CRANFIELD UNIVERSITY

CRANFIELD HEALTH

MSc by Research

Academic Year 2008-2010

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Analysis of Volatile Marker Compounds in Body Fluid Samples from Patients with Gastrointestinal Disease

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September 2010

Abstract

Causes of gastrointestinal diseases such as ulcerative colitis (UC) Crohn's disease (CD) and irritable bowel syndrome (IBS) are not yet completely understood and clinical investigation for diagnosis is invasive, costly and time consuming. Disease may originate from the host or in combination with commensal enteric bacteria, notably sulphate reducing bacteria. Examination of volatile compounds from clinical samples may provide indicators and better understanding into aetiology of these conditions and provide biomarkers for individual disease.

A total of 91 volunteers from Addenbrooke's Hospital consisting of controls and diseased patients pre and post treatment provided clinical samples of breath, blood, urine and faeces. Analysis using GC-MS and SIFT-MS provided a wealth of compounds for identification as potential markers which can be used as biomarkers to provide a rapid non-invasive, cost effective method for monitoring and disease diagnosis.

Clinical samples of breath, blood, urine and faeces from all 91 volunteers were analysed by GC-MS and compound identification was made for each sample with Automated Mass spectral Deconvolution and Identification System (AMDIS) and comparison with the National Institute of Standards and Technology library (NIST v.2). Compounds were identified for all the clinical matrices providing a vast pool

of information for data processing. From initial observation of all the clinical matrices faeces produced the largest numbers, typically several hundred on a single chromatogram. A method of data reduction for manageable data handling was required and a careful selection criterion to make accurate choices of compounds was produced. The three methods for selection to provide a list of 'candidate compounds' were:

1. Most abundant
2. Most probable based on learned literature
3. Visual inspection of a representative subset of chromatograms

The selection process provided a short-list of thirteen 'candidate compounds' chosen from pre and post treatment faecal samples. Initially the compounds were compared using Box and Whisker plots for each control and disease groups to show outliers, quartiles and median ranges. Kruskall-Wallis analysis of variance showed that the ethyl esters of propanoic and butanoic acid, butanoic acid, butanioc acid methyl ester, 3-methyl butanoic acid, 1-butanol, 1-propanol and indole were statistically significant. Mann-Whitney U test was performed on these compounds to show pair wise comparisons to identify differences between groups. All eight compounds showed a significant difference between pre treatment CD and the control group, and the number of compounds that had significance was less for UC and IBS. Far fewer significant differences were found between pairs of disease or disease/control groups for post treatment than

pre disease indicating the post treatment groups were more similar to the control groups and that these compounds are potential indicators for disease. The same candidate compound selection was made for breath, blood and urine and the same statistical analysis performed as for faeces. Unlike the faecal samples it was found that these compounds were absent in many of the breath, blood and urine samples and generally not often detected, therefore demonstrating that faeces provide the most reliable range of compounds for detection of disease states.

From the eight compounds two were chosen for GC-MS method development. Butanoic acid methyl ester (methyl butyrate) and propanoic acid ethyl ester having similar retention times between12 and 13 minutes. Pure samples of each were easily obtainable from Sigma-Aldrich. A range of dilutions of each compound was made to provide regression lines from which the lower limit of detection (LLOD) could be calculated. Accurate quantification can then be made from the regression line for each compound. The modified method provided a more rapid analysis time which would be favourable for a diagnostic test. These compounds showed potential as diagnostic markers for disease. The other six statistically significant candidate compounds could be treated in the same way to provide quantification, LLOD and retention time.

Acknowledgements

I would like to thank the following people for their help.

Members of the Wellcome Project for giving me the opportunity to study this subject in such detail.

- Professor John Hunter
- Professor Jeff Cole
- Dr Weining Ja
- Sarah Basser
- Claire Dawson
- The Wellcome patient volunteers for providing the samples!

To my supervisors for their patience and sharing of knowledge and the belief that I could get this far.

- Dr Claire Turner
- Dr Christopher Walton

Internal Examiners for listening to my first attempts.

- Dr David Aldred
- Dr Kal Karim

Colleagues for their tips, encouragement and keeping me on track.

- Mitesh Patel
- Dave Pitts
- Veronica Brown
- Dr Conrad Bessant

- Dr Michael Cauchi
- Rosemary Burns, (also doing an MRes and support for one another).

Family for their support and putting up with me and especially,

- My son James, who coincidentally was diagnosed with UC whilst I was doing the project and put up with me asking him questions.

Abbreviations

AMDIS	Automated Mass spectral Deconvolution and Identification System
ATD-GC-MS	Automatic Thermal Desorption Gas Chromatography Mass Spectrometery
CA	California
CARD 15	Caspase recruitment domains gene number 15
CD	Crohn's Disease
CHF	Congestive heart failure
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
E-nose	Electronic nose
GC	Gas Chromatography
GC-MS	Gas Chromatography Mass Spectrometry
GI	Gastrointestinal
HTRS	Human Tissue Record Sheet
HCN	Hydrogen cyanide
H_2S	Hydrogen sulphide
HTRS	Human tissue record sheet
IBD	Inflammatory Bowel Disease
IBS	Irritable Bowel Syndrome

Ltd	Limited
MAP	*Mycobacterium avium* subspecies *paratuberculosis*
MB	Methanogenic bacteria
MMOS	Mixed metal oxide semiconductor
MOS	Metal Oxide Semiconductor
MOSFET	Metal oxide semiconductor field effect transistor
MRB	Methane reducing bacteria
MRes	Masters by Researach
MUI	Multi Ion Mode
MUC	Mucin gene
m/z	mass to charge ratio
NIST	National Institute of Standards and Technology
NO	Nitric oxide
NOD2	nucleotide-binding oligomerization domain containing 2 gene
PC	Personal Computer
PDA	Personal Digital Assistant
ppb	parts per billion
ppm	parts per million
PTR-MS	Proton Transfer Reaction Mass Spectrometry
®	Registered trade mark
RHOD	Rhodanese
RT	Retention Time
SAW	Semi acoustic wave

SCFAs	Short-chain fatty acids
SIFT-MS	Select Ion Flow Tube Mass Spectrometry
SRB	Sulphate Reducing Bacteria
TD	Thermal Desorption
TMT	Thiol Methyltransferase
TIC	Total Ion Chromatogram
VOC	Volatile Organic Compound

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Chapter 1 and Literature Review

1.1 Introduction

Gastrointestinal disease is identified as a group of debilitating disorders that often leads to chronic ill health and can severely reduce quality of life and sometimes life expectancy. Aetiology of diseases in this investigation is, to date, poorly understood and treatment for patients is often not very effective. This Masters by Research (MRes) was undertaken as part of a larger project being funded by the Wellcome Trust principally for the investigation of sulphate metabolism by sulphate reducing bacteria in inflammatory bowel disease (IBD). It was hypothesised that IBD causes changes or is caused by changes in gut flora and that these changes can be detected through the signature of volatile organic compounds (VOCs).

VOCs are carbon containing compounds commonly produced by metabolic processes and found in the environment in the form of paint and adhesives that evaporate readily at ambient temperature and pressure. They are present as trace compounds in breath and above the headspaces of clinical samples and once captured can be detected by analytical equipment. Identification of specific

VOCs might enable them to be used as biomarkers for the development of alternative diagnostic tests.

IBD covered in this research appears to have a dysfunction in the metabolism of suphates and sulphides with the production of hydrogen sulphide (H_2S) which is cytotoxic in humans. This causes the epithelium to become inflamed resulting in structural damage to the gastrointestinal (GI) tract (Picton, *et al*., 2007).

Crohn's disease (CD), ulcerative colitis (UC), and irritable bowel syndrome (IBS) are collectively known as IBD and although each is aetiologically different, the symptoms can often appear similar. The causes of gastrointestinal disease have not yet been fully identified, but may involve a genetic susceptibility of the individual concerned or mutation or genetic modification of gastrointestinal bacteria. Environmental factors, lifestyle and stress appear to exacerbate the conditions and combinations of each of the factors involved make it difficult to single out a primary cause (Walter,*et al*., 2006).

Current investigation and diagnosis (See Section 1.4.6) for IBD is often time consuming, invasive and costly and a cheaper non-invasive alternative would be a great advantage. Technology into identification of VOCs has greatly improved, and a wealth of research has been carried out, potentially leading to new methods of disease monitoring and diagnosis (Smith and Španěl, 1996). Malodour from breath has been used as a health indicator for hundreds of years,

by physicians observing the smell. Advanced technology has identified biomarkers from breath, potentially for a rapid diagnosis of identifying disease by using portable devices in clinical practices (Miekisch and Schubert, 2006). Analytical tools, such as Gas Chromatogram-Mass Spectrometery (GC-MS) and Selected Ion Flow Tube-Mass Spectrometery (SIFT-MS) can detect many hundreds of volatile compounds from breath and other clinical samples such as blood and urine (Cao and Duan, 2006). Technology is moving towards VOC analysis as a potential route; portable monitoring devices may be seen in outpatient clinics to measure trace compounds from breath samples or from headspace above a clinical sample to provide a rapid and non-invasive test for IBD identification.

The samples for this study were provided by volunteers from Addenbrookes Hospital in Cambridge. Details are described in Chapter 2, Section 2.1.1. A list was subsequently sent to Cranfield with information on age and gender which has been interpreted and added to the Results and Discussion Chapters.

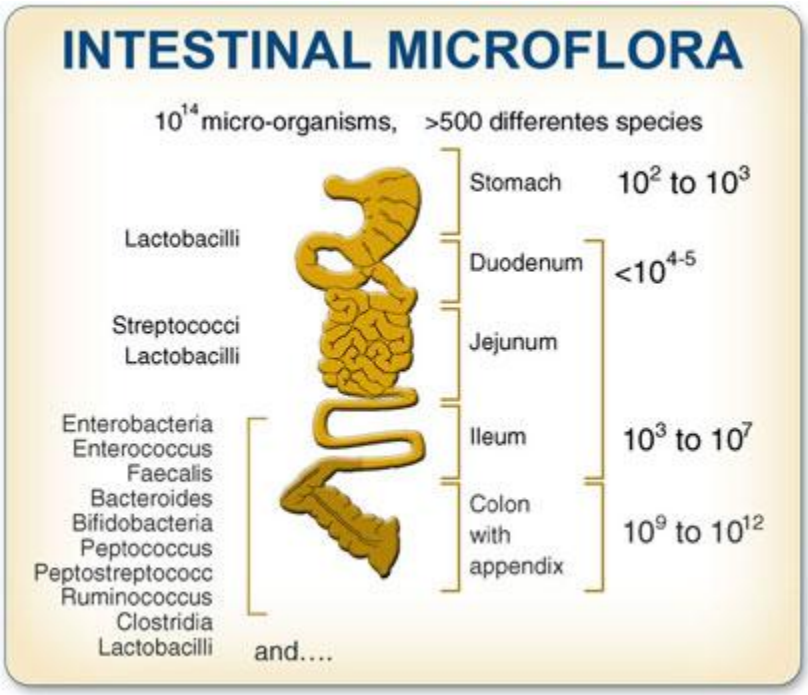
1.2 Healthy Gut

We generally do not take much notice of our digestive systems until something goes wrong; food goes in at one end and faeces come out of the other. The

gastrointestinal tract is designed to process food into simple soluble substrates to provide heat and energy for growth, and repair to the body. Digestion starts in the mouth where teeth cut and grind food, with enzymes such as amylases present in saliva to break down large molecules. The stomach processes food with peristaltic motion from its muscular walls and secretes gastric juices turning the food into a semi-fluid state called chyme. This partially digested food enters the first part of the intestine, the duodenum, where bile salts and pancreatic juices break down starches, proteins and fats into smaller molecules. The resulting watery emulsion, chyle, containing amino acids, fatty acids and glucose enters the small intestine where absorption of these small molecules occurs through the gut wall and into the bloodstream (Roberts,1976). The large intestine or colon, mainly absorbs water and some vitamins, and active transport moves sodium and chloride ions through the luminal wall. It is this part of the gastrointestinal tract that this project proposes to study. The structure of this organ has been well investigated, but there are still gaps in our knowledge of how it functions, identification of all gut bacteria and what causes disease to occur.

The colon is the final part of the digestive tract comprising of a convoluted tube of about 1.5 metres in length. Retention or transit time for food in the gastrointestinal tract is on average 60 hours for normal healthy individuals, (Cummings and Macfarlane, 1991).

