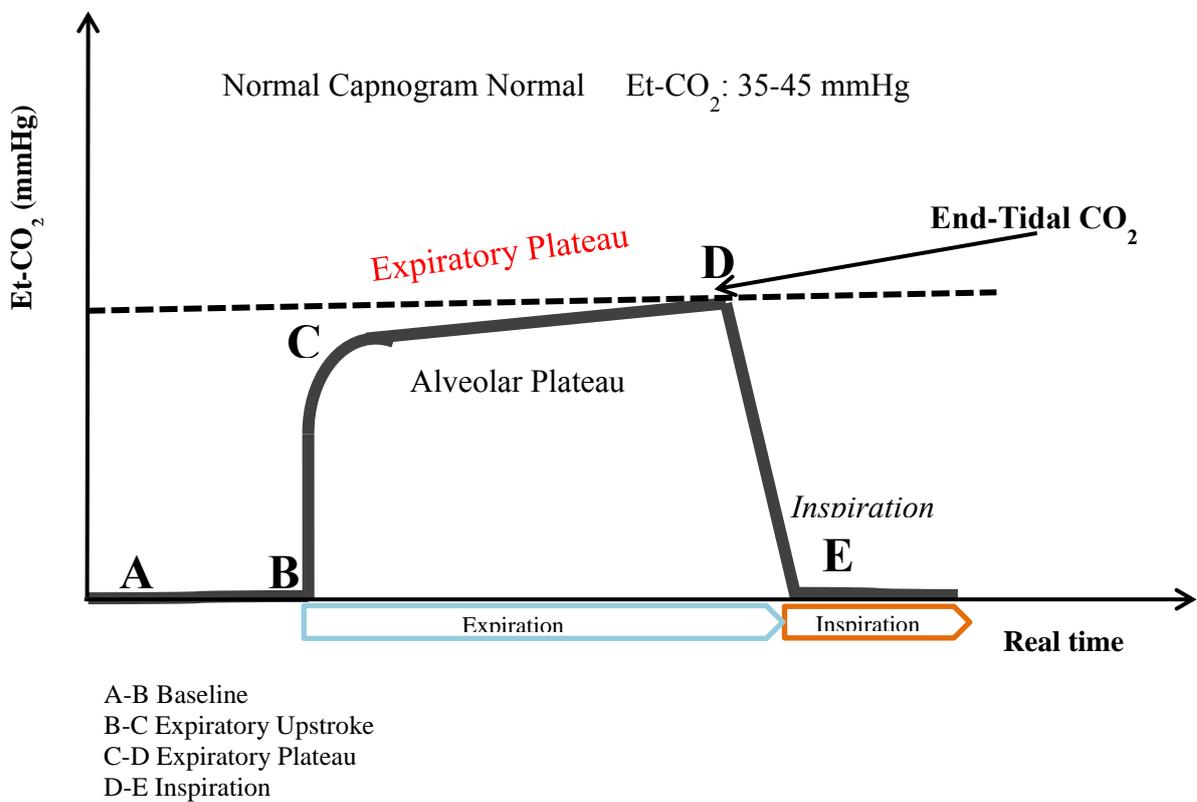


Figure 3.3 Phases in the expiratory and inspiratory cycle.

Figure 3.4 shows a picture of the capnometer used in this study to control the expiratory and inspiratory phases. It is a mainstream capnogard model 1265 from Novamatrix Medical Systems, INC (Wallingford, USA).



Figure 3.4 Capnogard 1265 from Novamatrix.

In figure 3.5 a screen close-up is shown in which the phases in the expiratory cycle can be clearly identified.

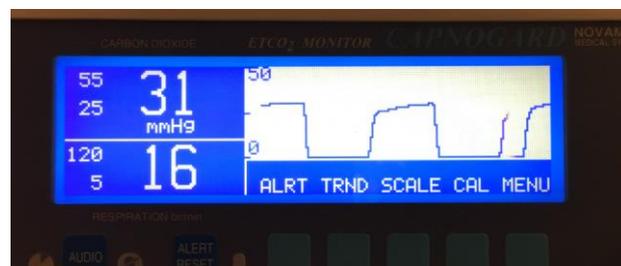


Figure 3.5 Photograph of a screen display on a capnograph.

This capnograph is a small and light portable instrument that completes a self-testing and auto-calibration within minutes. The capnograph provides a screen display with information of the end tidal CO₂ with an alarm set event.

3.5 Optimization of a Breath Sampling Protocol

The first aim was to improve breath-sampling techniques for off-line analysis. The main goal of this study was to establish the most suitable method for breath sampling collection and the optimal cleaning protocol for this investigation. There is no adopted breath collection method for the analysis of volatile analysis and uncontrolled breath sampling is not acceptable due to the high variability in the VOCs concentrations [64].

It was necessary to test and compared the suitability of flexFoil bags and glass syringes in order to establish the protocol for the collection of exhaled breath. The investigation was focused mainly on the issues of background effects.

3.5.1 Testing SKC flexFoil bags

The figure 3.6 shows a photograph of a SKC FlexFoil PLUS bag. The manufacturer of SKC bags state that they are ideal for collecting and storing human breath samples for short period of time and can store low-level (ppb) VOCs in addition to sulphur compounds and low molecular weight gases. The bag (SKC FlexFoil PLUS) has PTFE fittings attached. This fitting has a syringe port with PTFE-lined septum for taking samples for using in gas chromatography mass spectrometry analysis (GC-MS).



Figure 3.6 Photograph of a SKC FlexFoil PLUS bag and fitting.

For the collection of the breath samples in this investigation, SKC FlexFoil PLUS (SKC, USA) bags were tested. Prior to testing, the SKC bags were flushed three to four times with nitrogen to remove any remains of background contaminants and stored in an oven at a temperature of 45°C overnight.

Before the analysis, the bags were flushed again with nitrogen. The content of the bag was analysed after cleaning and compared with the analysis of the laboratory air to monitor contaminations. Figure 3.7 shows a mass scan spectrum of a SKC bag background versus lab air background. In the figure 3.7 the mass spectrum clearly indicates a high background coming from the SKC bags. The SKC bags were not suitable for this study and therefore were not chosen for the breath collection. Thus, glass syringes are preferred over bags and for breath sampling.

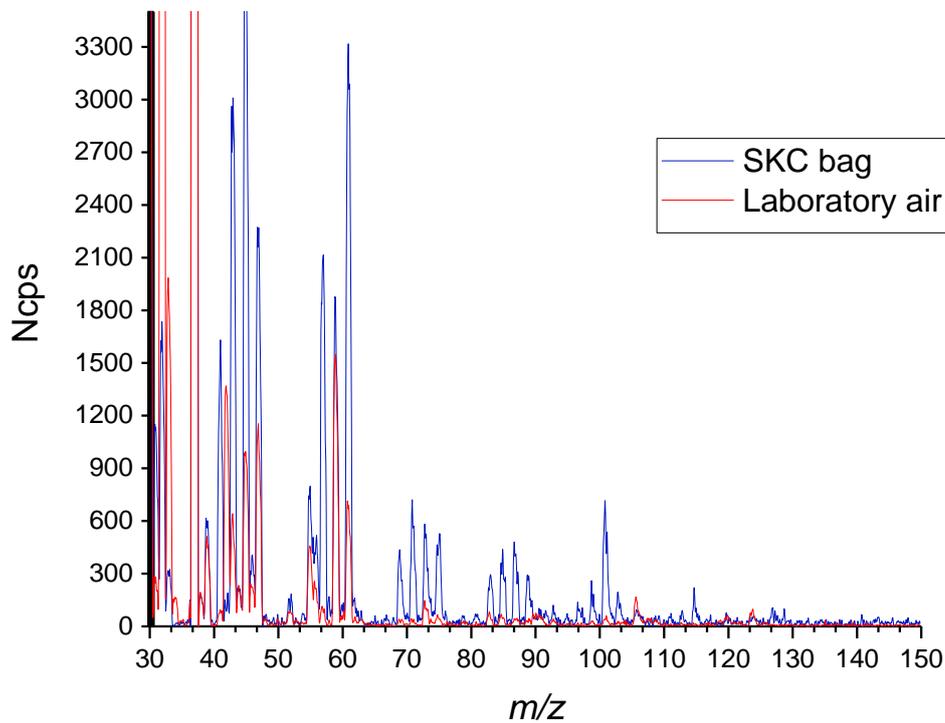


Figure 3.7 Mass scan spectrum of a SKC bag background versus laboratory air background.

3.5.2 Testing glass syringes

Glass syringes (Sigma-Aldrich) were tested and compared to SKC bags in order to probe their suitability to collect exhaled breath. The glass syringes were tested after a sterilization cycle. Glass syringes were flushed with N_2 several times and filled with lab air for the subsequent analysis. The content of the glass syringe was analysed immediately after the sterilization. Tests carried out showed that glass syringes were more suitable to use to collect breath in comparison to bags. The figure 3.8 shows a mass

scan spectrum of a glass syringe background compared to a mass scan spectrum of laboratory air.

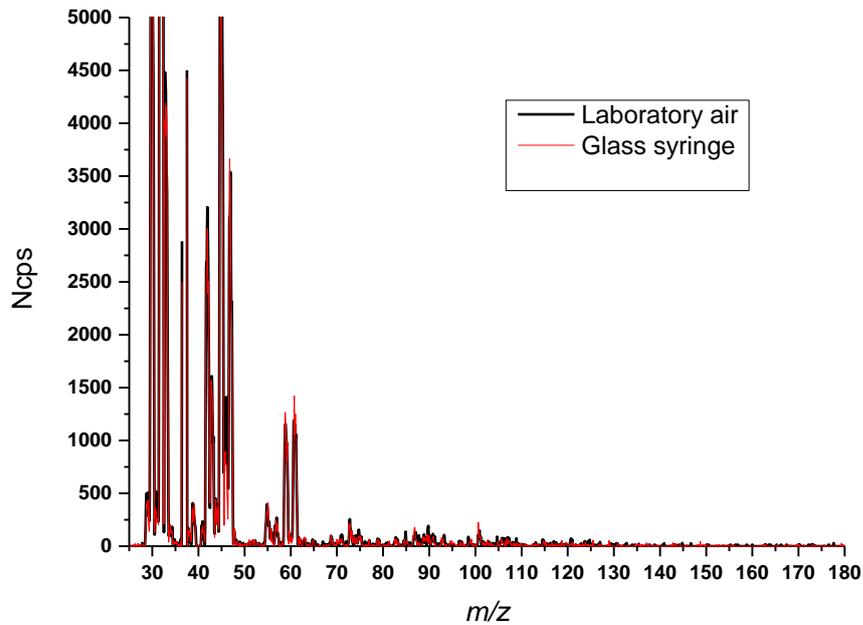


Figure 3.8 Mass scan spectrum of a glass syringe background versus laboratory air background.

For the collection of the breath samples, glass syringes (Sigma-Aldrich) with a capacity 100 ml (graduation line) and metal luer lock tip were required. Figure 3.9 shows a photograph of the type of glass syringe used in this investigation. Glass syringes have many advantages in comparison with Tedlar bags. For instance, the fortuna optima glass syringes are easy to clean and sterilize using autoclave systems avoiding the inconvenient of high backgrounds.



Figure 3.9 Photograph of a Fortuna® Optima glass syringe.

Glass syringes are considered more suitable for collecting and storing gases compared to bags [65]. They have a luer lock attachment that can be used to connect the glass syringe to the male or female luer or to small tubing of a respiratory system. The glass syringe has a plunger seal that creates a leak-free seal. Using the luer lock fittings the breath samples can be sealed reducing sample dilutions or contaminations with clinic or lab air. The cleaning method is essential factor for the reusability of the glass syringes. Glass syringes were tested after use and cleaning sterilization procedures. The results found that breath volatiles did not adsorb onto the glass. It also could not be found above the limit of detection any volatiles that could affect the analysis. All the concentrations measured during the study described in chapter 4, 5,6 were above the limit of detection of the PTR-MS instrument. The results of this investigation showed that glass syringes can be reused after a cleaning sterilization protocol.

Cleaning glass syringes in comparison to bags is quicker, safer for the material and more convenient. The cleaning procedure time for glass syringes sterilization takes only 3 minutes and the autoclave has the capability of sterilising up to 5 glass syringes in one cycle.

3.6 Breath Sampling Protocol

A 100 ml glass syringe (Sigma-Aldrich) was coupled to the respiratory sampling device tubing using a 3-way luer-lock stopcock (Braun Medical Limited) to seal the respiratory collection system. Figure 3.10 schematically shows the sampling system used for collecting the breath samples for this study.

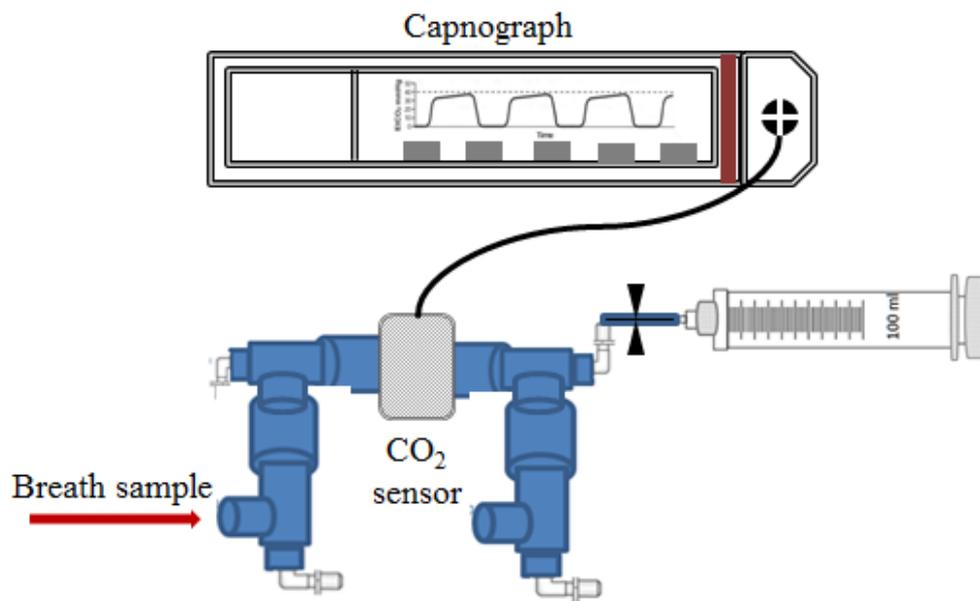


Figure 3.10 Schematic diagram of the breath sampling device. Breath samples are only drawn into the glass syringes once the capnograph shows that the alveolar phase of the exhaled breath has been reached. Typically 3-4 breaths are needed to fill a syringe to 100ml.

The figure 3.11 shows a photograph of a volunteer sitting down in a relaxed state. The subject was asked to breathe normally into a gas tight respiratory system containing an in-line CO₂ mainstream sensor connected to a fast-time response capnometer (Capnogard 1265 Novametrix Medical Systems Inc.).



Figure 3.11 Photograph showing a healthy volunteer giving a breath sample. In the right, the photograph shows a close up of the capnostat sensor with the airways adapter coupled to the respiratory breath tubing.

A capnometer was used to ensure that only the end-tidal breath was collected for each volunteer. When the alveolar plateau of CO_2 was displayed on the screen of the capnograph, the breath sample was manually drawn from the subject's breath stream into the glass syringe. Three to four alveolar breaths samples were sampled for each 100 ml syringe in order to increase the precision and to reduce the statistical error. Four replicates were collected to examine the repeatability of the samples, in cases of

instrument failure or due to inappropriate handling during the measurements or in cases of issues in transportation.

The breath samples collected were sealed using a 3-way stopcock connector (2 female luer locks) and stored in an incubator at 40°C to reduce condensation effects during the measurements. Following this, the content of the glass syringes were analysed by PTR-MS. Figure 3.12 shows how the glass syringe is coupled to the PTR-MS inlet using a Swagelok connection.

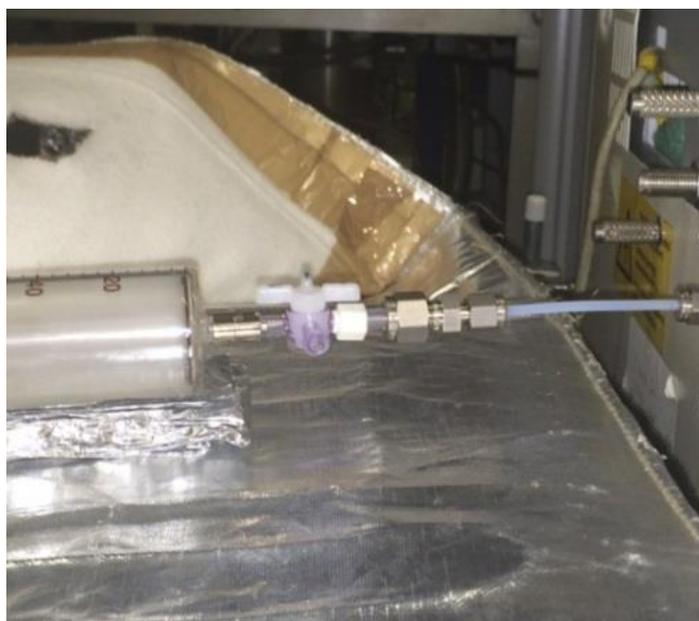


Figure 3.12 Photograph of a glass syringe attached to the PTR-MS

During the measurements, in order to minimize possible condensation of breath in the glass syringes, the glass syringes were covered with a plastic sheet and placed inside a heating blanket that was maintained at 45°C. Figure 3.13 shows the heating bag and plastic cover.



Figure 3.13 Heating blanket and plastic cover.

4 ANALYSIS OF VOLATILE ORGANIC COMPOUNDS IN BREATH FROM PATIENTS SUFFERING CIRRHOSIS AND THEIR COMPARISON PRE AND POST-TRANSPLANT

This chapter consists of a description of the analysis of breath volatiles for biomarker discovery and medical diagnosis relating to liver disease. The study involved patient recruitment, collection of the breath samples, mass spectral analysis of the breath samples using PTR-MS and analysis of the measurements. It is important to highlight that clinical research studies are challenging because of the need to recruit patients in clinical trials, to coordinate staff in the clinic and laboratory, liaise with physicians and patients, handle biological samples, manage and analyse large data sets and maintain secure database for the patient recruited.

4.1 Introduction

Liver disease is a major global health issue and the third most common cause of mortality and morbidity in the United Kingdom (UK), with cirrhosis accounting for 83% of liver related deaths [66], this rate is increasing every year being higher in the UK than in other European countries [67]. The publication of the 2014 Lancet Commission on liver disease has highlighted how the burden of liver disease in the UK has risen sharply over the past few decades and is the third biggest cause of premature mortality [68]. Liver disease has a widespread effect not only for the patient, encompassing physical and psychological morbidity and mortality, but also incurring significant societal costs.

Figure 4.1 shows the trend in mortality from liver disease in relation to the trend in mortality from other causes of death in the UK [69]. It is clearly shown that only liver disease has been increasing since 1970.

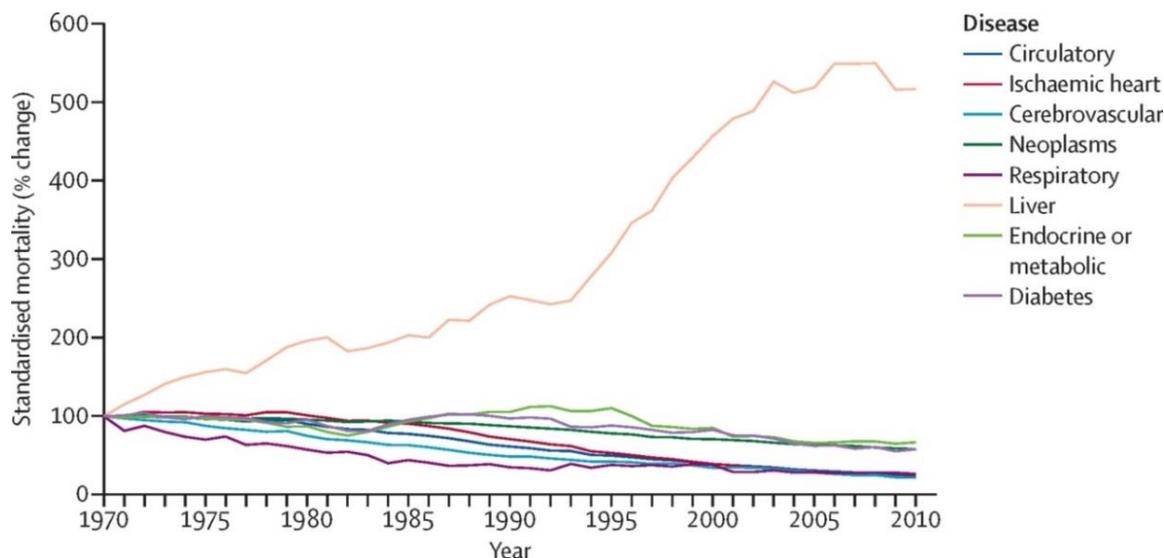


Figure 4.1 Standardized mortality rate data for common diseases in the UK. Data taken from the World Health Organization regional office for Europe. European health for all databases. WHO-HFA. Analysed by Nick Sheron [70].

It is relevant to mention that in Europe, the main causes of death among diseases of the digestive system are chronic liver disease and cirrhosis followed by stomach and intestinal ulcers [71]. Among the ten key recommendations detailed in the Lancet report is one to strengthen the detection of early-stage liver disease, which is essential to reduce disease progression. Analysis of volatiles in the breath has the potential of delivering this, but, only if chemical compounds can be found that are unambiguously associated with a diseased liver.

One of the main difficulties in the diagnosis of liver disease is that often patients suffering chronic liver disease do not present symptoms or a sign until the disease is advanced. Even then diagnosis is difficult and the symptoms and signs are often general and can be mistaken for other pathologies [70]. Unfortunately, end-stage liver cirrhosis is irreversible and to date patients suffering the disease can only survive with a liver transplant.

Liver evaluation methods can be categorised as between those that are non-invasive and the invasive methods. The non-invasive diagnostic techniques currently used are serum biomarkers, genetic tests and imaging techniques as fibroscan (FS) or transient elastography (TE). These techniques are not ideal and have limitations. Serum biomarkers are not liver specific and in case of inflammation, tend to be elevated.

TE results require an expert clinician for interpretation [72]. Liver biopsy is classified as an invasive method and remains to date as the “gold standard” to grade the staging of chronic liver disease and fibrosis.

One advantage of a liver biopsy is that a Consultant Hepatologist can obtain useful diagnostic information. Not only on cirrhosis but also on different pathologies related to the liver such as, liver necrosis, steatosis, liver cancer, hepatic deposits, cryptogenic and inflammation [73]. However, a liver biopsy has disadvantages such as, in sampling error, which can underestimate the grade of liver damage, and there is a risk of injury to the patient. Another disadvantage is that a liver biopsy is not suitable for follow up the disease. Cirrhosis can be missed on a single biopsy in 10%-30% of cases, increasing the percentage of a reliable diagnosis from 80% to 100% when several liver samples are analysed [74] [75].

Liver disease is an attractive target for breath analysis and hence has captivated the attention of the breath research community. A reason for this is that the liver plays a vital role in metabolism and performs critical functions in the human body. The liver is connected to two large blood vessels; the portal vein and the hepatic artery.

The portal vein is a vessel that delivers 70-75% of the total blood flow to the liver and the remainder coming from the hepatic arteries. The portal vein is different in comparison among other organs of the human body. It is unique due to the blood supply to the liver. The portal vein collects the blood which comes from the capillaries of the gastrointestinal track such as stomach, pancreas, intestines and spleens and delivers the blood to the liver tissues [76].

The hepatic portal vein does not carry blood into the heart. The function of the portal vein is to drain blood nutrients from the digestive track to the liver. The liver metabolizes and processes the metabolites in the blood before reaching the general circulation. Whereas the hepatic artery transports oxygenated blood to the liver.

Blood-based VOCs produced by impaired liver metabolism will undergo gas-exchange in the patient's lung and are exhaled in their breath and hence may provide a novel method to diagnose chronic liver disease. In the case of liver disease, previous studies investigating breath volatiles in patients suffering with chronic liver diseases have proposed a large number of possible biomarkers, but generally different studies report different volatiles [44, 45] [77].

Many of the biomarkers found are volatile sulphur compounds such as hydrogen sulphide, dimethylsulfide (DMS) or mercaptans [77]. The compound dimethyl sulfide has been reported to cause the distinctive odour found in the breath of patients suffering

chronic liver disease known as fetor hepaticus [78]. Van den Velde et al. proposed that the odour exhaled in the breath discriminated patients with liver disease and was mainly attributed to dimethyl sulphide [43]. Various GC-MS studies reported elevated levels of many volatiles in the breath of patients with liver disease, including dimethyl sulfide, acetone, 2-butanone, 2-pentanone, β -pinene, α -pinene, and limonene [79-82]. Studies using soft chemical ionization mass spectrometric techniques have reported volatiles such as acetaldehyde, ethanol, isoprene, benzene, methanol, 2-butanone, 2- or 3-pentanone, heptadienol, and a monoterpene (limonene) [44]. Friedman et al. reported levels of breath limonene in patients suffering chronic liver disease. However the authors did not conclude whether the elevated concentration found and the origin of limonene were a consequence of the chronic liver disease or coming from the diet intake [79].

Ammonia is another volatile compound that is present in breath as consequence of a hepatic disease [82] [83]. The concentration of blood ammonia is frequently measured to assess the severity and grade of hepatic encephalopathy (HE). HE occurs in patients suffering chronic liver disease. It is caused by the accumulation certain toxins and chemicals such as ammonia in the patient's blood stream. The liver is impaired and not able to convert the ammonia into urea that is excreted from the body in the urine. Patients with HE become drowsy and confused.

Spacek et al. reported differences in ammonia concentration between the breath ammonia and the venous blood ammonia in chronic cirrhotic patients. Concentration of ammonia in blood increases as a consequence of the disease, for instance, the presence of oral ammonia-producing bacteria, gamma-glutamyl transferase (GGT) and alanine aminotransferase activity (ALT) may be the reason as to why ammonia in blood is often over-estimated in cirrhotic patients [83].

Some volatiles, which have been proposed as biomarkers for liver disease, such as isoprene, acetone and ethanol, are not specific enough because they also result from other diseases or arise from numerous normal metabolic processes as previously mentioned.

In summary, although the results of previous studies are extremely encouraging, only several volatile organic compounds (VOCs) are common to more than two or three studies and it is not useful to have hundreds of putative markers.

If breath analysis is to progress to clinical utility, then markers must be definitively associated with the disease in question. This approach will provide a non-invasive method and tool to screen large populations, allowing the follow-up of patients suffering with chronic liver disease. All previous studies can be regarded as hypothesis-generating, in that they do not follow up in a second group to confirm the putative biomarkers.

The study presented here investigates the VOCs related to liver disease and demonstrates the diagnostic potential of breath analysis and its possibilities in the detection of chronic liver disease. This investigation directly connects biomarkers to a disease. Also, this work includes an independent study to confirm that the biomarker is linked to the disease.

A two-stage biomarker discovery process is reported:

- (i) Breath samples from a group of patients suffering from liver disease are first compared to breath samples from healthy controls.
- (ii) Post-transplant breath samples are then compared with a sub-cohort of these patients who went on to have a liver transplant.

A set of putative volatile markers is first determined by comparing patients with controls, and then pre- and post-transplant breath samples are examined to look for intra-individual differences in these volatiles. In this way, this study is hypothesis-led and uses patients as their own controls, thereby reducing the risk of false discovery.

Furthermore, the use of patients' companions as controls and of room air samples minimizes the influence of any exogenous volatiles present in the home and hospital as confounding factors. The findings and results from this study presented in this chapter 4 have been published in the biomedical journal of EBiomedicine supported by The Lancet [5].

4.2 Methods

4.2.1 Ethical approval

This study was established and conducted in collaboration with Dr. Andrew Holt and Dr. Tahir Shah, both consultant physicians employed at the liver transplant unit at the Queen Elizabeth Hospital Birmingham and Dr. Tony Whitehouse, Consultant Anaesthetist in the Intensive Care Unit (ICU) in the Department of Anaesthesia at the University Hospitals Birmingham NHS Foundation Trust.

The study was approved by the regional ethics committee of Camden and Islington (REC reference: 13/LO/0952). Provided in the appendix of this thesis are the protocol documents for the permission of the study and the ethical approval application. The study protocol was a crucial document providing all the requirements and design for the proposed research study.

The protocol consists of different details about the proposed project, such as goal of the study, participant selection and recruitment, actions, people and investigators involved in the study, locations, inclusion and exclusion criteria, and a record of consent and patient information forms.

This study was conducted in agreement of the ethical principles of Good Clinical Practice. The study was performed according to the ethics documents reviewed by the research ethics committee and any amendments made were submitted to and then approved by the research ethics committee.

4.2.2 Patient selection and recruitment

Patient and control recruitment is one of the most challenging, time-consuming and unpredictable phases of clinical trials. A successful recruitment involves a well-coordinated research team and plan that can be assessed. It is important to identify specific subjects with the right characteristic and profile, which is difficult sometimes. However, in the study presented, the patients recruited were well characterized by following the main inclusion criteria. Consultant physicians in liver medicine and hepatology were responsible for the edification and selection of potential participants in this clinical study. For this study, patients were recruited according to the approved protocol at the University Hospital Birmingham either from the transplant assessment clinic or in wards after being admitted with hepatic encephalopathy.

In the case of follow-up clinical trial of subjects, carefully planning and patient tracking is needed to enhance the follow-up and avoid data lost. A deficient follow-up restricts the suitability of the longitudinal data.

Once a right candidate was selected, a meeting was arranged during which it was discussed the goal of the study and provided all the necessary information about the research and all the details in clinic. In this way patients and healthy controls could make an informed consent.

It is important to highlight that in this study companions of patients were asked to act as healthy controls as they have been breathing the same air in the recent past as the patient. Confounding factors such as VOCs in the clinic air were therefore better controlled and minimized. More importantly, patients and companions often share a diet and home environment with the patients, therefore long-lived VOCs that can be stored in fat tissues or other body compartments were also controlled.

The candidates were also provided with either a patient or a volunteer information document regarding the aim, risks of the study, number of samples required (information documents are attached in the appendix of this thesis). Before patient recruitment, written informed consent was obtained from each volunteer. Volunteers had time to agree to take part in the study or not.

Candidates were asked to fill out an information sheet to provide details on their home environment, diet, smoking status, health and medications. During the collection of the breath samples a research nurse was present at the liver transplant assessment unit and was responsible for the collection of the consent forms and completing the patient case support forms, collecting patient data and ensuring the anonymity of the patients. Enrolled patients and controls were assigned with a unique alphanumeric code. All the personal data related to the patients and volunteers were held on the confidential password-protected databases and the master lists were not accessible outside the clinic.

4.2.3 Conditioning and cleaning of the sampling system

The glass syringes used to collect the breath samples were cleaned and sterilized in an autoclave prior to and after their use, following the autoclave standard operation procedure (SOP) to ensure that all infectious material and biological waste is inactive before re-used. Following the SOP cross-contaminations was prevented.

The procedure for autoclaving is described below:

All the material to be autoclaved was placed inside the autoclave for a period of 20 minutes at a temperature of 121°C. 3-way stopcocks, adult airways adapters attached to the capnostat CO₂ sensor gas sampling ports are not reusable. Sterilization will likely compromise system performance and they are designed for single use. Tubing connections were not reusable and were disposed of after use. Figure 4.2 shows a photograph of the connectors and adapters used for the breath collection system in this study.

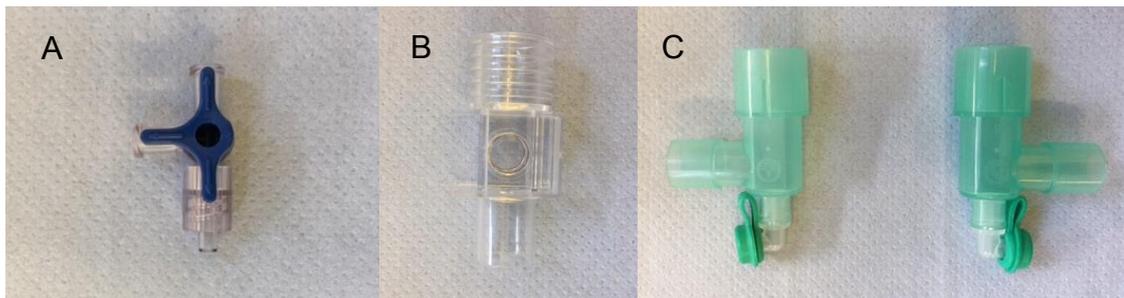


Figure 4.2 Photograph of connector and adapters. A. 3-way-stopcock (B. Braun Medical Limited, UK), B. airways adapter and C. Elbow 22M/15F-15M gas sampling port (Intersurgical, UK).

4.2.4 Breath sampling protocol

Prior to the collection of the breath samples, patients and companions spent approximately 10-15 minutes sitting quietly on a chair filling out the corresponding questionnaire. In such a way, it was ensured that the subjects were in a relaxed state throughout the collection of the breath sample. For improving breath analysis, CO₂

controlled sampling is recommended. Therefore, the protocol for breath collection previously described in chapter 3 was followed. In brief, the breath of the subjects was only drawn into the glass syringe once the capnograph showed that the alveolar phase of the exhaled breath had been reached.

During the collection of the breath samples in the case of mechanical ventilated patients in the intensive care unit (ICUs), an anaesthetist was responsible for attaching the capnometer sensor to the breathing circuit of the mechanical ventilator. Figure 4.3 shows a picture of an anonymous patient with mechanical ventilation during the collection of the breath samples after liver transplant in the ICU. The photograph shows the capnometer sensor coupled onto a breathing circuit tube of the ventilator.

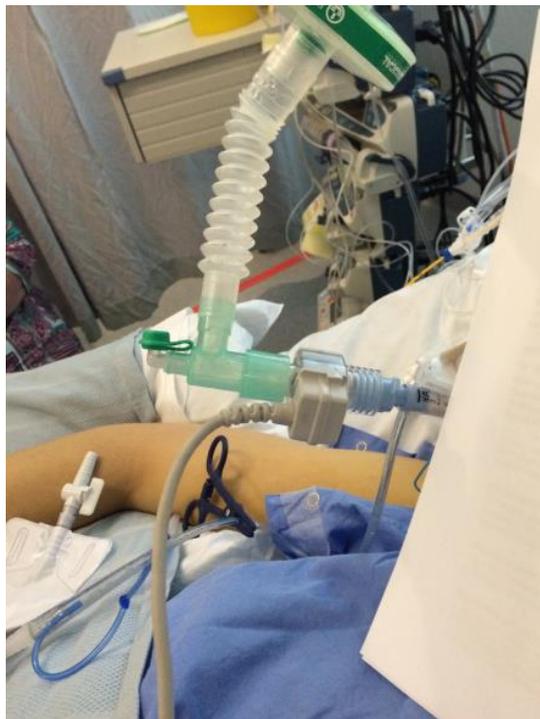


Figure 4.3 Photograph of a capnometer sensor attached to the breathing circuit tube.

After the collection, the syringes were sealed using a luer lock fitting. Each glass syringe was individually placed in an opaque bubble bag suitable for their transportation and storage. The samples were brought from the hospital to the laboratory of the Molecular Physics Group in a basket secured on a trolley. The time distance from hospital to the lab was a fifteen minutes outdoor walk. Once at the laboratory, the syringes were placed inside an incubator set at 40°C to reduce condensation of the samples. All samples were mass spectrometrically analysed within two hours of collection and the syringes were sterilized after used.

During the measurements, syringes were placed into a purpose designed heating bag to control the temperature at 45°C in order to limit condensation, which could otherwise cause volatile loss in water-soluble organic compounds [3].

The luer stopcock was coupled to a Swagelok fitting and connected directly to the inlet of the PTR-MS. The inlet flow was set at 10-15 ml/min and the drift tube and inlet lines were maintained at 45°C.

The syringes were gas tight and have minimal friction such that once the syringes are connected to the PTR-MS and the valve opened atmospheric pressure is sufficient to push the plunger in smoothly so that a breath sample was being drawn into the instrument at a constant flow during the measurements.

4.2.5 Analytical measurements and PTR-MS settings

The instrument used for the analysis of the samples was a first generation PTR-Quad-MS (IONICON Analytik GmbH). In chapter 2, the instrument has been described in detail. In this study, the voltage across the drift-tube was set at 600 V, to provide an E/N value of 136 ± 1 Td, which is sufficiently high to reduce water clustering to reagent and product ions by collision induced dissociation. The drift tube was controlled and maintained at a pressure of 2.07 ± 0.01 mbar and temperature of 45 ± 1 °C. The sequence followed to measure the breath samples in the lab was set following the collection time at the hospital, that is, from the earliest time of collection to the latest time of collection.

Before using the samples, background measurements were taken to obtain mass spectra under lab air conditions to check the performance of the instrument. By providing the instrumental background of the PTR-MS, it was ensured that the conditions of the PTR-MS were adequate for the analysis. After the background analysis, a glass syringe containing a breath sample was attached to the PTR-MS and subsequently measured. A mass scan spectrum was selected using the software to control the instrument and generally scanned a m/z range from 20 to 200 amu with a dwell time of 0.5 seconds per atomic mass unit. Mass spectra of the breath samples were recorded from the average of three cycles for each glass syringe measured, for every participant. As mentioned before, 4 glass syringes were used to collect breath samples for each participant. The resulting 12 mass spectra were averaged to provide one data set for each subject with the uncertainty in the intensity of each spectral peak expressed as the standard error of the mean for the four syringes.

The intensities of the product ion(s) associated with a given volatile were converted to volume mixing ratios (VMR) in units of nmol/mol by use of a standard procedure that relies on a calculated, compound-specific, collisional reaction rate

coefficient, determined using the effective translational temperature of the reagent ions [23]. The VMR was calculated by

$$\text{VMR} = \frac{i(\text{MH}^+)}{i(\text{H}_3\text{O}^+)} \frac{1}{k t} \frac{10^9}{N_d} \quad \text{Equation 4.1}$$

N_d is the number of density of the gas in the drift tube and it is determined from the pressure and the temperature of the measurements. The VMR is expressed in ppb and the N_d is given in unit of cm^{-3} . The VMR was calculated taking into account the protonated water dimer.

In this work, in order to identify and further understand the product ions of volatiles, pure samples of key volatiles were individually measured and analysed using PTR-MS to establish the m/z values of the product ions.

4.2.6 Details of the patients participating in the study

In this study, 31 patients suffering from liver disease were recruited in the pre-transplant assessment clinic (TAC) and participated in the pre-transplant measurements. The following notation was used in tables and figures to name the male volunteers M and female volunteers F subjects. (F/M 8/23, mean age 55 years, min-max 27-71 years).

Patients recruited were selected from the different types of liver stages. There were a number of aetiologies and some patients had more than one condition. Conditions include: alcoholic liver disease (ALD), hepatocellular cancer (HCC), cryptogenic, hepatitis C (CD), primary sclerosing cholangitis (PSC), primary biliary cirrhosis (PBC), autoimmune liver disease (AID), hepatitis B (HBV), non-alcoholic steatohepatitis (NASH), and non-alcoholic fatty liver disease (NAFLD). A summary providing the details of the 31 patients, including the different aetiologies, and the number of patients recruited with the same condition is given below in the table 4.1.

Table 4.1 Volunteer aetiology and number of patients recruited.

Aetiology	Patients recruited
Alcoholic liver disease	13
Hepatocellular cancer	10
Cryptogenic	4
Hepatitis C	5
Primary sclerosing cholangitis	4
Primary biliary cirrhosis	2
Autoimmune liver disease	1
Hepatitis B	3
Non-alcoholic steatohepatitis	1
Non-alcoholic fatty liver disease	1

In the second stage, 5 female and 8 male patients from the first group of 31 subjects had a liver transplant operation and had their breath collected and analysed after liver transplant. Table 4.2 shows the liver transplant patient details, including sex, age, initial diagnosis, histopathological results, the locations of the breath sampling pre and post-transplant, and the number of days pre and after transplant when breath samples were taken.

Table 4.2 Liver transplant patients' details. Including gender (female F, male M), age, initial diagnosis, histopathological results, location of pre-transplant and post-transplant breath sampling, and the number of days prior to and after transplant when breath samples were collected.