1.2.1 Gastrointestinal Bacteria



Key for Figure 1

Numbers represent micro-organisms/g luminal content

Figure 1 Diagram showing type of intestinal flora found in each section of the GI tract (from web page[1])

The environment within the large intestine is favourable to vast numbers of living bacteria with densities of up to 10^{12} cells/g of luminal content and up to 500 different species maintaining a dynamic microbial ecosystem of which two thirds of faecal matter is made up from bacteria (Guarner and Malagelada, 2003). As shown in Figure 1 the majority of bacteria and the greatest diversity of species are found in the colon. Many different types of gut bacteria have been identified but collecting a sample from a particular region of the GI tract as a practical

application of obtaining an 'uncontaminated' sample is not straightforward. It may be easy to obtain an excreted faecal sample to study, but obtaining samples from other areas of the gut is invasive as it would require a colonoscopy. Gut microorganisms are non-spore forming anaerobes and the most abundant are *Bacteriodes, Eubacterium* and *Bifidobacterium*. Also a number of gram-positive anaerobes such as *cocci*, *clostridia*, *enterococci* and *enterobacteriaceae* are common (Simon and Gorbach, 1984). Hydrogen metabolism in the gut often takes one of two routes: as observed from figures from the industrialised world, between 30 and 50% of people harbour methanogenic bacteria and others carry sulphate reducing bacteria. As a consequence people that have more methanogenic bacteria produce methane while sulphate reducing bacteria carriers produce hydrogen sulphide which is extremely toxic to humans, probably because it reacts with essential proteins (Tangerman, 2009). Strains of *Escherichia coli* have been observed to be more common in patients with IBD than in control patient groups (Xavier and Podolsky, 2007).

At birth the large colon is sterile, but soon becomes colonised with micro-flora from the mother and surrounding environment, determining the individuals' lifelong gut flora make-up. The balance between the species and numbers of micro-organisms generally remains in equilibrium throughout life and when the individual is in good health. Normally, the microorganisms have a symbiotic relationship with the host and many of which have been identified as having

useful functions in maintaining health by assisting the immune system. However, for some people there are factors that affect this balance, varying from stress to a genetic susceptibility (this can be the genetic make up of the host or from the resident bacteria), or from environmental triggers such as allergy to foods, medication, diet or infection resulting in bacterial species and numbers drastically altering and remaining so for periods of time. This often has a deleterious effect on the health of the gut lining and consequently the general health of the person.

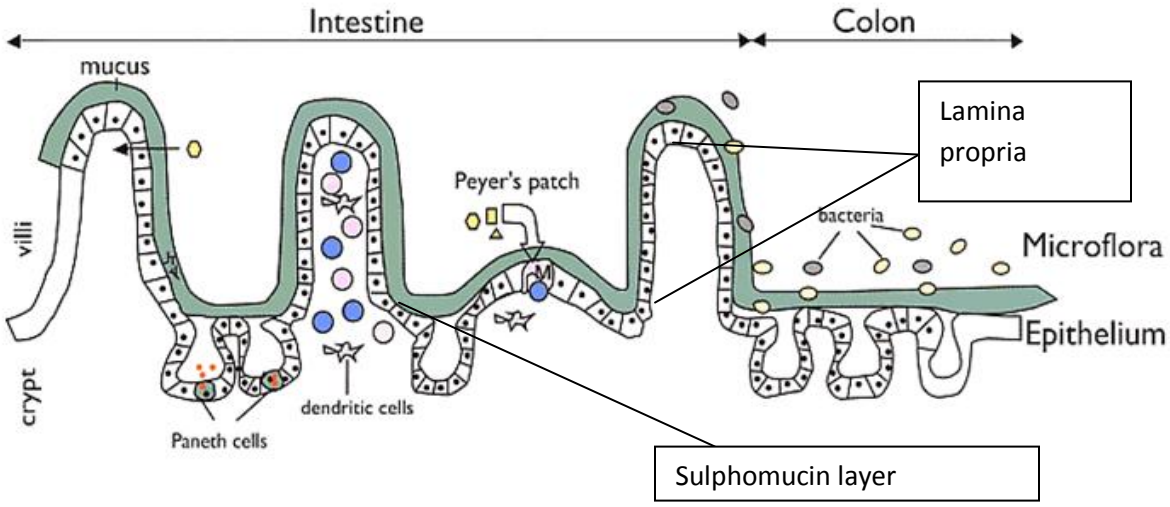


Figure 2 Cross-section diagram of the gut wall showing mucin layer (in green) covering the epithelial layer and bacteria most abundant in the colon (From Web page[2])

1.2.2 The Sulphomucin layer and the Immune Response

As seen from Figure 2 sulphomucin is a lubricating substance, providing a protective mucosal barrier for the luminal epithelial wall against direct contact from the passage of digestive material, digestive enzymes, bile salts, bacteria and the products of microbial fermentation. Protection is also required from digested materials such as hypertonic saline, ethanol and non-steroidal anti-inflammatory drugs (Allen, 1989)

The composition of sulphomucin is a protein backbone with large numbers of oligosaccharides attached at different points to the backbone with disulphide bridges as seen from the diagram of the sulphomucin molecule in Figure 3. There are two groups of mucin, one that is secreted and the other is membrane bound, the chemical composition also varies as some have acidic and some have sulphate groups attached to them. There are four major mucins present in the colon and rectum. They are MUC1, MUC2, MUC3 and MUC4 and have different functions and are apparent at different stages in life. Small peptide chains or trefoils within the mucin are involved in epithelial protection and mucosal healing (Shirazi, *et al.,* 1999).

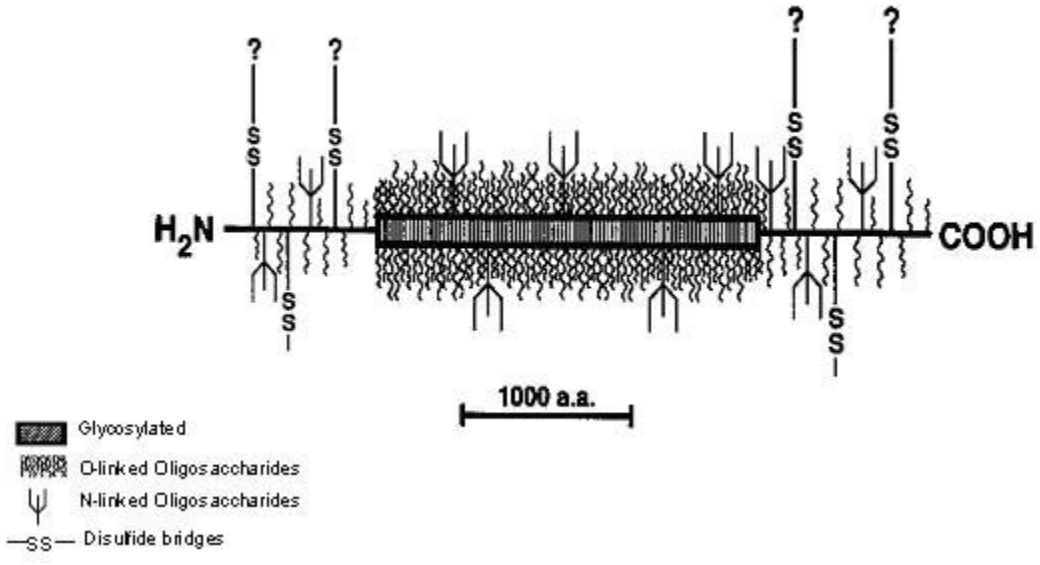


Figure 3 Diagram of a sulphomucin molecule (From Web page [3])

The gastrointestinal immune system has evolved for protection of the epithelium from bacterial invasion and for absorption of nutrients into the bloodstream (Helgeland and Brandtzaeg, 2000). A symbiosis exists between host and colonic bacteria which influences the health and immune response of the host, especially when products of bacterial metabolism protect the luminal lining (Garner, *et al*, 2007). Butyrate is an end product of colonic fermentation by bacteria and is associated with a beneficial effect on the mucosa and colonic health (Hamer, *et al,* 2010).

When symbiosis is disturbed, macrophages, neutrophils and T cells become activated and produce pro-inflammatory cytokines. In a normal state the gut is mildly inflamed due to everyday breakdown and repair of tissue. The equilibrium

is lost if the pro-inflammatory cells outnumber the anti-inflammatory benefit and inflammation to the gut occurs (Singleton, 2005). When inflammation is present the permeability of the epithelium is increased and the protective barrier is broken down. Pro-inflammatory cells have been shown to be produced during episodes of inflammatory bowel disease, consequently immunomodulators and immune-suppressive drugs are used for treatment (McFarland, 2008).

Characteristics of the sulphomucin layer have been observed to vary in disease, affecting thickness and composition by a defect of the fatty acid metabolism in colonocytes has been seen in patients with UC (Gibson, *et al.*, 1993). CD is known to have granulomas caused by aggregation of macrophages. It is commonly found in the terminal ileum and unlike UC is patchy and segmental and inflammation traverses through the epithelial layer (Xavier and Podolsky, 2007).

Coeliac disease and rheumatoid arthritis are both autoimmune inflammatory diseases. Coeliac disease affects the small intestine brought about by ingestion of gluten for susceptible individuals. Sometimes defined as 'gluten-sensitive enteropathy', it is lifelong and if left untreated can be very detrimental to health (Holmes,1987). Rheumatoid arthritis targets the joints and causes inflammation, and Parkinson's disease also has a deleterious effect on function and health of the gut. It appears that IBD can also be a result of a weakened immune system from other diseases with the gut becoming the 'victim' (Hvatum, 2006)

Inflammation can be found in other parts of the body such as arthritis in joints and uveitis an inflammation of the eye. It is thought that these too are due to a fault in the immune system (From web page[4]).

1.3 Sulphate-reducing bacteria and their potential to cause disease

Anaerobic bacteria such as *Clostridium difficile,* for example, present in the colon, produce metabolites such as (H_2S) which is extremely toxic as it inhibits butyrate oxidation and is implicated in the pathogenesis of UC (Roediger, *et al*.,1993). Synthesis and degradation of sulphur containing molecules is mediated by the sulphur anion metabolic pathway which involves the enzymes rhodanase (RHOD) and thiol methyltransferase (TMT). These enzymes are capable of removing H_2S. A study of patients with UC with impaired detoxification H_2S suggested that they have lower levels of these enzymes, (Picton, *et al*.,2007).

Sulphate in the form of sulphur dioxide is a preservative in many foodstuffs such as bread, beer, wine and cider. A diet rich in meat also produces faecal sulphides (Richardson, 1999). With the presence of SRBs and a greater amount of sulphur products available, they will compete with methanogenic bacteria and outnumber them (Christl, *et al*., 1992). Patients with ulcerative colitis have shown to have

more SRBs and sulphide in the faeces than healthy individuals (Gibson, *et al*., 1991). Although the Wellcome study did not arrive at the same conclusion because sulphide complexes were not found to this extent from the UC patients.

Some data is available for the absorption of the sulphate ion and a study of six healthy ileostomists and three normal subjects were chosen to measure absorption by addition of dietary sulphate. Urine samples found that the normal subjects had 12% more bound sulphate than the ileostomists. This is expected to have been derived from the metabolism of aromatic amino acids by gut bacteria. The sulphate content in faeces was low even from volunteers fed with higher doses of dietary sulphate (Florin, *et al.,* 1990).

In the last decade interest in SRBs and their significance in fermentation has become a focus for study; SRBs outcompete the MRBs in the gut. A comparison between two populations showed that a population from the UK had a higher incidence of SRBs than rural South Africans. Breath samples from the latter having a larger percentage of methane in breath compared to the UK population (Gibson, *et al*., 1988).

1.4 Characteristics for Inflammatory Bowel Disease

1.4.1 Diseased gut

It appears that good hygiene and twentieth century life-styles are having a deleterious effect on the function of the human gut, incidence of IBD is higher in industrialized countries than in the third world. Diet may be a significant factor. A pre-illness study found that sucrose, fat and refined carbohydrate intake was more common in CD and UC cases (Reif, *et al*.,1997).

The general terms for gastrointestinal disorders are structural or functional disorders. Structural disorders include CD, UC where sulphumucins protecting the gut epithelium is removed causing it to become inflamed and damaged, villi become worn down and absorption of nutrients is restricted (McFarland, 2008). Other disorders occurring in the GI tract are diseases such as diverticulitis, polyps and colon cancer (Morini, *et al*., 2008). Functional disorders include IBS; the gut goes into spasm causing pain altering the progress of the gut contents. A number of other inflammatory bowel diseases include infectious colitis, ischemic colitis and relatively newly discovered diseases such as eosinophilic oesophagitis (Attwood and Lamb, 2008).

1.4.2 Common Features

CD, UC and IBS have similar symptoms ranging from abdominal pain, bloating to general discomfort. Individuals have bouts of diarrhoea or constipation and generally feel quite unwell. It is not until the patient is investigated for these symptoms that a diagnosis can be made to identify which of these three IBD categories it is.

All conditions vary with intensity of physical symptoms and commonly found with one or a combination of inflammations of the lining of the intestine, bleeding and ulceration. Disease cycles includes bouts of remission and then flare up again. Ages between 15 and 35 appear to be the most susceptible to onset of disease and all conditions tend to be life long and have to be managed either by medication, diet, or change in lifestyle. The longer disease is present without intervention the greater damage occurs to the GI tract and surgery is required. Occasionally, disease is life threatening. Diagnosis of these conditions requires very invasive and uncomfortable investigations such as barium enemas, sigmoidiscopy and colonoscopy. Patients often resist going to the doctors due to the thought of these invasive procedures and may think they have something more serious.

Patients with IBD have more bacteria attached to the gut epithelium than do healthy people and this could be a key issue in causes to inflammation with regard to the reaction between the host and gut bacteria (Guarner and Malagelada, 2003).