Patient ID	Age (yr)	Initial Diagnosis	Histopathological Results	Location of pre-transplant breath sample	Pre-transplant breath sample: days before transplant	Location of post-transplant breath sample	Post-transplant breath samples: days after transplant
F1	27	AID	Severe steatohepatitis (PSC)	TAC	54	OPC	65
F2	49	Liver Failure	Severe hepatitis with multiacinar necrosis, seronegative hepatitis	ITU	0 ¹	OPC	3, 5, 130
F3	53	PBC	Cirrhosis (PBC)	TAC	74	OPC	45
F4	58	PSC	Cirrhosis (PSC)	TAC	83	Ward	5-8, 11-15, 18, 58
F5	53	ALD	Hepatocellular carcinoma, liver cirrhosis.(ALD, HCV)	-	-	Ward	2-6, 9-12
M1	54	ALD	Severe steatohepatitis	Ward	47	OPC	33
M2	45	ALD	Cirrhosis (ALD)	TAC	97	OPC	22
M3	53	ALD	Cirrhosis (ALD/HCV) Hepatocellular carcinoma (grade2)	TAC	179	Ward	4, 7, 48
M4	53	ALD, HBV, HCV	Cirrhosis (ALD, HCV, HBV)	TAC	21	OPC	126
M5	56	ALD, HCV, HCC	Cirrhosis (ALD, HCV, HCC)	TAC	125	OPC	61
M6	53	CD	Cirrhosis with mild steatohepatitis (etiology possibly NASH but uncertain)	TAC	154	OPC	22
M7	36	CD	Cirrhosis of uncertain etiology	TAC	180	Ward	2, 3, 6-8, 55
M8	67	ALD	Cirrhosis (ALD)	ITU	14	Ward	6

1. The pre-transplant breath sample for patient F2 was taken approximately 10 minutes before the patient went into surgery. This patient was admitted with liver failure and hepatic encephalopathy.

In the table 4.2 diseases include autoimmune liver disease (AID), alcoholic liver disease (ALD), cryptogenic disease (CD), hepatitis B (HBV), hepatitis C (HCV), hepatocellular cancer (HCC), primary biliary cirrhosis, (PBC) and primary sclerosing cholangitis (PSC). Breath samples were taken at various locations including the Intensive Treatment Unit (ITU), Out-Patient Clinic (OPC), Transplant Assessment Clinic (TAC), and in wards.

4.2.7 Data Analysis and Statistical Evaluation

In breath analysis, the concentration of exhaled breath dramatically changes from exposure to indoor spaces and pollution, which is a confounding factor, and which has been the subject of much discussion [84]. For instance in the case of e-cigarettes and cigarette smoke, certain harmful substances and toxic chemicals found in tobacco or in the smoke can also be detected in the exhaled breath of smokers and passive smokers [9] [85].

If the intensity of a product ion has a significant contribution coming from room air, then care must be taken when using it as a biomarker. There is no simple correction which can be applied to account for inhaled volatile concentrations, and it has been shown that simply subtracting the room air concentration is too simplistic [40] [63].

For this study, only the ion signal intensities in the breath sample that were at least twice that in the room air samples in at least half of the patients were retained for analysis. This resulted in a set of 40 product ions for analysis.

Table 4.3 shows m/z values and normalised counts per second, many of which are well known including m/z 33 (methanol), m/z 45 (acetaldehyde), m/z 47 (ethanol), m/z 59 (acetone) and m/z 69 (isoprene).

Table 4.3 m/z values and normalised counts per second, many of which are well known, including m/z 33 (methanol), m/z 45 (acetaldehyde), m/z 47 (ethanol), m/z 59 (acetone) and m/z 69 (isoprene). Median (lower quartile LQ, upper quartile UQ) and range (minimum – maximum) are provided. NCPS refers to normalising to 50 million counts per second for the reagent ions m/z 19 (hydronium) and m/z 37 (the protonated water dimer).

m/z	Patients (N = 31)		Controls (N = 31)		Room air (N = 21)	
	Median (LQ/UQ)	Range	Median (LQ/UQ)	Range	Median (LQ/UQ)	Range
33	77200 (64800 / 144700)	36600 - 334700	56900 (45100 / 68700)	32600 - 92200	10300 (8800 / 14600)	5800 - 22300
42	4300 (3950 / 6290)	3590 - 78060	4780 (3830 / 37440)	2810 - 98400	3030 (2800 / 5220)	1550 - 10050
43	86200 (75100 / 91200)	46700 - 186300	86100 (63700 / 98300)	45100 - 330700	29700 (21300 / 34400)	10000 - 236400
45	66400 (50000 / 84800)	21500 - 198200	65700 (39000 / 103100)	24400 - 281700	30800 (19800 / 40800)	8900 - 257700
47	34500 (31900 / 40400)	15200 - 64100	34000 (26800 / 37000)	22800 - 119800	16800 (12200 / 18700)	8300 - 29000
49	980 (720 / 1160)	320 - 3920	880 (720 / 1160)	310-1620	450 (220 / 630)	80-1310
51	3870 (2830 / 5890)	720 - 12430	2480 (1850 / 3150)	1270 - 6000	380 (160 / 550)	40-760
54	860 (580 / 1180)	290 - 2390	1050 (610 / 1570)	240 - 30380	260 (140 / 410)	40-610
55	85600 (71100 / 120400)	37200 - 208900	72900 (58200 / 117500)	39100 - 1114900	5900 (4500 / 7300)	2100 - 81300
57	12220 (10150 / 14690)	5300 - 20300	10530 (9670 / 12220)	6720 - 25720	4480 (3010 / 4960)	1970 - 9750
58	2750 (2060 / 5090)	990 - 11320	2710 (1890 / 4590)	1030 - 92120	650 (480 / 1130)	270-8640
59	359400 (268100 / 719700)	158200 - 1119800	269600 (219700 / 449200)	124600 - 1078500	31200 (20000 / 119000)	9200 - 1616500
61	50400 (42100 / 57300)	13600 - 138200	49500 (36800 / 61100)	26200 - 121200	13000 (9500 / 15700)	5200 - 23600
63	8580 (7370 / 10380)	3890 - 11780	7650 (5760 / 9320)	4760 - 13500	660 (510 / 1050)	220-1640
65	2340 (1960 / 2930)	820 - 4110	1930 (1660 / 2390)	940-29660	370 (230 / 520)	50-950
67	740 (590 / 940)	280 - 3330	660 (490 / 850)	280-1940	180 (110 / 360)	70 - 510
68	390 (320 / 470)	150 - 2160	430 (370 / 490)	130 - 4630	140 (60 / 270)	20 - 630
69	18250 (5800 / 25890)	2640 - 73030	11420 (7580 / 19410)	1890-43170	830 (550 / 970)	370 - 2800
71	8410 (5530 / 11200)	2580 - 16230	6020 (5040 / 8130)	2230-13980	780 (660 / 1090)	340 - 2460
72	720 (580 / 920)	430 - 1500	740 (460 / 910)	360-5940	230 (190 / 400)	40 - 890
73	11900 (9600 / 17300)	7500 - 30800	8900 (7300 / 12500)	5800-25600	3300 (2500 / 5000)	1900 - 16800
74	870 (780 / 1020)	630 - 1800	780 (610 / 980)	450-2020	370 (270 / 540)	120 - 980
75	4450 (3370 / 6830)	1490 - 13400	3060 (2460 / 5240)	1630-15240	1210 (890 / 1650)	390 - 4040
77	2590 (1840 / 3790)	1010 - 7470	1740 (1330 / 2920)	830-8660	360 (230 / 510)	150 - 2260

<i>m/z</i>	Patients (N=31)		Controls (N = 31)		Room air (N=21)	
	Median (LQ/UQ)	Range	Median (LQ/UQ)	Range	Median (LQ/UQ)	Range
79	1550 (1240 / 1990)	420 - 3150	1300 (980 / 2240)	600 - 13060	410 (300 / 560)	150 - 830
81	5000 (1700 / 14170)	800 - 61880	980 (830 / 1270)	540 - 2270	330 (260 / 450)	140 - 1880
83	1303 (1019 / 1905)	530 - 3084	949 (787 / 1556)	520 - 2959	479 (426 / 571)	224 - 1084
85	2110 (1070 / 3960)	650 - 5070	1800 (1140 / 2710)	610 - 6810	400 (360 / 620)	230 - 1380
87	7630 (5780 / 11400)	2650 - 33000	4030 (3470 / 4620)	2190 - 15410	830 (770 / 1360)	540 - 1630
89	1480 (980 / 3370)	540 - 10170	870 (690 / 1330)	570 - 12270	480 (460 / 630)	240 - 2750
93	1890 (1320 / 3450)	760 - 8300	1090 (890 / 2050)	660 - 6350	570 (400 / 760)	180 - 990
95	1810 (1200 / 3070)	770 - 10950	1110 (860 / 1580)	630 - 3730	450 (350 / 500)	280 - 1400
97	630 (550 / 930)	300 - 2010	570 (470 / 790)	310 - 1760	330 (250 / 410)	110 - 730
99	870 (750 / 1250)	450 - 5640	790 (630 / 980)	390 - 1550	440 (360 / 630)	80 - 820
101	2940 (2010 / 4420)	1180 - 6370	2140 (1820 / 3170)	1380 - 8750	1140 (700 / 1640)	500 - 2510
103	700 (620 / 880)	450 - 2690	690 (530 / 910)	300 - 3790	340 (230 / 430)	130 - 680
109	610 (470 / 860)	190 - 4920	560 (480 / 750)	230 - 4570	340 (240 / 530)	80 - 1690
115	610 (520 / 860)	410 - 2430	530 (460 / 650)	290 - 1520	400 (230 / 450)	120 - 630
135	550 (490 / 1100)	270 - 2300	450 (310 / 570)	170 - 3750	330 (240 / 450)	20 - 1620
137	3027 (830 / 9286)	278 - 40636	409 (333 / 506)	97 - 1309	243 (72 / 387)	43 - 855

***Note the number of room air samples are less than those for patients and controls because some patients were recruited in the same clinic at the same time.*

All the statistical data analysis was performed using SPSS version 22. In this study the data sets for each volatile of interest were assessed using a Shapiro-Wilks test. Shapiro Wilks tests were applied to determine the data classification and were found that the data set were not normally distributed. In this case, non-parametric tests were used.

Mann-Whitney U-tests determined which m/z values differed between the patients and controls. A Wilcoxon signed rank test was used to determine which volatile concentrations differed between pre- and post- transplant breath samples. To compare blood chemistry values with breath volatiles Kendall's tau-b correlation coefficients were measured. A medical diagnosis test evaluation is crucial in clinical practice to confirm the disease in a population and also to exclude the disease in healthy participants. In order to determine the performance of the method studied and to be able to achieve the diagnostic accuracy of the volatiles analysed, a receiver operating characteristic (ROC) curve was plotted using the software IBM SPSS version 22. The plot of the true positive rate (TPR) versus the false positive rate (FPR) generate a ROC curve [30]. The true positive rate is the sensitivity of the analysis and it is defined as the number of positives correctly identified, in this case for patients suffering liver disease.

The true negative rate is the specificity that measures the number of negatives correctly identified in the analysis, in this case volunteers who participated in the study and were correctly identified as controls, that is healthy subject with no condition. The area under the receiver operating characteristic curve (AUROC) determines the accuracy of the study. For instance an area equal to 1 represents that the test performance is perfect, whereas an area under the curve equal to 0.5 shows that the method applied is worthless. The AUROC is the most frequently chosen method to measure the discrimination, it is the capability of the test to categorize participants with and without a certain condition. Table 4.4 shows the traditional score and the accuracy of the diagnostic method [86] for a ROC analysis.

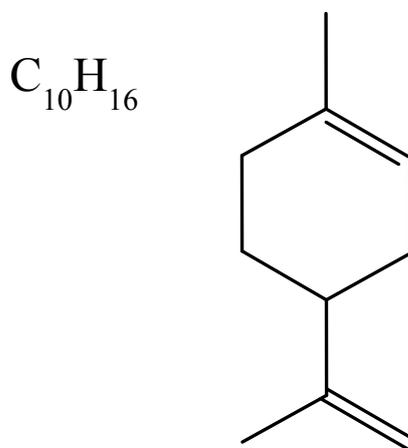
Table 4.4 Guide for classifying the accuracy of the diagnostic method.

AUROC	Accuracy test
0.90-1	Excellent
0.80-0.90	good
0.70-0.80	fair
0.60-0.70	poor
0.50-0.60	fail

4.3 Limonene

D-limonene (1-Methyl-4-(1-methylethenyl)-cyclohexene), $C_{10}H_{16}$, is the most common natural cyclic monoterpene found in nature. D-limonene is a flavour component of the essential oil extracted from citrus peels such as oranges and lemons, but it is also found in a large variety of other fruits and vegetables. Limonene is a chiral molecule with D-limonene enantiomer having a strong smell of oranges. Essential mint oils contain L-limonene enantiomer with a characteristic smell of piney. Limonene can be ingested or inhaled as it is a common additive in commercial food and drinks. Its citrus fragrance is used to give the fruit flavour to foods and is used in cosmetics, perfumes, aromatherapy and as a cleaning product solvent [87]. The figure 4.4 shows the chemical structure of limonene.

Figure 4.4 Chemical structure of limonene



In this study, limonene have been found in high concentration in patient's breath who were suffering from chronic cirrhosis in agreement with previous studies [44].

4.3.1 Limonene headspace analysis

In addition to this study and to better identify the fragment ions resulting from the reaction of limonene with H_3O^+ in a complex breath profile. A pure commercial sample of d-limonene was investigated as a function of the reduced electric field by PTR-MS.

D-limonene sample was prepared for analysis by adding 1 μl of D-limonene (Sigma Aldrich, UK) to a 100 ml glass bottle. A 100 ml glass syringe was coupled to the top part of the bottle and using a 3- ways luer lock stopcock and 10 ml of D-limonene headspace gas was sampled. The sample was diluted in the glass syringe with 90 ml of air.

For the D-limonene headspace analysis, the voltage across the drift-tube was varied from 400 V-600 V at intervals of 20 V, with a range of E/N (92-138 Td). Results from the PTR-MS analysis of D-limonene headspace are presented in figure 4.5.

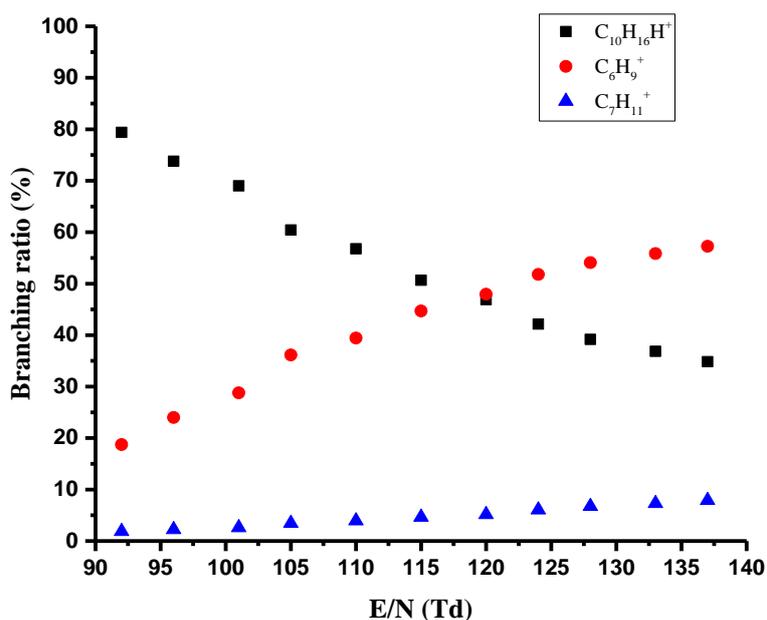


Figure 4.5 Product ions resulting of the reaction of D-limonene with H_3O^+ .

The protonated D-limonene $C_{10}H_{16}H^+$ was observed (m/z 137) for all of the values of the reduced electric field. From the results, D-limonene produces a product ion at m/z 81 ($C_6H_9^+$). In most of the previous studies carried out by PTR-MS, a signal is observed at these two masses [88]. Another ion fragment is produced with less intensity at m/z 95 ($C_7H_{11}^+$). However, the total contribution is less than 10% of the total ion signal. The ion peak at m/z 137 contributed to the total ion signal of 35% whereas the ion peak at m/z 81

contributed to the total ion signal of 55% when measured at E/N 136. This results agree well with the PTR-MS study carried out by Tani et al. [89].

4.4 Results

For the first stage measurements, a Mann Whitney U-test was used to compare the 40 product ion signal intensities found from the analysis between patients and controls.

Of these 40 products ions, eight ions showed significant differences in intensities between patients and controls. Their m/z values and significance (p-value in brackets) are shown in table 4.5.

Table 4.5 m/z values and the calculated probability (p-value) for the comparison between patients and controls.

m/z	p-value
<u>33</u>	<u>< 0.001</u>
73	0.004
77	0.035
<u>81</u>	<u>< 0.001</u>
<u>87</u>	<u>< 0.001</u>
89	0.03
135	0.019
<u>137</u>	<u>< 0.001</u>

In the second phase, the pre and post-transplant intensities of these eight ions were compared using a Wilcoxon Signed Rank Test for paired samples with a significance of 95%.

This eliminated m/z 89 and 135 from the putative marker set. Of the remaining ions, m/z 33 is assigned to be protonated methanol (CH_3OH_2^+) [90]. Based on previous GC and GC-MS studies [80, 81, 91], m/z 73 was tentatively identified as protonated 2-butanone ($\text{C}_4\text{H}_8\text{O.H}^+$), m/z 77 as protonated carbon disulfide (CS_2H^+), m/z 87 as protonated 2-pentanone ($\text{C}_5\text{H}_{10}\text{OH}^+$). CG-MS analysis confirmed m/z 81 and 137 as limonene (m/z 81 is a fragment ion (C_6H_9^+) resulting from dissociative proton transfer and m/z 137 is protonated limonene ($\text{C}_{10}\text{H}_{17}^+$). Alternative, based on previous PTR-MS studies [92], m/z 77 was tentatively identified as propyl mercaptan.

Figure 4.6 shows box plots which summarise the results for patients, control and clinic air samples (a) methanol and (b) carbon disulfide (c) 2-butanone (d) 2-pentanone (e) limonene. For limonene the sum of the intensities of the m/z 81 and m/z 137 product ions was used to plot the box and whisker plot. In these plots are shown the median, mean, lower quartile (LQ), and upper quartile (UQ) of the VMRs in units of nmol/mol for each volatile.

From the box plots it is clear that the presence of the volatiles in the clinic, in the wards and in ITCs has a negligible effect on the concentrations in the breath samples. Furthermore, the analysis is based on comparisons and the patients and controls should be affected similarly by the room air contaminations.

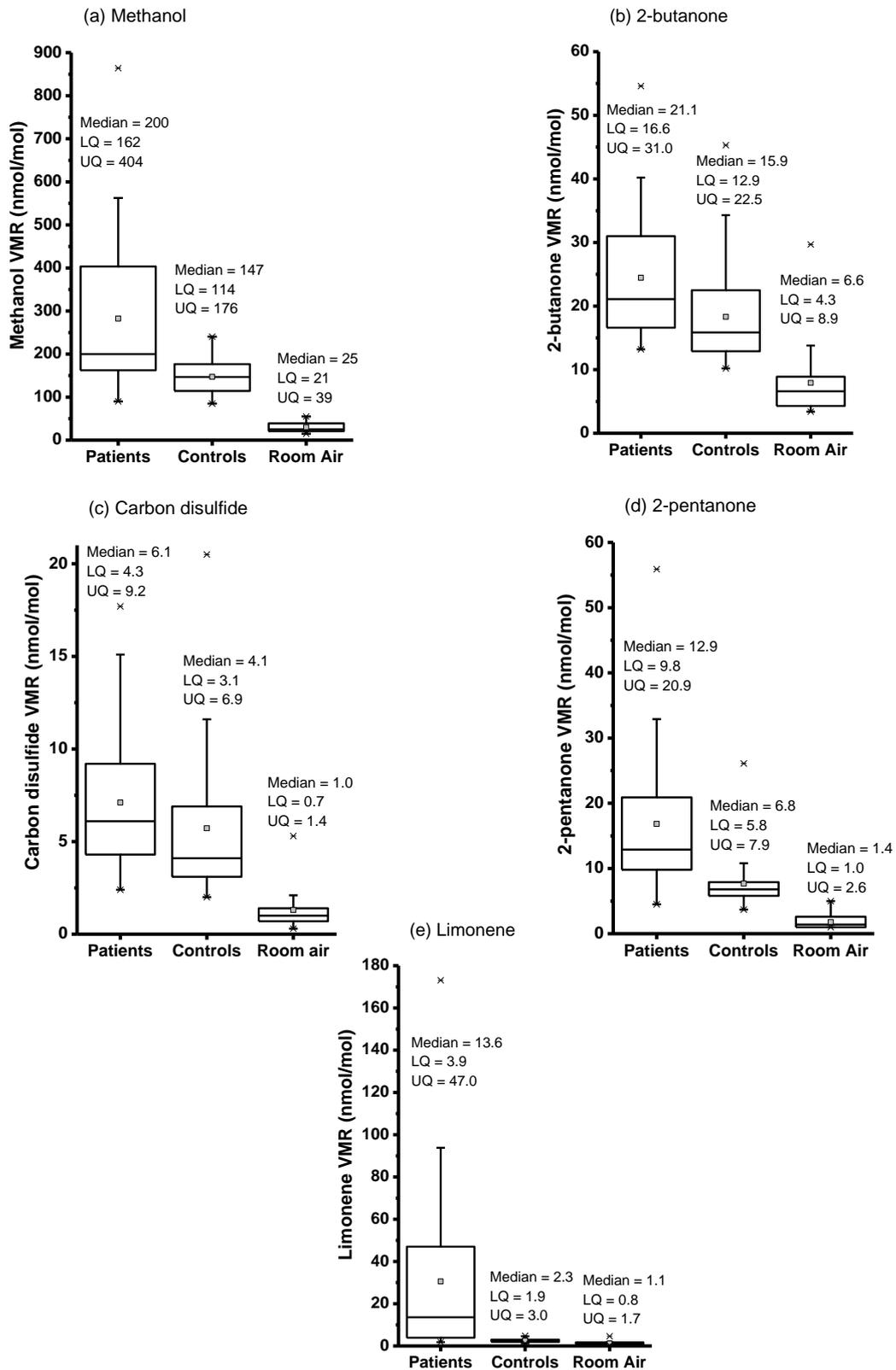


Figure 4.6 Boxplots showing lower quartile (LQ), median, mean and upper quartile (UQ) calculated volume mixing ratios (VMR) for (a) methanol, (b) 2-butanone, (c) carbon disulfide, (d) 2-pentanone, and (e) limonene for 31 patients with liver cirrhosis and 30 controls. Whiskers are 1.5 times the inter-quartile range and outliers are depicted by a star.

The results of the calculated volume mixing ratios (VMRs) are given in table 4.6 (methanol and 2-butanone) and table 4.7 (carbon disulfide, 2-pentanone and limonene). The post-transplant values correspond to those for the last post-transplant sample given in table 4.2. Measurement uncertainties are provided in brackets. The ratios of pre- to post-transplant concentrations are also provided.

Table 4.6 VMRs for the pre-transplant and post-transplant samples for methanol and 2-butanone for all of the participants who underwent liver transplants (4 females (F1-F4) and 8 males (M1-M8)).

Patient	Mean methanol VMRs nmol/mol			Mean 2-butanone VMRs nmol/mol		
	Pre	Post	Pre/Post	Pre	Post	Pre/Post
F1	200 (6)	190 (3)	1.1 (0.04)	18 (1.2)	11 (0.2)	1.6 (0.1)
F2	90 (9)	230 (6)	0.4 (0.04)	16 (1.1)	15 (1.9)	1.1 (0.2)
F3	530 (2)	78 (5)	6.8 (0.4)	21 (1.7)	12 (2.6)	1.8 (0.4)
F4	560 (18)	71 (3)	7.9 (0.4)	19 (1.2)	12 (0.4)	1.6 (0.1)
M1	170 (2)	120 (15)	1.4 (0.2)	26 (1.4)	14 (1.4)	1.9 (0.2)
M2	430 (36)	81 (1)	5.3 (0.4)	13 (2.2)	15 (1.7)	0.9 (0.2)
M3	320 (32)	86 (7)	3.7 (0.5)	55 (3.1)	46 (4.2)	1.2 (0.1)
M4	490 (37)	93 (6)	5.3 (0.5)	38 (5.9)	23 (7.5)	1.7 (0.6)
M5	190 (3)	320 (1)	0.6 (0.01)	15 (0.5)	17 (1.3)	0.9 (0.1)
M6	230 (12)	79 (5)	2.9 (0.2)	30 (2.2)	12 (3.3)	2.5 (0.7)
M7	510 (21)	160 (8)	3.2 (0.2)	19 (0.7)	24 (4.2)	0.8 (0.1)
M8	180 (5)	86 (2)	2.1 (0.07)	21 (1.0)	12 (0.4)	1.8 (0.1)

Table 4.7 VMRs for the pre-transplant and post-transplant samples for carbon disulfide, 2-pentanone and limonene for all of the participants who underwent liver transplants (4 females (F1-F4) and 8 males (M1-M8)).

Patient	Mean carbon disulfide VMRs (nmol/mol)			Mean 2-pentanone VMRs (nmol/mol)			Mean limonene VMRs (nmol/mol)		
	Pre	Post	Pre/Post	Pre	Post	Pre/Post	Pre	Post	Pre/Post
F1	4.7 (0.4)	2.0 (0.4)	2.4 (0.5)	9.0 (0.8)	5.3 (0.6)	1.7 (0.2)	7.5 (0.6)	3.5 (0.6)	2.1 (0.4)
F2	2.4 (0.5)	20 (2.9)	0.1 (0.03)	8.3 (0.8)	7.3 (0.6)	1.1 (0.1)	60 (4.7)	1.1 (0.1)	54 (6)
F3	11 (1.3)	2.3 (0.3)	4.8 (0.8)	10 (1.2)	5.7 (0.3)	1.8 (0.2)	14 (0.7)	2.3 (0.2)	6.1 (0.6)
F4	4.7 (0.2)	2.3 (0.4)	2.0 (0.4)	25 (3.3)	6.1 (0.3)	4.1 (0.6)	11 (0.5)	2.3 (0.1)	4.8 (0.3)
M1	9.3 (0.6)	2.1 (0.4)	4.4 (0.9)	10 (0.5)	17 (2)	0.6 (0.1)	32 (0.7)	3.3 (0.5)	9.7 (1.5)
M2	3.1 (0.1)	2.4 (0.5)	1.3 (0.3)	23 (0.6)	6.2 (1.5)	3.7 (0.9)	94 (3.4)	1.6 (0.2)	59 (8)
M3	18 (2.0)	1.8 (0.2)	10 (1.6)	29 (1.0)	13 (1.2)	2.2 (0.2)	170 (1)	5.7 (0.2)	30 (1)
M4	8.5 (1.0)	2.6 (0.5)	3.3 (0.7)	38 (4.3)	9.9 (0.9)	3.8 (0.6)	110 (6)	55 (4)	2.0 (0.2)
M5	4.0 (0.4)	3.6 (0.1)	1.1 (0.1)	21 (1.3)	6.7 (0.5)	3.1 (0.3)	9.2 (0.5)	4.1 (0.2)	2.2 (0.2)
M6	13 (1.9)	2.1 (0.4)	6.2 (1.5)	13 (1.3)	5.1 (0.2)	2.5 (0.3)	120 (8)	4.8 (0.3)	25 (2)
M7	4.9 (0.9)	4.5 (0.7)	1.1 (0.3)	14 (2.6)	7.5 (0.3)	1.9 (0.4)	47 (2)	2.6 (0.1)	18 (1)
M8	6.8 (0.6)	1.9 (0.3)	3.6 (0.7)	8.8 (0.3)	13 (1.6)	0.7 (0.1)	7.7 (0.6)	3 (0.2)	2.6 (0.3)

It should be noted that the number of days between collecting the pre-transplant and post-transplant breath samples is variable, because it is not possible to control when subjects are available or when a donor liver would be found. Only for one of the patients, F2, it was possible to collect a pre-transplant breath sample just prior to surgery.

However, and independent of when the pre and post-transplant breath samples were taken, the results in table 2 clearly demonstrate that the pre-transplant concentrations of these volatiles are, for the majority of patients, higher than the post-transplant levels for most patients. Limonene shows the largest average decrease in all patients post-transplant. Post-transplant concentrations of limonene dropped to within the normal control range (median (LQ, UQ) being 2.3 nmol/mol (1.9, 3.0)) within a number of days for all but one of the patients, M4, for whom limonene was found to be high even some months after transplant.

4.4.1 Longitudinal study

In order to investigate how the VMRs changed over a period of time and to gain an insight of the difference changes in concentration between the key volatiles such as methanol, carbon disulfide, 2-butanone, 2-pentanone and limonene, breath samples were collected after liver transplant. In the longitudinal study, five patients participated and were followed up (F2, F4, F5, M3 and M7). The VMRs changed over a period of time are presented in the figure 4.7. The data point at day 0 for F2 was taken just before transplant surgery.

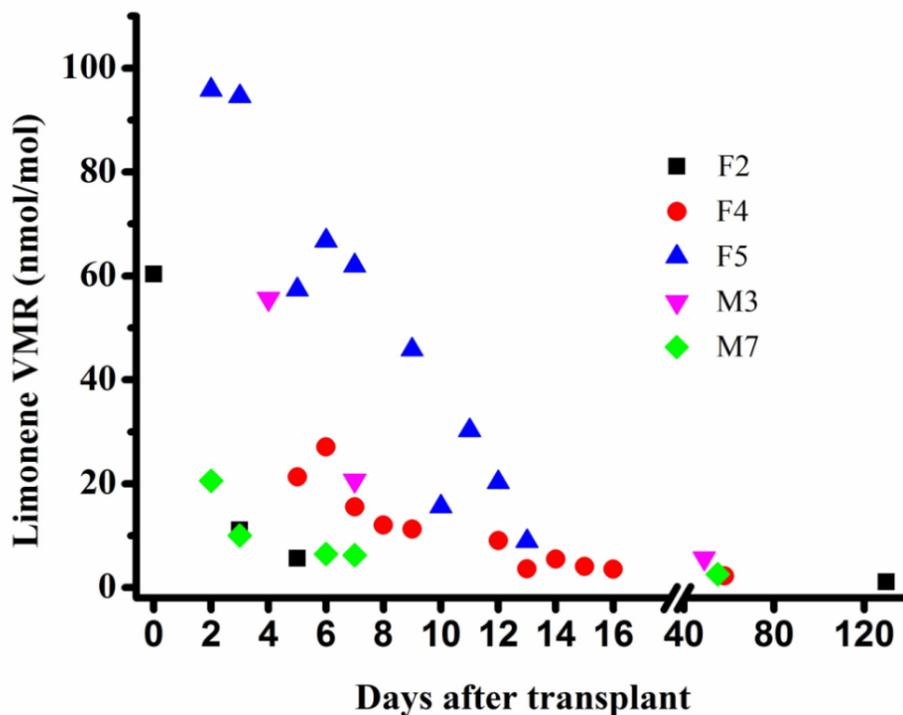


Figure 4.7 Longitudinal changes in volume mixing ratios (VMRs) in nmol/mol for limonene at given days after liver transplant for patients F2, F4, F5, M3 and M7. Error bars are smaller than the symbols.

The key result is that limonene VMRs dropped gradually following transplant surgery, as illustrated in figure 4.7. The same data for limonene presented as normalised to the highest intra-individual value are shown in figure 4.8. No concentration trend with time was observed for the case of methanol, carbon disulfide, 2-butanone and 2-pentanone, their VMRs were found to have dropped to within the normal range by the time of the first post-transplant measurement. Patient F5 showed the highest concentration of limonene in breath in relation to the other subjects followed for patient F2, both females.

Of note, also patient F2 showed the fastest limonene elimination in breath showing a concentration of limonene in breath comparable to healthy volunteers after only 5 days after transplant. This result clearly demonstrates that the concentration of limonene in breath decreased after liver transplant.

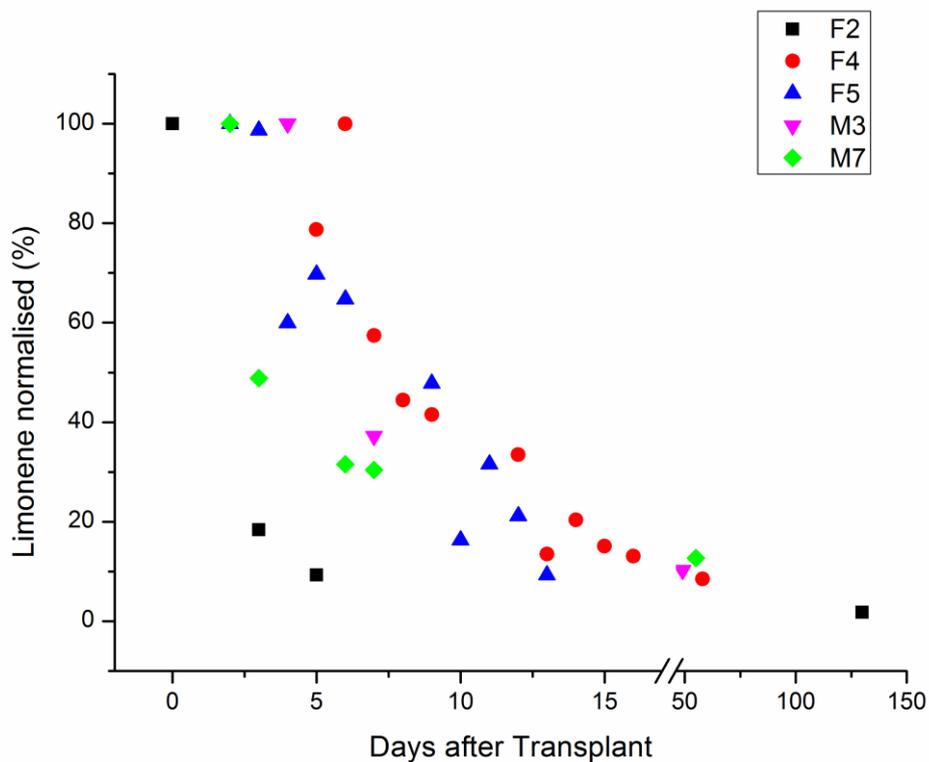


Figure 4.8 Longitudinal changes in volume mixing ratios (VMRs) normalized to the highest intra-individual value in nmol/mol for limonene at given days after liver transplant for patients F2, F4, F5, M3 and M7. Error bars are smaller than the symbols.

4.4.2 Prediction of liver cirrhosis

4.4.2.1 ROC analysis

In this study the receiver operating characteristic (ROC) curve displays the results of the breath study with the data taken together from the first and second study stage. The ROC curve is shown in figure 4.9. The ROC curve was obtained with a combination of the data from the box plots, the ratio of the pre- and post-transplant VMR values and the significance values given above imply that ions at m/z 33, 81, 87 and 137 are the ones that were most diagnostically useful.

The area under the ROC curve was also individually calculated for limonene and it was found that limonene provided the most predictive power (AUROC - 0.91 (standard error 0.04)). However, the best accuracy is achieved by combining the data from methanol, 2-pentanone and limonene.

The VMRs for limonene, methanol and 2-pentanone were normalised to the highest patient value for that volatile. These normalised fractions were simply added with no weightings. The model selected for the combination of these 3 volatiles predicted an area under the curve AUROC equal 0.95 (standard error 0.03) with a sensitivity of 97% and specificity of 70%.

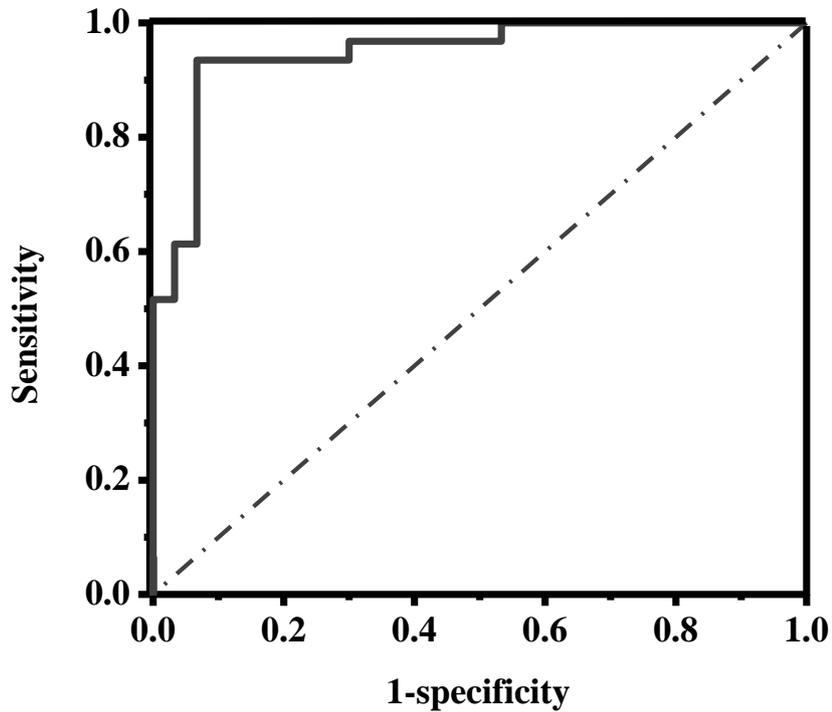


Figure 4.9 Receiver operating characteristic curve for a combination of methanol, 2-pentanone and limonene data in the study groups.

Often patients suffering from liver disease have liver function test (LFTs) to check abnormal results in blood. The LFTs are liver enzyme test that detect the health of the liver providing information of the organ such as inflammation and damage. Liver cells are destroyed and altered by the disease leaking out into the blood stream. The most common LFTS are aspartate aminotransferase (AST) and alanine aminotransferase (ALT). Another routine test is measuring bilirubin in blood, which is produced in the liver. High level of bilirubin may predict cirrhosis, whereas a low albumin levels in blood can be the cause of a liver or kidney disease [93]. The United Kingdom Model for End-Stage Liver Disease (UKELD) is a clinical scoring model used in the UK to determine and predict the best timing to have a liver transplant in cirrhotic patients [94]. It is a

calculation based on the clinical chemistry data. If the score is high, the higher the risk of death by the disease and the lower the time will be on the waiting list. For the study blood samples were routinely taken from patients and analysed in the clinical chemistry laboratory to diagnose the degree of injury and to check or monitoring the progression of the liver cirrhosis. Clinical chemistry data were available and was investigated for evaluation of possible correlations of common clinical chemistry values with the key volatiles such as limonene, methanol and 2- pentanone. The following correlations were checked, alanine aminotransferase, alkaline phosphatase, aspartate transferase, albumin, total bilirubin, creatinine, neutrophils, platelets, potassium, prothrombin/international normalized ratio, and UKELD.

Kendall's tau-b analysis showed only one correlation with a significance score below 0.05. This was for methanol with UKELD which had a Kendall's tau-b coefficient of 0.237 (significance 0.042). Over 33 correlations were tested and no multiple testing correction was applied so it is possible that this is a coincidental finding.

Correlations of key volatiles concentration with disease etiology were also examined. Owing to the small sample size and large number of etiologies, this was only feasible for the 13 patients with ALD versus the other 18 patients. The results showed that limonene was higher ($p = 0.020$) in the group with ALD than the rest, with median (LQ, UQ) of 19.7 nmol/mol (9.2, 63.9) for ALD versus 6.1 nmol/mol (2.9, 16.6) for all other etiologies. Methanol, 2-pentanone, 2-butanone and carbon disulfide showed no statistically significant difference.

Correlations between the 7 volatiles of interest in the putative marker set were examined both within the patient and the control group using a Kendall's tau-b test. In the patient group, there were 8 correlations with a significance score of < 0.05 . Results for all

21 correlations are shown in supplementary table 3. In the control group, only two were significant, limonene with m/z 135 ($p = 0.016$) and 2-butanone with carbon disulphide ($p < 0.001$).

These were also found in the patient group. Limonene correlated significantly with 2-butanone ($p = 0.004$), carbon disulfide ($p = 0.034$), m/z 89 ($p = 0.001$) and m/z 135 ($p < 0.001$) but not with methanol or 2-pentanone. This suggests that the mechanisms for the presence of limonene and that of methanol and 2-pentanone are independent.

21 correlations were examined with no multiple-testing correction applied; therefore some correlations may be coincidental.

Finally, the data was also checked for correlations between the concentrations of volatiles and demographic markers such as age, body mass index (BMI) and sex. The results showed that there were no significant correlations.

4.5 Discussion

The viability of breath analysis as a non-invasive technique for monitoring and diagnosing chronic liver disease has been demonstrated. Key volatiles in the patient's breath which are a consequence of the liver disease have been identified. In this investigation for the first time, VOCs have been analysed in the breath of patients with chronic liver disease pre and post liver transplant. The monitoring of volatiles in breath following a dramatic change in the condition of the patient, namely a liver transplant, has provided a method to attribute three diagnostically useful volatiles to the cirrhotic organ itself. These volatiles were methanol, 2-pentanone, and limonene, with that of limonene being the most significant.

Limonene has been found in previous breath volatile studies to be elevated in the breath of patients with cirrhosis compared with controls. It has also been observed in the breath of healthy volunteers; limonene levels found in the control group are comparable to those previously observed in healthy human volunteers [95].

Limonene is an exogenous volatile not produced in the human body and it is a common compound naturally found in many foods and drinks; hence it would be difficult to avoid ingesting. To note that, previous investigation has found that alveolar breath composition showed a lower concentration of exogenous volatiles [63]. This is not true for the case of limonene, which was found in high levels in the alveolar exhaled breath of patients.

Within the control and patient groups, it was found no association between breath limonene and diet and no correlation between having a self-reported large amount of fruit consumption and breath limonene concentrations.

Once in the blood stream, limonene is metabolized by the P450 enzymes CYP2C9 and CYP2C19 to the metabolites perillyl alcohol, trans-carveol and trans-isopiperitenol [96]. It has been found that levels of the enzyme CYP2C19 are reduced in patients with cirrhosis and that levels inversely correlate with severity of cirrhosis. Moreover, of four P450 enzymes tested in patients with liver disease, metabolism by CYP2C19 was found to decrease at the earliest stage of disease [97].

This is suggestive that the observed raised concentrations of limonene in breath arise from the inability of a cirrhotic liver to produce the appropriate metabolic enzyme [44]. Patient M4 is anomalous in this respect, as his breath limonene concentrations do not drop to the normal range post-transplant. Although his graft liver function blood tests

were found to be normal, this results suggest that this patient's new liver is not producing sufficient enzyme to metabolise fully limonene.

Owing to its lipophilic properties, it has been proposed that limonene which is not metabolized by the liver accumulates in the fat of patients suffering from liver disease. Limonene has a blood/air partition coefficient of 36 and an olive oil/blood partition coefficient of 140 [98]. Assuming that the olive oil/blood partition coefficient is close to a body fat/blood partition coefficient, a breath concentration of 1 part per billion by volume (ppbv) would translate to a fat concentration of approximately 5 parts per million by volume (ppmv).

In the set of data, the highest recorded breath VMR is 170 nmol/mol which implies a concentration in fat of the order of 850 ppmv. A study involving women with early-stage breast cancer taking a high oral dose of limonene (2 g/daily for 2-6 weeks before surgery) found that mean limonene concentration in breast tissue was 41.3 ± 49.9 $\mu\text{g/g}$ which is much higher than that found in a control group (0.08 ± 0.13 $\mu\text{g/g}$) [99]. Breast tissue is primarily composed of fat [100]. It has been also demonstrated that an intake of freshly lemonade which is rich in D-limonene implied a significant build-up of D-limonene in adipose tissue in comparison to plasma [101].

The longitudinal changes in breath limonene of patients on successive days in the 3 weeks after liver transplant supports the hypothesis that unmetabolised limonene accumulates in fat tissue. Following transplant, the metabolism of limonene increases, but it takes time for the limonene to be released from the fat into the blood stream. This explains the observed time dependence on limonene VMRs in the breath after transplant and its progressive elimination.

Of note, a similar wash-out behaviour is not observed for methanol and 2-pentanone presumably owing to their low solubilities in fat [102] [103].

Some medications are known to be CYP2C9 and CYP2C19 substrates and inhibitors. The possibility of the effect of medications in limonene concentrations was examined. Twenty patients were taking a CYP2C19 substrate (lansoprazole, omeprazole, propanalol, esomeprazole), 2 were taking a CYP2C9 substrate (naproxen, carvedilol), 6 were taking both a CYP2C9 and CYP2C19 substrate and 1 was taking a CYP2C9 inhibitor (sulfamethoxazole). This result showed no associations of any medications which are CYP2C9 and CYP2C19 substrates or inhibitors with VMRs of breath limonene. Moreover, nine patients who were taking enzyme substrates before transplant were still taking them after transplant.

Of interest is the correlation between limonene and m/z 135, because this product ion may come from perillyl alcohol ($C_{10}H_{16}O$), a metabolite of limonene. Unpublished results have shown that the reaction of H_3O^+ with perillyl alcohol leads to a dominant product ion $C_{10}H_{15}^+$ resulting from dehydration of the protonated parent. Previous studies has shown that the elimination of limonene from the body in healthy volunteer is fast and it takes 24 hours to be completely cleared from the body [104].

Dehydration following protonation is a common reaction process observed with many alcohols [105]. Morisco et al. also noted an ion at m/z 135 from patients with cirrhosis, but they assigned this to a terpene related compound [44]. The fact that a correlation of m/z 135 and limonene ($p < 0.001$) is also significant in the control group lends support to this assignment, because one would expect levels of a compound and its metabolite to be correlated in a group with well-functioning livers. It is also of interest to note that the correlation between limonene and m/z 89 has a very low p -value, but that

m/z 89 shows no discrimination between pre- and post- liver transplant. This is suggestive that m/z 89 arises from an independent process related to the patient's illness, but is not related to the cirrhosis itself.

The enhanced levels of methanol and 2-pentanone in pre-transplant patients could come from a number of sources, including diet. Elevated levels of methanol have been reported following consumption of alcohol or large quantities of fruit [90]. It is a product of the degradation of pectin by colonic bacteria, [106] and metabolism of the sweetener aspartame [107]. However, and in agreement with Morisco et al. [44], it was found that fruit consumption cannot explain the increased methanol concentrations in the breath of liver patients compared to controls. Alcoholic drinks are a source of methanol, but only one patient reported that he had drunk alcohol within the 24 hours prior to the breath sampling. Methanol is metabolised in humans in the liver, mainly by alcohol dehydrogenase, [108] so it is possible that this mechanism is impaired when a liver becomes cirrhotic. Morisco et al., also found elevated methanol in cirrhotic patients versus healthy controls [44]. The source of 2-pentanone in breath is unknown [109]. It has been found in human breath, faeces, skin and urine, [110] and it has been suggested that lung cells produce 2-pentanone [111]. 2-pentanone was suggested as a biomarker for liver disease by three previous studies [44] [80, 112].

4.6 Concluding Remarks

Importantly this study provides a set of biomarkers which can be used in a future trial to assess the potential of breath analysis for the non-invasive diagnosis of early-stage liver disease. A two-stage study was performed which compares volatiles in the breath of pre-transplant cirrhotic patients with controls followed by pre- and post- transplant breath samples. This has resulted in an assignment of methanol, 2-pentanone and limonene as markers in exhaled breath for the cirrhotic liver. It has been demonstrated that limonene can also be used for assessing liver function following transplant by monitoring wash-out. This study links limonene with the diseased organ itself, rather than simply the diseased patient as a whole. Breath volatiles have the advantage of the opportunity to assess the global function of the liver, rather than a localized test such as a biopsy.

This work suggests the possibility of a pharmacokinetic-based test study for a non-invasive assessment of liver function which could be used for diagnosing liver disease in early stages. For instance, the substrate, limonene could be isotopically labelled (^{13}C or D) and administered to the patient suffering different early stages of liver disease. ^{13}C is a natural, non-radioactive isotope, safe and innocuous for adults and can be safely used in children as well [113].

Since limonene is poorly metabolized in patients with liver disease, it will be seen in patients' breath. This will allow a quantification and monitoring of labelled limonene in breath without any background interference from the limonene ingested in the regular diet. In this way it should be possible to predict long term liver prognosis, monitoring the severity of liver disease pre liver transplant and then monitor the recovery with the biomarker disappearing in the breath post liver transplant surgery.

It can be also used to predict the best timing for a liver transplant in a patient or to followed up if a therapeutic regime is effective or not in a patient.

Currently, physicians monitor the progression of the liver disease pre and post-transplant using the current most common invasive liver test (LTs) such the alanine aminotransferase (ALT) and aspartate aminotransferase (AST), bilirubin and albumin [114]. These are enzymes found in blood and they are biomarker of liver damage. These enzymes can be also masked by other diseases or it can take long time to appear in the blood. Mass spectrometry would be the fastest and most suitable technology to detect the labelled ^{13}C isotope in patient's breath [115]. This will bring benefit to the patient and the NHS with faster and early diagnosis programs. The handicap is that PTR-MS has a high maintenance cost, but this can be solved by developing a new technology, for instance a portable device. For example, a device could possibly be developed based on the technique infrared spectroscopy (IR) to measure the isotopically labelled volatiles proposed in this study at more affordable cost.

5 ANALYSIS OF VOLATILE ORGANIC COMPOUNDS IN BREATH FROM PATIENTS SUFFERING WITH HEPATIC ENCEPHALOPATHY

The aim of this study was to establish the usefulness of PTR-MS for the on-line analysis of VOCs in exhaled breath of patients suffering Hepatic Encephalopathy (HE). This case study sought to obtain data to determine if there were any differences in the volatile breath profiles of patients suffering with liver disease between those with HE and those without. In this chapter, data comparison from patients suffering with hepatocellular cancer (HCC) and no hepatocellular cancer (no-HCC) are presented. It is also presented and examined the intra-individual variation in the key VOC biomarkers discussed in the previous chapter, limonene, methanol and 2-pentanone.

Finally, the results from a longitudinal study for two patients suffering from HE are presented which are used to examine changes in the above VOCs concentrations over an extended period of time.

5.1 Introduction

Hepatic encephalopathy (HE) refers to the neurocognitive changes and loss of brain function as a result of hepatic dysfunction. Approximately 50% of the patients with chronic or acute cirrhosis develop HE in the course of the illness [116]. Once the liver is damaged, episodes of loss of brain function may be triggered by the severity of cirrhosis. Depending on the severity of the illness, HE symptoms can vary but include confusion, disorientation, lethargy, sleep disturbance, severe personality changes, slurred speech and

cognitive impairment. Symptoms can begin slow and gradually get worse. Patients suffering HC can become unconscious and, in its extreme form, coma or death can result [117]. HE is caused by the action of neurotoxic substances, including ammonia and manganese, which are usually metabolised by the liver, persisting in elevated concentrations in the blood. These toxins can then cause glutamine induced changes in astrocytes that lead to the clinical manifestations of HE [118, 119]. HE is classified as overt or covert based on symptoms. Overt HE (OHE) is when the illness is clinically apparent whereas covert hepatic encephalopathy (CHE) is diagnosed only by cognitive test and the symptoms are mild. CHE was previously known as minimal HC (MHC) [120].

The most often and widely used scoring system to grade HE is the West-Haven criteria. This system has scores ranging from 0-4 depending on the severity and impairment of autonomy caused by the encephalopathy. Table 5.1 shows the West Haven criteria score system to grade a patient's mental state including a clinical description. However, it is very difficult and challenging to diagnose a patient with MHE or grade 1. In order to diagnose MHE correctly, psychometric tests and neurophysiological methods are required rather than a simple clinical assessment.

Table 5.1 West-Haven diagnostic criteria.

Clinical grade (0-4)	Diagnostic criteria, clinical symptoms
0	Normal
1	Mild impairment, mild intellectual function
2	Moderate impairment, inappropriate behavior
3	Severe impairment, disorientation, confusion
4	Coma, unconsciousness

HE can affect the patient's mental function intermittently, [121] so it is possible for them to pass a neurological test on the day of the examination, while still suffering from symptoms at other times.

Minimal HE (MHE) is difficult to diagnose and requires specialized testing. It is possible for patients to be suffering from undiagnosed MHE [122] [123]. There is no gold standard for the diagnosis of HE and many scoring systems have been criticized for lack of objectivity [122]. Current recommendations [124] include using the clinical HE staging scale (CHESS) [125], the HE scoring algorithm (HESA) [126] or modified orientation log (MO-log) to refine the West-Haven criteria, but their widespread use has yet to be adopted.

Estimates of the incidence of MHE range from 20% to 84% of cirrhotic patients, depending on which testing methods are used [121]. Another established method to grade the HE is to measure the ammonia levels in plasma. It has been shown that ammonia

concentration in arterial or venous blood correlate with the grade of the HE [127] and high levels of ammonia in blood indicates the beginning of liver dysfunction [128]. Although this is a method currently used to grade the HE the link between ammonia levels in plasma and the severity of HE remains questionable. A possible explanation for these results may be the fact that ammonia concentration changes during the course of a day.

Differences could also be attributed to the fact that the measurements of ammonia levels are strongly affected by the pre-analytical conditions [129]. Patients should be in a relaxed state, because difficulties in the blood sampling could increase the ammonia level in the plasma. Finally, the blood samples have to be quickly collected (within 15 minutes) and kept on ice. This is a crucial step, because it has been demonstrated that ammonia concentrations increase spontaneously in blood and plasma owing to the production of additional ammonia from the hemolysis of red blood cells [130].

Other methods such as imaging techniques are used to help in the diagnosis of HE. Brain scans may be used to rule out other conditions such as tumours in the brain. However, it is not possible to provide imaging procedures for all patients owing to time and costs.

HE not only degrades the patient quality of life, but it means a poorer prognosis for a patient [131]. In cirrhotic patients after the first symptom of HE the survival rate is 42% and, surprisingly, this rate has not improved in the last decade [132]. An early prognosis of HE would help to provide a better diagnosis and consequently a better treatment for the patient. There is therefore a need for an alternative method to monitor HE. This investigation puts forward the proposal that analysis of breath volatiles could aid in this.

Breath analysis for HE has been investigated in other pilot studies which used GC-MS involving patients with cirrhosis [112] but none of these reported limonene as a marker for HE. Khalid T et al. provided a model to distinguish HE in alcoholic cirrhotic patients with two volatiles. One volatile found was isothiocyanato-cyclohexane and it was related to the presence of HE. However, it is important to note that isothiocyanato-cyclohexane is a pollutant widely present in urban and industrial areas. The second volatile was methyl vinyl ketone and it was correlated with no symptoms of HE [82]. The study had a small number of participants and had no facilities to diagnose whether the patient suffered minimal HE or not. Vollnberg et. al performed a GC-MS study based on the analysis of breath air and tongue swabs and detected 150 compounds in the breath samples [112]. They identified a metabolite acetoin and 2-pentanone in patients with HE.

Another pilot study carried out by Arasaradnam et al. investigated HE using FAIMS (field asymmetric ion mobility spectrometry, portable e-nose) [133], but this technique unlike PTR-MS, was not able to determine precisely which VOCs were the cause of the discrimination. The technique is based on the comparison of VOC fingerprints with a database, providing an overall fingerprint of VOCs for the discrimination. The sample size was small in the study. Therefore, it was not possible to confirm whether the study detected limonene or not. Although extensive research on the subject has been carried out, it still remains the commitment for the “gold standard” test for grade and diagnosis HE because the current test based on the determination of the ammonia levels between cirrhotic patients without HE and with HE remains inconclusive with a poor prognosis.

In this chapter, the work of the study involving breath analysis and HE is presented. The findings, results and conclusions have been recently published in the IOPscience Journal of Breath Research [134].

5.2 Methods

5.2.1 Patient and control recruitment

Patients were recruited at the Queen Elizabeth Hospital Birmingham either from the transplant assessment clinic or in wards after being admitted with HE. For this study, 31 patients suffering from liver disease participated in the pre-transplant measurements (F/M 8/23, mean age 55 years, min-max 27-71 years). There were a number of etiologies and 11 patients had more than one condition. Full details of the patients and their diagnoses was given in chapter 4 [5]. Additional patient information is presented in the Appendix to this thesis.

5.2.2 Longitudinal measurements

For this investigation, two patients were recruited having been admitted as in-patients suffering from HE. Patient M1 was a 54-year-old male with a primary diagnosis of alcoholic liver disease (ALD). He gave four breath samples during an admission for HE before his liver transplant, then three further samples after transplant.

Patient M15 was also a 54-year-old male with a primary diagnosis of ALD. He gave breath samples on seven different days during an admission for HE. He did not receive a transplant so gave no post-transplant samples.

5.2.3 Breath sampling protocol

The breath sampling protocol used to collect the breath has been explained in detail in chapter 4. In brief, the subjects were sat down in a calm state and capnography controlled sampling was used to collect the alveolar part of the breath. A breath sample was taken from the subject using a 100 ml glass syringe. Four replicates were collected for each subject.

5.2.4 Proton Transfer Reaction Mass Spectrometry settings

The analytical measurement settings have been described in chapter 4. In summary, a m/z range of 20 to 200 amu was scanned with a dwell time of 0.5 seconds per atomic mass unit. The voltage across the drift tube was set at 600 V. The drift tube was maintained at a pressure of 2.07 ± 0.01 mbar and a temperature of 45 ± 1 °C. It should be noted that PTR-Quad-MS has a mass resolution of approximately 1 amu so it is possible that other compounds or fragments could contribute to observed peaks.

5.2.5 Statistical analysis

The data sets for each volatile of interest were assessed using the statistical analysis explained in chapter 4. In brief, IBM SPSS version 22 was used for all statistical analysis.

Mann-Whitney U-tests determined were used to determine whether the concentration of VOCs from the first stage were significantly different in patients suffering hepatic cellular carcinoma (HCC) with non-HCC patients. A Kruskal-Wallis one-way analysis of variance was used to compare the HE-now, History-HE and No-HE groups. The tests were calculated with a significance level of 0.95.

5.3 Results and Discussion

It has previously been reported in chapter 4 that there are 4 mass spectral peaks with statistical differences between patients and controls. The four mass spectral peaks are m/z 33, m/z 81, m/z 87 and m/z 137.

Limonene was tentatively identified by comparison with studies recently published in the literature on breath research and the assignment based on the results of the identification of the limonene key product ions at m/z 81 and 137 [44].

In order to confirm the identity of limonene, and in an attempt to determine the enantiomeric composition of limonene, 4 cartridges with breath samples were sent to the Max Planck Institute in Mainz (Germany) for analysis by GC-MS. The GC-MS only provided the confirmation of the findings of limonene presence and it was not possible to distinguish the two limonene enantiomers (D-limonene and L-limonene). The measurements were not successful due to the high water vapour content in the breath samples cartridges and the ensuing water degradation in the GC-MS column during the GC-MS measurements. It would be very interesting to know the limonene enantiomer in patient's breath, because previous investigations carried out in humans has indicated that limonene is the most abundant monoterpene in breath and blood [95].

In order to demonstrate how the key volatiles changed in each subgroup, the patient group was further subdivided into those with and without hepatocellular cancer (HCC and No-HCC). Figure 5.1 shows the box plots of limonene, methanol (m/z 33) and 2-pentanone (m/z 87) for patients with and without HCC, controls and room air. Mean (lower quartile (LQ)/upper quartile (UQ)) are given in the figure 5.1. It was found that there was a difference between patients with and without HCC only for limonene, with a Mann-Whitney U score of significance $p = 0.015$. Patients were categorized in different

groups according to the medical status and previous condition. Table 5.2 shows the patient classification according to the symptoms and previous history of HE.

Table 5.2 Patients HE classification.

<u>History-HE</u>	No current symptoms of HE but having a history of HE before
<u>HE-now</u>	Having HE symptoms presently
<u>No-HE</u>	No current symptoms of HE and no history of HE before

Figure 5.1 shows boxplots of volume mixing ratios (VMRs) calculated in units of nmol/mol and the comparisons for the different groups categorized by No-HE, History-HE, HE-now, controls and room air for (a) limonene, (b) methanol and (c) 2-pentanone. The box-plots were calculated for 21 patients with liver cirrhosis without HCC (No-HCC), 10 patients with cirrhosis and HCC (HCC), 30 controls and room air.

Figure 5.2 shows boxplots of volume mixing ratios (VMRs) calculated in units of nmol/mol and the comparisons for the different groups categorized by No-HE, History-HE, HE-now, controls and room air for (a) limonene, (b) methanol and (c) 2-pentanone for 11 patients without HE (No HE), 8 patients who have a history of HE but are not showing symptoms on the day of sampling (History HE), 12 patients who were suffering symptoms of HE on the day of sampling (HE-now), 30 controls and room air.

The study of the subgroups found that in the comparison of the three patient groups only one volatile organic compound limonene, was revealed to successfully differentiate the groups with a Kruskal-Wallis score of significance $p = 0.001$. Figure 5.2 shows a noticeable differentiation between HE-now and History-HE. It was observed for the subgroup of No-HE that there was an overlap with the HE-now group.

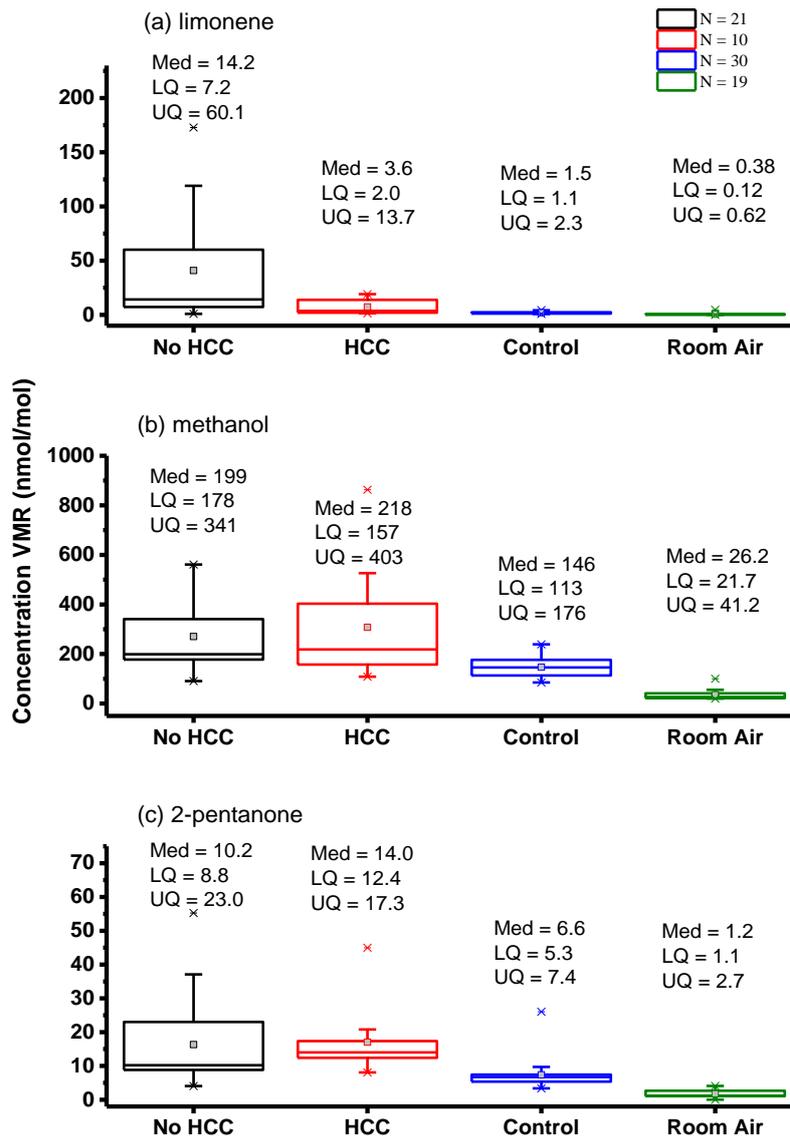


Figure 5.1 Box-plots representation showing volume mixing ratios (VMRs) measured for (a) limonene, (b) methanol and (c) 2-pentanone. The box-plots were calculated for 21 patients with liver cirrhosis without HCC (No-HCC), 10 patients with cirrhosis and HCC (HCC), 30 controls and room air. (Box represents lower quartile (LQ), median (Med), and upper quartile (UQ) in units of nmol/mol). Whiskers are 1.5 times the inter-quartile range and outliers are depicted by a star.

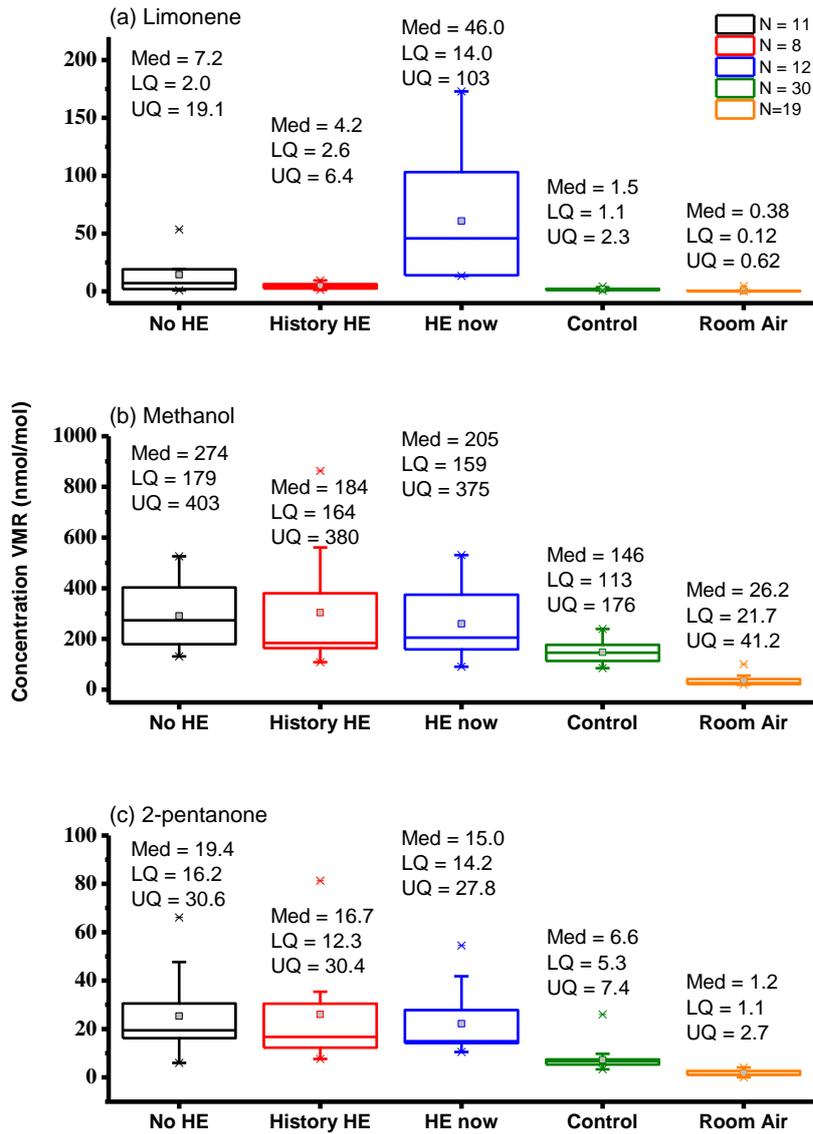


Figure 5.2 Boxplots representation showing volume mixing ratios (VMRs) measured for (a) limonene, (b) methanol and (c) 2-pentanone. 11 patients without HE (No HE), 8 patients who have a history of HE but are not showing symptoms on the day of sampling (History HE), 12 patients who were suffering symptoms of HE on the day of sampling (HE-now), 30 controls and room air. (Box represents lower quartile (LQ), median (Med), and upper quartile (UQ) in units of nmol/mol). Whiskers are 1.5 times the inter-quartile range and outliers are depicted by a star.

From previous results, the assumption of the differences observed in limonene concentration in patient's breath between No-HCC versus HCC groups could be a consideration of cirrhosis severity. The most interesting aspect of this assumption is that the initial assessment for a liver transplant is often based on the existence of a hepatocellular carcinoma (HCC) in the liver while the liver is still able to function adequately that is the liver is compensated. The analysis of the UKELD score for the different groups is corroborated by this consideration.

UKELD is an average score test which integrate specific clinical chemistry data to predict and access the preference for liver transplantation in patients with a prediction of 1-year mortality [135]. UKELD score is > 49 indicates that patients meet the criteria and they are considered for liver transplant except for the additional evidence for liver transplant such as HCC. In order to demonstrate the differences in the UKELD score in the groups and the severity of the liver, the UKELD score was calculated for the No-HCC and HCC groups. Table 5.3 shows the results for the calculation of the mean (range) UKELD score for the No-HCC and HCC groups. These were significantly different (Mann-Whitney $p = 0.002$).

Table 5.3 UKELD mean (range) score for the No-HCC and HCC groups

Patient ethiology	UKELD score mean (range)
No- Hepatic Cellular Cancer (No-HCC)	54 (48 – 63)
Hepatic Cellular Cancer (HCC)	49 (43 – 56)

In order to demonstrate whether the patients in the HE group had a worse outcome, a comparable analysis was performed for the HE classifications. Table 5.4 presents the results for UKELD mean (range) score for the three different groups (No-HE), history of HE and finally HE at the time of the study.

Table 5.4 UKELD mean (range) score for the HE groups.

Hepatic Encephalopathy history	UKELD score mean (range)
No- Hepatic Encephalopathy (No-HE)	49 (43 – 57)
History of HE	53 (46 – 63)
HE-now	55 (51 – 62)

The data analysis revealed that there was no significant difference between the UKELD scores for the History-HE and HE-now groups (Mann-Whitney $p = 0.21$), whereas there was a significant difference when comparing the No-HE groups and the HE-now groups (Mann-Whitney $p = 0.001$). This is evident, in the case of the group of patients who has never suffer symptoms of HE has a lower UKELD score in comparison to the group which was suffering HE symptoms when the samples were collected.

However, the considerable difference in limonene concentration between the History-HE group and the HE-now group cannot be explained by a difference in severity as judged by UKELD score as shown in table 5.4. Finally it should be noted that it was

previously reported in chapter 4, [5] that there was no correlation between the concentration of breath limonene and UKELD score.

Cordoba et. al performed a observational study to examine the survival data of 1348 cirrhotic patients admitted with acute liver decompensation. The data was compared and reviewed according the presence or absence of HE to investigate the risk factor for HE and to investigate the survival rate in patients with liver disease [136].

The results of this study determined that one of the most crucial risk aspect for the development of new episodes of hepatic encephalopathy in cirrhotic patient was previous HE. The study showed that HE was associated to acute chronic liver failure in younger cirrhotics, alcoholics, with severe liver failure and systemic inflammatory reaction. Whereas HE was not associated to acute chronic liver failure in older cirrhotics and inactive drinkers.

This study has determined that breath limonene is capable to discriminate between History-HE and HE-now groups. This outcome envisages the possibility to design monitoring tools to screen a high-risk population to improve therapies and treatments.

In chapter four was also reported that there was no association between self-reported consumption of fruit/fruit juice and elevated breath limonene [5]. As part of this study a pilot investigation was carried out with regards to the possible effect of fruit consumption (data not published). Three healthy young volunteers were asked to drink half a litre of concentrated orange juice. It was found that consumption of concentrated orange juice increases the concentration of limonene in exhaled breath, but this effect is due to mouth contamination. The levels of limonene decreased to normal levels comparable to the control group within 2-3 hours. In this study, it was not possible to find

any food intake in the patient diet or prior 24 hours of the collection of the breath samples that demonstrate the high levels of limonene found in their breath.

Isothiocyanato-cyclohexane was reported by Khalid et al. as indicator for HE in patients with symptoms of HE, and methyl vinyl ketone as an indicator to the absence of HE within a group of alcoholic cirrhotics [82]. Surprisingly, they used as a method the presence-absence analysis in which absolute concentrations were not analysed. The analysis was based on the comparison between groups of the signal intensity of presence/absence of a volatile compound above three times of the signal to noise ratio. Performing this method of analysis, the results would not have differentiated between patients and controls due to low concentrations of limonene were also observed in the control group, albeit at very low concentrations. Previous investigations have detected limonene in the blood and in the emissions from skin of healthy volunteers, and can be considered a normal volatile emission from healthy humans [137].

Mochalski et al. carried out a study to examine the constituents of human breath by GC-MS, limonene was found in the blood and breath of all 28 subjects [95]. The mean (range) concentration in breath was 1.46 ppbv (0.27-7.42 ppbv), similar to this present study. Up to now, only a small number of studies have investigate the use of volatile organic compounds in exhaled breath for the evaluation of markers for the detection of HE.

Vollnberg et al. carried out an investigation using tongue swabs and breath air to identify markers of HE [112] by GC-MS. Their work remains a conference abstract therefore insufficient details are known to make a comparison and in addition only a low number of patients suffering HE was used in the study. They found that acetoin (3-

hydroxybutanone) and 2-pentanone had a better correlation than blood ammonia for the prediction of HE.

This study presented, 2-pentanone was able to predict the presence of cirrhosis versus controls, but was not able to discriminate those with and without HE. During the study, volunteers were asked to indicate in the questionnaire when they last had something to eat or drink. This results showed that the levels of 2-pentanone were not associated with time since last meal. A number of studies has suggested that 3-hydroxybutanone (acetoin) give a protonated parent peak at m/z 89 as confirmed by PTR-MS measurements, and GC-MS has conclusively assigned this compound [138, 139].

In chapter 4, it was shown that in the initial case-control comparison, m/z 89 was significantly different between patients and controls. It did not, however, show a difference between patients pre- and post-transplant so it was ruled out as being a useful diagnostic volatile for liver cirrhosis.

Figure 5.3 shows the box plot for groupings of HE status for m/z 89. The boxplots are shown in normalized counts per second. The HE-now group is significantly different from the other groups, but the variation in room air is large and it overlaps with all other groups. An examination of the 12 patients who had a transplant shows that the concentration of m/z 89 in patients with and without HE is not significantly different. It is important to note that isomeric and isobaric compounds cannot be distinguished in the PTR-MS used in this study and a signal from acetoin may be confounded by the presence of another volatile which produced a product ion at the same m/z .

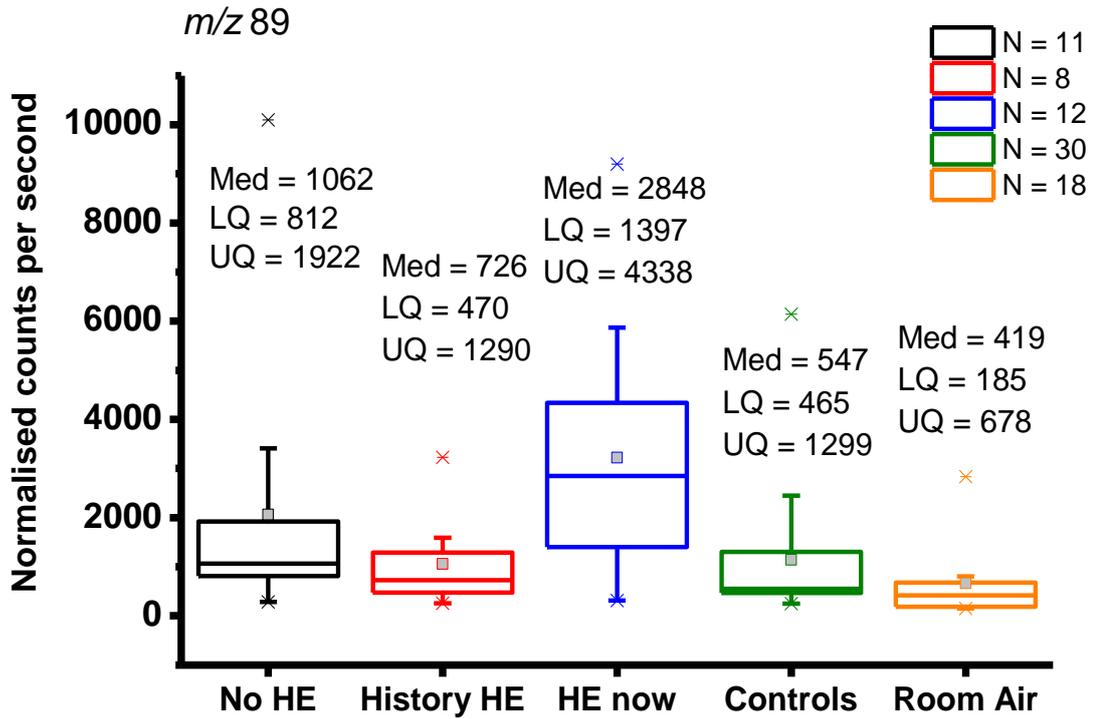


Figure 5.3 Boxplots representation showing volume mixing ratios (VMRs) measured for *m/z* 89 for 11 patients without HE (No-HE), 8 patients who have a history of HE but are not showing symptoms on the day of sampling (History HCC), 12 patients who were suffering symptoms of HE on the day of sampling (HE-now), 30 controls and room air. Whiskers are 1.5 times the inter-quartile range and outliers are depicted by a star. Note that only 18 room air samples are used as one sample gave a peak which could not be distinguished above the instrumental noise.

5.3.1 Longitudinal study for HE patients

In this investigation, the changes in concentration of the key volatiles previously identified in chapter 4, namely methanol, 2-pentanone and limonene were observed in the exhaled breath of two patients suffering from HE over a period of time. The differences were tracked in order to predict HE changes. For this cohort, two male patients (identification codes M1 and M15) consented and were enrolled in the study. The patients were recruited previously and had been admitted suffering with HE and were followed over the course of their stay in hospital.

Patient M15 had high grade HE, and had a very low mental capacity. M1 had liver transplantation during the course of the study so his results show four pre-transplant samples and three post-transplant. Figure 5.4 shows the results obtained for the changes in VMRs of limonene, methanol and 2-pentanone in breath of patient M15, M1 and room air in the longitudinal study. For M1, study days 1, 2, 3 and 4 correspond to 47, 44, 42 and 40 days prior to transplant, respectively. Study days 5, 6 and 7 correspond to 28, 29 and 33 days after transplant.

All the breath samples were taken on the wards, except for study day 7 which was taken in the out-patient clinic. For M15, if study day 1 is taken to be on day 1, study days 1 – 7 correspond to days 1, 2, 6, 7, 8, 9 and 15. The patient was discharged on day 15.

What is interesting in the data is the high degree of variability from day to day.

Despite this, from the data in figure 5.4 there is still a measureable decrease in breath limonene and methanol following transplant for M1. There is what appears to be an anomalous result for 2-pentanone for M1 on study day 6, which is, at present, unexplained as it was not present in the room air.

It is possible that the patient inhaled an environmental contaminant at high concentration some time before the breath test and it was still washing out.

What can be seen from the data is that the variability for M15 is more pronounced than for M1. This is due to the fact that the patient had grade 3 or 4 HE for the first 6 days of the study. On study day 7 his acute HE had resolved sufficiently to be discharged later that day, although it is likely that he was still suffering from minimal HE as he had a recent history of repeated hospital admissions.

It should be noted that there were difficulties in obtaining samples from M15 on some days of the study as his mental incapacity was such that he was unable at times to comply with instructions to breathe into the apparatus. It was, therefore, very difficult to obtain reliable end-tidal samples and it is possible that some of his samples were contaminated with dead-space air. The data presented must be interpreted with caution, because of the difficulties of the collection of samples during the course of the HE. From this limited evidence, the most important clinically relevant finding was that breath limonene cannot predict daily variations in symptoms of HE for an individual patient.