1.4.3 Crohn's Disease

Crohn's disease can affect any part of the digestive tract from the mouth to the rectum, but most commonly found by inflammation of the terminal ileum or small intestine and spreads through the whole wall of the intestine where mucosal lesions form over Peyer's patches as shown in Figure 2. Granulomas form from aggregated macrophages, giant cells and epithelioid cells which are surrounded by lymphoid, plasma and other inflammatory cells resulting in acute damage to the mucosa, with areas of inflammation patchy and segmental (Xavier and Podolsky 2007). One in 1500 people suffer from CD and it affects more women than men, and is most commonly present in young adults. Diet may be a contributory factor as people diagnosed with Crohn's disease often eat more sugar and fat than people without IBD (Hunter, 1998).

1.4.4 Ulcerative Colitis

Ulcerative colitis is a chronic inflammation and affects the large intestine (the colon and rectum) rather than the entire gastrointestinal tract as is the case for CD. About 1 in 1000 people develops UC in the UK; most tend to be young adults when disease is first apparent. The cause is unknown and can run in families. Although the condition is life long it has periods of remission. When active, the patient has symptoms of abdominal pain, bouts of diarrhoea that often contain blood. Stools and flatus have an unpleasant abnormal odour. The patient may also be anaemic due to the loss of blood and will lose weight. Malaise and general lethargy are common features. When a patient has a severe flare up, the whole colon can become ulcerated and it may perforate requiring surgery; this occurs in about 3 out of 10 patients. People diagnosed with UC sometimes go on to develop colon cancer (Web page[1)].

Inflammation of the gut wall is thought to be caused by the mucosal lining being damaged by enteric bacteria, either from a pathogenic source or from the normal commensal bacteria. Histopathological examination shows numbers of neutrophils found in the lamina propria and crypts (see Figure 2) causing ulceration to occur (Xavier and Podolsky, 2007). Patients with UC have been found to have SRBs with about 92% *Desulfovibrio* species present in faecal samples (Gibson, *et al*., 1991).

The hosts' own immune response can also be defective and inflammatory cells activate and damage the mucosal lining and/or lose tolerance to the normal enteric bacteria. These autoimmune mechanisms occur from genetic abnormalities and trigger an autoimmune response of overly aggressive T-cells to enteric bacteria and cause damage to the mucosal lining (Sartor, 2006).

Both CD and UC patients carry genetic disorders in the mechanism of regulation of inflammatory cells and cytokines are different from that in healthy people. A number of genes such as the CARD 15 formerly known as the NOD 2 gene have been identified in patients with IBD that are linked with disease (Sartor, 2006).

Experimental models, using rats and mice, to recreate the conditions of colitis to distinguish and show factors that contribute to disease and identification into the genes responsible have been studied (Elson, *et al*., 1996).

Studies into the guts response to environmental triggers and stresses have shown enhanced gastrointestinal inflammation causing development of colitis conditions in rats (Gué, *et al.,* 1997).

1.4.5 Irritable Bowel Syndrome

The symptoms of IBS are abdominal discomfort and pain, flatulence and bowel dysfunction and some IBS patients may also be genetically susceptible to inflammatory conditions (Collins, *et al*., 2001). It appears to be a functional

disorder of the gut, often with no actual abnormality to the structure. However, the muscular wall goes into spasm causing pain. Persistent malfunction may then increase the chance of inflammation and ulceration. IBS seems to present itself in three forms: diarrhoea-predominant (IBS-D), constipation-predominant (IBS-C) and alternating diarrhoea-constipation (IBS-A). The Rome III, Manning criteria has been developed to standardise the diagnosis of IBS (McFarland, 2008). It is not known what triggers the condition but there are several possibilities from food intolerance, altered bowel microflora, stress resulting in impaired mucosal blood flow, or induced by illness and/or emotional upset (Bowen, 2001).

1.4.6 Investigation and Diagnosis of Disease

Internal examination is performed to investigate and diagnose each condition and is often performed by a barium enema or a barium meal (depending on which part of the gut is affected) and X-ray; sigmoidoscopy, colonoscopy and/or a biopsy performed for histological examination, each of which is invasive and uncomfortable for the patient. From initial onset of the disease to the first visit to the doctor and eventual referral for investigation it can take over a year to pass before a diagnosis can be confirmed and before treatment can start (McFarland, 2008).

1.4.7 Medication and Treatment

Treatment for CD and UC includes medication such as steroids, and immunosuppressant agents such as 6-mercaptopurine and azathioprine which help to reduce the inflammation. Medication is not a cure and often has undesirable contra-indications and patients are usually on medication for life. Suppositories and enemas are also used as they treat the condition from the rectum upwards; however they are embarrassing and uncomfortable for the patient. Surgery is performed if the conditions persist or become more serious; badly damaged parts of the gut are removed and patients then require a colostomy. Although this outcome has its unpleasant side, it is often life saving (Singleton, 2005).

Finding the correct medication for IBS is somewhat more complicated than CD and UC as it covers a range of conditions. Anti-diarrhoeal, anti-spasmodic or anti-depressants can be prescribed, hopefully tailored for individual cases. However, the choice of treatment can be somewhat 'hit or miss' and it can take a long time to find effective relief. Surgery is usually not necessary for IBS, but invasive investigation such as endoscopy and colonoscopy are often carried out to eliminate the possibility of more serious illness as the disease presents itself in as similar way to CD or UC. When other conditions are ruled out, IBS is tentatively diagnosed, not always a satisfactory conclusion for the patient as it

can be difficult to prescribe effective treatment for the causes of the symptoms. All conditions, once present, tend to be long term and have to be managed.

1.4.8 Research into diagnosis and treatment

Novel techniques such as volatile analysis have been used to investigate disease from patients with IBD. Breath samples were found to have raised levels of alkanes and this has been observed as an indication of excessive lipid oxidation, also a marker for ageing and inflammatory conditions (Salminen, *et al*., 1998).

Peroxidation causes damage to the intestinal epithelial barrier and allows bacterial antigens to enter the submucosal layers initiating impairment to the immune response, discovered in 2007 but the causality is still unknown (Rezaie, *et al*., 2007).

Obtaining fluid samples during a colonoscopy examination for VOC analysis for comparison with a breath sample from the patient was one approach as the patient was already undergoing examination, 'colonoscopy-fluid' was easily obtained without further invasive treatment . Although the results were not altogether conclusive it did show that this may, with further work, have advantages as a diagnostic tool (Lechner and Rieder, 2007).

Individuals suffering from disease have been observed to have abnormal sulphurous smelling faeces; a cohort and longitudinal study was performed on faecal samples from healthy and diseased patients. The study included patients with CD, UC; and patients with *Clostridium difficile and Campylobacter jejuni* infection. Volatile compounds found for each condition were significantly different and manageable for statistical analysis to give clear differentiation between gastrointestinal diseases (Garner, *et al*., 2007).

Examination of the thickness of the mucosal layer was undertaken by patients with CD and UC and appeared to have differences in the thickness from normal healthy gut and it was thought that colonic contents may be responsible for the damage to the mucosal layer (Pullan, *et al.*, 1994). Both CD and UC show a higher risk to colon cancer (Burstein and Fearon, 2008).

Infection, antibiotics and non-steroidal anti inflammatory drugs implicated in causing inflammation by altering the mucosal barrier and the immune response, although the mechanisms that initiate conditions are not fully understood (Elson, *et al*., 2003).

A persistent pathogen infection of *Mycobacterium avium* subspecies *paratuberculosis* (MAP) which is found in pasturised cow's milk may cause development of CD (Ayele, *et al.,* 2004).

1.5 Benefit of breath testing to the Healthcare Service

Globally, visits to healthcare services have increase for people with symptoms of IBD. In the United States, 30,000 new cases are diagnosed every year and up to 15 million adults suffer with IBS. In the UK 10 to 15% of the adult population are affected by IBS. Due to the chronic nature of this illness reinvestigation and further treatment is often required; becoming costly to both patient and health care provider (Bassi, *et al.,* 2004). Identification of VOC biomarkers and using them in a portable device, or a version of the SIFT-MS that is cheaper and small enough for monitoring and diagnosis of disease would provide a rapid, reliable low cost method that could be used in a hospital out patients without the need of trained staff (Turner, *et al*., 2006).

1.6 Other Research into IBD with the Wellcome project and other establishments

Identification and characterisation of SRBs, to determine disease-specificity; and genomic screening to establish polymorphism in genomic DNA was part of the Wellcome project at the University of Birmingham.

The Gastroenterology Unit at Addenbrooke's Hospital, Cambridge is run by Professor John Hunter who is the honorary consultant physician and currently leads an active research team carrying out studies of the gut microflora and inflammatory bowel conditions.

HFL Sport Science Quotient Bioresearch Ltd at Fordham in Cambridgeshire funded analysis of the Wellcome urine samples analysis by Liquid Chromatography Mass Spectrometry initially for sulphur compounds.

University Hospital South Manchester, Department of Medicine, run by Peter Whorwell who is Professor of Medicine and Gastroenterology, has a particular interest in functional disorders of the gut and has written many papers on the subject. One of which concerns the risk of damage to the pelvic floor from constipation (Amselem, *et al.,* 2009).

The University of the West of England and Bristol University have worked together to find a rapid diagnosis method for identification of the chemical composition of faeces for a number of conditions including *Clostridium difficile* infection in the gut, run by Professor Norman Ratcliffe and Dr Chris Probert.

The MRC Mitochondrial Biology Unit formerly known as the Dunn Human Nutrition Unit in Cambridge has done extensive research on IBD (Gibson, Cummings and Macfarlane, 1993).

1.7 Susceptibility for IBD in other countries

IBD research shows Chinese people appear to have a lower incidence than Western populations for susceptibility (Singleton, 2005) and may be due to genetic differences between populations or differences in diet and lifestyle. People living in the tropics and the Indian subcontinent may have more microbes in the small intestine (Cummings and Macfarlane, 1991). Gastrointestinal disease has become more prevalent and is more common in the Western World than developing countries.

CD affects, proportionally, more people in Wales, New Zealand, Canada, Scotland, France, The Netherlands and Scandinavia, than the rest of the World. IBD is lower is Brazil, China, Korea, Greece, Japan, Malta and Slovakia (McFarland, 2008).

1.8 VOCs and diagnosis

VOCs have a potential as marker compounds from normal health to many disease situations. They arise from various sources. For example, as normal metabolic compounds produced from physiological processes and from compounds produced from bacteria which are either pathogenic or commensal. Gastro enteric bacteria are very numerous and varied, producing a large number of volatiles from their metabolism. When IBD is present the balance of good to bad bacteria becomes altered and volatiles produced may be useful indicators of gastrointestinal dysfunction. The host body also produces VOCs as a response to infection occurring from immunoregulation when an inflammatory response is triggered.

1.8.1 Breath as a medium for health monitoring

Using VOCs in breath to investigate and monitor a range of diseases could replace more invasive methods presently used in surgeries and hospitals, such as from blood or biopsy samples. A non-invasive method of breath analysis is

quick and painless and acceptable to children and elderly alike. The rapid time response of such a device for monitoring and diagnosis of disease would be more favourable than using blood and even urine and faeces as, although rapid tests are available, it can take up to a week for diagnosis. Costs would also be lowered for a technique where laboratory staff would not be required extensively for regular clinical testing. An instrument like the SIFT-MS but smaller and less costly to run and with a one step monitoring and testing application would be of great use in a hospital clinic (Smith and Španěl, 2010).

1.8.2 Limitations of breath for disease monitoring – other clinical matrices as providers for VOC biomarkers

Many compounds found in breath originate from blood; they pass across the blood/alveolar barrier from the lungs into breath (Van de Velde, *et al*., 2008). Urine too, contains many compounds that are found in blood these pass through from blood into the kidneys by renal filtration. Although not as straight forward as a breath sample it is still a useful 'medium' for extracting information from what is occurring within the body as it is non-invasive. Faecal samples give a 'coal face' analysis to what is happening, especially where gut disease is apparent, from within the lumen and luminal contents to provide information of bacterial balance and activity within the gut, but not the most suitable sample type to use for regular diagnostic testing, and patients do not like providing stool samples.

Storage and handling of these liquid and solid clinical samples also have a time and cost implication.

Breath simply by its ease of capture has many potential benefits but it also has disadvantages. Compounds present in breath are not always endogenous or systemic and become contaminated by metabolites present in mouth bacteria from the oral cavity. Mouth and nose-exhaled breath have been compared and shows that nose sampling would be preferred (Smith and Španěl, 2010). Breath also contains many exogenous compounds absorbed by the body from the environment. For example, xylene found in paint and substances such as benzene, trihalomethane, and many others from occupational exposure. Monitoring people to exposure of chemicals is another area where breath analysis can be of use, but this might compromise the effectiveness of using breath as contamination may interfere with the trace compounds of interest (Di Francesco,*et al*., 2004).

1.8.3 History and research diseases using breath analysis

Odour has been used to identify disease for thousands of years; the Greek physician Hippocrates knew that human breath may provide clues to poor health due to characteristic odours; some were recognised as indicators for disease such as the fruity smell of acetone for diabetes. We have since discovered that

VOCs found in breath and body fluids may be used as markers for disease. Breath VOC identification has been extensively researched but less literature has been found for VOCs from blood, urine and faeces.