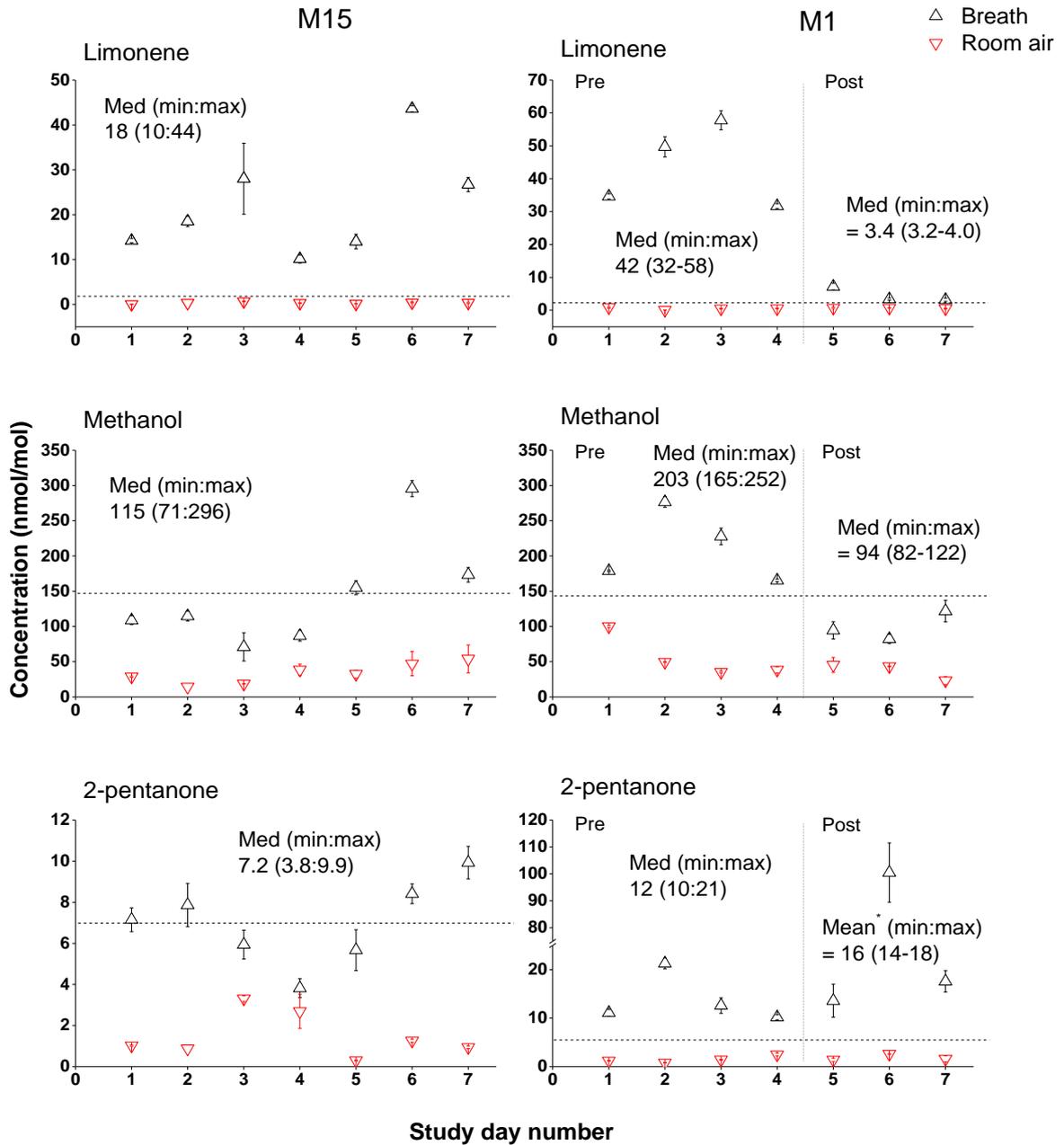


Figure 5.4 Scatter plots of volume mixing ratios (VMRs) for (a) limonene, (b) methanol and (c) 2-pentanone for patients M15 (left hand panel) and M1 (right hand panel). Breath concentrations are represented in black triangles and room air in red triangles. For M1, study days 1-4 are pre-transplant and days 5-7 are post-transplant. For patient M15, all days are pre-transplant, as he did not receive a transplant. The dashed horizontal line drawn in the figure 5.4 indicates the median of the concentration for healthy controls for each particular volatile. *The mean is given for M1 2-pentanone as it does not include the outlying point.

5.4 Conclusion and Final Remarks

The study has demonstrated the viability of on-line monitoring VOCs in exhaled breath by PTR-MS for the assessment of HE in patients with severe liver disease. It is important to highlight that for the first time this investigation presents a longitudinal study of VOCs in patients suffering HE.

It has been confirmed from the results that limonene is higher in a patient's breath with HC compared to No-HE patients, which supports the idea that limonene increase in breath is dependent on the severity of liver disease. Breath limonene has been reported higher in patients with No-HCC than with HCC. This seems to be associated with the severity of the cirrhosis and this idea is corroborated by analysis of the UKELD number. This investigation could have several applications. It could be useful for estimating disease severity and patient survival. It may also be useful as a timing indicator of liver transplant.

Although the current study is based on a small sample of patients, the findings suggest that limonene concentration in breath could be used to estimate the grade of liver disease since it seems to be related with the severity of hepatic dysfunction. More research is needed to investigate this further. Research questions that could be asked include the relationship between HE with the key volatiles such as limonene, methanol and 2-pentanone.

It is important to note that there have been reported cases of intoxication caused by tree tea essential oil in humans. The major components of the tree oil are terpenes and terpinoids and a low percentage of limonene. The symptoms reported in the case study were confusion, drowsiness and coma [140]. These are surprisingly similar to the symptoms of HE, and suggest that molecules may cross the blood/brain barrier to cause

neurological disorders. In all cases, the effects were transient and the patients recovered within a matter of hours. While this was not limonene, terpinoid oils consist of similar units and due to the lipophilic properties of limonene, it can be deposited in human fat tissue.

Although limonene has demonstrated low toxicity, there is a lack of information about chronic exposure for long periods [87]. For instance, if a patient suffering cirrhosis has high levels of limonene in plasma for months or years, it is uncertain if it will affect their health status.

These results support the hypothesis that high levels of limonene in breath are consequence of liver disease. This study was unable to investigate the intra and inter variability on a day-to-day basis. It would be very interesting to monitor changes in limonene levels pre liver transplant on a daily basis. This could be the subject of another study. This would only be possible for patients not suffering with HE. The main reason was due to the low number of participants in the longitudinal study. It is important to note that the incapability of one of the patients to follow the instructions at the breath sampling collection could have had implications on the high variability of the results.

This study set out to assess the importance of limonene, methanol and 2-pentanone to monitor of HE. This could be used to better help clinicians in HE prognosis and grading of HE. This study has thrown up many questions in need of further investigation such as the use of VOCs in breath to monitor HE. Further research should be undertaken with a higher number of participants to investigate the variability of exogenous limonene during HE in order to be able to discriminate between HE stages and limonene levels.

A cross-validation is needed using a new set of subjects from a distinct population and in multicentre institutions. Since there is need to have a “Gold standard treatment for HE” [122], the results presented provide a new insight into a non-invasive way to grade HE.

6 ELIMINATION CHARACTERISTICS OF POST-OPERATIVE ISOFLURANE LEVELS IN ALVEOLAR BREATH

The first part of this chapter presents a detailed description of the reaction of H_3O^+ with the anaesthetic isoflurane to facilitate the identification of the product ions in the absence of the complex breath matrix. The next section reports the first PTR-MS longitudinal study investigating isoflurane in the exhaled breath of patients following surgery. Data of temporal profiles of isoflurane concentration of 5 patients who had undergone a liver transplant are presented. The aim of this study was to investigate isoflurane wash out and the implication on the elimination profile based on the age of patients, body index and health following major surgery.

6.1 Introduction

Currently the majority of breath sampling and analysis for the health sciences is research based [113], but these studies show there is sufficient potential for the use of breath analysis for medical diagnosis and screening. Unlike many other diagnostics tests, the major benefit of breath analysis is that it is a non-invasive way of monitoring the patient condition. It also offers advantages such as real-time monitoring of VOCs changes in expiratory air and the potential of therapeutic monitoring of VOCs of intravenous anaesthesia for pharmacokinetic applications during surgery. Clinicians can obtain pharmacokinetic parameters of anaesthesia elimination from serum or blood measurements [141]. The main disadvantages are that sampling blood for routine analysis

requires a nurse or trained personnel and it is not the favoured patient preference option to choose.

Many research studies have focused on the investigation of anaesthesia monitoring because it provides continuous information and assessment of anaesthesia helping to keep the physiological state of the patient stable during anaesthesia. A considerable amount of literature has been published on either the monitoring of an intravenous anaesthetic, such as propofol [1, 4, 33, 34, 142, 143], or monitoring inhaled anaesthetics such as sevoflurane and isoflurane in the hospital environment, namely in an urological post-anaesthesia care unit [144] and sevoflurane in an operating theatre [145].

Some recent studies on anaesthetics pay particular attention to the investigation of the elimination of volatile anaesthetics from the body following surgery. Yasuda et al. [12] used gas chromatography with flame ionisation detection (GC-FID) to compare the kinetics of sevoflurane and isoflurane in humans. One interesting relevant finding is that they report a fast elimination of isoflurane and sevoflurane from the body, namely within a day the breath concentrations had decreased by an order of magnitude. However, their clinical trial used seven healthy male volunteers (mean age of 23 years with a standard deviation of 3 years). Thus, the rapid decrease in concentration may not be a true reflection of what happens with sick, obese and/or older people. A more recent paper by Ghimenti et al. [146] reports details on a study using GC-MS of post-operative elimination of sevoflurane and its metabolite, hexafluoroisopropanol, in exhaled breath of six very sick patients that had undergone a variety of surgeries. They used pharmacokinetic models for assessing liver function. In their investigations, mixed-expired breath samples (multiple deep breaths) were collected in disposable Nalophan bags. The samples were then transferred to desorption tubes for subsequent analysis.

They proposed that the metabolite hexafluorisopropanol is linked to CYP2E1 metabolism and their variation can be used as a marker for liver function efficiency.

The present study investigates the elimination characteristics of post-operative isoflurane in alveolar exhaled breath via breath analysis. Isoflurane (1,1,1-trifluoro-2-chloro-2-(difluoromethoxy)-ethane), $C_3H_2ClF_5O$ is a commonly used inhalation anaesthetic drug. Prior to being able to analyse the mass spectra of the post-transplant breath samples, it was necessary to establish which product ions result from the reaction of H_3O^+ with isoflurane in the drift tube environment of a PTR-MS, divorced from the complex chemical environment of breath. Therefore, as part of this investigation, a headspace analysis of isoflurane over a range of reduced electric fields 90-140 Td is presented.

For the purpose of analysis, the branching ratios have been determined using both clean air and CO_2 enriched clean air to mimic breath, as the buffer gases in the drift tube of the PTR-MS. It is important to check what happens in a CO_2 enriched air because the presence of a high percentage of CO_2 could influence the internal energies of the reagent ions leading to different product ion distributions. These need to be taken into account when analysing breath samples [28] [147].

To date, there has been no reliable evidence that isoflurane can be detected in exhaled breath weeks after being anaesthetised. As a result, questions are raised about the side effects of anaesthesia, including more serious complications such as, when the cognitive function fully returns to a patient.

A longitudinal study of post-transplant breath samples provides a unique opportunity to investigate the presence of isoflurane in the human body over time. These

findings have implications on how quickly patients can safely return to a normal life. The results from this investigation presented in this chapter have recently been published in the IOPscience Journal of Breath Research [148].

6.2 Methods

6.2.1 Isoflurane headspace analysis

Isoflurane (CAS Number 26675-46-7) was purchased from Sigma Aldrich (UK). Isoflurane samples were prepared for analysis by adding 1 μ l of isoflurane into a 100 ml glass bottle. Figure 6.1 shows the chemical structure of isoflurane.

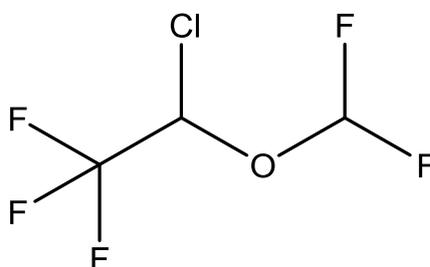


Figure 6.1 Chemical structure of isoflurane

A 100 ml glass syringe was then coupled to the top part of the bottle using a 3-way luer-lock stopcock and 10 ml of the isoflurane headspace gas was sampled. The isoflurane sample was then diluted in the glass syringe with 90 ml of clean air or CO₂ enriched clean air (4% carbon dioxide, 17% oxygen and 79% nitrogen (Scientific and Technical Gases

Ltd (STG, Staffordshire, UK)). The concentration of the isoflurane was sufficiently low that no depletion of the H_3O^+ signal was observed.

6.2.2 Ethical approval

The study was approved by the regional ethics committee of Camden and Islington (REC reference: 13/LO/0952).

6.2.2.1 Patient selection and recruitment

For this case study, 5 patients were recruited (3 females, 2 males) with a mean age of 50 years (min-max 36-58 years) at the University Hospital Birmingham from the transplant assessment clinic. Patients were informed in detail about the procedure of the research and writing informed consent was obtained.

Breath samples were collected from 5 patients who underwent liver transplantation and they were followed up several weeks following surgery. Table 6.1 summarises the details of these 5 patients, with each patient being just identified by sex (F or M) and a number, which was used in the previous study mentioned in Chapter 4 and retained here for comparison [1]. Note that the number of samples and the days at which breath samples were taken varies because of the availability and health of the patient. Patients were followed from the Intensive Care Unit after their transplant, through to the wards until their discharge. Some patients recovered and were discharged quicker than others so there are fewer data points for them.

Table 6.1 Patient identification and details for the isoflurane study. Including sex (female F, male M), age, location of post-transplant breath sampling (Out-Patient Clinic (OPC) or Ward), and number of days after transplant when breath samples were collected.

Patient ID	Age (year)	Location of post-transplant breath sample	Post-transplant breath samples: days after transplant
F2	49	OPC	3, 5, 130
F4	58	Ward	5-8, 11-15, 18, 58
F5	53	Ward	2-6, 9-12
M3	53	Ward	4, 7, 48
M7	36	Ward	2, 3, 6-8, 55

6.2.3 Breath sampling protocol

In this current study breath samples were collected following the protocol detailed in Chapter 4 [5]. In summary, capnometer controlled sampling was used to collect the patient's breath. Four glass syringes of 100 ml were taken for each patient. Within two hours after collection, breath samples were mass spectrometrically analyzed by PTR-MS, for which the syringes were maintained at a constant temperature of 40 °C using a heating bag (Infroheat, Wolverhampton). This was done to limit condensation, which could otherwise lead to volatile loss. The outlet from each syringe was connected directly to the inlet of the PTR-MS.

6.2.3.1 Investigation of glass syringes for the collection and storage of isoflurane breath samples

The study aimed to investigate the suitability of the use of glass syringes for the storage of isoflurane. For that investigation, 3 glass syringes were filled with isoflurane and stored at room temperature for a period of time to evaluate the stability of isoflurane. Analysis of the samples was performed at time 0, 1 hour, two hours and 48 hours. Figure 6.2 presents the results of the variability in concentration of isoflurane in glass syringes. It is important to highlight that storage condition greatly affects the decay of VOCs in breath. The results show that within two hours there was no significant loss of isoflurane in the glass syringe. Therefore, glass syringes are a convenient collection method to store isoflurane gas during a period of time less than two hours.

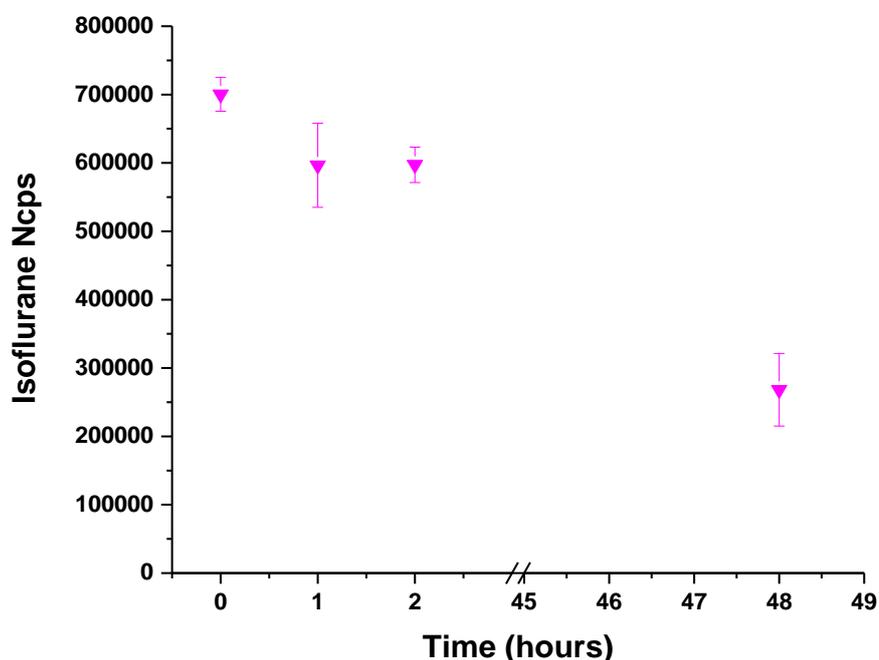


Figure 6.2 Isoflurane concentration (Ncps) versus storage time (hours).

In addition to taking breath samples from controls as described in [1], samples of air in the locations where the patients were seated were collected so that allowances for any background isoflurane could be taken into account. Hospital room air was collected every time breath samples were taken using glass syringes (2 syringes for every sample), which were analysed in the same way as the breath samples.

Data from the isoflurane concentration in the hospital environment show that the maximum concentration for any of the sampling days was at 4 ppbv. There was one exception to this when 13 ppbv was measured for one patient, F4, who was in a small enclosed room with poor ventilation, and therefore the patient was almost certainly contaminating the air with her own breath. Interestingly, any contribution to the isoflurane signal from the environment is negligible compared to that in the breath.

6.2.4 Analytical measurements and PTR-MS settings

The analysis of the samples was carried out using a PTR-Quad-MS (IONICON Analytik GmbH). The instrument has been described in detail in Chapter 2. In brief, the instrument setting for this investigation was a DT voltage at 600V to provide an E/N value of 136 ± 1 Td. All the breath samples were measured at a DT pressure of 2.07 ± 0.01 mbar and temperature of 45 ± 1 °C. For the headspace analysis the voltage across the drift-tube was changed from 400 V to 600 V at intervals of 20 V, E/N (96-138 Td).

Mass spectra of the breath samples were recorded and the intensity of the mass spectra converted to a volume mixing ratio (VMR) by use of a standard procedure that relies on the use of an experimentally measured reaction rate coefficient and reagent and product ion counts [23].

6.3 Results and Discussion

6.3.1 Headspace analysis of isoflurane

In this investigation, a headspace analysis of an isoflurane sample was analysed to obtain details on the product ions resulting from the reaction of isoflurane with H_3O^+ .

Product ions and their intensities were measured as a function of the reduced electric field. This study showed that for any value of the reduced electric field no protonated isoflurane (m/z 185) was observed. This agrees with the SIFT-MS results of Wang et al. who conclude that once formed the excited protonated parent spontaneously dissociates via various pathways [149]. However, in comparison to Wang et al. SIFT-MS (thermal energy) measurements, this study found substantial differences in product ions and their branching ratios. A possible explanation for this might be that due to the fact that reactions in PTR-MS are non-thermal. The results from the two studies are compared in table 6.2 for a reduced electric field of 138 Td. Assignments have been added by taking into account chlorine isotopes. For the PTR-MS studies, the product ion branching percentages have an error of approximately $\pm 20\%$.

Table 6.2 m/z values proposed product ions and branching ratio percentages resulting from the reaction of H_3O^+ with isoflurane using PTR-MS and SIFT-MS [149].

m/z	Proposed Product Ion	PTR-MS %	SIFT-MS [149] %
51	CHF_2^+	12	-
67	CF_2HO^+	-	15
67/69	CHFCI^+	50	-
99	$\text{CF}_3\text{CH}_2\text{O}^+$	-	10
117/119	CF_3CHCI^+	6	30
119/121	$\text{CF}_3\text{CFHOH}_2^+$	-	25
147*/149	$\text{CF}_3\text{HOCHCICH}^+$	-	10
163/165	$\text{CF}_3\text{CCIOCF}^+$	24	-
165/167	$\text{CF}_2\text{HOCHCICF}_2^+$	8	10

* In the paper by Wang et al. the ion at m/z 147 is incorrectly assigned as $\text{CF}_2\text{HOCHCICH}^+$.

Another ion involving isoflurane was observed at m/z 181 which is assigned as a secondary (association) reaction of $\text{C}_3\text{F}_4\text{OCl}^+$ with H_2O , but its intensity was low and is therefore not included in the table 6.2.

Wang et al. assigned their peak at m/z 67 to be CF_2HO^+ rather than CHFCI^+ . They also claim product ions which were not observed in this study at m/z 99 ($\text{CF}_3\text{CH}_2\text{O}^+$), m/z 119 ($\text{CF}_3\text{CFHOH}_2^+$), and m/z 147 ($\text{CF}_2\text{HOCHCICH}^+$).

To date, previous studies in breath volatiles have highlighted the importance to take into account factors such as the humidity and the CO₂ content for calibration purposes and samples storage [57, 147] to limit losses of VOCs concentrations [65] or avoiding systematic errors. For instance, humidity is considered a relevant factor in breath analysis. Humidity enhances volatiles losses depending on the chemical stability of the compound stored. For instance, humidity in breath can lead to solvation therefore there is a loss of volatiles by dilution. In Tedlar bags, volatiles losses are principally from diffusion through the surface of the bag [147]. On the other hand, it has been demonstrated that increasing CO₂ can lead to enhanced water clustering to the reagent ion in PTR-MS. The outcome is an overestimation of the concentration of the VOCs. Keck et al. [28] have already demonstrated that higher CO₂ concentrations in the drift tube buffer gas of a PTR-MS enhances the concentration ratio of the protonated water dimer to protonated water and changes the mass spectra of key breath gases such as methanol, ethanol, 1-propanol, 2-propanol, acetone and isoprene.

For this investigation and given that the product ion branching ratios were obtained using normal air in the drift tube, a question remains as to whether the presence of higher CO₂ concentrations in breath could affect these ratios through thermalisation effects as a result of collisions of H₃O⁺ with CO₂ prior to reaction with isoflurane. Therefore, a headspace analysis using CO₂ enriched (4%) air in the drift tube was carried out to determine if the product ion distributions associated with isoflurane would be altered when analysing the breath samples.

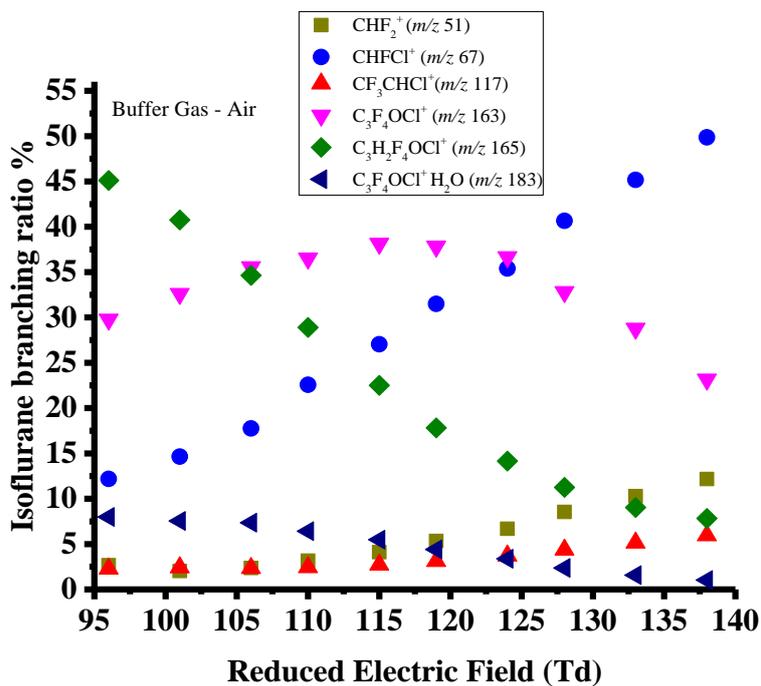
Table 6.3 shows the information of the product ion percentages resulting from the reaction of isoflurane anaesthetic gas with H₃O⁺ in buffer gas containing either (a) clean air or (b) 4% CO₂ enriched clean air as a buffer gas. The figure 6.3 summarises the results

of the product ion percentage branching ratios as a function of reduced electric field resulting from the reaction of H_3O^+ with isoflurane using (a) normal air and (b) CO_2 (4%) enriched air as a buffer gas. The data are provided because different PTR-MS users use different reduced electric fields and also because of the major differences observed in the product ions and branching ratios compared to the only other headspace analysis by Wang et al.

The results show that within experimental uncertainty, no significance difference in product ion percentage branching ratios is observed between normal and CO_2 enriched air buffer gases.

As previously stated, humidity strongly affects hydrophilic compounds in breath. For instance, anaesthetics with less blood gas solubility tend to equilibrate. In this study, an issue that was not addressed is whether the changes in humidity [149] will affect isoflurane concentration, but that will be investigated in future studies.

(a)



(b)

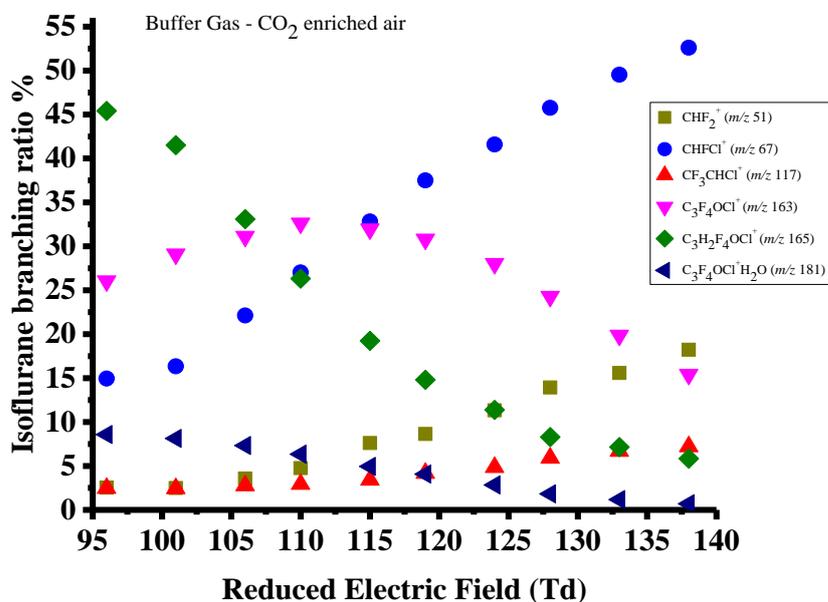


Figure 6.3 Product ion percentage branching ratios of isoflurane as a function of reduced electric field (a) normal air and (b) CO₂ (4%) enriched air.

Table 6.3 Product ion branching percentages resulting from the reaction of isoflurane anaesthetic gas with H_3O^+ in buffer gas containing either (a) clean air or (b) 4% CO_2 .

(a)

<i>E/N</i> (Td)	Product ion and ion branching ratio (%)					
	CHF_2^+ (<i>m/z</i> 51)	CHFCl^+ (<i>m/z</i> 67)	CF_3CHCl^+ (<i>m/z</i> 117)	$\text{C}_3\text{F}_4\text{OCl}^+$ (<i>m/z</i> 163)	$\text{C}_3\text{H}_2\text{F}_4\text{OCl}^+$ (<i>m/z</i> 165)	$\text{C}_3\text{F}_4\text{OCl}^+\text{H}_2\text{O}$ (<i>m/z</i> 181)
96	3	14	2	29	44	8
101	2	15	2	32	40	7
106	2	18	2	36	35	7
110	3	22	2	37	29	7
115	4	26	3	39	23	6
119	5	30	3	39	18	5
124	7	34	4	37	14	3
128	9	39	4	34	12	2
133	10	44	5	29	9	2
138	12	49	6	24	8	1

(b)

<i>E/N</i> (Td)	Product ion and ion branching ratio (%)					
	CHF_2^+ (<i>m/z</i> 51)	CHFCl^+ (<i>m/z</i> 67)	CF_3CHCl^+ (<i>m/z</i> 117)	$\text{C}_3\text{F}_4\text{OCl}^+$ (<i>m/z</i> 163)	$\text{C}_3\text{H}_2\text{F}_4\text{OCl}^+$ (<i>m/z</i> 165)	$\text{C}_3\text{F}_4\text{OCl}^+\text{H}_2\text{O}$ (<i>m/z</i> 181)
96	3	15	2	26	45	9
101	2	16	2	29	42	8
106	4	22	3	31	33	7
110	5	27	3	33	26	6
115	8	33	3	32	19	5
119	9	38	4	31	15	4
124	11	42	5	28	11	3
128	14	46	6	24	8	2
133	16	50	7	20	7	1
138	18	53	7	15	6	1

6.3.2 Isoflurane longitudinal study

This investigation presents for the first time the results of PTR-MS analysis of temporal profiles of isoflurane anaesthesia in alveolar exhaled breath of 5 patients (F/M 3/2, mean age 50 years, min-max 36-58 years) who had undergone liver transplant surgery. Isoflurane concentrations in exhaled breath were calculated by using the product ions and their branching ratios from the reaction of H_3O^+ with isoflurane at a reduced electric field of 138 Td, previously obtained in the headspace study described in section 6.2.1. The following products ions were used; m/z 51, m/z 67 (69), m/z 117 (119), m/z 163 (165), and m/z 165 (167). The m/z 69 signal intensity cannot be used directly owing to the presence of isoprene in human breath. Thus, the total ion signal associated with CHFCl^+ is determined only from the m/z 67 ion intensity. The contribution of isoflurane to the signal intensity at m/z 69 at the beginning of the longitudinal study was found to be significant and varied between 12% to 99%.

It is important to point out that since isoflurane in breath results in a spectral peak at m/z 69 ($\text{CHF}^{37}\text{Cl}^+$) there are consequences for any mass spectrometric studies which may monitor isoprene in the exhaled breath following a surgical procedure during which isoflurane is administrated.

Figure 6.4 presents the temporal changes in isoflurane concentrations (VMR) for five days after liver transplant for patients, F2, F4, F5, M3 and M7. A clinically relevant finding to highlight is that rapid decreases in isoflurane concentrations in patient's breath following surgery was not observed.

These results are contrary to those found by Yasuda et al. [12] who reported that the elimination of isoflurane from the human body was fast. Yasuda et al. showed that within one day the signal intensity had been observed to have dropped by about an order of magnitude, whereas in this investigation high concentrations of isoflurane in a patient's breath were found several days after the operation. Although the Yasuda et al. study used healthy young male subjects (age 23 ± 3 yrs (mean \pm sd)), which implied a higher pulmonary function. The observed temporal difference is still surprising given that the major elimination of isoflurane is through alveolar ventilation. Isoflurane is poorly metabolised (0.17%) by hepatic cytochrome P450 enzymes in the liver [150]. Only 0.17% of the isoflurane is broken down into the products trifluoroacetic acid (TFA) and inorganic F⁻ by the enzymes P-450. There is no transcutaneous elimination of isoflurane.

Previous research has established that isoflurane requires a longer time to equilibrate in the blood after induction in patients with high BMI (body mass index) such as obese patients, which implies a longer removal time. This effect is the consequence of a larger fat compartment in obese patients which facilitate the absorption and retention of isoflurane and consequently a slower release from the fat tissue [6].

This unexpected finding suggests a possible explanation for the differences observed between these results and those of Yasuda et al. by the fact that those discrepancies could be associated with the physical condition of the subjects/patients involved (BMI) and the duration of isoflurane anaesthesia exposure.

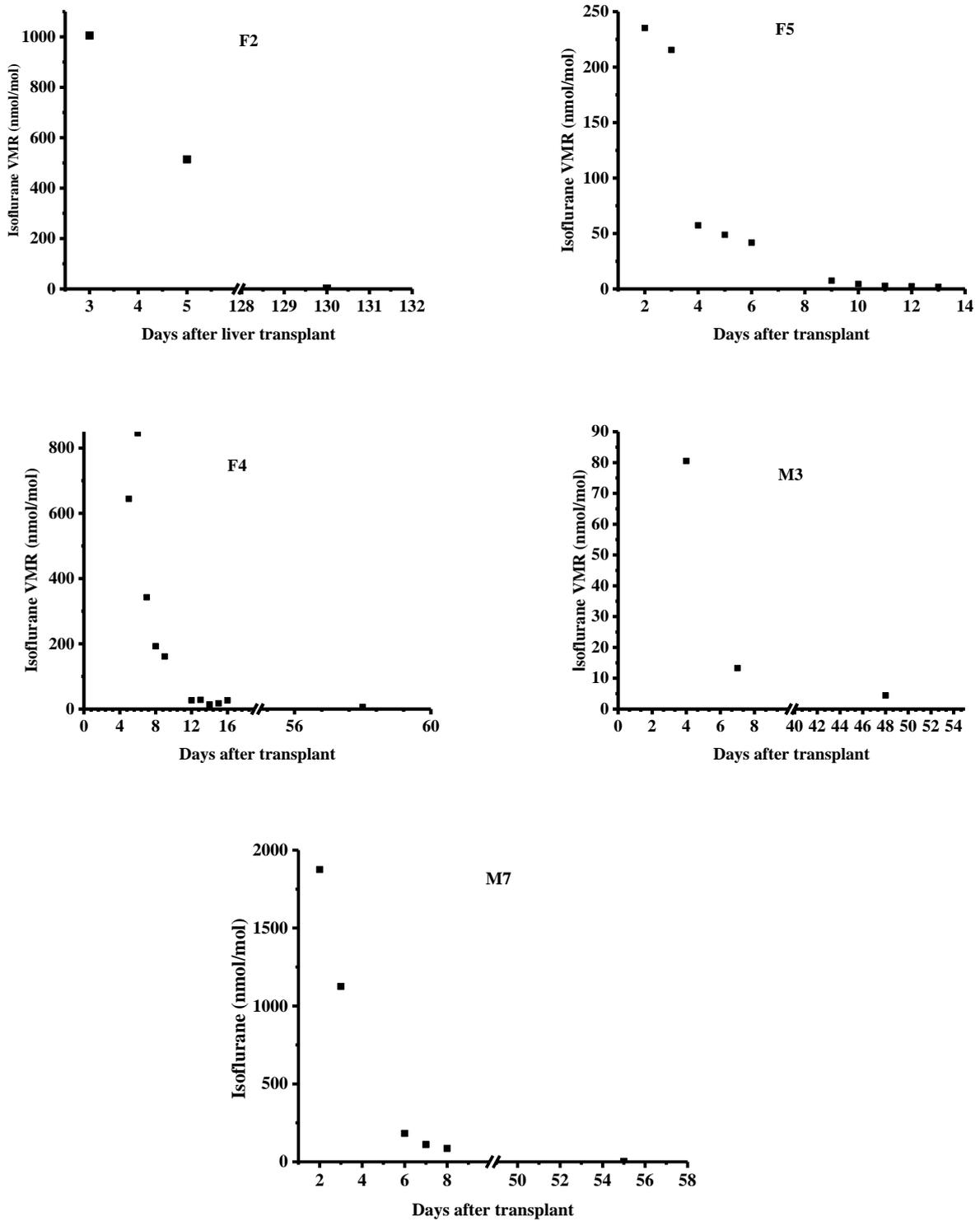


Figure 6.4 Longitudinal changes in VMR in nmol/mol for isoflurane for given days after surgery for patients F2, F4, F5, M3, and M7. Error bars are smaller than the symbols.

Yasuda et al. volunteers were healthy and had a mean body mass index (BMI) of 21.7 kg/m², i.e. all were within the normal (healthy weight) range. In comparison F2, F4, F5, M3 and M7 had BMIs of 31.2, 32.4, 28.0, 32.5, and 35.0 kg/m², respectively.

Table 6.4 provides further additional information of the 5 patients recruited in this study such as, patient ID, gender, estimated isoflurane dose administrated, duration of the surgery and body mass index.

Table 6.4 Patient information, body mass index, estimated isoflurane dose and duration of surgery.

Patient ID	Gender	Estimated Isoflurane dose (g)	Duration of Surgery (h)	Body Mass Index (kg/m ²)
F2	F	68.6	5	31.2
F4	F	90.4	7	32.4
F5	F	87.9	5	28
M3	M	138.3	6	32.5
M7	M	73.8	7	35

*M3 and F5 had desflurane administrated as maintenance agent during their operation.

Thus, F2, F4, F5, M3 and M7 were either overweight or obese. Although isoflurane has a low blood/gas partition coefficient of 1.45 ± 0.12 (mean \pm SD) [151], it has a high oil/blood partition coefficient of 97, and therefore it is proposed that for those patients a significant amount of the isoflurane was absorbed into fat tissue, only to be slowly released after surgery. It may be the case therefore that over time, isoflurane is deposited as a reservoir in body fat to be slowly released when the anaesthesia is stopped, and

thereby prolonging any effect on the neurocognitive functions and their later recovery [6, 152].

In addition to this, Yasuda et al. administered isoflurane for precisely 30 minutes, whereas in a transplant surgery patients underwent surgeries that lasted between 5 to 7 hours. These findings may be interpreted that doses of isoflurane administered and the duration is likely to prolong the elimination of the chemical from the patients. These findings are in agreement with another investigation which found that if isoflurane is administered for more than 2 hours, as would be the case for these patients, the adipose tissue becomes saturated and then the emergence from the anaesthetic is prolonged [153].

It is essential to determine any factor that can be affected by race or ethnicity. Previous investigations have suggested that Asians compared to Europeans with the same BMI have 3% to 5% higher total body fat, which could have consequences in isoflurane elimination from the body [154].

BMI is a reasonable score predictor of the body fat based on the height and weight of individuals. Table 6.5 shows the BMI score and the interpretation of BMI score. For European healthy normal weight subjects, BMI ranges goes from 18.5 to 25 whereas for overweight goes higher than 25 BMI [155].

Table 6.5 BMI classification and the correspondent weight status indicator.

BMI	Weight status
<18.5	Underweight
18.5-25	Normal
25-30	Overweight
>30	Obese

Finally, of all of the patients, patient M7 showed the fastest elimination of isoflurane even though he had the highest concentration in exhaled breath after surgery and was the most obese patient. However, the elimination time was still larger than found in the results of Yasuda's study. The rapid washout, in comparison to the other patients, could be a result of this patient being the youngest of the group involved at 36 years old. (F2, F4, F5 and M3 ages were 49, 58, 71 and 53 years, respectively.) M7 also recovered from surgery more quickly than the other patients in the group and was discharged within one week. However, with such a small number of patients it was not possible to conclude whether or not there is any correlation with age and perhaps gender with regards to isoflurane elimination from fatty tissue. Further studies should be undertaken with a higher number of patients to investigate the washout characteristic of different anaesthesia such as desflurane, sevoflurane, isoflurane, halothane between subjects with different BMI, age and ethnicities.

6.4 Conclusion and Final Remarks

This study has successfully demonstrated the use of PTR-MS for the non- invasive detection of isoflurane in the breath of patients after an operation, and highlights the importance of product ion identification in a complex breath matrix.

The headspace study has shown that the protonated parent ion is not observed, instead the resulting protonated parent ion rapidly dissociates to diagnostically useful ions with m/z values of 67 (69), 117 (119), m/z 163 (165) and m/z 165 (167), which are assigned to be CHFCl^+ , CF_3CHCl^+ , $\text{C}_3\text{F}_4\text{OCl}^+$ and $\text{C}_3\text{H}_2\text{F}_4\text{OCl}^+$, respectively. The present study makes several noteworthy contributions to the breath research community and suggests that m/z 69 cannot be used to monitor isoprene if patients have previously been

administrated with isoflurane anaesthesia. This investigation reports for the first time temporal changes in the concentrations of isoflurane in the breath of patients after major surgery and demonstrates that high levels of isoflurane remain in patients for several weeks.

In this investigation, isoflurane elimination is much slower than previously reported. This research provides a framework for the exploration of the implications of this prolonged release. Understanding the elimination of isoflurane is crucial for an appropriate administration and consequently improvement of patient safety. Also this work is beneficial for the use as conceptual premise for future clinical trials of metabolism recovery in different anaesthetics, such a sevoflurane, enflurane, halothane, desflurane and methoxyflurane.

Although the current study is based on a small sample of participants, it suggests that it could have an impact on a patient's ability to drive and use machines after isoflurane anaesthesia administration, especially after a long period of exposure to the anaesthesia such as for long surgeries.

Further studies need to be carried out in order to validate this investigation with a complete new set of patients and to estimate the average time to fully recover from the anaesthesia. Large controlled trials are required to assess the impact of isoflurane wash out in the neurocognitive function of patients focusing on current physiological factors such as age, ethnicity, etc.

7 FURTHER DISCUSSION

In the final chapter the significance of the major findings from the work presented in this thesis are summarised. The purpose here is to highlight the outcomes and the goals achieved. In the final section of this chapter, possible future directions are suggested for breath VOCs analysis.

7.1 Key Conclusions and Summary of Findings

7.1.1 Applicability of PTR-MS for the detection of gas trace volatiles in breath and headspace analysis

The work presented in this thesis confirms the potential of the analytical technique of PTR-MS as a useful tool for breath analysis to detect trace quantities of VOCs for medical applications. The fast response and high sensitivity allows for the quantification of trace volatiles and the subsequent temporal changes to be observed. In addition, it has been shown that PTR-MS was suitable to analyse headspace of anaesthetics.

7.1.2 Breath collection

In chapter 3, the use of a non-invasive sampling protocol for the off-line measurements of breath samples was demonstrated for the implementation and development of reproducible breath collection procedures.

Given the variability when measuring exhaled breath and the impact of confounding factors on their composition, in this investigation multiple breath samples were collected thereby improving the reliability of the analysis. This study showed a better-defined and reproducible composition of breath samples.

The key improvement was related to breath sampling procedures, with the breath sampling being controlled by a capnograph allowing only the collection of end-tidal phase of the exhaled breath sampled in order to minimize sample dilution. In addition to this, and for each individual measurement, the use of multiple alveolar breath samples collection improved the statistics.

7.1.3 Patient recruitment

A key improvement was the use of patient's companions as controls, thereby decreasing the risk of confounding factors such as exposure effects, and the recruitment of patients as their own controls for the second phase during the post-transplant study as described in detail in chapter 4.

7.1.4 Inclusion of a longitudinal study design of VOCs

The incorporation of a longitudinal design study allowed an in-depth observation of key VOCs and track their changes in concentration through time. For the first time this research was able to show wash out characteristics of VOCs in a cohort of patients after liver transplantation. This provided an improved accuracy to determine diagnostically useful VOCs.

Depending on the clinical study, it can be more important to measure longitudinal changes in VOC concentration than simply taking a breath sample at one particular time. The inclusion of a study to monitor changes in the key volatiles as a function of time unambiguously showed that the raised levels of limonene, methanol and 2-pentanone in the breath of patients pre-transplant are a direct result of chronic liver disease. Finally, this investigation highlights that breath analysis has the potential to assess liver function after liver transplantation.

7.1.5 Breath analysis for the detection of liver disease. Comparison of pre and post-breath samples

The detailed investigation focused on patients suffering from advanced liver disease. The study compared for the first time breath samples from pre and post-transplant patients in order to rule-out VOCs not resulting from the illness, but specific to liver disease.

This was a key procedure adopted in the liver disease study. The study has gone some way towards enhancing the understanding of why limonene is observed at elevated concentrations in the breath of patients with liver disease.

7.1.6 Breath analysis for the monitoring of HE

In this investigation, it has been demonstrated that breath analysis is a useful tool for the detection of complications caused by chronic liver disease such as hepatic encephalopathy. In addition to this, the findings complement those described in chapter 4. The study has gone some way towards enhancing the understanding of why limonene is

observed at elevated concentrations in the breath of patients with liver disease. In this investigation, limonene in exhaled breath is higher in patients who have current symptoms of HE compared with patient with liver disease but no HE. For the first time, the time dependence of VOCs was studied as the condition changed. HE is the end consequence of either chronic liver disease or acute liver damage, therefore high breath limonene may simply be a proxy for advanced liver disease.

Notwithstanding the relatively limited sample, this investigation offers valuable insights which will aid any further studies using a larger sample size for examining more closely the links between limonene and hepatic encephalopathy.

7.1.7 Breath analysis for anaesthesia monitoring

This study has shown that PTR-MS is ideal for the non-invasive detection of isoflurane in the breath of a patient after an operation. The investigation highlights the use of on-line monitoring mass spectrometry techniques to study longitudinal changes of anaesthesia in breath after surgery. The present study makes several noteworthy contributions by implying that in low-resolution quadrupole based PTR-MS instruments, the concentration of isoprene in breath after surgery is significant and confounded by the isoflurane concentration found in patient's breath. This investigation strongly suggests that isoflurane (or any other intravenous anaesthetic) should be carefully monitored after anaesthesia administration depending on the technology used. These results have serious repercussions for breath analysis investigations which correlate isoprene to any type of disease or use isoprene as a biomarker.

In addition to this, the study provided the product ions which can be used to identify the presence of isoflurane in the breath. Finally, this study has thrown up many questions in need of further investigation. Isoflurane has been found in high concentration in breath of patients several days after surgery. More research is required to determine the effects and impact of this to ensure that cognitive function has fully returned to a patient after intravenous anaesthesia administration, especially after a prolonged anaesthesia administration during long surgeries. Further work in a larger group of participants need to be carried out with a range of different ages and ethnicities to better understand factors that affect the elimination profile of isoflurane in exhaled breath.

The combination of these results provides support for the concept of study the metabolism of anaesthetics recovery, it might be possible to study different anaesthetics and investigate their subsequent biological effect with other drugs in humans in longitudinal studies.

7.2 Limitations of the Study

The investigations had a number of limitations. However, these can be overcome in any future work.

7.2.1 Population size, ethnicity and location

The limitation in this investigation was the low number of patients recruited, particularly in the follow-up study after liver transplant, the encephalopathy and isoflurane study. Owing to the clinical difficulties of conducting clinical research and the difficulty for

patient recruitment after liver transplant, only a small number of patients were involved. It would have been necessary to have more research personnel available to assist in the sampling collection and during samples measurements. A larger size study may have permitted more data to cross validate the results and to ensure a representative distribution of the affected population from liver disease. The next step towards the use of VOCs for liver disease detection requires larger multicentre clinical trials. Collaborative work between independent groups would contribute to a better understanding of liver disease. Although this study was limited in size, it was planned to avoid confounding results by using well-matched study patients. It is important to highlight that demographic aspects associated with liver disease may differ by ethnicity. Further investigations should be undertaken to understand the impact of ethnicity in liver disease.

7.2.2 Project time

It is also important to mention that clinical trials require a large amount of time to be completed. In this case, the time to demonstrate that the methodology was successful was limited. The clinical trial was limited to the time of the project funding. For instance, it was not possible to collect data from patients recruited for longer periods of time pre-transplant in order to investigate changes in key volatiles.

7.2.3 Off-line breath collection and sampling

The off-line breath collection during the study was a possible limitation and the significance of this finding is unclear. The off-line sampling has more risk due to the handling of samples, which can be difficult to estimate owing the complex nature of breath. It is well known that the chemical stability of breath can change due to condensation, evaporation and degradation effect affecting the results. This effect was minimised by measuring the samples within a short time period from collection and eliminating condensation effects by keeping warm all the samples stored.

7.3 Future Directions and Further Work

In this section recommendations for the improvement of the method, significance of the findings and future directions in breath analysis for the detection of liver disease are summarised. The work presented in this thesis demonstrates that breath VOCs analysis could be developed into a diagnostic procedure and used as a potential diagnostic tool for chronic liver disease. The results from this investigation are of direct impact and relevance for the diagnosis of end stage liver disease. An implication of this is that an exogenous compound can be used as a biomarker for chronic liver disease and that this compound can be used to assess liver function after liver transplantation. The findings are significant because of their potential to detect early stages of liver disease and hence reduce disease severity before the liver is irreversibly damaged.

This thesis strongly suggest an evaluation for the diagnostic accuracy of breath volatiles analysis for compensated liver cirrhosis and contribute considerably to the development and evaluation of liver disease techniques. Larger clinical trials using breath

analysis methods are required to establish whether early liver disease can be detected, for example in patients with compensated or fatty liver.

This study has set a pathway for the investigation and development of non-invasive technologies (such as a portable mass spectrometric user-friendly diagnostic point-of-care system) for screening, diagnosing and monitoring early stage liver disease.

This thesis supports the idea of further investigation on key volatiles in breath to predict the best timing for a liver transplant or even monitor whether the patient will suffer liver rejection after transplantation, which cannot be foreseen immediately after liver transplant. More broadly, research is needed to study key volatile changes during liver rejection. The use of these volatiles to follow-up liver function will serve as a basis for future studies in breath analysis. Further research should also be conducted to investigate the activity of the liver function by carbon-13 breath tests using key volatiles as the substrate to measure the concentration of limonene and subsequent metabolites such as perillyl alcohol in breath avoiding background interference from the diet.

Future large trials should take into account the impact of this investigation, and more work in this field would be of great help to establish a breath analysis test as a diagnostic procedure to detect liver disease. To conclude, it is important to mention that these advantages will benefit society by providing a personalized medical care improving the quality of life of patients and having an enormous and positive effect on savings to health providers.

Appendix I Correlations between volatiles for the 31 patients and 30 controls in the putative marker set. Significance scores below 0.05 are highlighted in bold.

Volatiles/ <i>m/z</i>		Patients (N = 31)		Controls (N = 30)	
		Kendall's tau-b statistic	Significance	Kendall's tau-b statistic	Significance
Limonene	Methanol	0.127	0.158	0.057	0.656
Limonene	2_pentanone	0.144	0.127	0.166	0.148
Limonene	2-butanone	0.333	0.004	0.147	0.253
Limonene	Carbon disulfide	0.230	0.034	0.078	0.544
Limonene	<i>m/z</i> 89	0.415	0.001	0.117	0.363
Limonene	<i>m/z</i> 135	0.492	0.000	0.310	0.016
Methanol	2-pentanone	0.288	0.012	-0.140	0.276
Methanol	2-butanone	0.062	0.311	-0.101	0.432
Methanol	Carbon disulfide	0.200	0.057	0.023	0.858
Methanol	m89	0.144	0.127	0.002	0.986
Methanol	m135	0.118	0.175	0.122	0.344
2_pentanone	2-butanone	0.127	0.158	0.087	0.498
2_pentanone	Carbon disulfide	0.028	0.413	-0.037	0.775
2_pentanone	<i>m/z</i> 89	0.290	0.011	0.067	0.605
2_pentanone	<i>m/z</i> 135	0.127	0.158	0.168	0.193
2-butanone	Carbon disulfide	0.544	0.000	0.452	0.000
2-butanone	<i>m/z</i> 89	0.058	0.323	0.032	0.803
2-butanone	<i>m/z</i> 135	0.153	0.114	0.110	0.392
Carbon disulfide	<i>m/z</i> 89	0.006	0.480	-0.074	0.568
Carbon disulfide	<i>m/z</i> 135	0.067	0.299	-0.032	0.803
<i>m/z</i> 89	<i>m/z</i> 135	0.320	0.006	0.182	0.159

Appendix II Pre-transplant blood chemistry for the 12 pre-patients who underwent liver transplant.

Blood chemistry includes values for alanine aminotransferase (ALT), alkaline phosphatase (ALP), aspartate transferase (AST), albumin, total bilirubin, creatinine, neutrophils, platelets, potassium, prothrombin/international normalized ratio (PT/INR), and the United Kingdom Model for End-Stage Liver Disease (UKELD).

Patient Identity	ALT (U/L)	ALP (U/L)	AST (U/L)	Albumin (g/L)	Total Bilirubin (̄mol/L)	Creatinine (̄mol/L)	Neutrophils 10 ⁹ counts/mL	Platelets 10 ⁹ counts/mL	Potassium mmol/L	PT/INR	Sodium mmol/L	UKELD
F1	149	160	212	30	26	42	0.6	57	4	1.8	137	53
F2	56	121	-	22	257	61	4.1	26	4.9	3.8	154	57
F3	46	194	88	38	143	30	0.6	48	3.3	1.7	129	62
F4	38	218	58	31	391	56	3.2	120	3.6	1.4	133	63
M1	28	164	-	29	39	124	4.7	151	5	1.4	131	58
M2	17	246	44	25	51	82	3.1	86	4.5	1.7	132	57
M3	44	257	71	30	137	79	3.9	61	3.8	2	144	55
M4	64	85	107	40	47	85	2.3	34	4.2	1.8	137	56
M5	34	101	-	29	21	75	3.4	87	4	1.2	138	50
M6	17	68	35	37	55	92	2.6	57	4.1	1.6	142	53
M7	39	105	73	25	63	67	0	40	4.8	2.4	138	57
M8	35	134	-	40	15	86	85	141	5.4	1.6	132	52

Appendix III Normalised count rates (area under the peak) for the 40 measured ion peaks which were included in the statistical analysis for patients, controls and room air. Median (lower quartile , upper quartile) and range (minimum – maximum). Count rates were normalised to 50 million counts per second of the areas under the ion peaks for the sum of the reagent ions m/z 19 (hydronium) and m/z 37 (the monohydrate cluster ion).

<i>m/z</i>	Patients (N = 31)		Controls (N = 30)		Room air	
	Median (LQ/UQ)	Range	Median (LQ/UQ)	Range	Median (LQ/UQ)	Range
33	77200 (64800 / 144700)	36600 - 334700	56900 (45100 / 68700)	32600 - 92200	10300 (8800 / 14600)	5800 - 22300
42	4300 (3950 / 6290)	3590 - 78060	4780 (3830 / 37440)	2810 - 98400	3030 (2800 / 5220)	1550 - 10050
43	86200 (75100 / 91200)	46700 - 186300	86100 (63700 / 98300)	45100 - 330700	29700 (21300 / 34400)	10000 - 236400
45	66400 (50000 / 84800)	21500 - 198200	65700 (39000 / 103100)	24400 - 281700	30800 (19800 / 40800)	8900 - 257700
47	34500 (31900 / 40400)	15200 - 64100	34000 (26800 / 37000)	22800 - 119800	16800 (12200 / 18700)	8300 - 29000
49	980 (720 / 1160)	320 - 3920	880 (720 / 1160)	310 - 1620	450 (220 / 630)	80 - 1310
51	3870 (2830 / 5890)	720 - 12430	2480 (1850 / 3150)	1270 - 6000	380 (160 / 550)	40 - 760
54	860 (580 / 1180)	290 - 2390	1050 (610 / 1570)	240 - 30380	260 (140 / 410)	40 - 610
55	85600 (71100 / 120400)	37200 - 208900	72900 (58200 / 117500)	39100 - 1114900	5900 (4500 / 7300)	2100 - 81300
57	12220 (10150 / 14690)	5300 - 20300	10530 (9670 / 12220)	6720 - 25720	4480 (3010 / 4960)	1970 - 9750
58	2750 (2060 / 5090)	990 - 11320	2710 (1890 / 4590)	1030 - 92120	650 (480 / 1130)	270 - 8640
59	359400 (268100 / 719700)	158200 - 1119800	269600 (219700 / 449200)	124600 - 1078500	31200 (20000 / 119000)	9200 - 1616500
61	50400 (42100 / 57300)	13600 - 138200	49500 (36800 / 61100)	26200 - 121200	13000 (9500 / 15700)	5200 - 23600
63	8580 (7370 / 10380)	3890 - 11780	7650 (5760 / 9320)	4760 - 13500	660 (510 / 1050)	220 - 1640
65	2340 (1960 / 2930)	820 - 4110	1930 (1660 / 2390)	940 - 29660	370 (230 / 520)	50 - 950
67	740 (590 / 940)	280 - 3330	660 (490 / 850)	280 - 1940	180 (110 / 360)	70 - 510
68	390 (320 / 470)	150 - 2160	430 (370 / 490)	130 - 4630	140 (60 / 270)	20 - 630
69	18250 (5800 / 25890)	2640 - 73030	11420 (7580 / 19410)	1890 - 43170	830 (550 / 970)	370 - 2800
71	8410 (5530 / 11200)	2580 - 16230	6020 (5040 / 8130)	2230 - 13980	780 (660 / 1090)	340 - 2460
72	720 (580 / 920)	430 - 1500	740 (460 / 910)	360 - 5940	230 (190 / 400)	40 - 890
73	11900 (9600 / 17300)	7500 - 30800	8900 (7300 / 12500)	5800 - 25600	3300 (2500 / 5000)	1900 - 16800

<i>m/z</i>	Patients (N = 31)		Controls (N=30)		Room air	
	Median (LQ/UQ)	Range	Median (LQ/UQ)	Range	Median (LQ/UQ)	Range
74	870 (780 / 1020)	630 - 1800	780 (610 / 980)	450 - 2020	370 (270 / 540)	120 - 980
75	4450 (3370 / 6830)	1490 - 13400	3060 (2460 / 5240)	1630 - 15240	1210 (890 / 1650)	390 - 4040
77	2590 (1840 / 3790)	1010 - 7470	1740 (1330 / 2920)	830 - 8660	360 (230 / 510)	150 - 2260
79	1550 (1240 / 1990)	420 - 3150	1300 (980 / 2240)	600 - 13060	410 (300 / 560)	150 - 830
81	5000 (1700 / 14170)	800 - 61880	980 (830 / 1270)	540 - 2270	330 (260 / 450)	140 - 1880
83	1303 (1019 / 1905)	530 - 3084	949 (787 / 1556)	520 - 2959	479 (426 / 571)	224 - 1084
85	2110 (1070 / 3960)	650 - 5070	1800 (1140 / 2710)	610 - 6810	400 (360 / 620)	230 - 1380
87	7630 (5780 / 11400)	2650 - 33000	4030 (3470 / 4620)	2190 - 15410	830 (770 / 1360)	540 - 1630
89	1480 (980 / 3370)	540 - 10170	870 (690 / 1330)	570 - 12270	480 (460 / 630)	240 - 2750
93	1890 (1320 / 3450)	760 - 8300	1090 (890 / 2050)	660 - 6350	570 (400 / 760)	180 - 990
95	1810 (1200 / 3070)	770 - 10950	1110 (860 / 1580)	630 - 3730	450 (350 / 500)	280 - 1400
97	630 (550 / 930)	300 - 2010	570 (470 / 790)	310 - 1760	330 (250 / 410)	110 - 730
99	870 (750 / 1250)	450 - 5640	790 (630 / 980)	390 - 1550	440 (360 / 630)	80 - 820
101	2940 (2010 / 4420)	1180 - 6370	2140 (1820 / 3170)	1380 - 8750	1140 (700 / 1640)	500 - 2510
103	700 (620 / 880)	450 - 2690	690 (530 / 910)	300 - 3790	340 (230 / 430)	130 - 680
109	610 (470 / 860)	190 - 4920	560 (480 / 750)	230 - 4570	340 (240 / 530)	80 - 1690
115	610 (520 / 860)	410 - 2430	530 (460 / 650)	290 - 1520	400 (230 / 450)	120 - 630
135	550 (490 / 1100)	270 - 2300	450 (310 / 570)	170 - 3750	330 (240 / 450)	20 - 1620
137	3027 (830 / 9286)	278 - 40636	409 (333 / 506)	97 - 1309	243 (72 / 387)	43 - 855

Appendix IV Demographic information for the patient group. Medications are those which they were taking at the time of the pre-transplant breath sample, except for F5, who was only sampled post-transplant. BMI = body mass index. The weight used for the BMI calculation is the one given in the patient's notes closest to the date for the pre-transplant sample. AID = Autoimmune liver disease, ALD = Alcoholic Liver Disease, CD = Cryptogenic disease, HBV = Hepatitis B virus, HCC = Hepatocellular cancer, HCV = Hepatitis C virus, LF = liver failure, NAFLD = Non Alcoholic Fatty Liver Disease, NASH = non-alcoholic steatohepatitis, PBC = Primary biliary cirrhosis, PSC = Primary sclerosing cholangitis.

Study ID	Sex	Age	BMI	Diagnosis	Smoker	Medications
F1	F	27	25.8	AID	yes	prednisolone, tacrolimus (prograf), azathioprine, lansoprazole, ursodeoxycholic acid, sulfasalazine
F2	F	49	31.2	LF	no	esomeprazole before and after transplant, hydrocortisone, flucanazole, metaclopramide, mycophenolate
F3	F	53	26.4	PBC	no	ursodeoxycholic acid, paracetamol, ranitidine hydrochloride, vitamin B, thiamine, lactulose.
F4	F	58	32.4	PSC	no	spironolactone, omeprazole, ciprofloxacin, thiamine, calcium carbonate
F5	F	71	28.0	CD	no	carvedilol, lactulose, omeprazole
F6	F	52	21.5	PBC	no	deoxycholic acid
F7	F	65	28.0	NASH, HCC	no	carvedilol, omeprazole, rifaximin, spironolactone, gabapentin, fruesamide, novomix 30 (insulin)
F8	F	54	22.1	HCV, HCC	yes	clamiparin, omeprazole
M1	M	54	33.7	ALD	yes	lansoprazole
M2	M	45	21.1	ALD	yes	Lactulose, Lansoprazole, Mirtazapine, paracetamol, Propanolol, Adcal D-3, ciprofloxacin, Furosemide, spironolactone
M3	M	53	32.5	ALD	no	Thiamine, Omeprazole, bisodol, vitamin B, amitryptiline, lactulose, topical steroid for psoriasis
M4	M	53	28.1	ALD, HVC, HVB	yes	aspirin, azathioprine, coamoxiclave, cotrimoxazole, enoxaparin, entecavir, lansoprazole, Morphine Sulfate, nystatin, paracetamol, piperacillin/tazobactam, prednisolone, prograf (BD tacrolimus), senna, sertraline, testogel, tramadol, (cyclizine, lidocaine, morphine sulfate, ondansetron, paptac, tramadol as required)
M5	M	56	27.2	ALD, HCV, HCC	gave up 6 weeks before breath sample	None
M6	M	53	33.1	CD	no	rifaximin, ursodeoxycholic acid, citalopram, Lactulose, carvedilol, spironolactone, buscopan, enlive plus, omeprazole, micolax enema, peppermint oil
M7	M	36	35.0	CD	no	furosemide, amiloride, carvedilol
M8	M	67	25.7	ALD	no	beclomethasone, vitamins A and D, lactulose, enoxaparin, hydrocortisone, omeprazole, paracetamol, fresnius renalyte acid concentrate, fresnius BiB ag, calogen extra, Build Up (sweet), ciprofloxacin, rifaxi

Study ID	Sex	Age	BMI	Diagnosis	Smoker	Medications
M9	M	55	24.0	PSC	no	paracetamol, codeine phosphate
M10	M	53	42.3	ALD	no	lenograstim, cotrimoxazole, neomycin, vitamin B, thiamine, propranolol, lansoprazole
M11	M	67	24.5	ALD	no	omeprazole, folic acid, furosamide, spironalactone, tamazepam, carvedilol
M12	M	50	24.1	PSC	no	vitamin K, calcium, vitamin D
M13	M	42	21.8	PSC	no	omeprazole, septrin (sulfamethoxazole), creon, questran (Cholestyramine)
M14	M	64	35.9	ALD	no	propranolol, spironalactone, vitamin D (rocaltrol), omeprazole, erythromycin,
M15	M	54	29.8	ALD	no	Fortimel, rifaximin, lactulose, thiamine, build up, vitamin B, lansoprazole, phosphate enema, piperacillin/tazobactam, ciprofloxacin, trimethoprim
M16	M	45	31.6	ALD	yes	none
M17*	M	65	31.2	NAFLD, HCC	no	paracetamol
M18	M	69	37.0	ALD, HCC	no	omeprazole, propranolol, thiamine, fluoxetine, vitamin B
M19	M	57	27.8	HBV, HCV, HCC	gave up 2 months before breath sample	metformin, omeprazole, ramipril, amitriptyline, naproxen, viagra, bendrofluazide
M20	M	66	29.4	ALD, HCC	yes	furosemide, spironolactone, lactulose, vitamin B, thiamine
M21	M	50	27.2	HBV, HCC	yes	tenofovir
M22	M	71	24.5	CD, HCC	no	furosemide, amiloride
M23	M	55	28.2	HCV, HCC	yes	metformin, insulin, paraxatone

*Only patient M17 reported that they still drank alcohol

Appendix V Protocol for ethical approval

BREAth Testing for Hepatic Encephalopathy

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Research Protocol Version 2
30/09/2013

Sponsor Number
RG-13-119

Principal Investigator Dr Chris Mahyew

School of Physics and
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CHIEF INVESTIGATOR SIGNATURE PAGE

BREATHE

This Protocol is approved by:

Dr Chris Mayhew

Signature:

Date:

Chief Investigator

PROTOCOL SIGNATURE

I have thoroughly read and reviewed the study

protocol: BREATHE

I have read and understood the requirement and condition of the study protocol.

I am aware of my responsibilities as an Investigator under the guidelines of Good Clinical Practice (GCP), local regulation and the study protocol and I agree to conduct the study according to these guidelines and to appropriately direct and assist the staff under my control who will be involved in the study.

I agree to use the study material only as specified in the protocol.

I understand that changes to the protocol must be made in form of an amendment, which has to be approved by the relevant Ethics committee and the Research and Development department prior to its implementation.

I understand that any violation of the protocol may lead to early termination of the study.

INVESTIGATOR'S NAME

.....
SIGNATURE

.....
DATE

STUDY SYNOPSIS

Background

Liver Disease incidence has increased in the UK population in recent years. Diagnosis of cirrhosis is difficult and usually invasive. There is no cure; patients must have a transplant, but, if caught early enough, some liver damage may be reversed with lifestyle changes. Hepatic Encephalopathy is a common side effect of liver cirrhosis and causes significant morbidity. Patients may suffer from acute episodes, in which they are extremely confused, and if not treated, may lapse into a coma. These episodes often arrive without warning and necessitate hospital treatment. Diagnosis of encephalopathy is by cognitive tests to assess the level of confusion, as well as blood ammonia. These tests require knowledge of the patient's normal behaviour and have to be adjusted for educational level.

The measurement of endogenous volatile organic compounds (VOCs) in human breath for the diagnosis of disease is an emerging field; the Proton-Transfer-Reaction Mass-Spectrometer (PTR-MS) is a widely used tool for this purpose due to its high sensitivity and speed of analysis. Some previous studies have measured VOCs in the breath of liver disease patients. One conference abstract describes blood and breath measurements in patients with hepatic encephalopathy.

No published study has examined the evolution of breath VOCs during a period in which a patient is receiving therapy for Hepatic Encephalopathy.

Gastrointestinal Cancers (GI cancers) are common, with liver cancer often being a consequence of cirrhosis and hepatitis infections. Neuroendocrine tumours (NET) is a type of cancer commonly found in the gastrointestinal tract, including liver and pancreas. GI cancers are often not diagnosed until the tumour is advanced, thus reducing the chance of curative treatment. Paracetamol overdose is the most common poisoning event in the UK and leads to death by liver failure caused by the toxic effect of the metabolite *N*-acetyl-*p*-benzoquinoneimine (NAPQ).

AIMS

We propose to measure VOCs in the breath of patients with acute encephalopathy while they are receiving therapy to treat the condition. We wish to examine which VOCs are elevated in the breath of patients with Hepatic Encephalopathy and which change over time in association with the severity of symptoms. We also propose to measure VOCs in the breath of patients with liver cirrhosis and GI cancers and compare them to identify biomarkers of cirrhosis and cancer. Patients with cancer will give a sample both before and after tumour removal to further aid in biomarker identification. Breath samples will be taken from patients with paracetamol overdose to determine if the toxic metabolite NAPQ can be detected.

Entry Criteria

Main inclusion criteria

- Age \geq 18
- Admitted for treatment of hepatic encephalopathy in accordance with usual care
 - or attending clinic for hepatic cirrhosis in accordance with usual care
- Or attending clinic for GI cancer in accordance with usual care
- Or admitted as an emergency for paracetamol overdose

Recruitment

Patients will be recruited from the Queen Elizabeth University Hospitals NHS Foundation Trust Birmingham

ABBREVIATIONS

EEG	Electroencephalogram
GC-MS	Gas Chromatography Mass Spectrometry
HE	Hepatic Encephalopathy
GI	Gastrointestinal
NET	Neuroendocrine Tumour
NHS	National Health Service
PTR-MS	Proton Transfer Reaction Mass Spectrometer
UK	United Kingdom
VOC	Volatile Organic Compound
NAPQ	<i>N</i> -acetyl- <i>p</i> -benzoquinoneimine
NIHR	National Institutes of Health Resear

1. Context and Features of Disease

1.1. Hepatic Encephalopathy and Liver Cirrhosis

The burden of liver disease in the UK has risen sharply and poses a major public health issue. Deaths from chronic liver disease have increased eight fold in men aged 35-44 and seven fold in women over the past three decades. Liver disease already kills more people than diabetes and road accidents combined, but if rates of increase continue, deaths are predicted to double in 20 years. Hospital episode statistics for 2000/01 show over 15,000 admissions for alcoholic liver disease alone (129,000 bed days) and almost 4000 admissions for other conditions related to fibrosis and cirrhosis of the liver (25,000 bed days). The mean length of stay for patients with cirrhosis/fibrosis is 12 days serving to emphasise the economic cost to the NHS.

Early stages of liver disease are often difficult to recognize as the liver can function normally even when a quite large proportion is diseased. Often patients do not present until the disease is quite advanced. Even then, diagnosis is difficult and symptoms are often general and can be mistaken for other causes. The frustration for the liver physician, is that, if caught early enough, much liver damage is completely reversible with appropriate lifestyle changes such as abstaining from alcohol. However, most patients do not present until the damage is too far gone because early stage liver disease may present like general flu-like or tiredness symptoms. Even when it is quite badly cirrhotic, the liver compensates and can continue to function enough to support life, although the patient will become increasingly ill. Cirrhosis then inevitably worsens until the liver can no longer support life and the organ fails completely. Before this outcome, the only hope of survival for the patient is a liver transplant.

Liver blood tests are not very specific for liver disease and diagnosis is not straightforward. Conventional methods of establishing and identifying liver diseases, such as biopsy, can cause complications and even death, which limits their applicability. Biopsy is expensive and carries non-negligible risk, with approximately 1 in 10000 patients dying as a result. Moreover, complications of liver disease such as encephalopathy and cancer require time and resource- intensive radiological and clinical techniques for detection. The use of non-invasive breath analysis to identify VOCs that could identify groups of patients at risk of cirrhosis or cancer is, therefore, appealing. If breath biomarkers of cirrhosis or cancer could be found, this may lead to earlier diagnosis.

HE is a common side effect of liver cirrhosis. It is caused by the build up of toxins in the blood which are normally cleared by the liver. These toxins can cross the blood-brain barrier and cause neurological symptoms. Encephalopathy is graded from 1 – 4, with 4 being the most severe. Patients with lower grade HE do not usually require hospital treatment and are monitored in their usual clinic visits. In general these are patients in the earlier stages of liver cirrhosis. Their symptoms are shortened attention span and difficulty with simple numerical tasks. HE is a condition characterised by variability in symptoms; some patients will vary between ever present minimal HE and acute, others can be precipitated into an acute episode from being symptom- free. As cirrhosis worsens, so the symptoms of HE become more apparent and begin to interfere with the

patient's everyday life. Patients with grades 3 and 4 may become confused and disoriented, similar to symptoms of advanced dementia. These patients cannot be safely left on their own, as they are a danger to themselves during an acute episode and there is no warning when such an episode will occur. This constitutes a considerable burden on family/carers.

Acute episodes can occur spontaneously or may be precipitated by various factors such as: gastrointestinal bleeding, dehydration (caused by vomiting, diarrhoea or use of diuretics), constipation, use of certain medications, hyponatraemia, surgical intervention and transjugular intra-hepatic porto-systemic shunting (TIPS). In its most severe forms HE can be life-threatening and may require ventilatory support.

A differential diagnosis must be made for HE and other causes for the symptoms must be ruled out. Diagnosis of HE is by cognitive tests such as number connection, as well as blood ammonia and EEG studies. Cognitive tests are time consuming and have to be adjusted for educational ability. Blood ammonia testing is invasive and it takes time to receive results. A breath marker for HE would speed up diagnosis and thus lead to better management.

HE is managed principally using diet to reduce the intake of dietary protein, which thus limits the production of ammonia and by use of laxatives to expel toxins. Other therapeutic interventions are bowel acidification and sterilisation.

Another advantage of finding a VOC biomarker for HE is that it may provide insight into the biochemical aetiology of the condition. It is not fully understood how the neurological symptoms of HE are caused.

1.2. Gastrointestinal Cancers

1.2.1 Liver Cancers

One of the complications of chronic liver disease is liver cancer, a tumour that is often clinically silent and frequently missed. Treatment is by transplant, ablation surgery, trans-arterial chemo-embolisation or oral chemotherapy with sorafenib. Patients who are listed for non-transplant therapy will provide samples in clinic before and after treatment.

1.2.2 Neuroendocrine Cancer

Neuroendocrine Tumours (NET) may arise in any organ where neuroendocrine cells are located, but they are most frequently found in the gut, pancreas and liver. Although this type of cancer is not one of the most common, UHB is a national centre for treatment and 100 patients are treated each year. Treatment is by surgery, chemotherapy or radionuclide therapy. Patients who are listed for tumour eradication treatment will be asked to give a breath sample in clinic before and after their treatment. This tumour has well recognised plasma biomarkers with which to compare any VOC biomarkers which may be discovered.

Biomarkers from a non-invasive procedure such as breath analysis would potentially help in early recognition of GI cancers. Early treatment improves the chances of survival

as treatments are available but are less successful once the tumour has had time to grow and metastasised.

These cancers are typically treated within the same clinics as general liver so patients will be available to give samples in the same setting.

1.3. Paracetamol Overdose

Paracetamol is the most common drug taken in overdose in the UK and accounts for 120 deaths per year on average. The cause of liver failure following paracetamol overdose is the build up of the hepatotoxic metabolite *N*-acetyl-*p*-benzoquinoneimine (NAPQ). At therapeutic doses, paracetamol is mostly converted to non-toxic metabolites via the Phase II metabolism by conjugation with sulphate and glucuronide. Only a small proportion of it is metabolized via the cytochrome P450 enzyme system to NAPQ. In overdose, the phase II metabolism pathways are saturated and more paracetamol is shunted to the cytochrome P450 system, leading to an overproduction of NAPQ. Normally, NAPQ would be cleared by the liver's natural antioxidant, glutathione. In overdose, glutathione cannot be produced quickly enough to cope with the oversupply of NAPQ, therefore, excess NAPQ directly damages the hepatocytes. Treatment depends on the time at which the patient presents following overdose. Treatment is mostly with acetylcysteine but the correct dosage must be calculated using the plasma paracetamol concentration and an estimate of the time of overdose. Patients may not be able to say exactly when they overdosed and may not even be able to identify if it was paracetamol which they have taken. Plasma tests take time to return from the lab, therefore, an instant diagnostic test for the presence of paracetamol metabolites would be beneficial. For those patients whose treatment is not successful, their only chance of survival is a liver transplant.

2. VOC Analysis-Scientific Background

2.1. VOC Breath Analysis - Instrumentation

Proton Transfer Reaction Mass Spectrometry (PTR-MS) is a very sensitive chemical ionisation mass spectrometry technique that can be used to measure and quantify many trace gases. The application of PTR-MS technology to the detection of volatile organic compounds (VOCs) on exhaled human breath is our main area of interest. Breath analysis is not a new science; the measurement of exhaled fractions of administered compounds (both stable isotope and radioactive) is well established, a well-known example being the C-14 breath test for *Helicobacter Pylori* bacteria.

A newer approach, which has been growing over the past two decades, is to measure endogenous VOCs as indicators of disease or metabolism. This field has grown so much in recent years that it now has its own Institute of Physics journal, the *Journal of Breath Research*, which was founded in 2008. There are hundreds of VOCs on the human breath, most of them products (metabolised or not) of the ambient environment, with the remainder produced by metabolic processes in the body, or by bacteria in the gut. These latter endogenously produced VOCs measured on the breath are of particular interest to us, as they can act as indicative markers of metabolic processes related to various diseases.

The most common and best established technique used to measure the VOCs is Gas Chromatography Mass Spectrometry (GC-MS), which has been used extensively by

Michael Phillips in his pioneering work in the field. Although GC-MS is well established, it has numerous drawbacks; samples cannot be directly measured, they must first be preconcentrated, usually on Tenax tubes. This leads to time consuming trapping and concentration of large volumes of breath, as well as transportation to and analysis in specialised laboratories. This introduces a risk of errors due to issues of surface adsorption, contamination and permeability; this can make the breath samples unreliable. Furthermore, GC-MS uses electron impact to ionise molecules, which invariably leads to considerable fragmentation and the production of many daughter ions. This can greatly complicate the analysis, particularly given the highly complex chemical environment and low concentrations of the molecules to be detected.

More recent newcomers to breath analysis include other forms of mass spectrometry including ion mobility mass spectrometry (IMS-MS), such as selected ion flow tube mass spectrometry (SIFT-MS) and solid phase micro extraction (SPME), laser spectroscopy and electronic noses. Numerous reviews give an overview of current methods and applications of endogenous VOC breath analysis.

Each technique has its advantages and disadvantages, but the potential of PTR-MS to human breath analysis comes mainly from its superior sensitivity and real-time capability. This offers the opportunity to do direct real-time measurement of exhaled breath as well as near instantaneous analysis, without the need to pre-concentrate the sample. There exists now a large body of work on the application of PTR-MS to medical applications.

2.2. VOC Breath Analysis - Liver Disease

It has been noted that sulphur compounds are associated with liver disease and these have been found to be responsible for the well known odour of the breath of cirrhotics known as *foetor hepaticus*. Other groups have measured VOCs in the breath of patients with liver disease. These studies used other techniques, either gas chromatography mass spectrometry (GC-MS) or Ion Molecule Reaction Mass spectrometry (IMS-MS). These studies all found some VOCs that may be indicative of certain liver diseases. There is some agreement between some of these studies in terms of which biomarkers are implicated, however, full validation studies in a different set of patients have not been performed.

Two published studies have examined HE specifically as distinct from other liver diseases. Ammonia is well known to be excreted in the breath as it passes readily from the blood into the alveoli in the lungs. One previous study has attempted to correlate breath and arterial blood ammonia in patients with HE. The results were inconclusive, however, the patients assessed had only minimal HE; the authors admit this limitation and suggest that more severe grades of HE should be assessed. Another study has measured breath VOCs using gas chromatography mass spectrometry in patients admitted to hospital for severe HE and noted a number of volatile compounds which were associated with symptoms of HE. This remains only a conference abstract and a full paper has not been published, nor has a validation study been performed.

2.3 Breath Analysis Cancer

VOC breath analysis has principally focused on lung cancer although breast, and head and neck cancer have also been investigated. Despite large numbers of studies in lung cancer, inconsistent results have been reported and robust second phase clinical trials have not been reported to test phase one diagnostic models. No diagnostic tests have been developed for use in clinical settings. Previous studies have compared patients with other groups, as we are doing in our cirrhosis study. However, no cancer-specific biochemical pathways have been suggested which would account for changes in breath VOC. Rather, the pathways proposed are linked to oxidative stress in general, and may be markers of general inflammation rather than cancer. Since a great many other processes may lead to such oxidative stress, and may be present in the patient group both we do not think that comparison of groups should be used in isolation as way to proceed in biomarker identification for cancer. For this reason, we will use patients as their own controls by examining breath samples before and after treatment. In this way, we would hope to limit the impact of inter-individual variability which can confound attempts to identify biomarkers.

3. Research Questions

3.1. Hepatic Encephalopathy

The current study seeks not only to compare breath VOCs in patient with disease with controls, but we also propose to investigate the time dependence of those markers as the severity of the condition changes. In this, we will emulate the work of Halbritter et al who measured breath VOCs in women being tested for gestational diabetes in response to a glucose challenge. They found that absolute concentrations of VOCs were not the best predictors of whether the women were subsequently found to have gestational diabetes; rather the time constant of the clearance of breath VOCs was of better diagnostic value. One of the most difficult problems in biomarker discovery is the natural variation in concentrations between individual humans, high levels of a particular marker may be 'normal' for that individual, whereas for another they may indicate disease. By examining the change over time in a situation where the condition varies from acute back to normal (or, normal for that patient), we hope not only to rule out confounding VOCs which may be coincidentally high but not actually related to encephalopathy, but also to examine how the rate of change of VOCs may be of diagnostic potential. In summary, the research questions are

1. Are there breath VOCs which are elevated initially in HE but which then reduce as the patient responds to therapy?
2. Do concentrations of any breath VOCs relate to other diagnostic metrics such as blood ammonia in patients with HE?

3.2 Cirrhosis and GI Cancer

The broad aim is to find VOCs in human breath which can be used as biomarkers of cirrhosis and GI cancer. Two approaches will be used, comparing a disease group (cirrhosis) with normal, and comparing patients before and after treatment to remove tumours (GI cancer). Cirrhosis patients will give one sample and GI cancer patients will give breath samples before and after surgery for tumour ablation.

Samples from all patients and healthy controls will be collected in clinic. It is intended to ask companions of patients to act as healthy controls as they will have been breathing the same air in the recent past as the patient; VOCs in the air in the clinic will, therefore, be controlled for. Moreover, they may well share a diet and home environment with the patient, so long lived VOCs which may be retained in fat or other body compartments, may also be controlled for.

There will be three groups; cirrhosis, GI cancer and healthy controls. Comparisons will be made between all of these groups to establish any putative biomarkers for the disease conditions. Further, the pre-therapy GI cancer and post-therapy GI cancer groups will be compared to examine any differences in VOC concentration as a consequence of the removal of the tumour. The difference with this comparison is that the patient will be acting as their own control. In theory, this should mean that there is less natural variability in the breath VOC composition and a difference should be more easily detectable.