During the 1970's Pauling *et al* detected more than 200 compounds in human breath with the use of gas chromatography. Antony Manolis, working in the same area discovered 200 compounds in breath of healthy subjects but at the time in 1983 did not know which metabolic pathways produced those (Manolis, 1983) and many are still not known. Separation of compounds and accurate identification became more reliable in the 1980s and 1990s with the progress of analytical methods (Miekisch, *et al*., 2004). During the 1990s identification of breath components in cross-sectional and longitudinal studies investigated exhaled biomarkers for assessment as predictive markers for disease (Cao and Duan, 2006). Latterly, GC-MS and more recently SIFT-MS which is capable of measuring several compounds simultaneously, can analyse VOCs from exhaled air, within a few seconds (Smith and Španěl, 2010). It has a potential as a tool for non-invasive disease diagnosis, and the advantage that it can be used for real time, on line breath sampling for a variety of diseases with identification of compounds down to parts per billion (Turner, *et al*., 2006).

A study of patients with cystic fibrosis and asthma found elevated levels of hydrogen cyanide (HCN) in breath from *Pseudomonas aeruginosa* infection in

children with cystic fibrosis and conversely those with asthma had lower levels of HCN but higher levels of nitric oxide (NO) in their breath (Smith and Španěl, 2010).

Breath screening was used for patients diagnosed with type 2 diabetes with samples being collected into Tedlar bags for GC-MS analysis. Findings showed acetone levels elevated in people with diabetes (Da Silva *et al*., 2008). VOCs such as aromatic hydrocarbons from breath samples have been found in higher concentrations for patients with lung cancer (Poli, *et al*., 2008). Patients with congestive heart failure (CHF) have been shown to have breath acetone levels four times higher than control subjects (Kupari, *et al*., 1995).

Breath contains a number of alkanes such as ethane and pentane found to be markers for oxidative stress and present when disease is active (Aghdassi and Allard, 2000). Pro-inflammatory cells and bacteria produce oxidised compounds from metabolism. Isoprene in breath is implicated in cholesterol metabolism and organ failure (Kuzma, *et al*., 1995).

1.8.4 Headspace Analysis – for clinical matrices

A gas sampling technique known as headspace analysis was used for clinical samples. Liquid or solid sample forms equilibrium within a headspace over the

sample matrix, contained and isolated from the ambient environment inside a sample bag. The equilibrium theory was proved by William Henry in 1803 and his law (Henry's Law) states that, (Pysanenko, *et al.,* 2009).

'*At a constant temperature, the amount of a given gas dissolved in a given type and volume of liquid is directly proportional to the partial pressure of that gas in equilibrium with that liquid'*

VOC analysis using methods to obtain a representative sample of volatile compounds from liquids and solids was a novel technique in the late 1990s; and a method to trap the gas phase in equilibrium with a liquid or solid was developed in 1996 for a number of applications (Creasy and Capuano, 1996). This technique also known as headspace analysis is used with SIFT-MS and ATD-GC-MS. Since then headspace analysis has been used for a variety of applications including volatile fractions from cows' milk analysed by this novel technique (Toso, *et al*., 2002), and urinary headspace with SIFT-MS (Abbott, *et al*., 2003).

1.9 Analytical Techniques for VOCs in breath and clinical matrices

1.9.1 Collection of breath samples

A Breathotron device was used at Addenbrooke's Hospital to collect breath and ambient air samples. Direct breath samples were taken from patients in a matter of minutes via a facemask as shown in Figure 4 where breath is passed onto a TD tube containing Tenax® for analysis by GC-MS.

Figure 4 Particle mask (3M model no 750) adapted with tube for attachment to Breathotron

1.9.2 Gas chromatography – Mass spectrometry

GC-MS is an important analytical method for the analysis of VOCs, it is semi-quantitative and very sensitive for the determination of individual substances in complex mixtures down to ppb/ppt. The sample in its gaseous phase is passed onto a column by carrier gas, usually helium, where the separated components elute from the column at different times, then each is analysed by the mass spectrometer where components are broken into ionised fragments and detected by their mass to charge ratio (*m/z)*. The fragments are compared with stored libraries of compounds for identification.

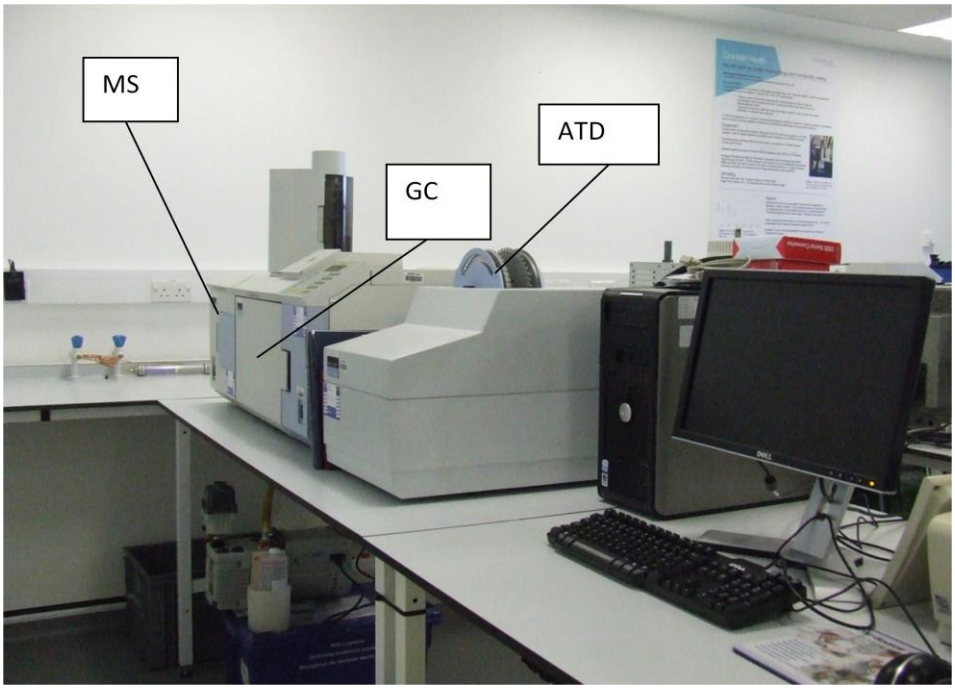


Figure 5 Cranfield Health Department ATD GC-MS System

Automatic thermal desorption (ATD) with GC-MS (and apparatus shown inFigure 5) is a standard technique for the analysis of VOCs especially non polar analytes

with low molecular weight. Thermal desorption tubes containing adsorbent materials such as Tenax® and Carbotrap® are used to trap VOCs passed from a headspace containing a liquid or solid or breath sample. The headspace sample was passed onto the tube through a flow pump and the breath sample passed onto the TD tube via the Breathotron device. This technique was useful for pre-concentration of sample so that trace compounds at ppt concentrations could be detected (Knobloch, 2009), and for transport and storage prior to analysis.

GC-MS does although have some disadvantages. Water vapour can provide erroneous results and has to be removed prior to analysis. It is very expensive to perform sample analysis and the equipment is not portable, although samples are collected on the TD tubes. The analysis time is slow and only semi-quantitative compared with the SIFT-MS.

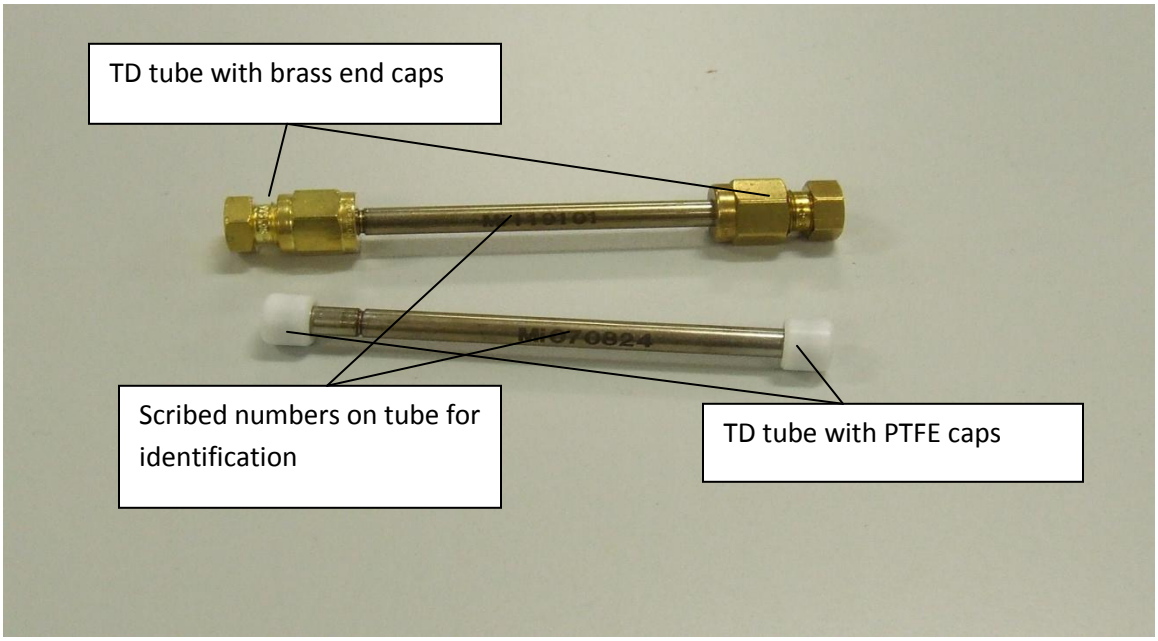


Figure 6 TD tubes showing permanent and temporary caps

Figure 6 shows two TD tubes, the upper one with permanent brass end caps; these caps are in place while tubes are being stored or in transit. The white PTFE temporary caps are used while the tubes are on the ATD. The actual size of TD tube is 9cm long.

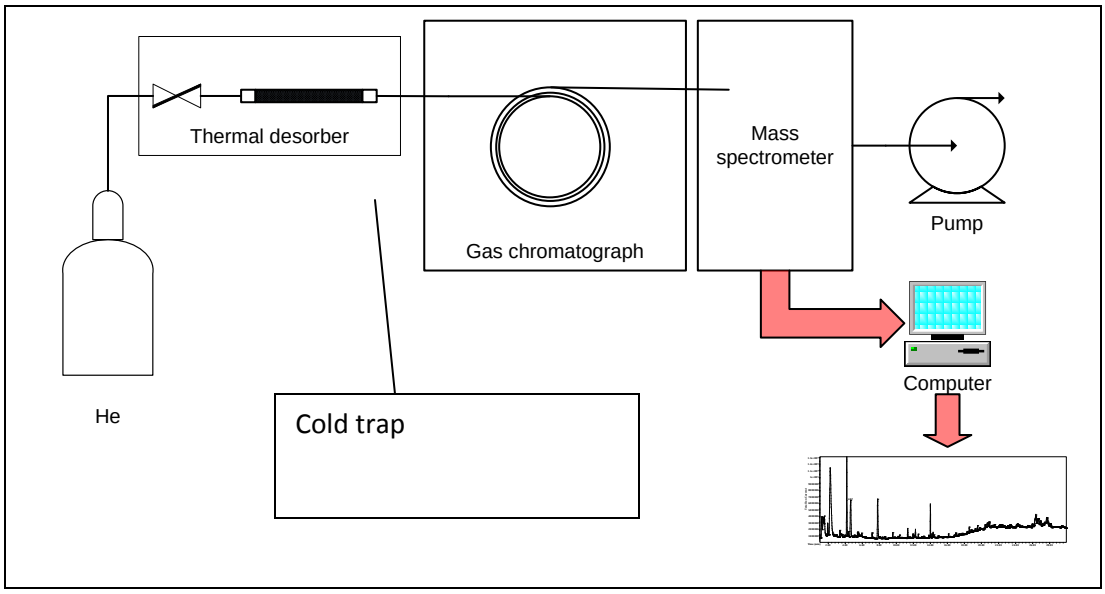


Figure 7 Schematic of the GC-MS showing different components and processes from desorption to the TIC

1.9.3 Selected ion flow tube – mass spectroscopy

The SIFT-MS, shown in Figure is a Model Profile 3, manufactured by Instrument Science Ltd, (Crewe, Cheshire); it is being developed as a method of analysing breath from patients; and determines on-line real-time measurements of volatile compounds down to ppb (Smith, Španěl 2005). The capabilities of breath analysis by this technique once established have many advantages (Miekisch and Schubert, 2006). Non-invasive breath testing for these conditions is much less embarrassing for patients than existing diagnostic tests and may encourage people with symptoms to seek help leading to faster treatment. The high

moisture content of a breath is well tolerated by this method producing quantitative analysis of many compounds.