The cirrhosis and GI cancer group will act as disease controls for each other. It is important in breath analysis to have a disease control group, as some breath markers may result from general inflammation or immune system response processes. For example, short chain alkanes such as pentane and ethane are known to arise from oxidative stress; elevated pentane has been noted as elevated in conditions as diverse as sleep apnea, schizophrenia, asthma, lung cancer, sepsis and increased age of subject.

In summary the research questions are:

1. Are there breath VOC biomarkers of cirrhosis or GI cancer?
2. Do putative breath VOC biomarkers of GI cancer change follow tumour removal.

3.3. Liver Failure from Paracetamol overdose

The aim is to determine whether the toxic metabolite NAPQ is detectable by PTR- MS. It is similar in chemical structure and behavior to another quinine metabolite of the anaesthetic agent propofol, which has been measured using PTR-MS. At this stage, measurements would be a simple proof of principle to establish whether any NAPQ is detectable in the patient's breath.

4. Method and Materials

4.1. Breath Sampling

End expired air must be collected as this contains the highest proportion of alveolar air. The collection of mixed expired air leads to unacceptable variability in the VOC concentrations within and between subjects. Our own work has shown that a rebreathing protocol gives low levels of variability and allows for an equilibrium to be established between concentration of VOCs in alveolar blood and lung air. A rebreathing protocol is not, however, appropriate for patients in this particular clinical setting. It can be time consuming and difficult for patients to follow instructions. Instead, end-alveolar samples will be collected using a capnometer which will control the breath being collected such that breath will only be collected if the CO₂ concentration is above a minimum threshold. This consists of plastic ventilation tubing with an inline capnometer. A plastic mouthpiece is attached to the end, and this is the only part of the apparatus that the patient has contact with. The sample bag is attached to the tubing. When the capnometer registers a CO₂ level of above a specified percentage, the researcher will place their gloved hand over the open end of the tubing so that the patient's breath is redirected into the sample bag. Patients will also be asked to exhale a full breath into a separate bag as this will help to identify exogenous VOCs (see 'Confounding Variables')

For the HE in-patients, if possible, we will collect a sample before the patient has begun treatment; this will depend on how quickly the researchers are informed about the patient being admitted. Once treatment has started, one sample bag will be collected every half hour for the first three hours, then on an hourly basis thereafter. If the patient has not been discharged, breath samples will be taken on the following day on a two hourly basis.

For cirrhosis and GI cancer patients, breath samples will be collected in clinic. For paracetamol overdose patients, samples will be collected at the bedside.

4.2. Infection Control

The breath sampling apparatus consists of a plastic mouthpiece connected to some plastic tubing with an inline medical capnometer to measure carbon dioxide, and a removable Tedlar bag attached to the tubing to collect the breath. The patient will only come into contact with the mouthpiece. A fresh mouthpiece will be used for each subject. Used mouthpieces will be sterilised by soaking overnight in a solution of sodium dichloroisocyanurate. Infection control is doubly important in this patient group as immune system function is compromised in advanced liver disease.

4.3. Breath Gas Measurement

Bags of breath will be taken immediately to the university and analysed on the same day. The sample is fed directly into the PTR-MS and the act of analysis destroys the sample, so samples will not be stored after analysis. The air sample (breath) to be measured is fed directly into the apparatus. Inside, it reacts with ionised water molecules from an internal water supply, such that a proton (hydrogen atom) from the ionised water attaches itself to the VOC. A large number of the VOCs in the breath sample will become charged by way of this reaction, and their charge to mass ratio is then determined by a mass

spectrometer. Bags containing the breath sample will be attached directly to the instrument and will be kept inside a heated outer bag at all times to prevent condensation. A full mass scan up to at least 240 m/z will be measured for each bag. Bags awaiting measurement will be kept in an oven to prevent condensation.

4.4. Blood samples

Patients in clinic routinely have blood taken as part of their disease monitoring. We would ask for one extra vial of blood to be stored in liquid nitrogen for possible future studies of plasma VOC components. This would not involve an extra venepuncture as the patient will have a canula inserted anyway for their routine bloods. This would be done for the cirrhosis and GI cancer subjects. The samples would be stored within the National Institutes of Health Research (NIHR) facility at QEH.

4.5. Confounding Variables

It is well known that there are other sources of breath VOCs; diet, gut microbes and pollution being the main ones. It is not a simple matter to simply subtract these exogenous VOCs which are in the room air because some VOCs may be stored in different body compartments, eg fat tissue and released over a longer time, perhaps up to weeks or months. Some of the VOCs which are emitted by gut bacteria and diet may also be the same ones which are associated with the disease, so it is not feasible to simply exclude them. Even within subjects there may be large variability in the concentration of breath VOCs. For example, it is well known that breath acetone levels vary widely depending on the time since a person last ate. There are some measures we can take to minimize the confounding effect of these markers:

1. Smoking markers have been well characterized and there are ones which are well known to be associated with smoking behavior: these VOCs will be removed from the analysis.
2. Patients' companions will be asked to supply control samples; as these people often share a diet and environment with the patient, some VOCs associated with these may be controlled.
3. A questionnaire about diet and environment will ask about known sources of breath VOCs such as garlic and fragrances in air freshener products, and what and when they have eaten on the day of sampling.
4. One full mixed-expired breath will be collected, it has been shown that the ratio of VOCs in end-expired and mixed-expired air can give indications as to whether or not the VOC is endogenous. This is because mixed expired air contains air from the upper respiratory tract which has been inhaled but has not taken part in gas exchange in the lungs. Comparison of mixed expired and end-expired air can help to identify VOCs which are in ambient air so that they can be excluded from the analysis.

5. Patients

5.1. Recruitment and Consent

For HE in-patients and paracetamol overdose a member of the care team on the ward will ask the patient or their relative if they wish to participate and alert the scientific research team to come to the hospital to take a breath sample. A member of the care team will obtain informed consent from all participants. The study will be explained to prospective participants by either a member of the care team, or a study researcher. For patients in clinic (cirrhosis and GI cancer), their consultant physician will explain the research to them and obtain consent. Subjects will be given 15 minutes to think over the study before consent is sought. They will be given a copy of the patient information leaflet and consent form if they take part. The issues of consent will be discussed further under 'Ethical Considerations'.

The participants will be asked to fill in a questionnaire to inquire about previous smoking history, diet and other conditions from which they may be suffering. They will be taken through the questionnaire by a study researcher. The subjects will be told that they should terminate the procedure at any time if they feel uncomfortable. Study researchers will supervise the taking of breath samples. It is expected that the whole procedure including filling in the questionnaire and giving the breath sample will take approximately 10 - 15 minutes. The collection of subsequent (if any) breath samples will take around 2-4 minutes.

5.2. Anonymisation

The patient will be assigned an anonymous code by the researchers which will be entered on the questionnaire and against any electronic files generated from their breath data. Another file will be created which will relate the coded name to the participant's name and hospital number. This will be kept separately from the data files.

Data about patients and from the breath samples will be kept on the computers of the researcher in the University of Birmingham. This is on a secured server which is backed up to avoid data loss. No patient data will be kept on laptops, personal computers or flash drives.

Questionnaires and consent forms will be kept in the locked offices of the researchers in the Molecular Physics group office in the University of Birmingham. Only researchers involved directly with the study will have access to any patient data.

5.3. Inclusion Criteria

5.3.1. Patients

Subjects will be adults over the age of 18 of both genders. For HE, they will have a confirmed diagnosis of liver cirrhosis and have been admitted to hospital suffering from acute hepatic encephalopathy. Cirrhosis patients will have a confirmed diagnosis of liver cirrhosis and will be attending their usual clinic. GI cancer patients will have a confirmed diagnosis of GI cancer and will be either those scheduled for tumour removal

or those returning after tumour removal having already given a pre-tumour removal sample.

5.3.2. Control Groups

Control group subjects will preferably be the companion of the patient who has accompanied them into hospital. In cases where no companion is available or who does not wish to participate, a member of the ward team will act as control.

5.4. Exclusion Criteria

Any subjects who have pre-existing severe respiratory conditions such as asthma, lung cancer or COPD, or who are pregnant will be excluded. Controls will be excluded if they are suffering from liver disease or cancer of any type.

6 Statistics

Power calculations are not applicable at this time as we don't know how many variables to expect, and what the variability on them will be. Although this is principally a biomarker discovery study, we will also seek to obtain more information by assessing VOC levels over time in response to therapy. Ideally, we would then seek to model the decrease in concentration and assign a time dependent parameter to the wash out curve. This will depend on the patient's response to therapy and the rapidity with which VOCs clear from the bloodstream.

As with all biomarker discovery studies, the more patients, the more power the study has. At present, we regard this study as a pilot, which is to identify putative markers which can then be validated in future work.

6.1. Sample Sizes

As with any biomarker discovery, the more samples the better the chances of finding markers which are representative of the population rather than just the current sample. We will seek to measure as many patients and controls as feasibly possible up to a maximum of 50 in each patient group (except paracetamol overdose). We will not wait until we reach this maximum number, however, if it is apparent that recruitment is insufficient within the timescale of the project. It is expected that, on average, two or three patients per week will be admitted for acute encephalopathy. Liver clinics see around 40 patients per week with cirrhosis and around 20 patients per week with GI cancer. For paracetamol overdose, the intention is simply to detect the metabolite, so a maximum of 5 patients will be recruited. Around 50 patients per year are treated at the QEH for paracetamol overdose.

7. Ethical considerations

Acute encephalopathy is a very distressing illness both for the patient and their relatives/carers. Patients in ITU post-surgery are in a vulnerable state. Researchers must be sensitive to the condition of the patient and their relatives/carers. Where patients are being admitted for the first instance of acute HE, they and their relative/carer are likely to be highly anxious and will require to be treated with extra consideration. It is necessary to obtain breath samples from patients in the acute stages of HE because of the transient nature of the illness. Previous work on breath ammonia was inconclusive probably because only lower grades of HE were investigated. When patients are having an acute episode, levels of VOCs which are associated with HE are more likely to be elevated and, therefore, measurable. Moreover, we are deliberately attempting to measure a dynamic situation; biomarker research, in general, is fraught with the difficulty that a single measurement taken at any one time gives only a snapshot view of the metabolism. By taking measurements over time we hope to exclude VOCs which may be randomly elevated and find only those VOCs which exhibit change accordance with the changing condition of the patient. We can then have greater confidence that these VOCs are related to HE. Because confusion is a defining characteristic of acute HE, there is necessarily a risk that patients may not be in a fit mental state to consent at the outset of the study. It will be necessary for patients to have some awareness and ability to cooperate in order to obtain a breath sample from them, however, we would not automatically assume full mental capacity just because they are able to follow the instructions to breathe into the apparatus. It is difficult to judge to what extent an encephalopathic patient truly understands what is being said to them. It is, however, expected that once therapy is underway a patient's mental capacity will return. We will check with the patient and their relative/carer and the patient's care team to ascertain when the patient is deemed to have regained their mental capacity and obtain consent at that time. In the first instance when it is not clear if the patient can give consent or not, we will consult with the patient's relative/carer to ascertain whether or not this is the kind of research that the patient would consent to. It is expected that the patient will have a companion with them when they are admitted, as they are generally unable to arrange for admission without assistance, such is their degree of confusion. It is possible that being interviewed for the questionnaire may cause the patient some stress. This is unlikely but it is possible. If a patient is showing signs of anxiety, then the interview will be stopped.

8. Research benefits

There is no direct benefit to the participants from taking part in this study, but they may gain satisfaction from knowing that they have contributed to research in this field. The benefits of the identification of a biomarker for HE are improved diagnosis, quicker diagnosis and better management. At present, a differential diagnosis must be done and this takes time. Ultimately, it is hoped that the identification of breath biomarkers would lead to the development of a home test that patients could use to monitor their own condition. At present, patients have to try to closely manage their intake of fluids, salt, protein and laxatives to prevent toxin build-up. Although a laxative, usually

lactulose, is a cheap and simple treatment, many people find it unpleasant to drink and it can lead to a distressing urgency in the need to go to the toilet.

Patients are understandably reluctant to take too much lactulose, but too little can lead to HE episodes. It is difficult for patients to manage the correct dose of lactulose, while simultaneously managing their intake of salt, protein and fluids. It is a complex set of variables and can easily be thrown off balance by an infection, or something as simple as not drinking enough. Currently, patients have no yardstick by which to judge whether they are on track to prevent an episode, or whether an episode is imminent. An HE episode can strike suddenly; a patient may feel and behave completely normally but within a few hours could become mentally incapable. If they had a quick, non-invasive test that they could do several times a day, they could monitor the change in levels of biomarkers. Increases in levels of VOCs may have predictive value, allowing a patient to increase their dose of lactulose, or give the opportunity to call someone to attend to them. At present, patients who are at risk of acute episodes cannot be left alone, which is a significant burden on carers.

This has the potential to relieve the burden on carers and improve the quality of life for the sufferer. This would not only be of benefit to the patient in terms of time, distress and convenience, it would reduce the need for in-patient stays and, therefore, save NHS resources.

Patients with complex illnesses such as liver disease and cancer endure many venepunctures and radiological procedures in the course of assessing their illness, often over many years. A non-invasive method of diagnosis would improve the patient's experience and reduce distress. Treatment for paracetamol overdose must be timed correctly and the dose is dependent on the plasma concentration of paracetamol. The lab tests take time to do, so a quicker method of establishing the metabolite load would be beneficial.

9. Resources and cost

The research will be funded by a Daphne Jackson Fellowship (jointly funded by the EPSRC and the University of Birmingham) and a Marie Curie International Training Network grant. Consumables are sample bags and mouthpieces which will be bought from the Marie Curie grant.

6. Have you ever smoked in the past?

If yes:

How many cigarettes a day? For how long did you smoke?

How long ago did you stop?

Years/months/weeks

7. Have you been exposed to other people smoking in the past week?

8. Do you have an open fire, or have you recently been exposed to any other source of open fire?

9. Are you taking any prescribed medication other than the Pill?

10. Are you taking any preparations or medications that have not been prescribed by a GP, eg herbal remedies?

11. Are you taking any food supplements such as vitamin pills?

12. Have you been eating normally in recent days?

13. When did you last have something to eat or drink

- 14. What did you last eat or drink
- 15. Have you eaten or drunk any of the following things in the past 24 hours?

Garlic

Coffee

Alcoholic drinks

- 16. How many cups of coffee do you usually drink? (including decaffeinated coffee)

Never

1-3 per month 1 per day

2 - 3 per day

4 - 5 per day

>5 per day

1 per week

5 – 6 per week

- 17. Have you used nasal decongestants or any product containing menthol in the past 24 hours?

- 18. Do you normally eat food or drink with citrus flavour eg orange, lemon, grapefruit, including artificially flavoured drinks?

- 19. Have you eaten citrus fruit, or any food with citrus flavour in the past 24 hour

- 20. Have you drunk any drink with citrus flavour in the past 24 hours?

- 21. Have you been anywhere where citrus or pine scented air fresheners have been used in the past week?

Your signature

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Research Paper

Volatile Biomarkers in Breath Associated With Liver Cirrhosis — Comparisons of Pre- and Post-liver Transplant Breath Samples



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ABSTRACT

Background: The burden of liver disease in the UK has risen dramatically and there is a need for improved diagnostics.

Aims: To determine which breath volatiles are associated with the cirrhotic liver and hence diagnostically useful.

Methods: A two-stage biomarker discovery procedure was used. Alveolar breath samples of 31 patients with cirrhosis and 30 healthy controls were mass spectrometrically analysed and compared (stage 1). 12 of these patients had their breath analysed after liver transplant (stage 2). Five patients were followed longitudinally as in-patients in the post-transplant period.

Results: Seven volatiles were elevated in the breath of patients versus controls. Of these, five showed statistically significant decrease post-transplant: limonene, methanol, 2-pentanone, 2-butanone and carbon disulfide. On an individual basis limonene has the best diagnostic capability (the area under a receiver operating characteristic curve (AUROC) is 0.91), but this is improved by combining methanol, 2-pentanone and limonene (AUROC curve 0.95). Following transplant, limonene shows wash-out characteristics.

Conclusions: Limonene, methanol and 2-pentanone are breath markers for a cirrhotic liver. This study raises the potential to investigate these volatiles as markers for early-stage liver disease. By monitoring the wash-out of limonene following transplant, graft liver function can be non-invasively assessed.

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1. Introduction

The publication of the 2014 Lancet Commission on liver disease has highlighted how the burden of liver disease in the UK has risen sharply over the past few decades and that it poses a major public health issue (Williams et al., 2014). It is the only major cause of mortality and morbidity which is on the increase in England, while at the same time decreasing in most other European countries, with cirrhosis accounting

for 83% of deaths (Davies, 2012). It is the third biggest cause of premature mortality, with three quarters of liver deaths due to alcohol (Williams et al., 2014). Liver disease has a widespread effect not only to the patient, encompassing physical and psychological morbidity and mortality, but also incurring significant societal costs. One of the main difficulties is that often patients do not present symptoms or signs until the disease is advanced. Even then diagnosis is difficult and the symptoms and signs are often general and can be mistaken for other pathologies. Non-invasive diagnostic techniques currently used, namely serum biomarkers and transient elastography (TE) are not ideal. Serum biomarkers are not liver specific and TE results require an expert clinician for interpretation (Castera et al., 2015).

Among the ten key recommendations in a recent Lancet report is to strengthen the detection of early-stage liver disease, which is essential to reduce disease progression (Williams et al., 2014). Analysis of volatiles in the breath has the potential to deliver this, but only if chemical compounds can be found that are unambiguously associated with a diseased liver.

To date, the use of breath volatiles for medical diagnosis has met with limited success. Confounding factors, such as volatiles present in the environment, contamination in the sampling procedures and poor sampling methods, have meant that there is a great deal of uncertainty in volatile discovery (Kwak and Preti, 2011). Problems of bias and false

Abbreviations: AID, autoimmune liver disease; ALD, alcoholic liver disease; AUROC, area under receiver operator curve; CD, cryptogenic disease; GC, gas chromatography; HBV, hepatitis B virus; HCC, hepatocellular cancer; HCV, hepatitis C virus; ITU, intensive treatment unit; LQ, lower quartile; MS, mass spectrometry; OPU, out-patient clinic; PBC, primary biliary cirrhosis; PSC, primary sclerosing cholangitis; ppbv, parts per billion by volume; ppmv, parts per million by volume; PTR-MS, proton transfer reaction mass spectrometry; ROC, Receiver operating characteristics; TAC, transplant assessment clinic; TE, transient elastography; UKELD, United Kingdom model for end-stage liver disease; UQ, upper quartile; VOC, volatile organic compounds; VMR, volume mixing ratio; BMI, body mass index.

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discovery in biomarker discovery research have been widely reviewed (Broadhurst and Kell, 2006; Ransohoff, 2005).

Previous studies investigating breath volatiles in patients suffering with liver disease have proposed a large number of possible biomarkers (Millonig et al., 2010; Morisco et al., 2013; Sehnert et al., 2002; Solga et al., 2006; Tangerman et al., 1983; Van den Velde et al., 2008; Dadamio et al., 2012; Friedman et al., 1994; Hanouneh et al., 2014; Khalid et al., 2013, 2014; Shimamoto et al., 2000; Vollnberg et al., 2009; Verdam et al., 2013), but generally different studies report different volatiles. Various GC–MS studies found raised levels of many volatiles in the breath of patients with liver disease, including dimethyl sulfide, acetone, 2-butanone, 2-pentanone, β -pinene, α -pinene, and limonene (Van den Velde et al., 2008; Dadamio et al., 2012; Friedman et al., 1994; Khalid et al., 2013). Studies using soft chemical ionization mass spectrometric techniques have reported volatiles such as acetaldehyde, ethanol, isoprene, benzene, methanol, 2-butanone, 2- or 3-pentanone, heptadienol, and a monoterpene (limonene) (Morisco et al., 2013). Although the results of these studies are extremely encouraging, few volatile organic compounds (VOCs) are common to more than two or three studies and it is not useful to have hundreds of putative markers. Furthermore some volatiles, which have been proposed as biomarkers for liver disease, such as isoprene, acetone and ethanol, are not specific enough because they are possible biomarkers for other diseases or arise from numerous normal metabolic processes. If breath analysis is to progress to clinical utility, then markers must be definitively associated with the disease in question.

All previous studies can be regarded as hypothesis-generating, in that they do not follow up in a second group to confirm the putative biomarkers. We report here a two-stage breath biomarker discovery process: breath samples from a group of patients suffering from liver disease are first compared to breath samples from healthy controls; post-transplant breath samples are then compared with a sub-cohort of these patients who went on to have a liver transplant. A set of putative volatile markers is first determined by comparing patients with controls, and then pre- and post-transplant breath samples are examined to look for intra-individual differences in these volatiles. In this way, this study is hypothesis-led and uses patients as their own controls, thereby reducing the risk of false discovery. Furthermore, the use of patients' companions as controls and of room air samples minimizes the influence of any exogenous volatiles present in the home and hospital as confounding factors.

2. Methods

2.1. Patients, Controls and Hospital Room Air

Patients were recruited at the University Hospital Birmingham from either the transplant assessment clinic or in wards after being admitted with hepatic encephalopathy. 31 patients suffering from liver disease participated in the pre-transplant measurements (F/M 8/23, mean age 55 years, min–max 27–71 years). There were a number of etiologies and 11 patients had more than one condition: alcoholic liver disease (N = 13), hepatocellular cancer (N = 10), cryptogenic (N = 4), hepatitis C (N = 5), primary sclerosing cholangitis (N = 4), primary biliary cirrhosis (N = 2), autoimmune liver disease (N = 1), hepatitis B (N = 3), non-alcoholic steatohepatitis (N = 1), and non-alcoholic fatty liver disease (N = 1). Of these 31 patients, 12 went on to have a liver transplant (F/M 4/8, mean age 48 years, min–max 27–58 years). One additional patient (F, age 53 years) was recruited into the post-transplant study. Table 1 summarises the details of the 13 patients who had a liver transplant (12 from the pre-group), with patients being identified by sex (F or M) and a number. In addition to pre-transplant diagnostics, definitive diagnoses by histopathological examination of the explanted liver are provided. All but one (F2) were diagnosed with cirrhosis by standard liver function tests and biopsy. F2 was admitted suffering with hepatic encephalopathy, and histopathology of the explanted

liver gave a diagnosis of severe hepatitis with multiacinar necrosis. Supplementary Table 1 shows demographic information for all patients including medications they were taking at the time of the pre-transplant sample.

For 28 pre-transplant measurements, breath samples from the patients' companions were taken. For the other three, two came alone to clinic and the other's companion declined to take part. Two additional controls were therefore recruited, one was a ward nurse and the other was a visitor to the hospital in ITU. These controls, while not related to the patient, had been in the same room for several hours prior to sampling so that confounding factors associated with volatiles present in the room environment were taken into consideration. In total 30 controls (F:M 23:7, mean age 44 years, min–max 20–75 years) took part in the study. The larger number of females in the control group arose due to the tendency of men coming to the clinic accompanied with their wives. While this means the control group is not ideally matched, there is no consistent evidence of dependences of volatile breath composition on sex (Kwak and Preti, 2011; Ellis and Mayhew, 2014). In confirmation of this we also found no correlation between sex and VOCs in either our control or patient groups. We consider that inhaled VOCs have a greater potential to confound biomarker discovery. As the majority of the companions were living with the patients, they provided an ideal control for exposure to exogenous volatiles in the home environment. VOCs inhaled at home, or in transit, may well still be present in breath for hours or days after inhalation and the biological half-life of inhaled VOCs is not well known (Beauchamp, 2011).

All study subjects were asked to complete a detailed questionnaire which included details on their home environment, diet, smoking status, health and medications. Participants were asked if they had consumed fruit and fruit juices and fruit flavoured drinks as a normal part of their diet, and, if so, to provide details on quantity and how long before the breath sampling these had been consumed.

Hospital room air was collected every time breath samples were taken so that any exogenous volatiles, such as isopropanol coming from hand gels resulting in product ions at m/z 43 and m/z 61, could be taken into consideration.

2.2. Breath Sampling Protocol

There is no agreed standard for the collection of breath for volatile analysis and uncontrolled breath sampling has been shown to be unreliable (Schubert et al., 2001; O'Hara et al., 2008). Therefore, capnography controlled sampling was used to collect only the alveolar phase of the breath. Subjects were in a relaxed state throughout the measurements and were either in a seated or lying position. They were asked to breathe normally into a gas tight respiratory system (Intersurgical Limited) containing an in-line CO₂ mainstream sensor connected to a fast-time response capnometer (Capnogard 1265 Novamatrix Medical Systems Inc.). A 100 ml glass syringe (Sigma-Aldrich) was coupled to the tubing using a 3-way luer-lock stopcock (Braun Medical Limited). When the alveolar plateau on the capnograph was observed, a breath sample was manually drawn from the subject's breath stream into the syringe. Three to four breaths samples were collected for each 100 ml syringe, and four replicates of these were taken for each subject. Glass syringes were used, because our tests showed that they have no contaminating volatiles. Fig. 1 schematically shows the sampling system used.

After collection, the syringes were sealed using the luer lock fitting. They were transported from hospital to laboratory (a 10 minute outdoor walk) in an opaque storage box. Once at the laboratory, the syringes were placed inside an incubator set at 40 °C.

All samples were mass spectrometrically analysed within 2 h of collection. For the measurements, syringes were taken out of the incubator and immediately placed into a purpose designed heating bag (Infroheat, Wolverhampton) maintained at a constant temperature of 40 °C in order to limit condensation, which could otherwise lead to volatile

Table 1
Liver transplant patient details, including sex (female F, male M), age, initial diagnosis, histopathological results, location of pre-transplant and post-transplant breath sampling, and the number of days prior to and after transplant when breath samples were collected. Diseases include autoimmune liver disease (AID), alcoholic liver disease (ALD), cryptogenic disease (CD), hepatitis B (HBV), hepatitis C (HCV), hepatocellular cancer (HCC), primary biliary cirrhosis (PBC) and primary sclerosing cholangitis (PSC). Breath samples were taken at various locations including the intensive treatment unit (ITU), out-patient clinic (OPC), transplant assessment clinic (TAC), and in wards.

Patient ID	Age (Yr)	Initial diagnosis	Histopathological results	Location of pre-transplant breath sample	Pre-transplant breath sample: days before transplant	Location of post-transplant breath sample	Post-transplant breath samples: days after transplant
F1	27	AID	Severe steatohepatitis (PSC)	TAC	54	OPC	65
F2	49	Liver Failure	Severe hepatitis with multiacinar necrosis, seronegative hepatitis	ITU	0 ¹	OPC	3, 5, 130
F3	53	PBC	Cirrhosis (PBC)	TAC	74	OPC	45
F4	58	PSC	Cirrhosis (PSC)	TAC	83	Ward	5–8, 11–15, 18, 58
F5	53	ALD	Hepatocellular carcinoma, liver cirrhosis.(ALD, HCV)	-	-	Ward	2–6, 9–12
M1	54	ALD	Severe steatohepatitis	Ward	47	OPC	33
M2	45	ALD	Cirrhosis (ALD)	TAC	97	OPC	22
M3	53	ALD	Cirrhosis (ALD/HCV)	TAC	179	Ward	4, 7, 48
M4	53	ALD, HBV, HCV	Hepatocellular carcinoma (grade2)	TAC	21	OPC	126
M5	56	ALD, HCV, HCC	Cirrhosis (ALD, HCV, HBV)	TAC	125	OPC	61
M6	53	CD	Cirrhosis with mild steatohepatitis (aetiology possibly NASH but uncertain)	TAC	154	OPC	22
M7	36	CD	Cirrhosis of uncertain aetiology	TAC	180	Ward	2, 3, 6–8, 55
M8	67	ALD	Cirrhosis (ALD)	ITU	14	Ward	6

The pre-transplant breath sample for patient F2 was taken approximately 10 min before the patient went into surgery. This patient was admitted with liver failure and hepatic encephalopathy.

loss (Beauchamp et al., 2008). The luer stopcock was coupled to a Swagelok fitting and connected directly to the inlet of the analytical device, a proton transfer reaction mass spectrometer (PTR-MS). The inlet flow was set at 10–15 ml/min and the drift tube and inlet lines were maintained at 45 °C. The syringes are gas tight and have minimal friction such that atmospheric pressure is sufficient to push the plunger in smoothly so that the breath sample is being drawn into the instrument at a constant flow.

2.3. Analytical Measurements

PTR-MS is a platform technology designed to detect low concentrations of volatiles (less than parts per billion by volume). Hence it has found use in many analytical applications ranging from drug detection through to industrial pollution (Ellis and Mayhew, 2014; Lindinger et al., 1998; Agarwal et al., 2011; de Gouw et al., 2011; Jurschik et al., 2012). Details of the instrument used, a PTR-Quad-MS (IONICON Analytik GmbH), and how it operates are described in detail in the literature (Ellis and Mayhew, 2014; O'Hara et al., 2008; Lindinger et al., 1998). In brief, it exploits the reactions of protonated water with neutral volatiles (M), usually leading to a protonated parent (MH⁺). If dissociative proton transfer occurs then it is not extensive in terms of the number of resulting product ions. Operational parameters used for this investigation were those previously reported (O'Hara et al., 2009a,b). Namely, the drift-tube was maintained at a pressure of 2.07 ± 0.01 mbar and temperature of 45 ± 1 °C. The voltage across the drift-tube was set at 600 V, which is sufficiently high to reduce water clustering to reagent and product ions by collision induced dissociation.

A *m/z* range of 20 to 200 amu was scanned with a dwell time of 0.5 s per atomic mass unit. Mass spectra of the breath samples were recorded from the average of three cycles for each of the four syringes, for every participant. These four spectra were averaged to provide one data set for each subject with the uncertainty expressed as the standard error of the mean for the four syringes.

The intensities of the product ion(s) associated with a given volatile were converted to volume mixing ratios (VMR) in units of nmol/mol by use of a standard procedure that relies on a calculated, compound-specific, collisional reaction rate coefficient, determined using the effective translational temperature of the reagent ions (Ellis and Mayhew, 2014).

To help identify product ions, pure samples of key volatiles were individually measured using PTR-MS to establish the *m/z* values of the product ions.

2.4. Data Analysis and Statistics

Room air contamination is a potential confounding factor in breath analysis and has been the subject of much discussion. If the intensity of a product ion has a significant contribution coming from room air, then care must be taken when using it as a biomarker. There is no simple correction which can be applied to account for inhaled volatile concentrations, and it has been shown that simply subtracting the room air concentration is too simplistic (Schubert et al., 2005; Spanel et al., 2013; Pleil et al., 2013). For this study, a filter was applied such that only ion signal intensities in the breath sample that were at least twice that in the room air samples in at least half of the patients were retained for analysis. This resulted in a set of 40 product ions for analysis. (The *m/z* values and normalised counts per second are provided in Supplementary Table 2, many of which are well known including *m/z* 33 (methanol), *m/z* 45 (acetaldehyde), *m/z* 47 (ethanol), *m/z* 59 (acetone) and *m/z* 69 (isoprene).

The data sets for each volatile of interest were assessed using a Shapiro–Wilks test and were found not to be normally distributed so non-parametric tests were used. IBM SPSS version 22 was used for all statistical analysis. Mann–Whitney U-tests determined which *m/z* values differed between the patients and controls. A Wilcoxon signed

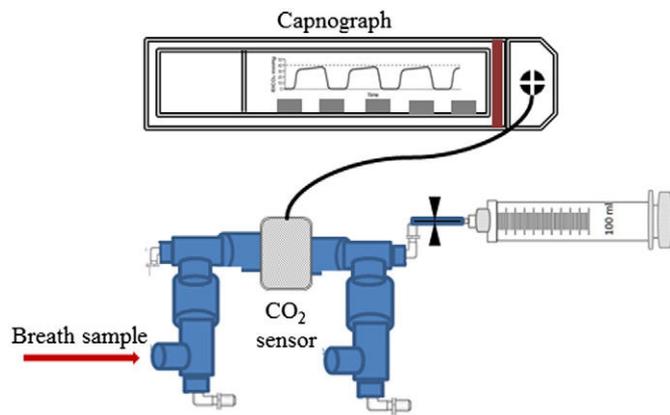


Fig. 1. Schematic of the breath sampling device. Breath samples are only drawn into the glass syringe once the capnograph shows that the alveolar phase of the exhaled breath has been reached. Typically 3–4 breaths are needed to fill a syringe to 100 ml.

rank test was used to determine which volatile concentrations differed between pre- and post-transplant breath samples. To compare blood chemistry values with breath volatiles Kendall's tau-b correlation coefficients were measured. Receiver Operating Characteristic (ROC) curves were used to determine the diagnostic accuracy of volatiles.

3. Results

For the first stage of our study, a Mann–Whitney U-test with a significance level of 95% was used to compare the 40 product ion signal intensities used in our analysis between patients and controls. Of these, eight showed significant differences in intensities between patients and controls. Their m/z values and significance (p value in brackets) are 33 (<0.001), 73 (0.004), 77 (0.035), 81 (<0.001), 87 (<0.001), 89 (0.03), 135 (0.019) and 137 (<0.001). In the second phase, pre- and post-transplant intensities of these eight ions were compared using a Wilcoxon Signed Rank Test for paired samples with a significance of 95%. This eliminated m/z 89 and 135 from the putative marker set. Of the remaining ions, m/z 33 is assigned to be protonated methanol (CH_3OH_2^+) (Lindinger et al., 1997). Based on previous GC and GC–MS studies (Sehnert et al., 2002; Van den Velde et al., 2008; Dadamio et al., 2012), we tentatively identify m/z 73 as protonated 2-butanone ($\text{C}_4\text{H}_8\text{OH}^+$), m/z 77 as protonated carbon disulfide (CS_2H^+), m/z 87 as protonated 2-pentanone ($\text{C}_5\text{H}_{10}\text{OH}^+$), and m/z 81 and 137 as limonene. (m/z 81 is a fragment ion (C_6H_9^+) resulting from dissociative proton transfer and m/z 137 is protonated limonene ($\text{C}_{10}\text{H}_{17}^+$.) VMRs of these volatiles in room air, patient and control samples are shown in Fig. 2 for (a) methanol, (b) carbon disulfide, (c) 2-butanone, (d) 2-pentanone, and (e) limonene (sum of the intensities of the m/z 81 and m/z 137 product ions). The median, mean, lower quartile (LQ), and upper quartile (UQ) of the VMRs in units of nmol/mol for each volatile are shown. It is clear from Fig. 2 that the presence of the volatiles in room air has a negligible effect on the concentrations in the breath samples. Furthermore, the analysis is based on comparisons and the patients and controls should be affected similarly by the room air contaminations.

The pre-transplant and post-transplant VMRs for methanol, carbon disulfide, 2-butanone, 2-pentanone and limonene for all of our participants who underwent liver transplants (4 females (F1–F4) and 8 males (M1–M8)) are provided in Table 2. It should be noted that the number of days between collecting the pre-transplant and post-transplant breath samples is variable, because it is not possible to control when subjects are available or when a donor liver would be

found. Only for one of the patients, F2, were we able to collect a pre-transplant breath sample just prior to surgery. However, and independent of when the pre- and post-transplant breath samples were taken, the results in Table 2 clearly demonstrate that the pre-transplant concentrations of these volatiles are, for the majority of patients, higher than the post-transplant levels for most patients. Limonene shows the largest average decrease and also decreased in all patients post-transplant. Post-transplant concentrations of limonene dropped to within the normal control range (median (LQ, UQ) being 2.3 nmol/mol (1.9, 3.0)) within a number of days for all but one of the patients, M4, for whom limonene was found to be high even some months after transplant.

In order to gain an insight on how the methanol, carbon disulfide, 2-butanone, 2-pentanone and limonene breath VMRs changed over a period of time after transplant, five patients (F2, F4, F5, M3 and M7) participated in a longitudinal study. The key result is that limonene VMRs dropped gradually following transplant surgery, as illustrated in Fig. 3. (The same data for limonene presented as normalised to the highest intra-individual value are shown in Supplementary Fig. 1.) This concentration time dependence was not observed for methanol, carbon disulfide, 2-butanone and 2-pentanone. Their VMRs were found to have dropped to within the normal range by the time of the first post-transplant measurement.

Taken together, the box plots (Fig. 2), the ratio of the pre- and post-transplant VMR values and the significance values given above imply that ions at m/z 33, 81, 87 and 137 are the ones that are most diagnostically useful. This is confirmed by ROC curve analyses. Individually, limonene is found to provide the most predictive power (AUROC – 0.91 (standard error 0.04)). However, the best accuracy is achieved by combining the data from methanol, 2-pentanone and limonene. The VMRs for limonene, methanol and 2-pentanone were normalised to the highest patient value for that volatile. These normalised fractions were simply added with no weightings. Fig. 4 shows a ROC curve for the combined data. The AUROC is 0.95 (standard error 0.03) and achieves a sensitivity of 97% with a specificity of 70%.

Clinical chemistry data for the patients for whom blood data were available were analysed for possible correlations with limonene, methanol and 2-pentanone. Correlations were checked for alanine aminotransferase, alkaline phosphatase, aspartate transferase, albumin, total bilirubin, creatinine, neutrophils, platelets, potassium, prothrombin/international normalised ratio, and the United Kingdom Model for End-Stage Liver Disease (UKELD). Kendall's tau-b analysis showed only one correlation with a significance score below 0.05. This was for methanol with UKELD which had a Kendall's tau-b coefficient of 0.237 (significance 0.042). Over 33 correlations were tested and no multiple testing correction was applied so it is possible that this is a coincidental finding.

Volatile concentrations were examined for correlations with disease etiology. Owing to the small sample size and large number of etiologies, this was only feasible for the 13 patients with ALD versus the other 18 patients. Limonene was higher ($p = 0.020$) in the ALD group than the rest, with median (LQ, UQ) of 19.7 nmol/mol (9.2, 63.9) for ALD versus 6.1 nmol/mol (2.9, 16.6) for all other etiologies. Methanol, 2-pentanone, 2-butanone and carbon disulfide showed no statistically significant difference.

Correlations between the 7 volatiles of interest in the putative marker set were examined both within the patient and the control group using a Kendall's tau-b test. In the patient group, there were 8 correlations with a significance score of <0.05. Results for all 21 correlations are shown in Supplementary Table 3. In the control group, only two were significant, limonene with m/z 135 ($p = 0.016$) and 2-butanone with carbon disulfide ($p < 0.001$). These were also found in the patient group. Limonene correlated significantly with 2-butanone ($p = 0.004$), carbon

Fig. 2. Boxplots showing in units of nmol/mol lower quartile (LQ), median, mean and upper quartile (UQ) calculated volume mixing ratios (VMRs) for (a) methanol, (b) 2-butanone, (c) carbon disulfide, (d) 2-pentanone, and (e) limonene for 31 patients with liver cirrhosis, 30 controls and room air samples. Whiskers are 1.5 times the inter-quartile range and outliers are depicted by a star.