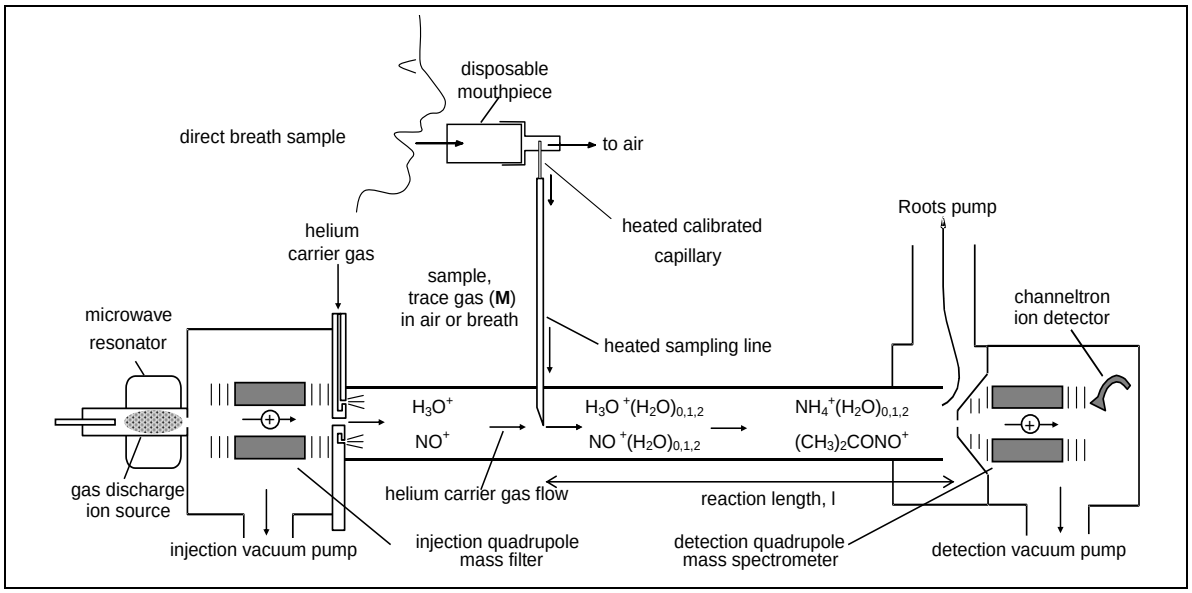


Figure 8 Diagram of the SIFT-MS

Breath and headspace samples pass through a heated capillary and into the flow tube where three precursor ions; H_3O^+, NO^+ and O_2^+ are generated from the upstream injection quadrupole (see Figure 8) used to ionize the trace gases in the sample. The precursor ions and product ions are then carried down the flow tube by helium onto detection quadrupole mass spectrometer and the ions are counted by the mass detector producing a mass spectrum. Two modes of operation are used; the full scan mode which is semi-quantitative where the ions are analysed over a selected mass range, and secondly a quantitative multiple

ion monitoring mode in which individual ions are selected for monitoring (Turner, *et al*., 2006).

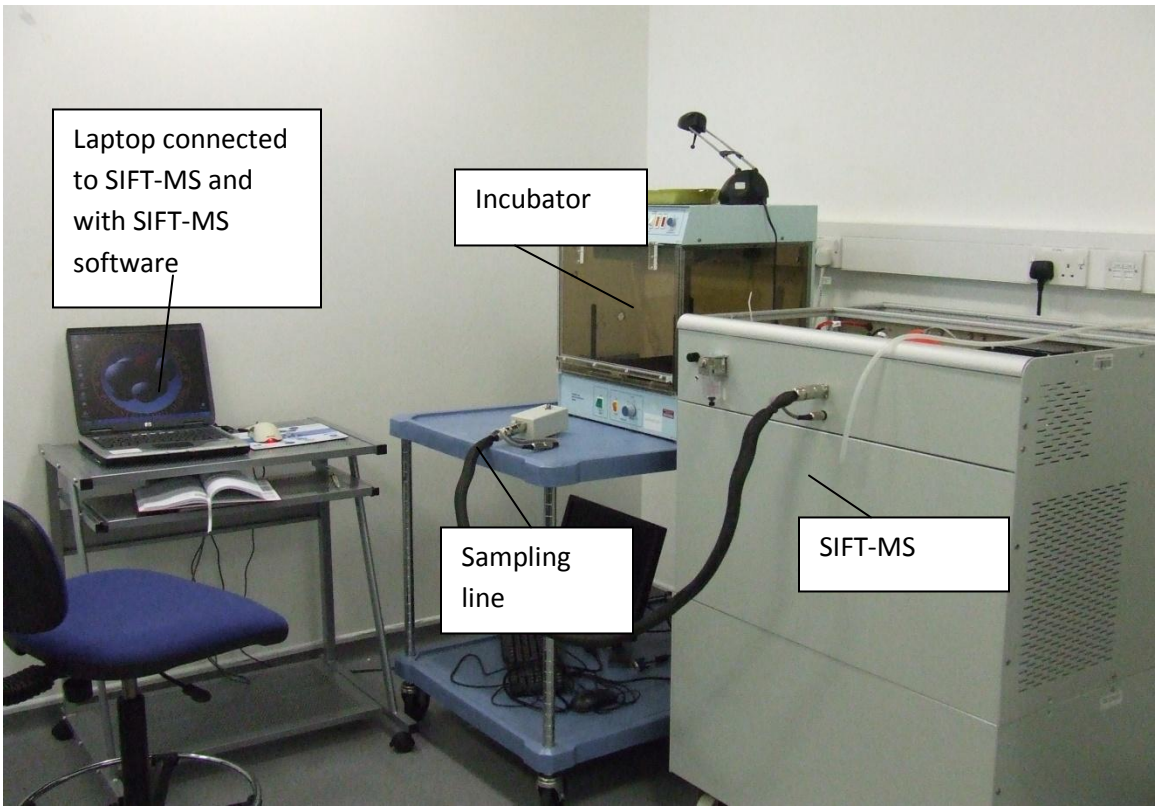


Figure 9 SIFT-MS with incubator and laptop

Figure 9 shows the SIFT-MS used in this study. Development of the SIFT-MS, or a similar device, such as Proton Transfer Reaction Mass Spectrometry (PTR-MS) measures VOCs in real time and produces mass spectra within seconds, is much quicker than GC-MS analysis.

1.10 Aim

The aim of this project was to determine whether VOC markers could be found from breath, blood, urine and faeces and distinguish between CD, UC, IBS and healthy volunteers. VOC markers have a potential to be used as a rapid diagnostic test for monitoring and identification of IBD conditions.

Chapter 2

2.1 Materials and Methods

2.1.1 Patient Recruitment and sample collection

A total of 91 volunteers recruited from the Gastroenterology department at Addenbrooke's Hospital in Cambridge provided samples for this study to produce the data required for analysis. From the volunteers 24 were diagnosed with CD, 19 with UC and 28 with IBS and 20 healthy volunteers were used as controls.

When the volunteers had verbally agreed to donate samples they were given a letter explaining why the trial was being conducted and what they would need to do.

Patient confidentiality was required for data protection and each volunteer was given a unique code number that was attached to each of their sample containers; this also enabled the samples to be analysed blind which was a prerequisite for the trial. Addenbrooke's is the keeper of the patient list and cross reference to the unique codes which identifies disease conditions. This

was not released to the project partners, Cranfield, until patient recruitment and sampling was complete to ensure that the study was carried out blind.

All volunteer recruits were given a consent form to sign and asked to fill in a questionnaire for details on diet, sleep, exercise, alcohol and nicotine intake, medication and general health. Samples were taken as soon as possible after consent was given the date and time of this was filled out on the consent forms.

Breath, urine and blood samples were taken from each volunteer. Sample pots were provided for faecal samples to fill at the next opportunity for patients and then handed in for storage, prior to analysis. The samples were stored at Addenbrooke's, frozen at -40˚C until they were collected for delivery to the University of Birmingham and Cranfield University. At Cranfield the samples were stored at -80˚C prior to analysis. Freezing the samples inhibits biochemical and bacterial processes from occurring. Vapour emissions from human urine samples were not significantly changed by freezing, for samples analysed by GC-MS and SIFT-MS (Peakman and Elliot, 2008).

Patients with disease conditions were asked to supply a second batch of samples after they had received treatment. The protocol for sample collection was the same as for the first set of samples.

Ethical consent was given for this Wellcome Project by the National Research Ethics Service. Human Tissue Sample Record Sheets (HTRS) were kept for

blood, urine and faecal samples. The reference numbers were added to the HTRS when samples were collected from patients. The date was entered when the sample was analysed, and finally a date was entered when the sample was destroyed in accordance with instructions for the protocol for ethical consent.

Information of age and gender was sent to Cranfield towards the latter part of the study and was analysed to show trends.

2.1.2 Breath Sampling – Breathotron and TD sample collection

The volunteer was given the opportunity to relax before sampling started, subsequently they were given a standard 3M mask; Model no. 750 to wear, that was modified with a PTFE tubing arranged to carry the breath to the Breathotron. The patient was asked to breathe normally, for about five minutes until a volume of 500ml had passed across the TD tube.

The mask was generally well tolerated by patients they were asked to relax and breathe normally while the breath sample was being collected. MMOS sensor data were stored on a PDA attached to the Breathotron. These data were collected for the Wellcome study but was not required for the Masters project.

Breath compounds were collected onto a TD tube containing 50% Tenax TA and 50% Carbotrap (Markes International Ltd, Llantrisant, UK) via the Breathotron.

2.1.3 Breath – Bag Samples

Breath samples were collected into pre-prepared breath sample bags. The prepared bags were manufactured from a metre strip of 'Nalophan NA' (polyethylene terephthalate) purchased from Kalle GMbH & Co. KG. Rheingaustrabe, 190-196 D-65203, Wiesbaden, Germany, with a 70mm length of polypropylene tube and a Swagelok closure fitted into the apical opening. The Nalophan NA was carefully fan folded to wrap around the tube and then tied with two tie-wraps to secure the tubing; the basal end was also fan folded and then approximately two centimetres was folded over and secured with two tie-wraps, this made an adequately air tight container. Patients were asked to relax and then breathe normally into the bag through the uncapped Swagelok closure until it was full and then closure screwed tightly shut. Breath bags were labelled with unique patient ID number.
The breath bags and TD tubes were then despatched to Cranfield via courier and breath bags were analysed by SIFT-MS upon arrival. TD tubes were refrigerated at 4°C prior to analysis with GC-MS.

2.1.4 Headspace Analysis

Bags for the liquid and solid clinical samples were prepared from slightly smaller Nalophan NA tubing than the breath bags. These were prepared from 100mm wide Nalophan NA cut to 500mm lengths, and similar to the breath bags with a Swagelok fitting with tube attached to the apical end with tie wraps and the basal end was left open for the sample to be added. When the bag was sealed and zero grade air

added this created an environment for a headspace above the sample for VOCs to be released from the liquid or solid phase (Figure 10).

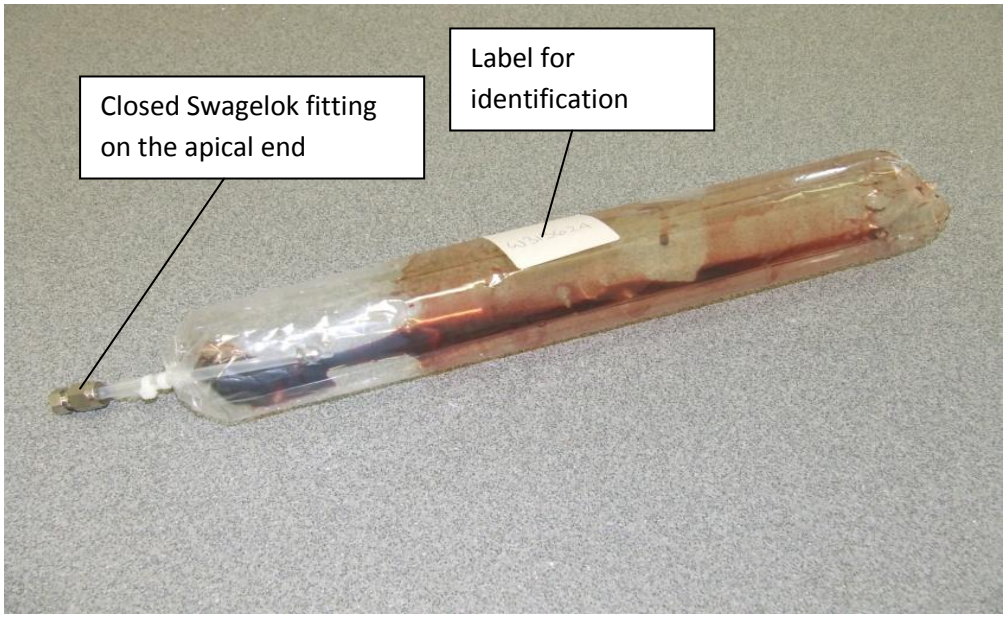


Figure 10 Headspace bag containing a blood sample and filled with zero grade air.

The blood, urine and faecal sample aliquots were transferred into a bag. The actual amount of sample placed into each bag was not critical. All blood, urine and faecal samples required thawing from -80°C to room temperature before preparation. An aliquot of blood was dispensed by pipetter into the open, basal end of the Nalophan bag which was then folded and tie-wrap sealed. The bag was then attached to line with zero grade air via the Swagelok fitting and filled with sufficient air to make it rigid. A similar aliquot of urine was placed into the Nalophan bag or approximately a 3g 'splodge' of faeces placed into the bag. The following procedure was then the same for the urine and faecal samples. The bag was labelled with the corresponding patient Wellcome number and then placed in an incubator at 40°C for 10 minutes to

allow the headspace to equilibrate. A bag filled with zero grade air and without sample was used as a blank. Table 14 in the appendix shows list of compounds.

2.2 Analytical Methodology

2.2.1 Capture of volatiles onto TD tube from headspace

Liquid and solid clinical samples: - Headspace from urine, blood and faecal samples was drawn from the bag by using a FLEC® constant-flow pump purchased from Markes International, Llantrisant, Wales using a flow rate of 100ml for 5 minutes and passed onto a TD tube. The tube was then stored at 4°C prior to GC-MS analysis.

Breath samples:- The breath TD tube samples, on arrival at Cranfield, were checked to make sure TD tube number inscribed on the tube and patient Wellcome number corresponded to the record sheet included then stored at 4°C until a batch of approximately 50 TD tubes was ready for a run on the GC-MS. Standard operating procedure (SOP) for the preparation of the TD tubes for addition of standard and GC-MS analysis is described in the Appendix 5.4.

2.2.2 GC-MS Instrument Settings and Conditions

A Perkin Elmer system was used for volatile analysis, combining a TurboMass MS 4.1(Software version), Autosystem XL GC and Automatic Thermal Desorption system ATD 400 (Perkin Elmer, Wellesley, MA). The carrier gas was CP grade helium (BOC gases, Guildford, UK) passed through a combined trap for removal of

hydrocarbons, oxygen and water vapour. The trap was changed after every fourth helium cylinder. A wall-coated Zebron XB624 chromatographic column was used (Phenomenex, Torrance, CA), with dimensions 30m x 0.4mm x 0.25mm (internal diameter), the liquid phase comprising of a 0.25µm layer of 6% cyanopropylphenyl and 94% methylpolysiloxane.

TD tubes were desorbed by purging for 2 min at ambient temperature then for 5 min at 300°C. Volatiles purged from the TD tubes were captured on a cold trap which was initially maintained at 30°C. The ATD valve was set to 180°C and TD tubes were desorbed onto the secondary cold trap which was initially maintained at 30°C. This purges the tubes to remove water and other contaminants. Once desorption was complete, the trap was heated to 320°C using the fastest heating rate and then maintained for 5 min whilst the effluent was transferred to the GC via a transfer line heated to 210°C. The GC oven was maintained at 50°C for 4 min after injection and then raised at increments of 10°C per minute until reaching 220°C and then held for 9 min. The eluted products were transferred by a heated line held at 240°C to the MS where the compounds were ionised to carry out a full scan of mass/charge ratios from 33 to 350*m/z* with a scan time of 0.3s and 0.1s delay producing mass spectra with a total ion chromatogram (TIC).

2.3 Compound Identification

Compounds were identified by Automated Mass spectral Deconvolution and Identification System (AMDIS) Version 2.62, allowing identification of target

compounds from raw GCMS data by comparing with the mass spectral library from the National Institute of Standards and Technology (NIST). A representative part of all the compounds deconvoluted from each clinical sample was copied onto an Excel spreadsheet as shown on Table 1. The NIST library, Version 2.0c, was used to compare with the AMDIS identification for the best of the three retention time matches and shown in Table 2. The first one from each three with the same retention time. This was then used to process the data and was a much quicker method than checking all three matches with the same retention time with the NIST library to make a choice.

Table 1 AMDIS data showing sample of set compounds found (Name) with retention time for that compound (R.T.) and Amount in %. Table 2 is representative of a full list of several hundred compounds.

Name	R.T. (minutes)	Amount
argon	3.88	0.13%
propyne	3.88	0.13%
borane carbonyl	3.88	0.13%
nitrous Oxide	4.011	1.40%
carbon dioxide	4.011	1.40%
phenol, 4-[2-(methylamino)ethyl]-	4.011	1.40%
sulfur dioxide	4.569	0.54%
aminomethanesulfonic acid	4.569	0.54%
2-aminoethyl hydrogen sulfate	4.569	0.54%
ethylene oxide	4.781	1.41%
alanine	4.781	1.41%
cyclopropyl carbinol	4.781	1.41%
1-propene, 2-methyl-	4.94	0.09%
1-butene	4.94	0.09%
cyclobutane	4.94	0.09%
acetaldehyde	5.236	1.56%
formic acid, ethenyl ester	5.236	1.56%
ethylene oxide	5.236	1.56%
hydroxyurea	5.414	5.07%
DL-cystine	5.414	5.07%
cystine	5.414	5.07%

butane, 2-methyl-	5.92	0.11%
pentane	5.92	0.11%
isobutane	5.92	0.11%
trichloromonofluoromethane	6.288	0.18%
2,4-dioxabicyclo[3.2.0]hept-6-en-3-one, 1,5-dichloro-6,7-dimethyl-	6.288	0.18%
phosphorus trichloride	6.288	0.18%
trichloromonofluoromethane	6.292	0.38%
2,4-dioxabicyclo[3.2.0]hept-6-en-3-one, 1,5-dichloro-6,7-dimethyl-	6.292	0.38%
phosphorus trichloride	6.292	0.38%
pentane	6.362	0.64%
isobutane	6.362	0.64%
butane, 2-methyl-	6.362	0.64%
pentane	6.3623	0.64%
ethyl alcohol	6.541	1.92%
hydrazine, methyl-	6.541	1.92%
methane, nitroso-	6.541	1.92%
trimethylene oxide	7.114	0.02%

Table 2 Amdis data again showing first choice from R.T. The additional column is the Absolute amount calculated from the Absolute Amount of Toluene-D8 (ng/l).

Name	R.T. (minutes)	Amount	Absolute Amount ng/l
acetaldehyde	5.236	1.56%	42
hydroxyurea	5.414	5.07%	137
butane, 2-methyl-	5.92	0.11%	3
trichloromonofluoromethane	6.288	0.18%	5
trichloromonofluoromethane	6.292	0.38%	10
pentane	6.362	0.64%	17
pentane	6.3623	0.64%	17
ethyl alcohol	6.541	1.92%	52
acetone	7.259	4.45%	120
dimethyl sulfide	7.401	3.02%	82
acetic acid, hydrazide	7.747	0.29%	8
cyclopropane, ethyl-	8.095	17.50%	473
butane, 2-methyl-	8.683	0.10%	3

butane, 2-methyl-	8.688	0.10%	3
1-propanol	9.083	0.49%	13
2,3-butanedione	9.661	0.60%	16
2-butanone	9.897	0.62%	17
methanesulfonyl chloride	9.936	0.15%	4
2-butanol	10.08	0.15%	4
1,3-dioxolane, 2-methyl-	10.919	0.12%	3
isobutane	10.942	0.19%	5
acetic acid	11.177	1.00%	27
benzene	11.283	0.24%	7
propane	11.423	0.28%	7
1-butanol	11.953	0.32%	9
propanoic acid	13.655	0.34%	9
disulfide, dimethyl	13.896	2.62%	71
toluene-D8	14.109	3.70%	100
cyclobutene, 2-propenylidene-	14.219	0.14%	4
butanoic acid, ethyl ester	14.675	0.02%	1
ether, 2-chloro-1-propyl isopropyl	15.062	0.15%	4
butanoic acid	15.79	0.60%	16
butanoic acid, 3-methyl-	17.056	0.09%	2
1,4-methano-1H-cyclopropa[d]pyridazine, 4,4a,5,5a-tetrahydro-6,6-dimethyl-, (1à,4à,4aà,5aà)-	19.035	0.09%	2
dimethyl trisulfide	19.811	0.17%	5
benzaldehyde	19.918	0.19%	5
1,7-octadiene, 3-methylene-	20.143	0.19%	5
phenol	21.065	0.25%	7

2.3.1 Biomarker Selection

Hundreds of different compounds were produced from analysis from each of the clinical matrices. An approach to find a manageable number of compounds for data handling that was not time consuming was required. The faecal samples were chosen first as they had an abundance of compounds and seemed more likely to be

associated with disease. Three batches of four GC-MS chromatograms with control, pre-treatment CD, UC and IBS were displayed together to enable a visual comparison. Differences in compound peak heights from the chromatograms were compared with the AMDIS deconvolution list with the corresponding retention time (see Table 2). Compounds with peaks showing a difference from controls and disease groups were chosen to go onto a short-list. The list in Table 3 was then drawn up of compounds from which the short-list selection was made.

Scoring for Table 3 was made by 0 being no peak visible and 3 being the greatest peak size. A total possible score was 9; for example 3 for the highest peak, times 3 for the frequency that compound was seen in each of the three batches. Figure 11 shows the four GC-MS chromatograms the upper one is a pre treatment CD sample, then UC, IBS and a control sample providing a good method to visually compare chromatograms.

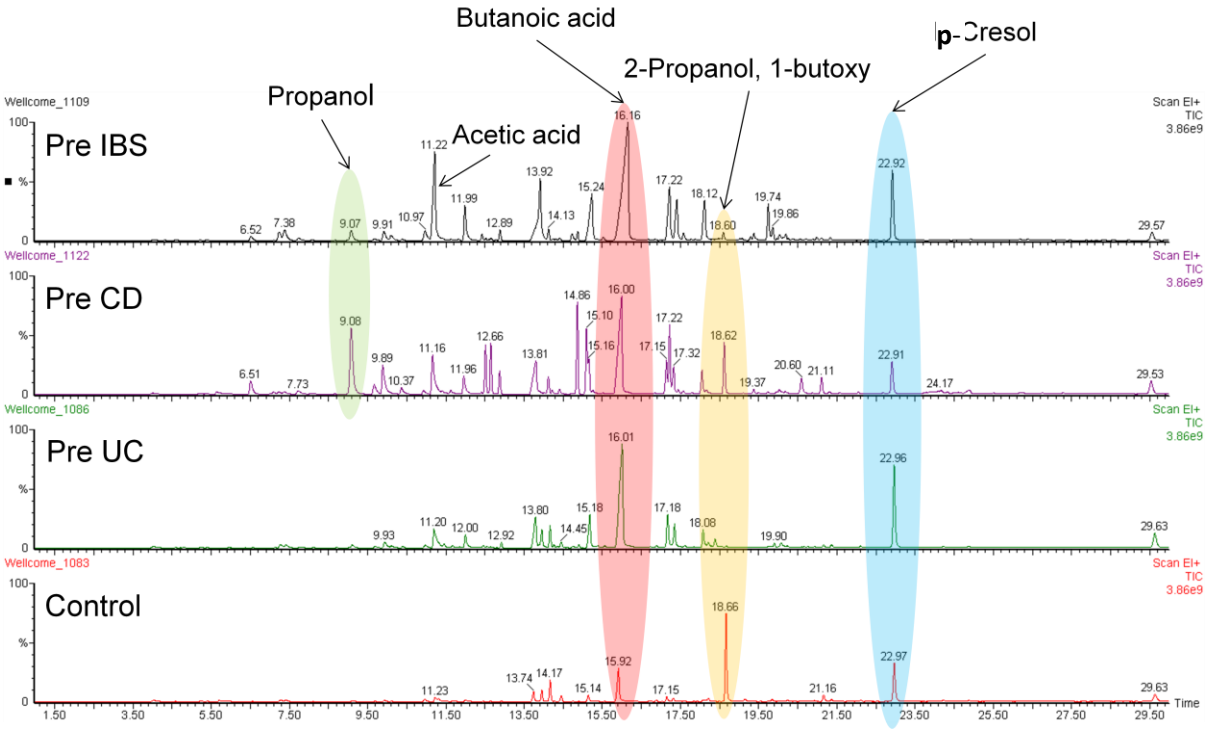


Figure 11 TIC alignment for control and pre treatment disease groups, some of the compounds have been highlighted where differences were found.

Table 3 List of compounds selected from the visual inspection method for Candidate Compounds. Peak height being given a score from 0 to 3 and this was multiplied by the frequency that compound from the three groups.