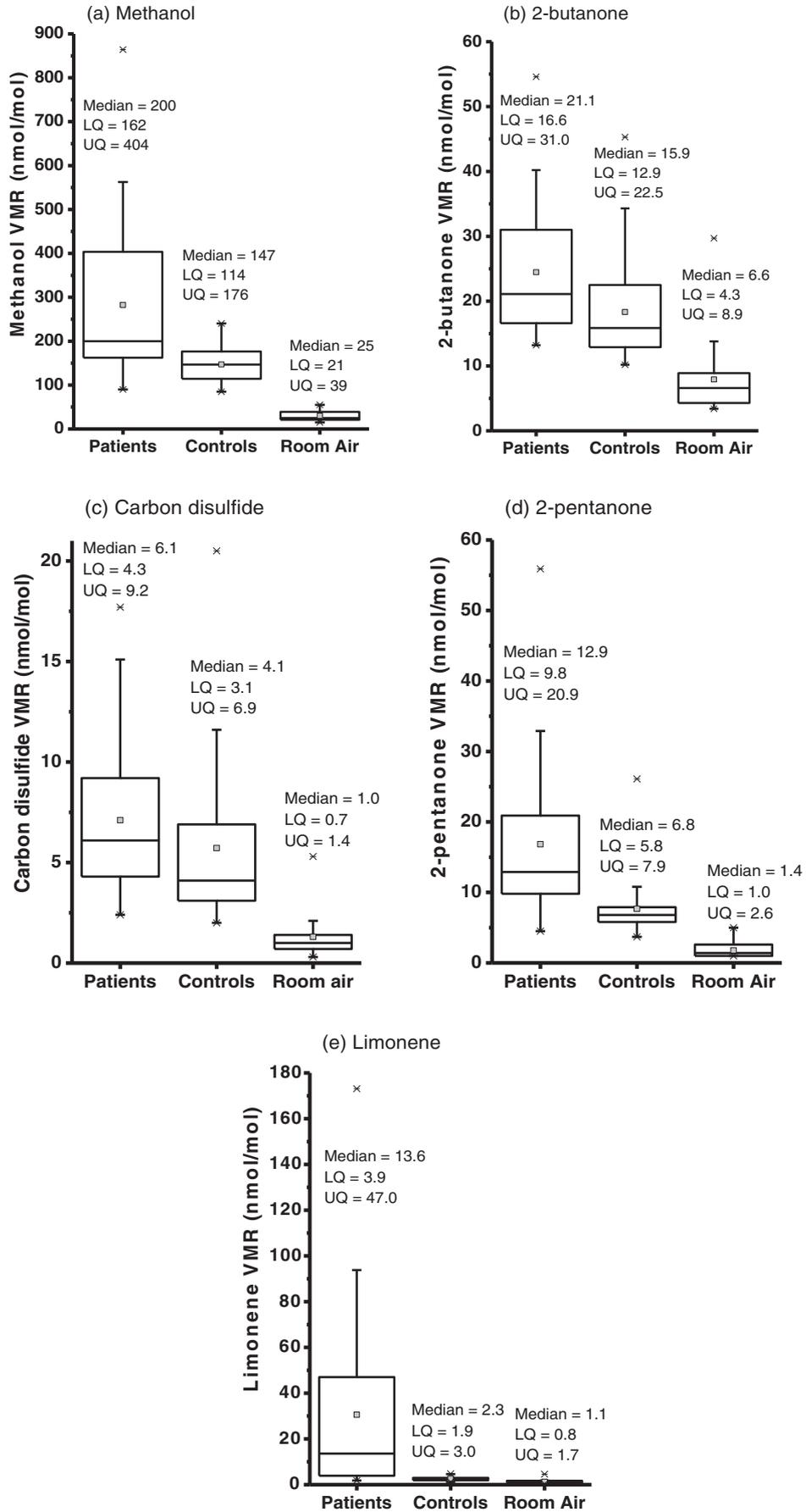


Table 2
Calculated mean volume mixing ratios (VMRs) for methanol, 2-butanone, carbon disulfide, 2-pentanone, and limonene, in units of nmol/mol for pre- and post-transplant breath samples. The post-transplant values correspond to those for the last post-transplant sample given in Table 1. Measurement uncertainties are provided in brackets. The ratios of pre- to post-transplant concentrations are also provided.

Patient	Mean methanol VMRs nmol/mol			Mean 2-butanone VMRs nmol/mol			Mean carbon disulfide VMRs nmol/mol			Mean 2-pentanone VMRs nmol/mol			Mean limonene VMRs nmol/mol		
	Pre	Post	Pre/post	Pre	Post	Pre/post	Pre	Post	Pre/post	Pre	Post	Pre/post	Pre	Post	Pre/post
F1	200 (6)	190 (3)	1.1 (0.04)	18 (1.2)	11 (0.2)	1.6 (0.1)	4.7 (0.4)	2.0 (0.4)	2.4 (0.5)	9.0 (0.8)	5.3 (0.6)	1.7 (0.2)	7.5 (0.6)	3.5 (0.6)	2.1 (0.4)
F2	90 (9)	230 (6)	0.4 (0.04)	16 (1.1)	15 (1.9)	1.1 (0.2)	2.4 (0.5)	20 (2.9)	0.1 (0.03)	8.3 (0.8)	7.3 (0.6)	1.1 (0.1)	60 (4.7)	1.1 (0.1)	54 (6)
F3	530 (2)	78 (5)	6.8 (0.4)	21 (1.7)	12 (2.6)	1.8 (0.4)	11 (1.3)	2.3 (0.3)	4.8 (0.8)	10 (1.2)	5.7 (0.3)	1.8 (0.2)	14 (0.7)	2.3 (0.2)	6.1 (0.6)
F4	560 (18)	71 (3)	7.9 (0.4)	19 (1.2)	12 (0.4)	1.6 (0.1)	4.7 (0.2)	2.3 (0.4)	2.0 (0.4)	25 (3.3)	6.1 (0.3)	4.1 (0.6)	11 (0.5)	2.3 (0.1)	4.8 (0.3)
M1	170 (2)	120 (15)	1.4 (0.2)	26 (1.4)	14 (1.4)	1.9 (0.2)	9.3 (0.6)	2.1 (0.4)	4.4 (0.9)	10 (0.5)	17 (2)	0.6 (0.1)	32 (0.7)	3.3 (0.5)	9.7 (1.5)
M2	430 (36)	81 (1)	5.3 (0.4)	13 (2.2)	15 (1.7)	0.9 (0.2)	3.1 (0.1)	2.4 (0.5)	1.3 (0.3)	23 (0.6)	6.2 (1.5)	3.7 (0.9)	94 (3.4)	1.6 (0.2)	59 (8)
M3	320 (32)	86 (7)	3.7 (0.5)	55 (3.1)	46 (4.2)	1.2 (0.1)	18 (2.0)	1.8 (0.2)	10 (1.6)	29 (1.0)	13 (1.2)	2.2 (0.2)	170 (1)	5.7 (0.2)	30 (1)
M4	490 (37)	93 (6)	5.3 (0.5)	38 (5.9)	23 (7.5)	1.7 (0.6)	8.5 (1.0)	2.6 (0.5)	3.3 (0.7)	38 (4.3)	9.9 (0.9)	3.8 (0.6)	110 (6)	55 (4)	2.0 (0.2)
M5	190 (3)	320 (1)	0.6 (0.01)	15 (0.5)	17 (1.3)	0.9 (0.1)	4.0 (0.4)	3.6 (0.1)	1.1 (0.1)	21 (1.3)	6.7 (0.5)	3.1 (0.3)	9.2 (0.5)	4.1 (0.2)	2.2 (0.2)
M6	230 (12)	79 (5)	2.9 (0.2)	30 (2.2)	12 (3.3)	2.5 (0.7)	13 (1.9)	2.1 (0.4)	6.2 (1.5)	13 (1.3)	5.1 (0.2)	2.5 (0.3)	120 (8)	4.8 (0.3)	25 (2)
M7	510 (21)	160 (8)	3.2 (0.2)	19 (0.7)	24 (4.2)	0.8 (0.1)	4.9 (0.9)	4.5 (0.7)	1.1 (0.3)	14 (2.6)	7.5 (0.3)	1.9 (0.4)	47 (2)	2.6 (0.1)	18 (1)
M8	180 (5)	86 (2)	2.1 (0.07)	21 (1.0)	12 (0.4)	1.8 (0.1)	6.8 (0.6)	1.9 (0.3)	3.6 (0.7)	8.8 (0.3)	13 (1.6)	0.7 (0.1)	7.7 (0.6)	3 (0.2)	2.6 (0.3)

disulfide ($p = 0.034$), m/z 89 ($p = 0.001$) and m/z 135 ($p < 0.001$) but not with methanol or 2-pentanone. This suggests that the mechanisms for the presence of limonene and that of methanol and 2-pentanone are independent. 21 correlations were examined with no multiple-testing correction applied, therefore some correlations may be coincidental.

Correlations between the concentrations of volatiles and demographic markers such as age, BMI and sex were also checked. No significant correlations were found.

4. Discussion

A major aim of this study is to determine the viability of breath analysis as a non-invasive technique for monitoring/diagnosing liver disease by identifying volatiles in the breath which are a consequence of the disease. In this investigation, the monitoring of volatiles in breath following a dramatic change in the condition of the patient, namely a liver transplant, has provided a method to attribute three diagnostically useful volatiles to the cirrhotic organ itself. These are methanol, 2-pentanone, and limonene, with that of limonene being the most significant.

Limonene has been found in previous breath volatile studies to be elevated in the breath of patients with cirrhosis compared with controls (Morisco et al., 2013; Dadamio et al., 2012; Friedman et al., 1994). It has also been observed in the breath of healthy volunteers; limonene levels found in our control group are comparable to those previously observed in healthy human volunteers (Mochalski et al., 2013). Limonene is not produced in the human body. It is a common compound naturally found in many foods and drinks; hence it would be difficult to avoid ingesting. Within the control and patient groups, we found no association between breath limonene and diet and no correlation between having a self-reported large amount of fruit consumption and breath limonene concentrations. Once in the blood stream, limonene is metabolised by the P450 enzymes CYP2C9 and CYP2C19 to the metabolites perillyl alcohol, trans-carveol and trans-isopiperitenol (Miyazawa et al., 2002). It has been found that levels of the enzyme CYP2C19 are reduced in patients with cirrhosis and that levels inversely correlate with severity of cirrhosis. Moreover, of four P450 enzymes tested in patients with liver disease, metabolism by CYP2C19 was found to decrease at the earliest stage of disease (Frye et al., 2006). This is suggestive that the observed raised concentrations of limonene in breath arise from the inability of a cirrhotic liver to produce the appropriate metabolic enzyme (Morisco et al., 2013). Patient M4 is anomalous in this respect, as his breath limonene concentrations do not drop to the normal range post-transplant. Although his graft liver function blood tests were found to be normal, our results suggest that this patient's new liver is not producing sufficient enzyme to fully metabolise limonene.

Owing to its lipophilic properties, we propose that limonene which is not metabolised by the liver accumulates in the fat of patients suffering from liver disease. Limonene has a blood/air partition coefficient of 36 and an olive oil/blood partition coefficient of 140 (Falk et al., 1990). Assuming that the olive oil/blood partition coefficient is close to a body fat/blood partition coefficient, a breath concentration of 1 part per billion by volume (ppbv) would translate to a fat concentration of approximately 5 parts per million by volume (ppmv). Our highest recorded breath VMR is 170 nmol/mol which implies a concentration in fat of the order of 850 ppmv. A study involving women with early-stage breast cancer taking a high oral dose of limonene (2 g/daily for 2–6 weeks before surgery) found that mean limonene concentration in breast tissue was $41.3 \pm 49.9 \mu\text{g/g}$ which is much higher than that found in a control group ($0.08 \pm 0.13 \mu\text{g/g}$) (Miller et al., 2013). Breast tissue is primarily composed of fat (Boston et al., 2005). This supports our hypothesis that unmetabolised limonene accumulates in fat tissue. Following transplant, the metabolism of limonene increases, but it takes time for the limonene to be released from the fat into the blood stream. This, we propose, explains the observed time dependence on limonene VMRs in the breath after transplant. A similar wash-out behaviour is not observed for methanol and 2-pentanone presumably owing to their low solubilities in fat (Griffin et al., 1999; Sangster, 1989).

Some medications are known to be CYP2C9 and CYP2C19 substrates and inhibitors. We therefore looked into the possibility that medications could affect limonene concentrations. Twenty patients were taking a CYP2C19 substrate (lansoprazole, omeprazole, propanalol, esomeprazole), 2 were taking a CYP2C9 substrate (naproxen, carvedilol), 6 were taking both a CYP2C9 and CYP2C19 substrate and 1 was taking a CYP2C9 inhibitor (sulfamethoxazole). Our results show no associations of any medications which are CYP2C9 and CYP2C19 substrates or inhibitors with VMRs of breath limonene. Moreover, nine patients who were taking enzyme substrates before transplant were still taking them after transplant.

Of interest is the correlation between limonene and m/z 135, because this product ion may come from perillyl alcohol ($\text{C}_{10}\text{H}_{16}\text{O}$), a metabolite of limonene. Studies by us (unpublished) have shown that the reaction of H_3O^+ with perillyl alcohol leads to a dominant product ion $\text{C}_{10}\text{H}_{15}^+$ resulting from dehydration of the protonated parent. Dehydration following protonation is a common reaction process observed with many alcohols (Brown et al., 2010). Morisco et al. (2013) also noted an ion at m/z 135 from patients with cirrhosis, but they assigned this to a terpene related compound. The fact that a correlation of m/z 135 and limonene ($p < 0.001$) is also significant in the control group lends support to our assignment, because one would expect levels of a compound and its metabolite to be correlated in a group with well-functioning livers. It is also of interest to note that the correlation between limonene and m/z 89 has a very low p -value, but that m/z 89

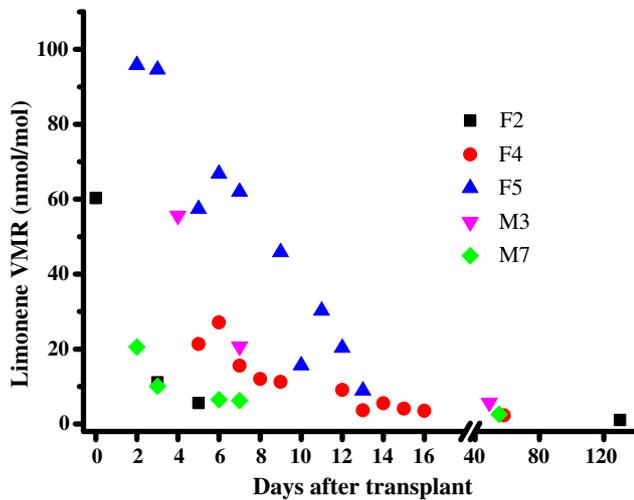


Fig. 3. Longitudinal changes in volume mixing ratios (VMRs) in nmol/mol for limonene at given days after liver transplant for patients F2, F4, F5, M3, and M7. The data point at day 0 for F2 was taken just before transplant surgery.

shows no discrimination between pre- and post-liver transplant. This is suggestive that m/z 89 arises from an independent process related to the patient's illness, but is not related to the cirrhosis itself.

The enhanced levels of methanol and 2-pentanone in pre-transplant patients could come from a number of sources, including diet. Elevated levels of methanol have been reported following consumption of alcohol or large quantities of fruit (Lindinger et al., 1997). It is a product of the degradation of pectin by colonic bacteria (Siragusa et al., 1988), and of metabolism of the sweetener aspartame (Stegink et al., 1989). However, and in agreement with Morisco et al. (2013) we find that fruit consumption cannot explain the increased methanol concentrations in the breath of liver patients compared to controls. Alcoholic drinks are a source of methanol, but only one patient reported that he had drunk alcohol within the 24 h prior to the breath sampling. Methanol is metabolised in humans in the liver, mainly by alcohol dehydrogenase (Skrzydłowska, 2003), so it is possible that this mechanism is impaired when a liver becomes cirrhotic. Morisco et al. (2013), also found elevated methanol in cirrhotic patients versus healthy controls. The source of 2-pentanone in breath is unknown (King et al., 2010). It has been found in human breath, faeces, skin and urine (de Lacy et al., 2014), and it has been suggested that lung cells produce 2-pentanone

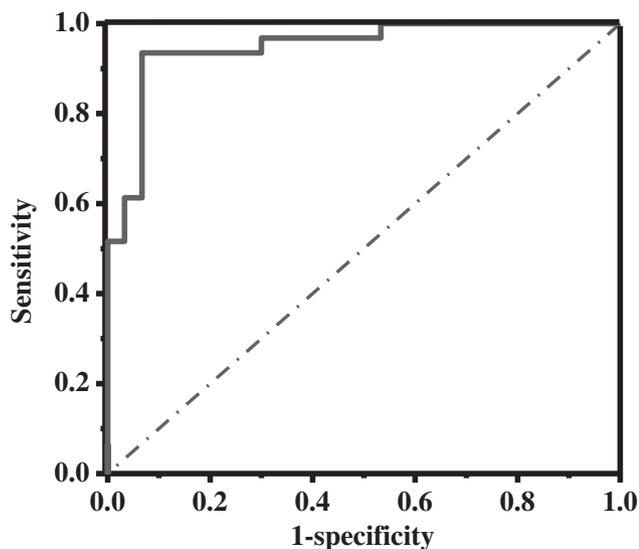


Fig. 4. Receiver operating characteristic curve for a combination of methanol, 2-pentanone and limonene data in the study groups.

(Filipiak et al., 2010). 2-pentanone was suggested as a biomarker for liver disease by three previous studies (Morisco et al., 2013; Van den Velde et al., 2008; Vollnberg et al., 2009).

In conclusion, we have performed a two-stage study which compares volatiles in the breath of pre-transplant cirrhotic patients with controls followed by pre- and post-transplant breath samples. This has resulted in an assignment of methanol, 2-pentanone and limonene as markers in exhaled breath for the cirrhotic liver. We have demonstrated that limonene can also be used for assessing liver function following transplant by monitoring wash-out. Our study links limonene with the diseased organ itself, rather than simply the diseased patient as a whole. Breath volatiles have the advantage of offering non-invasive testing, but also offer the opportunity to assess the global function of the liver, rather than a localised test such as a biopsy. Our study raises the possibility of a pharmacokinetic-based test for assessing liver function which could be used for diagnosing liver disease, i.e. where a known quantity of limonene is administered and its wash-out in breath is assessed over time. Importantly, this study provides a set of biomarkers which can be used in future studies to assess the potential of breath analysis for the diagnosis of early-stage liver disease.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.ebiom.2015.07.027>.

Conflicts of Interest

All authors have declared no conflicts of interest.

Contributions

MEOH and CAM in discussion with AH and TS were responsible for the conception of this study. RFdR was responsible for the design and development of the breath sampling system, taking the majority of the breath samples, for proposing the longitudinal study, and for undertaking most of the mass spectrometric measurements. RFdR and MEOH were responsible for the data analysis of the mass spectrometric files. MEOH was responsible for obtaining ethical approval, patient recruitment, study management, statistical analyses of the data and assisted with some of the sample collection and measurement. CAM, MEOH and RFdR wrote the paper with input from PP, AH, TS, and TW. PP, AH, TS, and TW were responsible for the provision of patients and for providing the medical context. The final paper has been approved by all authors.

Ethics Approval

The regional ethics committee of Camden and Islington, London approved this study (REC reference: 13/LO/0952). Informed consent was obtained from each volunteer.

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Isoflurane (1-chloro-2,2,2-trifluoroethyl difluoromethyl ether), $C_3H_2ClF_5O$, is a commonly used inhalation anaesthetic. Using a proton transfer reaction mass spectrometer (PTR-MS) we have detected isoflurane in the breath of patients several weeks following major surgery. That isoflurane is detected in the breath of patients so long after being anaesthetised raises questions about when cognitive function has fully returned to a patient. Temporal profiles of isoflurane concentrations in breath are presented for five patients (F/M 3/2, mean age 50 years, min–max 36–58 years) who had undergone liver transplant surgery. In addition, results from a headspace analysis of isoflurane are presented so that the product ions resulting from the reactions of H_3O^+ with isoflurane in PTR-MS could be easily identified in the absence of the complex chemical environment of breath. Six product ions were identified. In order of increasing m/z (using the ^{35}Cl isotope where appropriate) these are CHF_2^+ (m/z 51), $CHFCl^+$ (m/z 67), CF_3CHCl^+ (m/z 117), $C_3F_4OCl^+$ (m/z 163), $C_3H_2F_4OCl^+$ (m/z 165), and $C_3F_4OCl^+ H_2O$ (m/z 183). No protonated parent was detected. For the headspace study both clean air and CO_2 enriched clean air (4% CO_2) were used as buffer gases in the drift tube of the PTR-MS. The CO_2 enriched air was used to determine if exhaled breath would affect the product ion branching ratios. Importantly no significant differences were observed, and therefore for isoflurane the product ion distributions determined in a normal air mixture can be used for breath analysis. Given that PTR-MS can be operated under different reduced electric fields (E/N), the dependence of the product ion branching percentages for isoflurane on E/N (96–138 Td) are reported.

Introduction

In a recent paper we reported a study of the volatile biomarkers in breath associated with liver disease using proton transfer reaction mass spectrometry (PTR-MS) [1]. The results of that study strongly suggest that three volatiles, methanol, 2-pentanone and limonene, are related to a diseased liver. The risk of false discovery was minimised by using a two stage process to reduce the variable set so that we did not have to rely on unsupervised multivariate analysis. Importantly, for this present work 12 of the 31 patients investigated in the first stage, which involved comparing breath samples of patients suffering with

chronic liver disease with 30 healthy controls (usually partners of the ill patients), had their breath analysed post liver transplant. Of these 12 patients, 5 were followed longitudinally as in-patients during the post-transplant period for a number of weeks. For all 12 patients, a number of ions were observed in the proton transfer reaction mass spectra of the post-transplant breath samples, which were not present in the pre-transplant breath samples. We considered that these result from a reaction of H_3O^+ with the anaesthetic, isoflurane, ($C_3H_2ClF_5O$) used during surgery. In agreement with this, one of the ions that we observe at m/z 165, has been assigned to be $CF_2HOCHClCF_2^+$ from a Selected Ion Flow Tube-MS (SIFT-MS) analysis

of the headspace of isoflurane with H_3O^+ as the reagent ion [2].

Given our longitudinal measurements, which provide mass spectra of post-transplant breath samples for several weeks after the operation, we had a unique opportunity to investigate how long isoflurane remains in the body of unhealthy patients following major surgery. This may have consequences in terms of the intellectual function of patients by providing details on how quickly patients can safely return to a normal life.

Other PTR-MS studies have either concentrated on monitoring an intravenous anaesthetic, propofol, during surgery [3–8] or for monitoring sevoflurane and isoflurane in the hospital environment, namely in an urological post-anaesthesia care unit [9] and for sevoflurane in an operating theatre [10].

There have been several other studies investigating the elimination of volatile anaesthetics from the body following surgery. Yasuda *et al* [11] used gas chromatography with flame ionisation detection (GC-FID) to compare the kinetics of sevoflurane and isoflurane in humans. Of relevance to our study they report a fast elimination of isoflurane from the body, namely within a day the breath concentrations had decreased by an order of magnitude. However, their work used seven healthy male volunteers (mean age of 23 years with a standard deviation of 3 years). Therefore, the rapid decrease in concentration may not be a true reflection of what happens with sick, obese and/or older people. A more recent paper by Ghimenti *et al* [12] reports details on a study using GC-MS of post-operative elimination of sevoflurane and its metabolite, hexafluoroisopropanol, in exhaled breath of six very sick patients that had undergone a variety of surgeries. They used pharmacokinetic models for assessing liver function. In their investigations mixed-expired breath samples (multiple deep breaths) were collected in disposable Nalophan bags. The samples were then transferred to desorption tubes for subsequent analysis. Here we report the first PTR-MS longitudinal study investigating isoflurane in the breath of patients following surgery. Prior to being able to analyse the mass spectra of the post-transplant breath samples, it was necessary to establish which product ions result from the reaction of H_3O^+ with isoflurane in the drift tube environment of a PTR-MS, divorced from the complex chemical environment of breath. Therefore, as part of this study we present a headspace analysis of isoflurane at the reduced electric field of 136 Td used in our previous liver study. Given that the product ions found in this study differ from those by Wang *et al* [2], we also present the production branching ratios over an extended reduced electric field from approximately 90 Td through to 140 Td. These have been determined using both clean air and CO_2 enriched clean air, to mimic breath, as the buffer gases in the drift tube of our PTR-MS. The use of CO_2 enriched air is important because the presence of a high percentage of CO_2 could influence the proton transfer

reaction leading to different product ion distributions, which need to be taken into account when analysing breath samples [13, 14].

Experimental details and methods

Headspace analysis

Isoflurane (CAS Number 26675-46-7) was purchased from Sigma Aldrich (UK).

Isoflurane samples were prepared for analysis by adding 1 μl of isoflurane into a 100 ml glass bottle. A 100 ml glass syringe was then coupled to the top part of the bottle using a 3-way luer-lock stopcock and 10 ml of the isoflurane headspace gas was sampled. The isoflurane sample was then diluted in the glass syringe with 90 ml of clean air or CO_2 enriched clean air (4% carbon dioxide, 17% oxygen and 79% nitrogen (Scientific and Technical Gases Ltd (STG, Staffordshire, UK)). The concentration of the isoflurane was sufficiently low that no depletion of the H_3O^+ signal was observed.

Patients

Patients were recruited at the University Hospital Birmingham from the transplant assessment clinic. Of the 31 patients who consented to take part in the clinical trial, 12 went on to have liver transplant. Of these we were able to follow five patients for several weeks following surgery (F/M 3/2, mean age 50 years, min–max 36–58 years). Table 1 summarises the details of these five patients, with each patient being just identified by sex (F or M) and a number, which was used in our previous study and retained here for comparison [1]. Note that the number of samples and days at which breath samples were taken varies because of the availability and health of the patient. Patients were followed from the Intensive Care Unit after their transplant, through to the wards until their discharge. Some patients recovered and were discharged quicker than others so there are fewer data points for them.

In addition to taking breath samples from controls as described in [1], samples of air in the locations where the patients were in, were collected so that allowances for any background isoflurane could be taken into account.

Hospital room air was collected every time breath samples were taken using glass syringes (2 syringes for every sample), which were analysed in the same way as the breath samples. Our data relating to isoflurane concentrations in the hospital environment show that the maximum concentration for any of the sampling days was at 4 ppbv. There was one exception to this when 13 ppbv was measured for one patient, F4, who was in a small enclosed room with poor ventilation, and therefore the patient was almost certainly contaminating the air with her own breath. Thus any contribution to the isoflurane signal from the environment is negligible compared to that in the breath.

Table 1. Liver transplant patient details, including sex (female F, male M), age, location of post-transplant breath sampling (Out-Patient Clinic (OPC) or Ward), and number of days after transplant when breath samples were collected.

Patient ID	Age (year)	Location of post-transplant breath sample	Post-transplant breath samples: days after transplant
F2	49	OPC	3, 5, 130
F4	58	Ward	5–8, 11–15, 18, 58
F5	53	Ward	2–6, 9–12
M3	53	Ward	4, 7, 48
M7	36	Ward	2, 3, 6–8, 55

Breath sampling protocol

A detailed description of the breath sampling protocol used can be found in our earlier paper dealing with volatiles associated with liver disease [1]. In brief, capnography controlled sampling was used to collect only the alveolar phase of the breath. Subjects were seated and in a relaxed state and were asked to breathe normally into a gas tight respiratory system (Intersurgical Limited) containing an in-line CO₂ mainstream sensor connected to a fast-time response capnometer (Capnograph 1265 Novamatrix Medical Systems Inc.). A 100 ml glass syringe (Sigma-Aldrich) was coupled to the tubing using a 3-way luer-lock stopcock (Braun Medical Limited). When the alveolar plateau on the capnograph was observed, the breath sample was manually drawn from the subject's breath stream into the syringe. Three to four breaths samples were collected into each 100 ml syringe, and four replicates of these were taken for each subject. Glass syringes were used, because our tests showed that they minimize surface adsorption. We avoided the use of bags owing to potential problems associated with losses and ageing effects [15].

Within two hours after collection, breath samples were mass spectrometrically analyzed by PTR-MS, for which the syringes were maintained at a constant temperature of 40 °C using a heating bag (Infroheat, Wolverhampton). This was done to limit condensation, which could otherwise lead to volatile loss [16]. The outlet from each syringe was connected directly to the inlet of the PTR-MS. Within two hours we have found that there is no significant loss of isoflurane in the syringe.

Analytical measurements

PTR-MS is a technology designed to detect low concentrations of volatiles (less than parts per billion by volume). Hence it has found use in many analytical applications ranging from drug detection through to industrial pollution [17]. Details of the instrument used, a first generation PTR-Quad-MS (IONICON Analytik GmbH), and how it operates are described in detail in the literature [17, 18]. In brief, it exploits the reactions of protonated water with neutral volatiles (M), often leading to a protonated parent (MH⁺). If dissociative proton transfer occurs then it is not extensive in terms of the number of resulting product ions. The drift tube was maintained at 2.07 ± 0.01 mbar and temperature

of 45 ± 1 °C. For the breath analysis the drift-tube voltage was set at the standard recommended value of 600 V. For the headspace analysis the voltage across the drift-tube was changed from 400 V to 600 V at intervals of 20 V, E/N (96–138 Td).

Mass spectra of the breath samples were recorded and the areas under the spectral peaks converted to a volume mixing ratio (VMR) by use of a standard procedure that relies on the use of an experimentally measured reaction rate coefficient and reagent and product ion counts [17].

Results and discussion

Headspace analysis of isoflurane

No protonated isoflurane (m/z 185) was observed for any value of the reduced electric field. This agrees with the results of Wang *et al* who conclude that once formed the excited protonated parent spontaneously dissociates via various pathways [2]. However, in comparison to Wang *et al* SIFT-MS (thermal energy) measurements, we find substantial differences in product ions and their branching ratios. In our measurements we have observed primary product ions at m/z 51, m/z 67, m/z 117, m/z 163, and m/z 165. By taking into account isotopic abundance we have assigned these to be CHF₂⁺, CHFCl⁺, CF₃CHCl⁺, C₃F₄OCl⁺, and C₃H₂F₄OCl⁺, respectively. Another ion involving isoflurane is observed at m/z 181 which is assigned as a secondary (association) reaction of C₃F₄OCl⁺ with H₂O. The product ion percentage branching ratios at a commonly used reduced electric field of 138 Td are presented in table 2 and compared to the measurements of Wang *et al*.

Wang *et al* observed no m/z 51 ions. This almost certainly results from the differences in collisional energies and possibly the internal energy of the H₃O⁺ ions, both of which are considered to be at thermal energies in SIFT-MS systems. Certainly we only observe a significant branching ratio for this ion at 138 Td. The supplementary information (stacks.iop.org/JBR/10/046006/mmedia) provides details on the product ion branching ratios as a function of reduced electric field. This is provided because different PTR-MS users use different reduced electric fields and also because of the major differences observed in the product ions and branching ratios compared to the only other headspace analysis by Wang *et al* also observed product ions at m/z

Table 2. m/z values (dominant ion peak), proposed product ions and branching ratio percentages resulting from the reaction of H_3O^+ with isoflurane using PTR-MS (reduced electric field of 138 Td) and SIFT-MS [2].

m/z	Proposed product ion	% PTR-MS	% SIFT-MS [2]
51	CHF_2^+	12	—
67	CF_2HO^+	—	15
67	CHFCl^+	50	—
99	$\text{CF}_3\text{CH}_2\text{O}^+$	—	10
117	CF_3CHCl^+	6	30
119	$\text{CF}_3\text{CFHOH}_2^+$	—	25
147 ^a	$\text{CF}_3\text{HOCHClCH}^+$	—	10
163	$\text{CF}_3\text{CClOCl}^+$	24	—
165	$\text{CF}_2\text{HOCHClCF}_2^+$	8	10

^a In the paper by Wang *et al* the ion at m/z 147 is incorrectly given as $\text{CF}_2\text{HOCHClCH}^+$.

Note: The percentages have taken into account the ^{37}Cl isotope for the chlorine containing ions. For the PTR-MS studies the product ion branching percentages have an error of approximately $\pm 20\%$.

67, but assign it to be CF_2HO^+ rather than CHFCl^+ , m/z 99 (assigned to be $\text{CF}_3\text{CH}_2\text{O}^+$), which we did not observe, m/z 117 (CF_3CHCl^+ , in agreement with our assignment), m/z 119 ($\text{CF}_3\text{CFHOH}_2^+$), which we do not observe other than the ^{37}Cl isotope of CF_3CHCl^+ , m/z 147 ($\text{CF}_2\text{HOCHClCH}^+$), which we do not observe, and m/z 165 ($\text{C}_3\text{H}_2\text{F}_4\text{OCl}^+$).

Given that the product ion branching ratios were obtained using normal air in the drift tube, a question remains as to whether the presence of higher CO_2 concentrations in breath could affect these ratios through thermalisation effects as a result of collisions of H_3O^+ with CO_2 prior to reaction with isoflurane. This is an important issue for breath analysis using PTR-MS. Keck *et al* [14] have already demonstrated that higher CO_2 concentrations in the drift tube buffer gas of a PTR-MS enhances the concentration ratio of the protonated water dimer to protonated water and changes the mass spectra of key breath gases such as methanol, ethanol, 1-propanol, 2-propanol, acetone and isoprene. We therefore undertook a headspace analysis using CO_2 enriched (4%) air in the drift tube to determine if the product ion distributions associated with isoflurane would be altered when analysing the breath samples. We find that within experimental uncertainty no significance difference in product ion percentage branching ratios is observed between normal and CO_2 enriched air buffer gases (see supplementary information). There is still a question of changes in humidity [2], but that will be the subject of another study.

Longitudinal measurements of isoflurane in breath samples post liver transplant

By using the product ions and their branching ratios determined in the headspace analysis we can determine the isoflurane concentrations in breath by adding the intensities of all product ions that result from the reaction of H_3O^+ with isoflurane at a reduced electric

field of 138 Td, namely m/z 51, m/z 67 (69), m/z 117 (119), m/z 163 (165), and m/z 165 (167). It should be noted that the m/z 69 signal intensity cannot be used directly owing to the presence of isoprene in human breath. Therefore the total ion signal associated with CHFCl^+ is determined only from the m/z 67 ion intensity. However, it is important to point out that since isoflurane in breath results in a spectral peak at m/z 69 ($\text{CHF}^{37}\text{Cl}^+$) there are consequences for any PTR-MS studies which monitor isoprene in the breath following a surgical procedure during which isoflurane is used. The contribution of isoflurane to the signal intensity at m/z 69 was found to be significant and varied between 12% to 99% at the beginning of our longitudinal study, being dependant on the concentration found in a patient's breath.

Figure 1 shows the temporal changes in isoflurane concentrations for the five patients: F2, F4, F5, M3 and M7. The important observation is that we did not observe rapid decreases in isoflurane concentrations following surgery. This is different from the results of Yasuda *et al* who found that the elimination of isoflurane from the human body was fast [10], i.e. within one day the signal intensity had been observed to have dropped by about an order of magnitude, whereas in our study we still found high concentrations of isoflurane in the breath several days after the operation. Although the Yasuda *et al* study used healthy young male subjects (age 23 ± 3 years (mean \pm sd)), the observed temporal difference is still surprising given that the major elimination of isoflurane is through breath. Isoflurane is poorly metabolised (0.17%) by hepatic cytochrome P450 enzymes in the liver [19].

A possible explanation for the observed difference between our results and those of Yasuda *et al* could be associated with the physical condition of the subjects/patients involved and the duration of isoflurane administration. Yasuda *et al* volunteers were healthy and had a mean body mass index (BMI) of 21.7 kg m^{-2} , i.e. all were within the normal (healthy weight) range. In comparison F2, F4, F5, M3 and M7 had BMIs of 31.2, 32.4, 28.0, 32.5, and 35.0 kg m^{-2} , respectively. Thus our patients were either overweight or obese. Furthermore, Yasuda *et al* administered isoflurane for precisely 30 min, whereas our patients underwent surgeries that lasted between 5 to 7 h. Although isoflurane has a low blood/gas partition coefficient of 1.45 ± 0.12 (mean \pm SD) [20], it has a high oil/blood partition coefficient of 97, and therefore we propose that for our patients a significant amount of the isoflurane was absorbed into fat tissue, only to be slowly released after surgery. In support of this argument, it has been found that if isoflurane is administered for more than 2 h, as would be the case for our patients, the adipose tissue becomes saturated and then the emergence from the anaesthetic is prolonged [21].

Finally, we comment that of all our patients, patient M7 showed the fastest elimination of isoflurane even though he had the highest concentration in exhaled

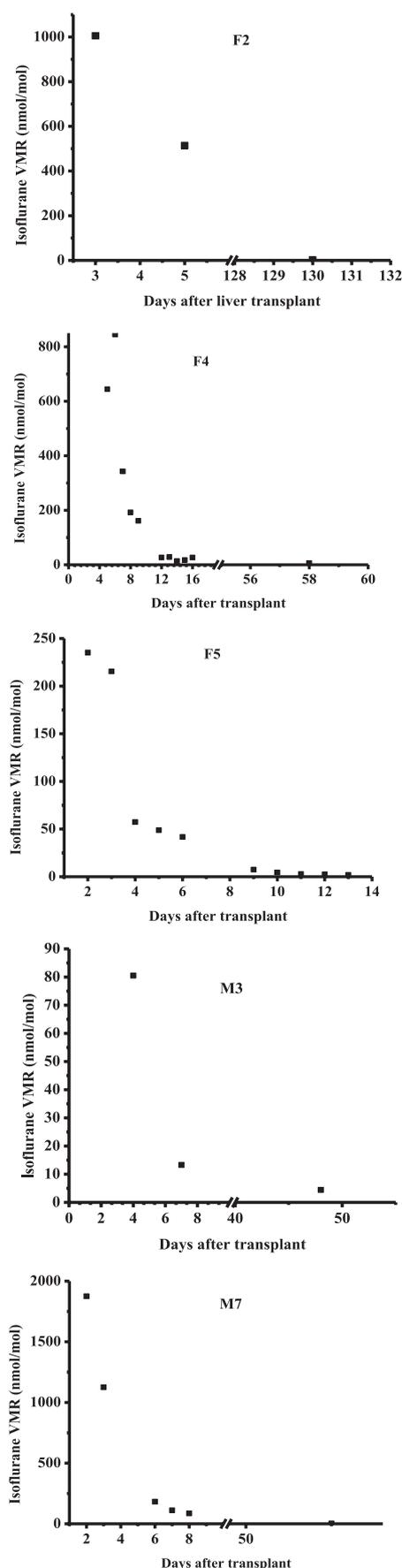


Figure 1. Longitudinal changes in volume mixing ratios (VMR) in nmol/mol for isoflurane for given days after liver transplant for patients F2, F4, F5, M3, and M7. Error bars are not shown as they are smaller than the data points.

breath after surgery and was the most obese patient. However, the elimination was still much less than found in the study of Yasuda's study. The rapid washout, in comparison to the other patients, could be a result of this patient being the youngest of the group involved at 36 years old. (F2, F4, F5 and M3 ages were 49, 58, 53 and 53 years, respectively.) M7 also recovered from surgery more quickly than the other patients in this group and was discharged within one week. However, with such a small number of patients we cannot state whether or not there is any correlation with age and perhaps gender with regards to isoflurane elimination from fatty tissue. More detailed studies are therefore required.

Conclusions

An aim of this study was to determine which product ions result from the reaction of H_3O^+ with isoflurane in order to monitor isoflurane in the breath. No protonated parent was observed, instead the protonated parent rapidly fragments to diagnostically useful ions with m/z values of 67 (69), 117 (119), 163 (165) and 165 (167), which are assigned to be CHFCl^+ , CF_3CHCl^+ , $\text{C}_3\text{F}_4\text{OCl}^+$ and $\text{C}_3\text{H}_2\text{F}_4\text{OCl}^+$ respectively. Using these ions, the wash out characteristics of isoflurane following liver transplant have shown that a patient will still have high levels of isoflurane in their blood even several days after the operation. This could have an impact on a patient's ability to drive and use machines after isoflurane anaesthesia administration, especially after a long operation, when isoflurane will be stored in fat tissue.

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Ethics approval

The regional ethics committee of Camden and Islington, London approved this study (REC reference: 13/LO/0952). Informed consent was obtained from each volunteer.

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PAPER



Limonene in exhaled breath is elevated in hepatic encephalopathy

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Keywords: breath analysis, cirrhosis, limonene, hepatic encephalopathy, PTR-MS, volatile organic compound

Abstract

Breath samples were taken from 31 patients with liver disease and 30 controls in a clinical setting and proton transfer reaction quadrupole mass spectrometry (PTR-Quad-MS) used to measure the concentration of volatile organic compounds (VOCs). All patients had cirrhosis of various etiologies, with some also suffering from hepatocellular cancer (HCC) and/or hepatic encephalopathy (HE). Breath limonene was higher in patients with No-HCC than with HCC, median (lower/upper quartile) 14.2 (7.2/60.1) versus 3.6 (2.0/13.7) and 1.5 (1.1/2.3) nmol mol⁻¹ in controls. This may reflect disease severity, as those with No-HCC had significantly higher UKELD (United Kingdom model for End stage Liver Disease) scores. Patients with HE were categorized as having HE symptoms presently, having a history but no current symptoms and having neither history nor current symptoms. Breath limonene in these groups was median (lower/upper quartile) 46.0 (14.0/103), 4.2 (2.6/6.4) and 7.2 (2.0/19.1) nmol mol⁻¹, respectively. The higher concentration of limonene in those with current symptoms of HE than with a history but no current symptoms cannot be explained by disease severity as their UKELD scores were not significantly different. Longitudinal data from two patients admitted to hospital with HE show a large intra-subject variation in breath limonene, median (range) 18 (10–44) and 42 (32–58) nmol mol⁻¹.