Faecal samples - Candidate Compounds abundance					
Compound	**R.T. (minutes)**	**Control**	**Pre UC**	**Pre CD**	**Pre IBS**
trimethylamine	5.729	0	1	0	0
ethyl alcohol	6.522	2	1	4	4
acetone	7.24	3	7	4	6
1-propanol	9.076	1	3	8	3
propan-2-ol	7.42	1	1	2	2
2,3-butanedione	9.672	1	2	2	1
2 butanone	9.914	2	3	4	3
acetic acid	11.167	1	5	3	5
ethyl acetate	9.944	0	0	2	0
acetonitrile	7.674	0	1	0	0
1-butanol	11.976	1	2	5	5
2 butanol ®	10.117	1	0	1	1
2-pentanone/2,3-pentanedione	12.422	0	1	1	1
methyl propionate	10.461	0	0	1	1
1,3-dioxolane, 2-methyl/1-propanol, 2-methyl	10.962	1	2	2	2
propanoic acid	13.825	3	2	5	6
propanoic acid ethyl ester	12.518	0	1	4	0
propanoic acid , 2-methyl	15.159	2	6	6	5
butanoic acid	15.966	3	9	6	6
n-propyl acetate	12.709	0	0	2	1
butanal, 2-methyl	11.642	1	2	2	1
hexane	11.6835	0	1	1	1
disulfide, dimethyl	13.961	3	4	3	1
butanoic acid, methyl ester	12.929	0	0	1	2
butanoic acid, ethyl ester	14.911	0	0	2	1
butanoic acid, 2-methyl ester	17.321	0	0	1	0
butanoic acid, 3-methyl ester	17.157	0	0	1	0
butanoic acid, 2 methyl	17.315	2	5	3	3

butanoic acid, 3-methyl	17.137	2	6	5	4
styrene	17.581	0	0	1	1
formic acid	13.816	0	2	1	1
pentanoic acid	18.052	1	6	3	4
2-propanol, 1-butoxyl	18.624	3	0	2	2
propanoic acid, 2-methyl, butyl ester	19.402	0	1	0	0
hexanoic acid	20.107	0	4	1	1
5-hepten-2-one, 6-methyl	19.741	0	0	0	3
dimethyl trisulfide	19.858	1	1	0	2
phenol	21.122	2	1	3	4
phenol, 4-methyl	22.919	4	0	5	5
indole	29.546	3	6	8	6

2.3.2 Candidate Compounds – short list

The list on Table 3 gave the initial choice for further selection criteria based on three key ideas;

- The 'most abundant' was selected from the list in Table 3. Then going back to the Table 2 list which shows the 'amount' column abundance was then identified and ones with the most abundance chosen.
- The 'literature sourced' were compounds sourced from the literature. For example, likely metabolites, which come from the host or bacteria resident in the gut. Acetone is produced by lipolysis (Turner, 2006). Aliphatic acids can inhibit growth of gut bacteria. Carboxylic and short chain fatty acids such as propanoic and butanoic acid are produced by anaerobic fermentation of undigested carbohydrates by colonic bacteria. Intestinal mucosa is a major site for fatty acid ethyl ester synthesis and they may be involved in organ injury. Methanol as a

product from MRB can become trapped as an ester by gut flora and thought to have health implications (Garner, *et al.,* 2007). Phenolic compounds such as indole, p-cresol and phenol are markers for protein fermentation (De Preter *et al.,* 2009). A total of 13 Candidate Compounds was chosen and Table 4 shows them with their corresponding GC-MS retention time.

- Visual inspection, these were chosen from the list in Table 3 as ones with a difference between control and pre treatment disease groups.

Table 4 Candidate compound Short –list in the groups of choice showing retention times for identification.

Group	Compound	Retention Time (minutes)
Most abundant	acetone	7.24
	propanoic acid	13.64
	butanoic acid	15.84
Literature sourced	1-propanol	9.083
	propanoic acid, ethyl ester	12.47
	butanoic acid, methyl ester	12.88
	butanoic acid, ethyl ester	14.88
	p-cresol	22.92
	indole	29.59
	disulpide dimethyl	13.89
Visual inspection	1-butanol	11.97
	butanoic acid, 3-methyl	17.12
	phenol	21.12

2.3.3 Calculations of sample for semi-quantitative measurements

An internal standard of D8 toluene was used for quantification. Calculation and parameters used are shown below.

Absolute amounts of Faecal Candidate Compounds were calculated from:

Absolute amount = % of sample / % of D8 toluene x volume of standard, nano grams(ng) x dilution of sample, litre (l).

Absolute amount = ng/l

Volume of standard =50ng*$_1$

Dilution of sample = 0.5 litre*^{2}

% of sample and D8 Toluene was the value given in the 'Amount' column

*$_1$ Volume of standard dispensed by Hamilton syringe into the TD tube for 20seconds with a flow rate of 500mls/min.

*$_2$ 100 ml/minute for 5 minutes

2.3.4 Data Collection for Candidate Compounds

Data for the 13 candidate compounds short-list in Table 4 was gathered from faeces for control and the pre and post treatment groups. The same candidate compounds were looked for in blood, urine and breath.

2.3.5 Statistical Analysis

Box and Whisker plots were a good method to visually compare the skewedness of the data. Then non-parametric Kruskal Wallis test done to find significant differences between groups and the Mann Whitney U-test was used as a *Post hoc* test to determine differences between pairs within the groups where Kruskal Wallis test was significant (control and pre diseases groups), there were six pairs to compare candidate compounds; Control vs. CD, Control vs. UC, Control vs. IBS, CD vs. UC, CD vs. IBS and UC vs. IBS.

2.3.6 Limit of detection for Candidate Compounds

Two of the compounds from the candidate compound list were used for quantitative analysis to produce calibration curves and determination of the LOD.

2.4 Method Development

The GC-MS method used predominantly in this study was a general screening method for volatile analysis and was based on screening for as many compounds as possible for identification. The general method provided the basis for compound identification and from the compiled short-list. From the short-list two compounds were chosen for use in method development to improve a number of parameters; faster analysis time, better quantification, improve peak separation and to determine

the limit of detection. The compounds chosen to use with method development were ethyl propionate and methyl butyrate, pure samples purchased from Sigma-Aldrich. They were chosen as their performance in the general method was having similar retention times and both eluted at mid-point in the analysis run time.

2.4.1 Apparatus and method modification

Apparatus modifications:

Tenax TA® mesh size 35/60, (catalogue number C1-AAXX-5033) thermal desorption tubes were used. These are stainless steel tubes containing a porous polymer resin based on 2.6-diphenylene oxide, specifically designed for trapping volatiles or semi-volatiles from air and in headspace (Sato, *et al*., 2001).

A Zebron® ZB-WAX (30m x 0.25mm id x 0.25 film thickness) column was fitted into the GC oven. It is a polar column used for low molecular weight organic acids, both the column and TD tubes were used to find volatile substances and specifically propanoic acid and butanoic acid (similar to the candidate compounds chosen for method development) from a paper on 'malodour-causing substances of human waste' (Sato, *et al.,* 2001).

GC-MS Method modifications:

TD tubes were desorbed by purging for 2 min at ambient temperature then for 10 min at 250°C. Volatiles purged from the TD tubes were captured on a cold trap with was initially maintained at 10°C then set to a fast heating rate to 250°C with a hold

time of 5 min. (Conditions based on Markes Recommendations Thermal Desorption)*[i]. The GC oven was maintained at 35°C for 5 min then raised at increments of 10°C per min to a maximum of 200°C. (The conditions were initially based on parameters from Zebron recommendations) *[ii] and then adjusted to the above to give a retention time between 2 and 5 min for both ethyl propionate and methyl butyrate.) Scan, Time 0 to 21.50 min with no solvent delay, selected to capture peaks with a low retention time.

2.4.2 Candidate compound Internal Standards

The two candidate compounds were purchased from Sigma-Aldrich; methyl butyrate (butanoic acid, methyl ester) – Sigma-Aldrich product no. 19358. Formula $C_5H_{10}O_2$. Molecular weight 102.13g/mol. Boiling point 102-103°C. Density 0.898g/mLat 25°C. CAS no. 623427.

Ethyl propionate (propanoic acid, ethyl ester) – Sigma-Aldrich product no. 96727. Formula $C_5H_{10}O_2$. Molecular weight 102.13g/mol. Boiling point 99°C. CAS no. 105373.

Stock solution of each compound was prepared at 200ng/l and serial dilutions of each prepared; - 100, 50, 25, 12.5, 6.25, 3.12 and 0ng/l all diluted in methanol. This range was first chosen to see what peak size would be found from GC-MS analysis with the modified method. A 100µl aliquot of each concentration was dispensed into glass vial and sealed. The solutions were then stored at -80°C until required. With a 1µl Hamilton syringe the Tenax TA tube was injected with 0.5µl of each standard and

a flow of helium at 500ml/min passed across each tube, analysis by GC-MS was using the modified method. All dilutions were prepared in the same way.

On finding peaks from an initial run further batchs of solutions of ethyl propionate and methyl butyrate were made to cover the range of values found from the faecal samples. These ranged from 4000 to 0 ng/l for methyl butyrate and 1000 to 0 ng/l for ethyl propionate in methanol. Dilutions for ethyl propionate were 1000, 250, 62.5, 15.62, 3.9, 0.97, 0.49 and 0.12 ng/l. Dilutions for methyl butyrate were 4000, 1000, 250, 62.5, 15.62, 3.9, 0.97, 0.49 ng/l. Several repeats of each dilution were analysed on the GC-MS to prepare a standard curve for the quantification of these compounds in an actual sample.

2.4.3 Limit of Detection for the Standards

The limit of detection in analysis is the smallest measurable concentration of a substance that can be detected with reasonable certainty against the absence of that substance for a given method. This is often described as the LOD (Lower limit of detection). Standard curves for methyl butyrate and ethyl propionate were prepared to quantify the LOD (a value of 3SD from the mean gives a 5% or less of a probability of the result being wrong). Calculation of the value of 3SD from the lowest mean value the LOD was taken as the lowest concentration relating to the blank or intercept as shown in Figures 23 and 24.

2.4.4 Comparing retention time for general method and modified method

Analysis from a faecal sample from the general method was picked out for comparison. There was still some of the frozen sample left to do further analysis for the modified method to compare methods. The faecal headspace was drawn across a Tenax TA TD tube and analysed with the modified method, this sample analysed from the original method contained both methyl butyrate at 10 ng/l and ethyl propionate at 243 ng/l. This was run alongside TD tubes with a similar range containing 250, 75 and 1.17 ng/l ethyl propionate and methyl butyrate for comparison.

2.4.5 Spiking faecal sample for analysis

Headspace from the same faecal sample was passed onto a further three Tenax TA TD tubes and spiked with 250, 75 and 1.17 ng/l respectively of both methyl butyrate and ethyl propionate, and control TD tubes of the same concentrations was also analysed to compare with the spiked samples.

2.4.6 Details from patient volunteers – age and gender

Information on age and sex was provided by Addenbrooke's Hospital for the control and disease groups after completion of the analysis.

Chapter 3

3.1 Results from patient volunteers - age and gender

Age group and disease type, CD, UC and IBS was plotted into a bar chart as seen in Figure 12. Each disease is more prevalent in the 26 to 35 age range and CD continues to be high in the 36 to 45 age range.

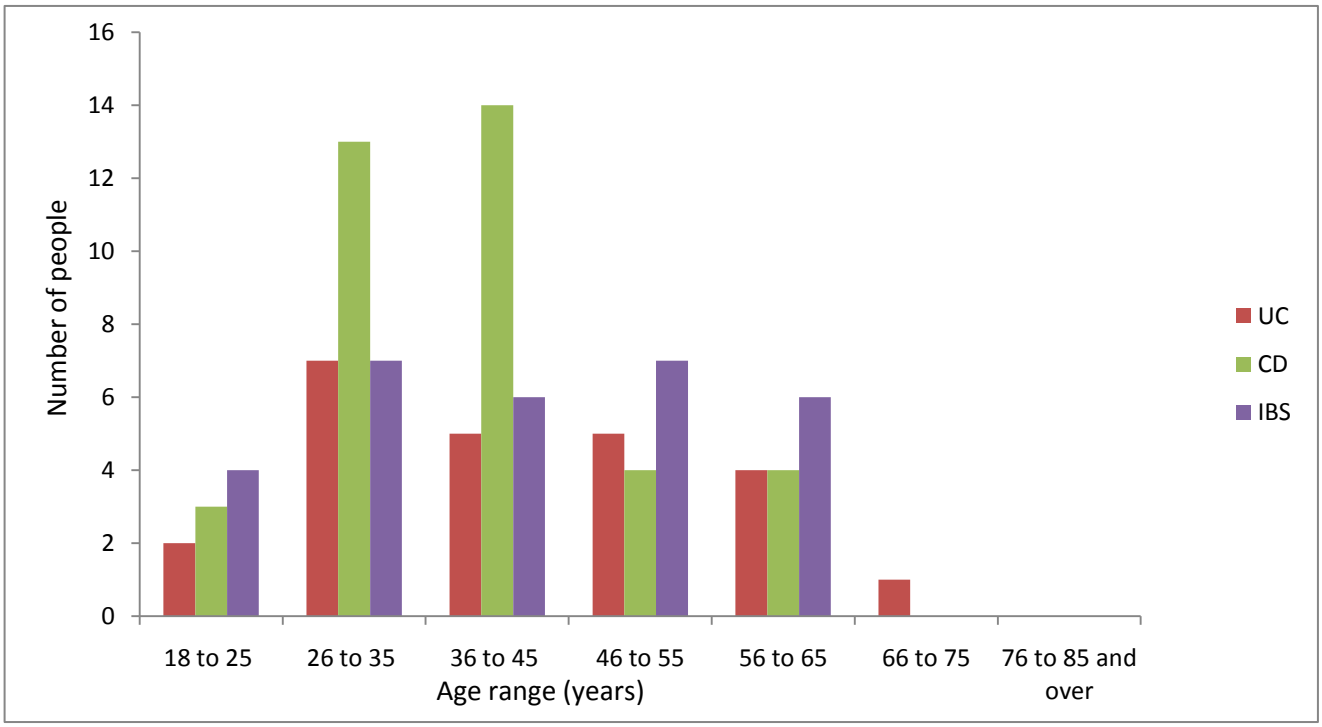


Figure 12 Bar chart showing disease type against age.