Abbreviations

ALD	Alcoholic liver disease
GC	Gas chromatography
HE	Hepatic encephalopathy
HCC	Hepatocellular cancer
ITU	Intensive treatment unit
LQ	Lower quartile
MS	Mass spectrometry
MHE	Minimal hepatic encephalopathy
OHE	Overt hepatic encephalopathy
OPU	Out-patient clinic
ppbv	Parts per billion by volume
PTR-MS	Proton transfer reaction mass spectrometry
UKELD	United Kingdom model for End-stage Liver Disease
UQ	Upper quartile
VOC	Volatile organic compounds
VMR	Volume mixing ratio

1. Introduction

Volatile organic compounds (VOCs) are produced in the body as a result of various metabolic processes and endogenous VOC analysis in the breath has been proposed as a potential diagnostic tool in human disease. Breath analysis has attracted clinical and scientific attention as a potential means for delivering non-invasive, real-time rapid screening and diagnosis of complex diseases. Recent overviews of the field are provided in a book published in 2014 [1] and in numerous review papers [2–6].

Breath VOCs have been measured in patients with liver disease in a number of studies [7–21] and several VOCs have been suggested as biomarkers for cirrhosis. In particular, sulphur compounds have been suggested as being associated with liver disease and are responsible for the characteristic odour of patient's breath called Foetor Hepaticus [19, 22]. Previous work

has included patients with various etiologies, different sampling methods and different analytical methods. It is, therefore, difficult to compare results. There is some agreement as to which potential biomarkers are associated with liver disease, but not all studies find the same volatile markers. As previously reported [7], these issues were addressed by conducting a two phase biomarker discovery study. Limonene, methanol and 2-pentanone were identified as biomarkers of liver cirrhosis. These VOCs not only discriminated cases from controls but were also significantly different before and after liver transplantation in a sub-group of patients who had received a transplant.

Pilot data [23] was obtained by our group prior to the published study from patients in out-patient clinic. Twelve had cirrhosis or fibrosis, four had neuroendocrine disease with liver tumours and five had well-functioning graft livers following previous liver transplants due to Hepatitis C virus. This study found that a monoterpene, which was assumed to be limonene, had elevated concentrations in the breath of patients with cirrhosis, and in particular, those with hepatic encephalopathy (HE). Limonene in the breath of patients with liver cirrhosis has been observed in two other studies using GC-MS [9, 10] and tentatively identified in a proton transfer reaction—time of flight-mass spectrometric (PTR-TOF-MS) study [13]. Limonene is a monoterpene of dietary origin, typically found in citrus fruits but also naturally occurring in many vegetables. There is no evidence to suggest that it can be produced in the human body. It is used in the food and drink industry to give a citrus flavor and in perfumes and air fresheners. As such, it is a ubiquitous exogenous compound and it would be difficult to avoid in the diet in general. Limonene is metabolized in the liver by the P450 enzymes CYP2C9 and CYP2C19 [24] to the metabolites perillyl alcohol, trans-carveol and trans-isopiperitenol. It has been noted that levels of the enzyme CYP2C19 are reduced in patients with cirrhosis and, that levels inversely correlate with severity of cirrhosis [25]. This presents the possibility that elevated levels of limonene in the body may be associated with liver disease owing to a reduced ability of the liver to produce the appropriate metabolic enzymes, as has already been noted [13].

The present work will discuss these results further in respect to HE, a co-morbidity of advanced liver cirrhosis. Comparisons of cirrhosis with and without hepatocellular cancer (HCC) and HE will be shown. Results of longitudinal data on patients with HE to examine intra-individual variation in these three VOCs of interest will also be shown.

HE is a spectrum of neuropsychiatric abnormality associated with liver dysfunction. Symptoms vary in severity depending on the stage of the illness and include confusion, lethargy, sleep disturbance, personality change and cognitive impairment. In its extreme form, it can lead to coma and even death [26]. Overt HE (OHE) can be diagnosed clinically, usually using

a scoring system such as the West-Haven criteria, and can be episodic or persistent. HE can affect the patient's mental functioning intermittently [27], so it is possible for them to pass a neurological test on the day of the examination, while still suffering from symptoms at other times.

Minimal HE (MHE) is difficult to diagnose and requires specialized testing. It is possible for patients to be suffering from undiagnosed MHE [28]. There is no gold standard for the diagnosis of HE and many scoring systems have been criticized for lack of objectivity [28]. Current recommendations [29] include using the clinical HE staging scale (CHESS) [30], the HE scoring algorithm (HESA) [31] or modified orientation log (MOD) to refine the West-Haven criteria but their widespread use has yet to be adopted.

Estimates of the incidence of MHE range from 20% to 84% of cirrhotic patients, depending on which testing methods are used [27]. HE not only degrades quality of life, but it means a poorer prognosis for the patient [32]. HE is thought to be caused by the action of neurotoxic substances including ammonia and manganese, which are usually metabolised by the liver, persisting in elevated concentrations. These toxins can then cause glutamine induced changes in astrocytes that lead to the clinical manifestations of HE [33, 34]. Usual treatment is through the use of laxatives and antibiotics which act to prevent the accumulation of toxins. In the advanced stages, patients must be admitted to hospital for management of episodes of OHE.

Breath analysis for HE has been investigated previously in two other pilot studies which used GC-MS in patients with cirrhosis [20, 35], but neither of these reported limonene as a marker for HE. One pilot study investigated HE using an e-nose [36], but this technique is unable to specify exactly which VOCs are responsible for the discrimination so it is not possible to determine whether it is based on limonene.

2. Methods

2.1. Patients, controls and hospital room air

Patients were recruited at the University Hospital Birmingham from either the transplant assessment clinic or in wards after being admitted with HE. The regional ethics committee of Camden and Islington, London approved this study (REC reference: 13/LO/0952). Written informed consent was obtained from each volunteer.

Thirty-one patients suffering from liver disease participated in the pre-transplant measurements (F/M 8/23, mean age 55 years, min–max 27–71 years). There were a number of etiologies and 11 patients had more than one condition. Full details of the patients and their diagnoses are given in our earlier publication which reported volatiles associated with liver cirrhosis.

For the longitudinal data, two patients were recruited having been admitted as in-patients suffering from HE. Patient M1 was a 54 years old male with a

primary diagnosis of alcoholic liver disease (ALD). He gave four breath samples during an admission for HE before his liver transplant, then three further samples after transplant. Patient M15 was also a 54 years old male with a primary diagnosis of ALD. He gave breath samples on seven different days during an admission for HE. He did not receive a transplant so gave no post-transplant samples.

For 28 pre-transplant measurements, breath samples from the patients' companions were taken. For the other three, two came alone to clinic and the other's companion declined to take part. Two additional controls were therefore recruited, one was a ward nurse and the other was a visitor to the hospital in ITU. These controls, while not related to the patient, had been in the same room for several hours prior to sampling so that confounding factors associated with any volatiles present in the room environment could be taken into consideration. In total 30 controls (F:M 23:7, mean age 44 years, min-max 20-75 years) took part in the study. The larger number of females in the control group arose because the majority of patients were men and often attended the clinic with their wives. While this means the control group is not ideally matched, there is no consistent evidence of dependences of volatile breath composition on sex [1, 37]. In confirmation of this, no correlation was found between sex and VOCs in either our control or patient groups. It was considered that inhaled VOCs have a greater potential to confound biomarker discovery. As the majority of the companions were living with the patients, they provided an ideal control for exposure of exogenous volatiles in the home environment. VOCs inhaled at home, or in transit, may well still be present in breath for hours or days after inhalation; the biological half-life of inhaled VOCs are not well known and in any case may be patient-specific [38].

All study subjects were asked to complete a detailed questionnaire which included details on their home environment, diet, smoking status, health and medications. Participants were asked if they had consumed fruit, fruit juices and fruit-flavoured drinks as a normal part of their diet, and, if so, to provide details on quantity and how long before the breath sampling these had been consumed.

Hospital room air was collected every time breath samples were taken so that any exogenous volatiles, such as isopropanol coming from hand gels, resulting in product ions at m/z 43 and m/z 61, could be taken into consideration. There are fewer room air samples than patient-control pairs because the same room was used for consecutive patient-control pairs on the same day in some cases.

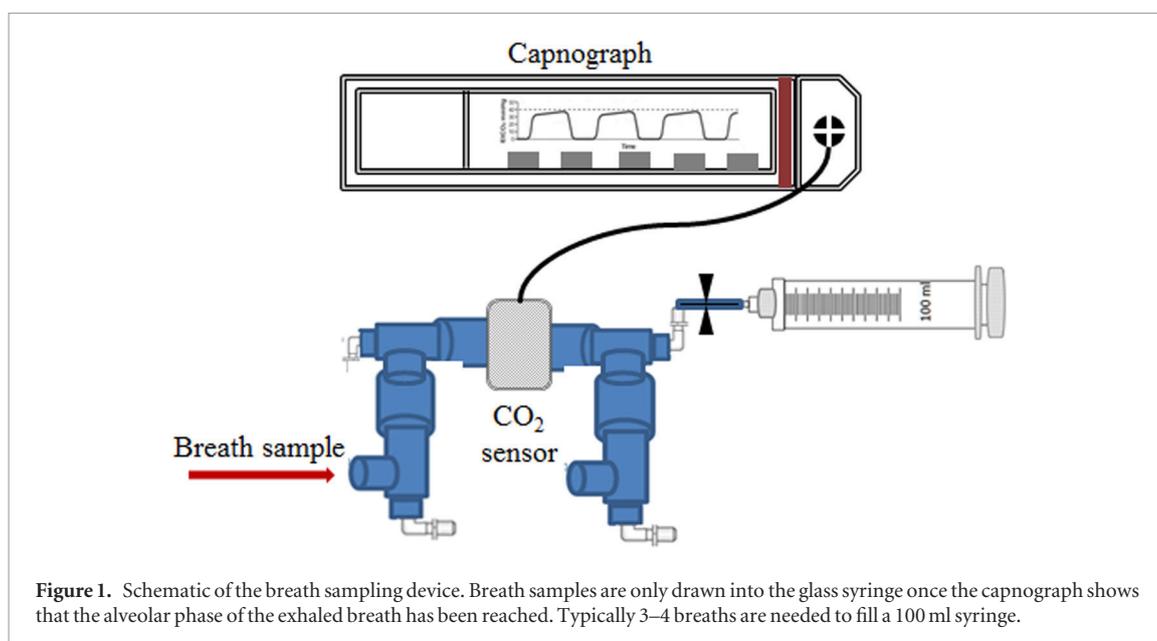
Diagnosis of HCC was based on previous investigations including radiology and histology. For analysis of HCC, the groupings are **HCC** ($N = 10$): those with at least one hepatocellular tumour, and **No-HCC** ($N = 21$): those with no hepatocellular tumours. HE status was assessed both from clinical diagnosis using the West Haven criteria and also from questioning of the

patient and the relative with whom they were attending clinic. Patients and relatives were questioned as to whether the patient was suffering from any symptoms of HE at the present time and were classed as having HE at the present time if they answered yes. Some patients and relatives reported having had HE in the past, sometimes following a trigger such as an infection, but that the patient had recovered and was not symptomatic on the day of the breath sample. These patients were classed as having a history but not currently suffering. The final group of patients had never had HE and did not have symptoms on the day of sampling according to their medical records and their own and their companions' assessment of their mental function. These patients are classed as 'No-HE'. For analysis of HE, the grouping are: 1. **HE-now**: suffering symptoms of HE on the day the breath samples were taken ($N = 11$); 2. **History-HE**: had suffered from HE in the past but were not suffering symptoms on the day of sampling ($N = 7$), and 3. **No-HE**: those who were not suffering symptoms on the day of sampling, nor had any history of HE ($N = 11$). All patients were ambulatory and were seen in clinic, apart from four who had been admitted to hospital for HE, one of whom had acute liver failure. Only one patient (ID code M15) had HE above grade 2. In the No-HE and History-HE groups, no patients were taking medication for HE. One patient in each of these two groups was taking lactulose, but in each case this was for constipation, not for symptoms of HE. Of the 11 HE-now patients, eight were taking medication for HE: three were on lactulose and rifaximin, one on rifaximin only and four on lactulose only.

2.2. Breath sampling protocol

There is no agreed standard for the collection of breath for volatile analysis, and uncontrolled breath sampling has been shown to be unreliable [39, 40]. Therefore, capnography controlled sampling was used to collect only the alveolar phase of the breath. Subjects were in a relaxed state throughout the measurements and were either in a seated or recumbent position. They were asked to breathe normally into a gas tight respiratory system (Intersurgical Limited) containing an in-line CO₂ mainstream sensor connected to a fast-time response capnometer (Capnogard 1265 Novamatrix Medical Systems Inc.). A 100 ml glass syringe (Sigma-Aldrich) was coupled to the tubing using a 3-way luer-lock stopcock (Braun Medical Limited). When the alveolar plateau on the capnograph was observed, a breath sample was manually drawn from the subject's breath stream into the syringe. Three to four alveolar breath samples were collected for each 100 ml syringe, and four replicates of these were taken for each subject. Glass syringes were used, because our tests showed that they have no contaminating volatiles. Figure 1 schematically shows the sampling system used.

After collection, the syringes were sealed using the luer lock fitting. They were transported from hospital to laboratory (a 10 min outdoor walk) in an opaque



storage box. Once at the laboratory, the syringes were placed inside an incubator set at 40 °C.

All samples were mass spectrometrically analyzed within 2 h of collection. For the measurements, syringes were taken out of the incubator and immediately placed into a purpose designed heating bag (Infroheat, Wolverhampton) maintained at a constant temperature of 40 °C in order to limit condensation, which could otherwise lead to volatile loss [40]. The luer stopcock was coupled to a Swagelok fitting and connected directly to the inlet of the analytical device, a proton transfer reaction mass spectrometer (PTR-MS). The inlet flow was set at 10–15 ml min⁻¹ and the drift tube and inlet lines were maintained at 45 °C. The syringes are gas tight and have minimal friction such that atmospheric pressure is sufficient to push the plunger in smoothly so that the breath sample is being drawn into the instrument at a constant flow.

2.3. Analytical measurements

PTR-MS is a platform technology designed to detect low concentrations of volatiles (less than parts per billion by volume). Hence it has found use in many analytical applications ranging from drug detection through to industrial pollution [1, 41–44]. Details of the instrument used, a PTR-Quad-MS (IONICON Analytik GmbH), and how it operates are described in detail in the literature [1, 40, 41]. In brief, it exploits the reactions of protonated water with neutral volatiles (M), usually leading to a protonated parent (MH⁺). If dissociative proton transfer occurs then it is not extensive in terms of the number of resulting product ions. Operational parameters used for this investigation were those previously reported [45, 46]. Namely, the drift-tube was maintained at a pressure of 2.07 ± 0.01 mbar and temperature of 45 ± 1 °C. The voltage across the drift-tube was set at 600 V, which is sufficiently high to reduce water clustering to reagent and product ions by collision induced dissociation.

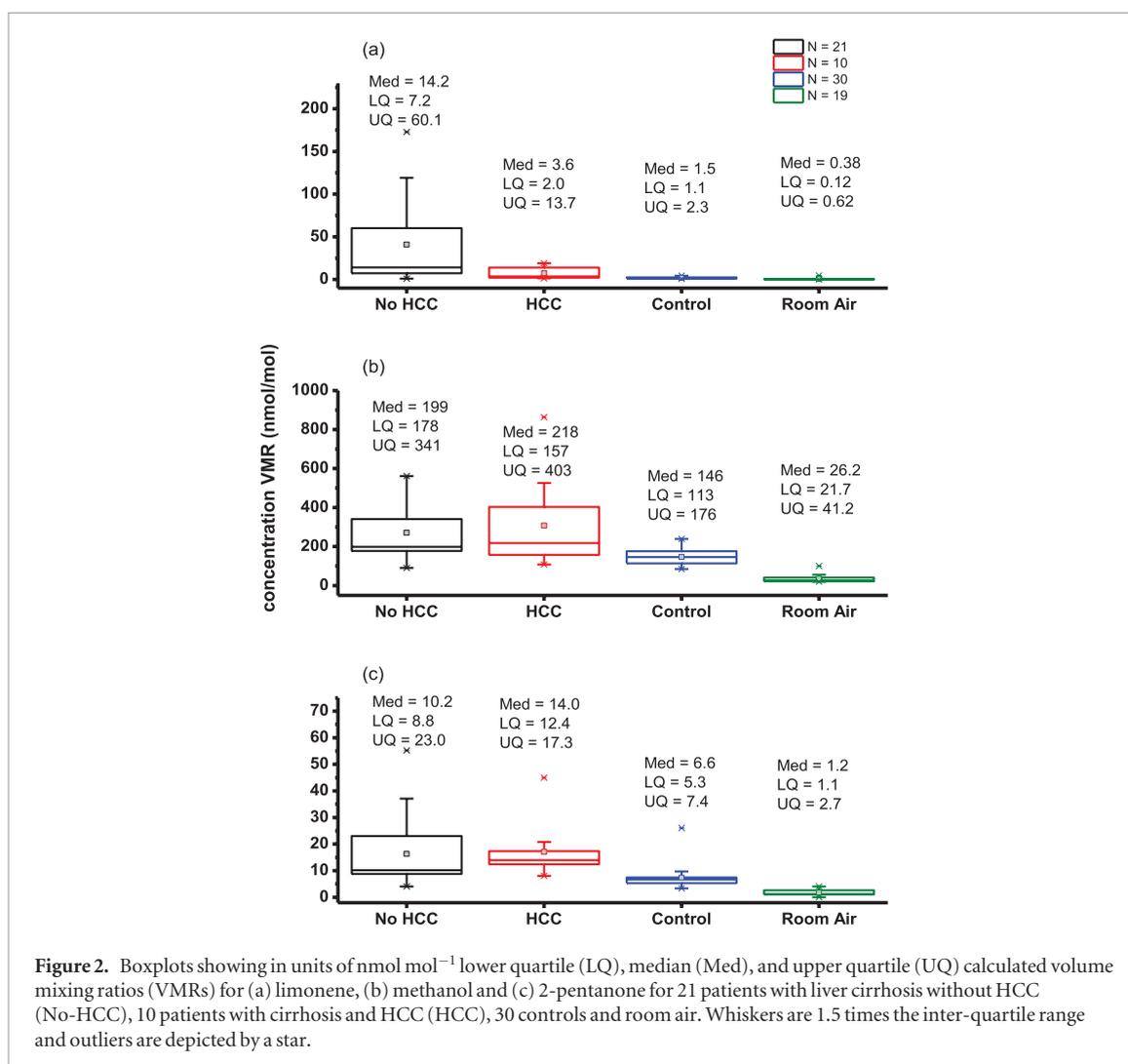
A *m/z* range of 20–200 amu was scanned with a dwell time of 0.5 s per atomic mass unit. Mass spectra of the breath samples were recorded from the average of three cycles for each of the four syringes, for every participant. These four spectra were averaged to provide one data set for each subject with the uncertainty expressed as the standard error of the mean for the four syringes.

The intensities of the product ion(s) associated with a given volatile were converted from normalized counts per second (cps) to volume mixing ratios (VMR) in units of nmol mol⁻¹. Normalisation was to 50 million cps of the sum of the area under the peak of the H₃O⁺ and the (H₃O⁺) · (H₂O) ions. Conversion to VMR was by use of a standard procedure that relies on a calculated, compound-specific, collisional reaction rate coefficient, determined using the effective translational temperature of the reagent ions [1]. It should be noted that PTR-Quad-MS has a mass resolution of approximately 1 amu so it is possible that other compounds or fragments could contribute to observed peaks.

To help identify product ions, pure samples of key volatiles were individually measured using PTR-MS to establish the *m/z* values of the product ions.

2.3.1. Statistical analyses

The data sets for each volatile of interest were assessed using a Shapiro–Wilks test and were found not to be normally distributed so non-parametric tests were used. IBM SPSS version 22 was used for all statistical analysis. Mann–Whitney U-tests determined which *m/z* values differed between the patients and controls in the original study, and to assess whether concentrations of volatiles were significantly different between HCC with non-HCC patients. A Kruskal–Wallis one way analysis of variance was used to compare the HE-now, History-HE and No-HE groups. All tests were done using a significance level of 0.95.



3. Results and discussion

3.1. Case control study

As previously reported [7], differences between patients and controls were found for four mass spectral peaks: m/z 33, m/z 81, m/z 87 and m/z 137. Peaks at m/z 81 and m/z 137 result from limonene; the protonated parent is at 137 and m/z 81 is a fragment ion (C_6H_9) [47, 48]. The area under the peaks for both m/z 81 and m/z 137 were summed and used to determine the concentration of limonene in the breath samples. PTR-MS is unable to differentiate isomers, and there is a possibility that other monoterpenes could have contributed to these peaks. However, previous work in humans has indicated that limonene is the most abundant monoterpene in human breath and blood [49]. GC-MS confirmation was performed with one of the breath samples to confirm the presence of limonene by colleagues at the Max Planck Institute in Mainz, Germany. The methodology has been previously reported [50, 51]. Limonene has also been shown to be elevated in the breath of patients with liver disease in other studies [10, 13].

The patient group has been further subdivided into those with and without hepatocellular cancer (HCC and No-HCC, respectively). Box plots of limonene, methanol (m/z 33) and 2-pentanone (m/z 87) for

patients with and without HCC, controls and room air are shown in figure 2. Figures for mean, (lower quartile (LQ)/upper quartile (UQ)) are given in the figure. There is a difference between patients with and without HCC only for limonene, with a Mann–Whitney U score of significance $p = 0.015$.

Figure 3 shows comparisons for the groups No-HE, History-HE, HE-now, controls and room air. Comparison of the three patient groups revealed that only one VOC, limonene, was able to successfully differentiate the groups with a Kruskal–Wallis score of significance $p = 0.001$. Figure 3 shows a clear difference between HE-now and History-HE. For No-HE, there is overlap with the HE-now group, but this is highly skewed by just three patients.

It was conjectured that the difference in means between breath limonene in the No-HCC versus HCC groups may be a reflection of severity of cirrhosis. Often patients are considered for transplant on the basis of the presence of a hepatocellular tumour while the rest of the liver is still compensating adequately. This conjecture is supported by analysis of the UKELD (United Kingdom model for End-stage Liver Disease) score for the groups. UKELD is a scoring algorithm which combines a selection of clinical chemistry values and gives a prediction of 1 year mortality [52]. It is used to select patients for

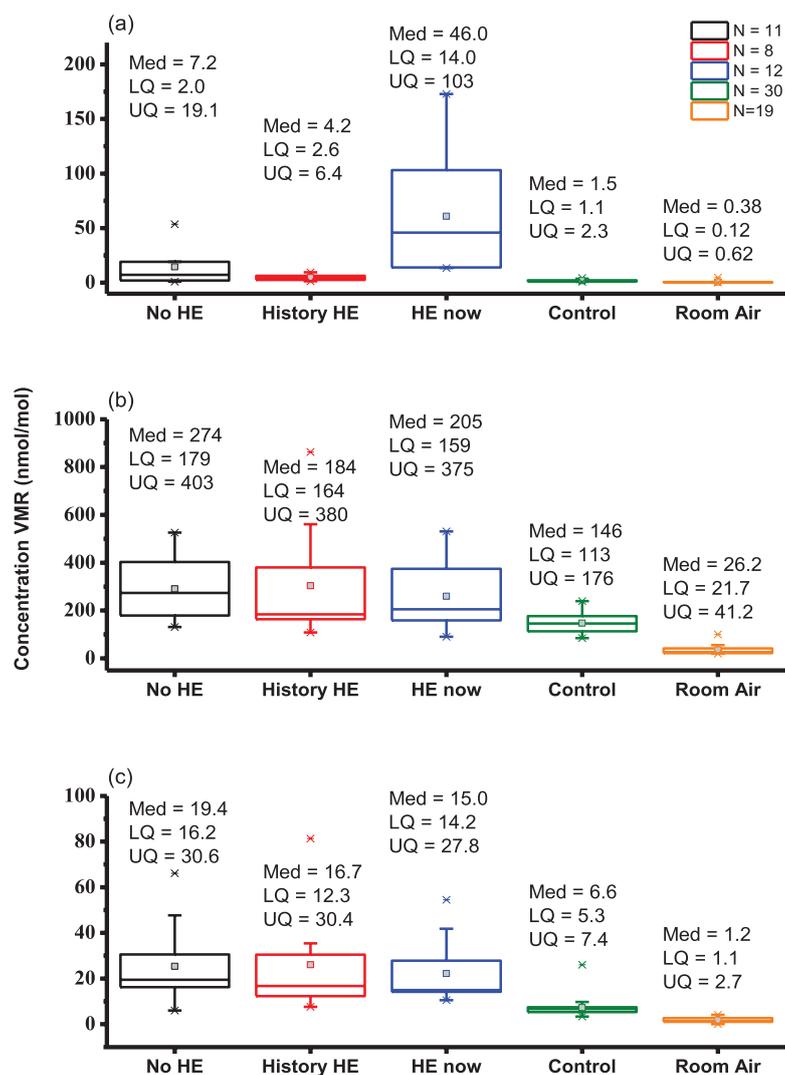
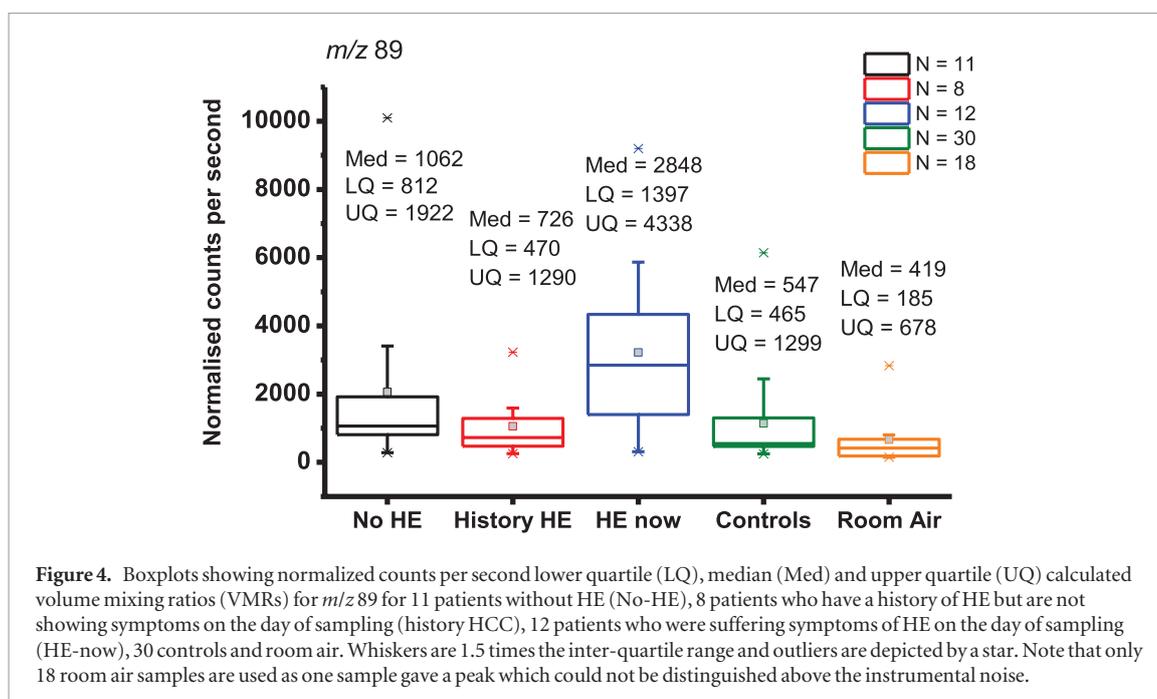


Figure 3. Boxplots showing in units of nmol mol^{-1} lower quartile (LQ), median (Med) and upper quartile (UQ) calculated volume mixing ratios (VMRs) for (a) limonene, (b) methanol and (c) 2-pentanone for 11 patients without HE (No HE), 8 patients who have a history of HE but are not showing symptoms on the day of sampling (History HE), 12 patients who were suffering symptoms of HE on the day of sampling (HE-now), 30 controls and room air. Whiskers are 1.5 times the inter-quartile range and outliers are depicted by a star.

transplant in the United Kingdom and patients are usually considered for transplant when their UKELD score is >49 , unless there is an additional indication for transplant such as HCC. The mean (range) of the UKELD score for the No-HCC and HCC groups is 54 (48–63) and 49 (43–56), respectively. These are significantly different (Mann–Whitney $p = 0.002$). A similar analysis was done for the HE classifications to determine whether patients in the HE-now group had more severe disease. UKELD scores for the three groups were 49 (43–57) for No-HE, 53 (46–63) for History-HE and 55 (51–62) for HE-now. There is no significant difference between the UKELD scores for the History-HE and HE-now groups (Mann–Whitney $p = 0.21$), but there is a significant difference between the No-HE groups and the HE-now groups (Mann–Whitney $p = 0.001$). It is perhaps not surprising that the group which has never experienced HE has a lower UKELD score than the group which was suffering symptoms at the time of sampling. However, the large difference in limonene

between the History-HE group and the HE-now group cannot be explained by a difference in severity as judged by UKELD score. Overall, as previously reported [7], there is no correlation between the concentration of breath limonene and UKELD score. A prospective observational multicentre study of 1348 patients in 29 liver units in eight European countries was recently undertaken to investigate risk factors for the development of HE and assess survival in patients with liver cirrhosis [53]. This found that previous HE was the most important risk factor for the development of HE, with a poor relationship between traditional precipitating factors such as bacterial infections, active alcoholism or gastrointestinal haemorrhage. Our present study has found that limonene is able to distinguish between History-HE and HE-now groups, which presents the possibility of a screening or monitoring tool to enable targeting of prophylactic therapies in at-risk populations.

As previously reported [7], there was no association between self-reported consumption of fruit/fruit juice



and elevated breath limonene. Studies by us (unpublished) have shown that in normal healthy people, consumption of pure, concentrated orange juice is followed by a small increase in breath limonene which returns to baseline within half an hour. There is nothing in the diet or previous 24 h diet of the patients which would explain elevated breath limonene.

Khalid *et al* suggested isothiocyanato-cyclohexane as indicating the presence of HE, and methyl vinyl ketone as indicating absence of HE within a group of alcoholic cirrhotics [35]. They used a presence/absence analysis in which absolute concentrations were not measured; rather only the presence or absence of a compound above three times the signal to noise ratio was compared across groups. Using this analysis, our previous results would not have shown a difference between cases and controls because limonene was also observed in controls, albeit at very low concentrations. Limonene has also been found in the blood and in the emissions from skin of healthy volunteers, and can be considered a normal volatile emission from healthy humans [54, 55]. A previous study using GC-MS to examine the normal volatile constituents of human breath reported limonene in the venous blood and breath of all 28 subjects [49]. The mean (range) concentration in breath was 1.46 ppbv (0.27–7.42), which is similar to our findings.

To our knowledge, Vollnberg *et al* [20] remains, until now, a conference abstract rather than a full paper so insufficient details are known to make a comparison, but they noted acetoin (3-hydroxybutanone) and 2-pentanone as having predictive utility for HE [20]. The present study has found 2-pentanone as predictive for the presence of cirrhosis versus controls, but did not find a difference between those with and without HE. Participants had been asked to indicate when they last had something to eat or drink, but there was

no association with time since last meal and concentration of 2-pentanone. Acetoin would give a protonated parent peak at m/z 89. In our initial case-control comparison, m/z 89 was significantly different between patients and controls. It did not, however, show a difference between patients pre- and post-transplant so it was discounted as being a useful diagnostic volatile for liver cirrhosis. Figure 4 shows the box plot for groupings of HE status for m/z 89. Only normalized counts per second are shown as we are not confident about assigning an identity to this peak. The HE-now group is significantly different from the other groups, but the variation in room air is large and it overlaps with all other groups. An examination of the 12 patients who had a transplant shows that the concentration of m/z 89 in patients with and without HE is not significantly different. It is important to bear in mind that isomeric and isobaric compounds cannot be distinguished in our system and a signal from acetoin may be confounded by the presence of another volatile which produced a product ion at the same m/z .

3.2. Longitudinal study

Two patients (ID codes M1 and M15) who had been admitted suffering with HE were followed over the course of their stay in hospital. Only M15 had high grade HE, and had very low mental capacity. M1 received a liver transplant during the course of the study so his results show four pre-transplant samples and three post-transplant. Figure 5 shows concentrations of limonene, methanol and 2-pentanone in breath and room air over the course of the measurement period. For M1, study days 1, 2, 3 and 4 correspond to 47, 44, 42 and 40 d prior to transplant, respectively. Study days 5, 6 and 7 correspond to 28, 29 and 33 d after transplant. All measurements were taken in wards, except for study day 7 which was taken in the out-patient clinic. For M15,

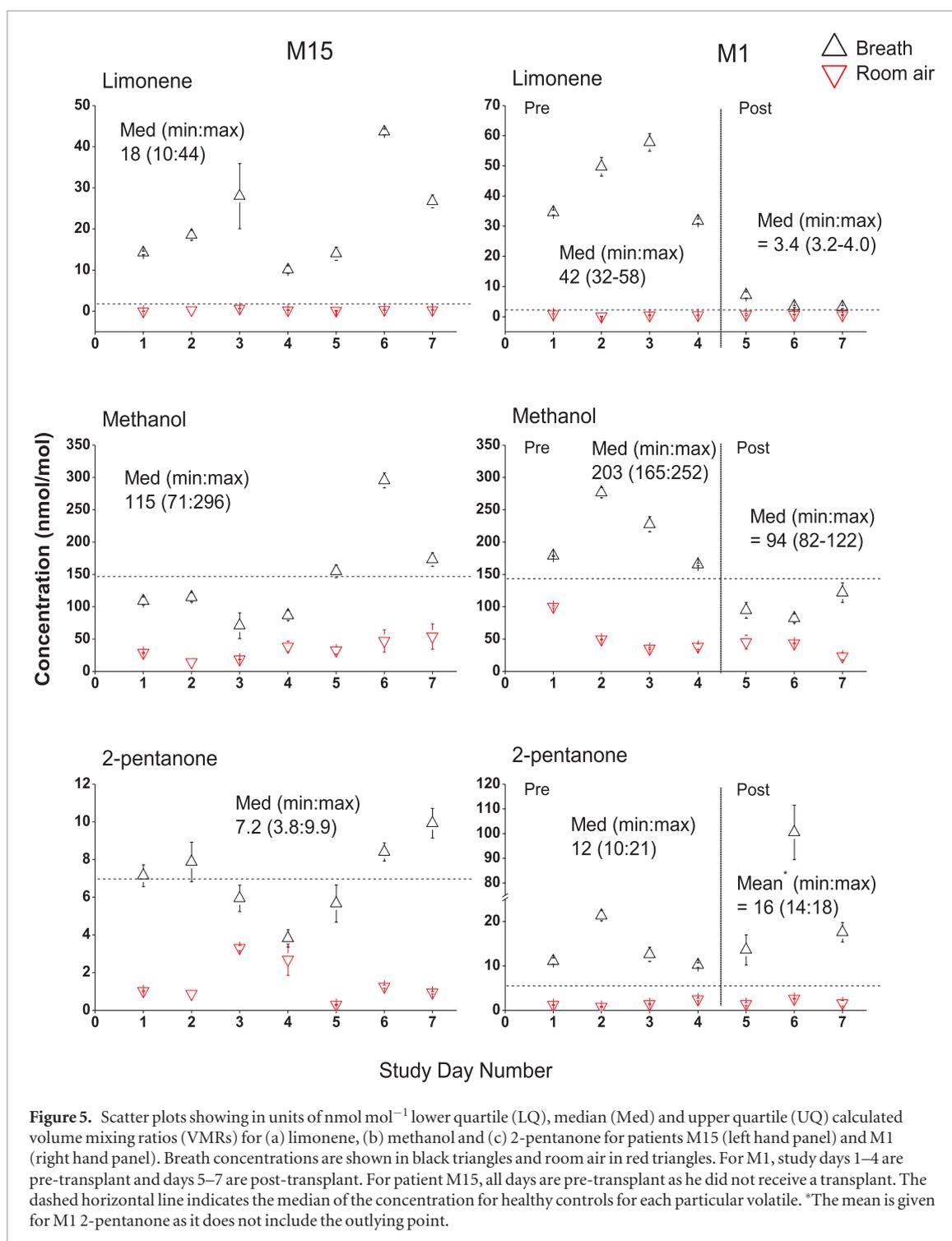


Figure 5. Scatter plots showing in units of nmol mol^{-1} lower quartile (LQ), median (Med) and upper quartile (UQ) calculated volume mixing ratios (VMRs) for (a) limonene, (b) methanol and (c) 2-pentanone for patients M15 (left hand panel) and M1 (right hand panel). Breath concentrations are shown in black triangles and room air in red triangles. For M1, study days 1–4 are pre-transplant and days 5–7 are post-transplant. For patient M15, all days are pre-transplant as he did not receive a transplant. The dashed horizontal line indicates the median of the concentration for healthy controls for each particular volatile. *The mean is given for M1 2-pentanone as it does not include the outlying point.

if study day 1 is taken to be on day 1, study days 1–7 correspond to days 1, 2, 6, 7, 8, 9 and 15. The patient was discharged on day 15.

The main thing to note is the high degree of variability from day to day. Despite this, there is still a measurable drop in breath limonene and methanol following transplant for M1. There is what appears to be an anomalous result for 2-pentanone for M1 on study day 6, which is, at present, unexplained as it was also not present in the room air. A corresponding peak in the mass spectrum at m/z 89 suggests that it may have been a chlorinated compound. It is possible that the patient inhaled an environmental contaminant at high

concentration some time before the breath test and it was still washing out.

The variability for M15 is more pronounced than for M1. This patient had grade 3 or 4 HE for the first 6 d of the study. On study day 7 his acute HE had resolved sufficiently to be discharged later that day, although it is likely that he was still suffering from minimal HE as he had a recent history of repeated hospital admissions. There were difficulties in obtaining samples from this patient on some days of the study as his mental incapacity was such that he was unable at times to comply with instructions to breathe into the apparatus. It was, therefore, very difficult to obtain reliable end-tidal

samples and it is possible that some of his samples were contaminated with dead-space air. From this limited evidence, it does not appear that breath limonene can predict daily variations in symptoms of HE for an individual patient.

4. Conclusions

Limonene, methanol and 2-pentanone have previously been shown to be elevated in the breath of cirrhotic patients compared with controls and in patients pre-transplant compared with the same patients after transplant [7]. Limonene, but not methanol or 2-pentanone is able to discriminate patients currently suffering symptoms of HE from patients with no current symptoms. Patients with HCC had lower levels of limonene than those without HCC but this may be a reflection of the fact that patients with HCC are usually considered for transplant at an earlier stage of liver decompensation. This is supported by the fact that the UKELD scores for patients with HCC were significantly lower than patients without HCC.

It is possible that breath limonene is higher in more severe liver disease, and because HE is a complication of advanced disease, high breath limonene may simply be a proxy for advanced disease. However, there was no correlation between limonene and any clinical chemistry value. Nor was there a correlation with UKELD score. This leaves the possibility that limonene is itself a causative agent in HE. Limonene is highly lipophilic; this, together with its low molecular mass, make it possible for it to cross the blood–brain barrier. There have been cases of acute poisonings with tea tree oil, which is composed of terpenes and terpinoids, including limonene at low levels. The symptoms were confusion, drowsiness and coma [56]. These are strikingly similar to the symptoms of HE, and suggest that molecules from the oil crossed the blood–brain barrier to cause neurological symptoms. In all cases, the effects were transient and the patients recovered within a matter of hours. While this was not pure limonene, terpinoid oils are composed of similar units and it is not known what biochemical reactions will occur if limonene is resident in the human body for long periods. While limonene has not been shown to be acutely toxic in humans [57], information is lacking about chronic exposure. If a cirrhosis patient lives with a high blood concentration of limonene for months or even years, it is not known how this will affect their health.

It has been shown that limonene concentrates in human breast tissue in women given a large daily dose of limonene for 2 weeks before undergoing surgery for breast cancer [58]. Breast tissue is composed mainly of fat [59] so this lends weight to our previously stated hypothesis [7] that limonene, being highly lipophilic, accumulates in the fat tissue of patients with liver disease. The wash-out curves for patients who gave breath samples on consecutive days in the 3 weeks after liver transplant presented in our previous paper [7] support this hypothesis. The brain has a high fat content, so it

would be expected that limonene would partition into it should it cross the blood–brain barrier.

The main limitation of this study is the small sample size. The practical difficulty of requiring a patient with severe HE to comply with instructions would have to be addressed in future studies. The evidence presented here suggests that breath limonene is an exogenous compound which may serve as an indicator of an elevated risk of HE in patients with cirrhosis of the liver. The hypothesis that limonene is itself in the causative pathway of HE remains to be explored.

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Conflicts of interest

All authors have declared no conflicts of interest.

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