Key to Figure 12

UC = ulcerative colitis

CD = Crohn's disease

IBS = irritable bowel syndrome

The distribution of males vs. females for groups is shown on Table 5.

Table 5 Number of males to females for each group.

Group	Male	Female
Control	12	8
IBS	4	27
UC	13	9
CD	13	16

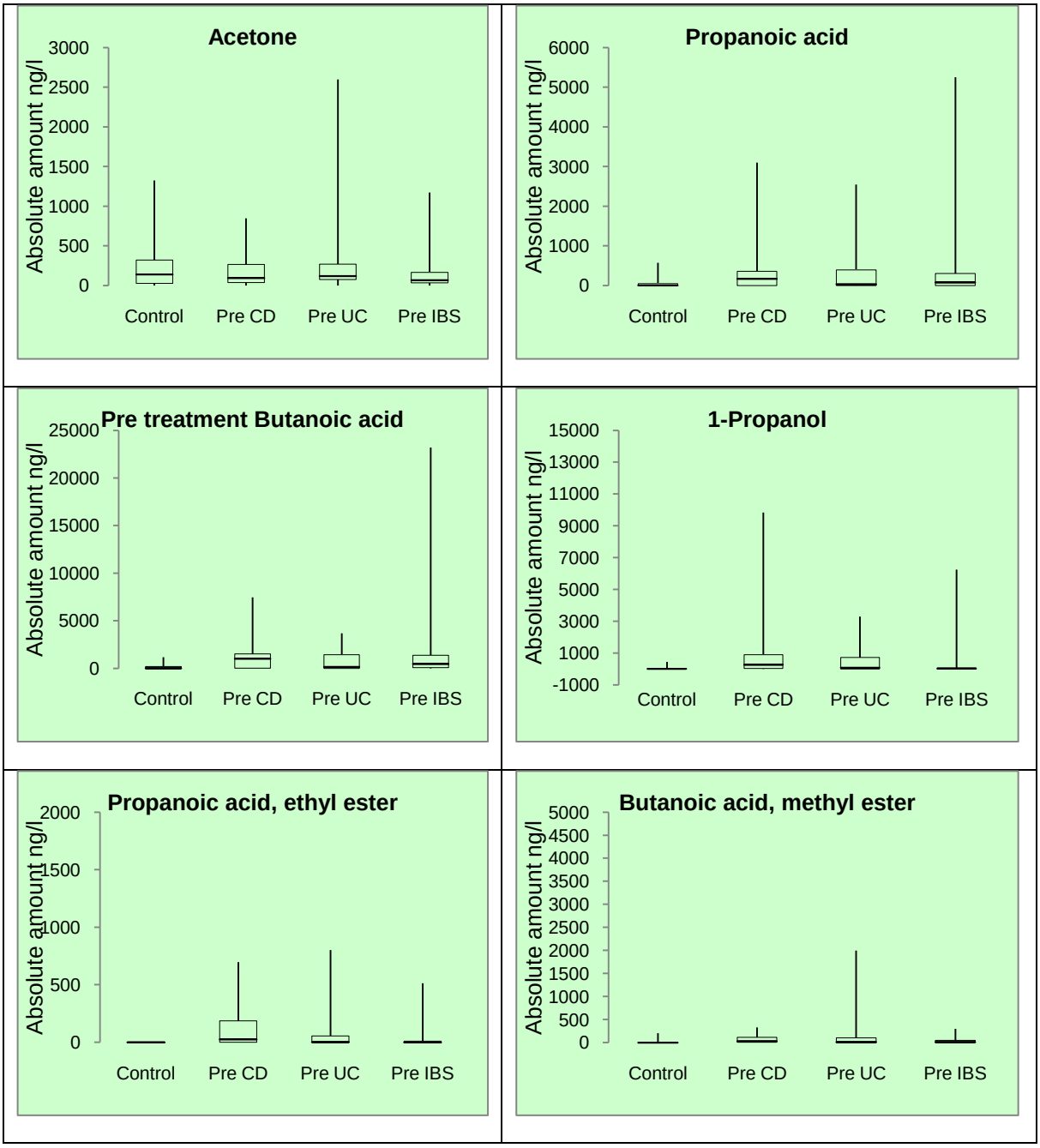
3.2 Candidate compound results

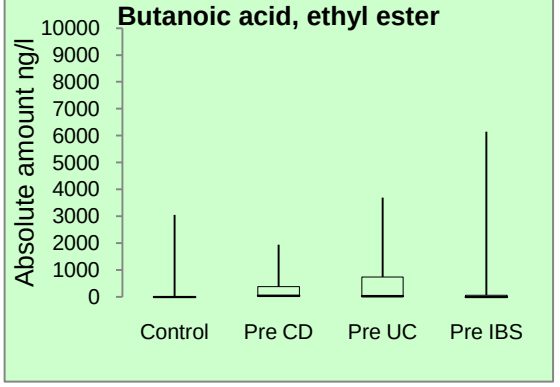
The candidate compound short-list was statistically analysed. This was carried out on pre-treatment faeces initially, as this sample matrix was the basis for choosing candidate compounds. The post treatment faeces groups were then examined for the same compounds, followed by breath, blood and urine for the same faecal compounds. There was only one control sample group and this was the same for pre and post treatment disease groups.

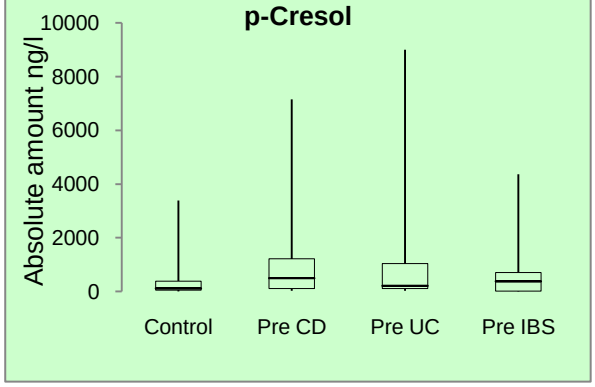
3.2.1 Faecal samples pre treatment disease groups and controls

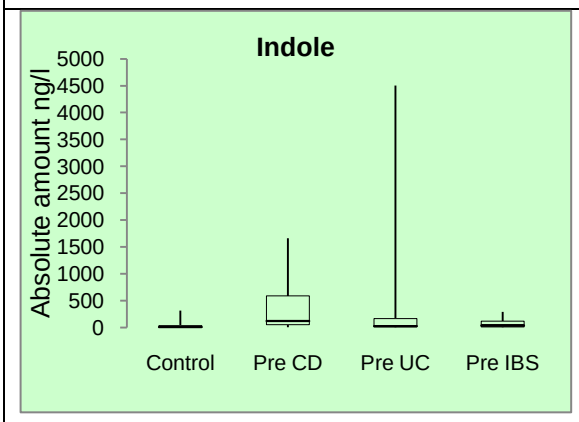
Box and whisker plots are a statistically descriptive way to show the data, Figure 13 shows the data range for each of the compounds on the short-list chosen on potential biomarkers.

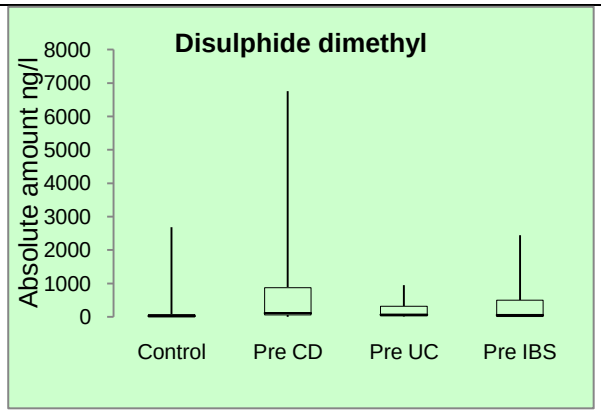
Figure 13 Box and whisker plots for the candidate compound short-list for faeces to compare between control and pre treatment disease groups.

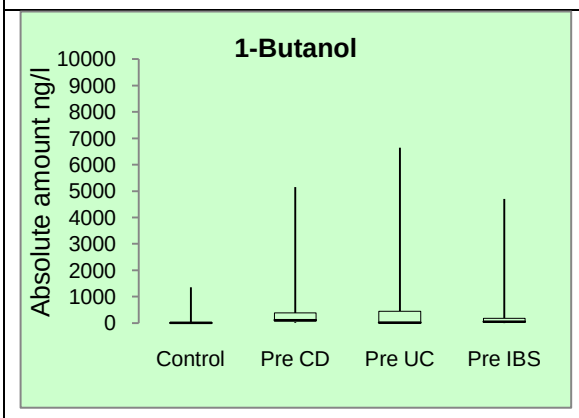


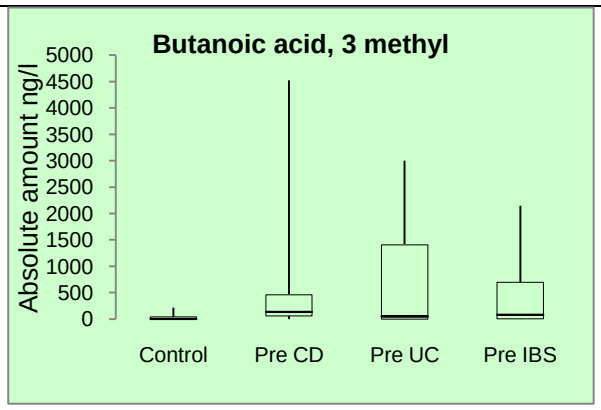

Butanoic acid, ethyl ester
Absolute amount ng/l
10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
Control
Pre CD
Pre UC
Pre IBS

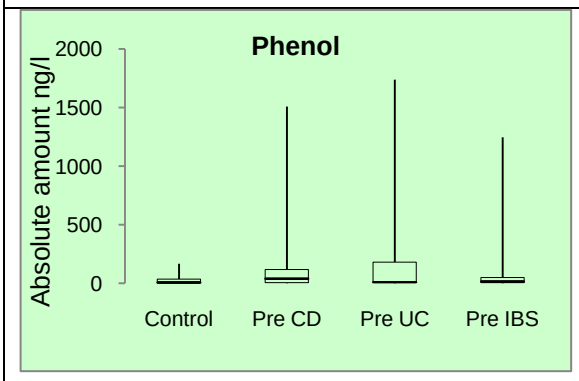

p-Cresol
Absolute amount ng/l
10000
8000
6000
4000
2000
0
Control
Pre CD
Pre UC
Pre IBS


Indole
Absolute amount ng/l
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
0
Control
Pre CD
Pre UC
Pre IBS


Disulphide dimethyl
Absolute amount ng/l
8000
7000
6000
5000
4000
3000
2000
1000
0
Control
Pre CD
Pre UC
Pre IBS


1-Butanol
Absolute amount ng/l
10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
Control
Pre CD
Pre UC
Pre IBS

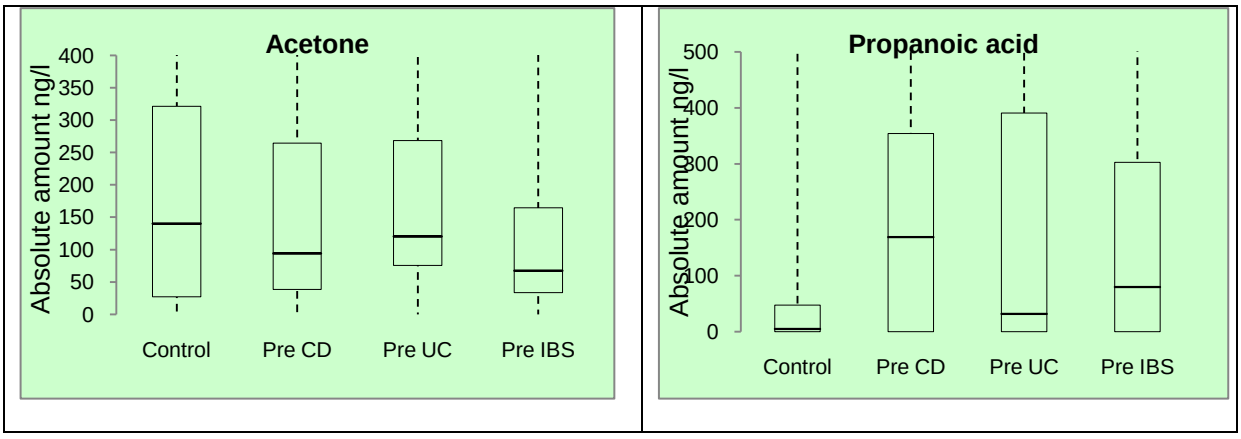

Butanoic acid, 3 methyl
Absolute amount ng/l
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
0
Control
Pre CD
Pre UC
Pre IBS

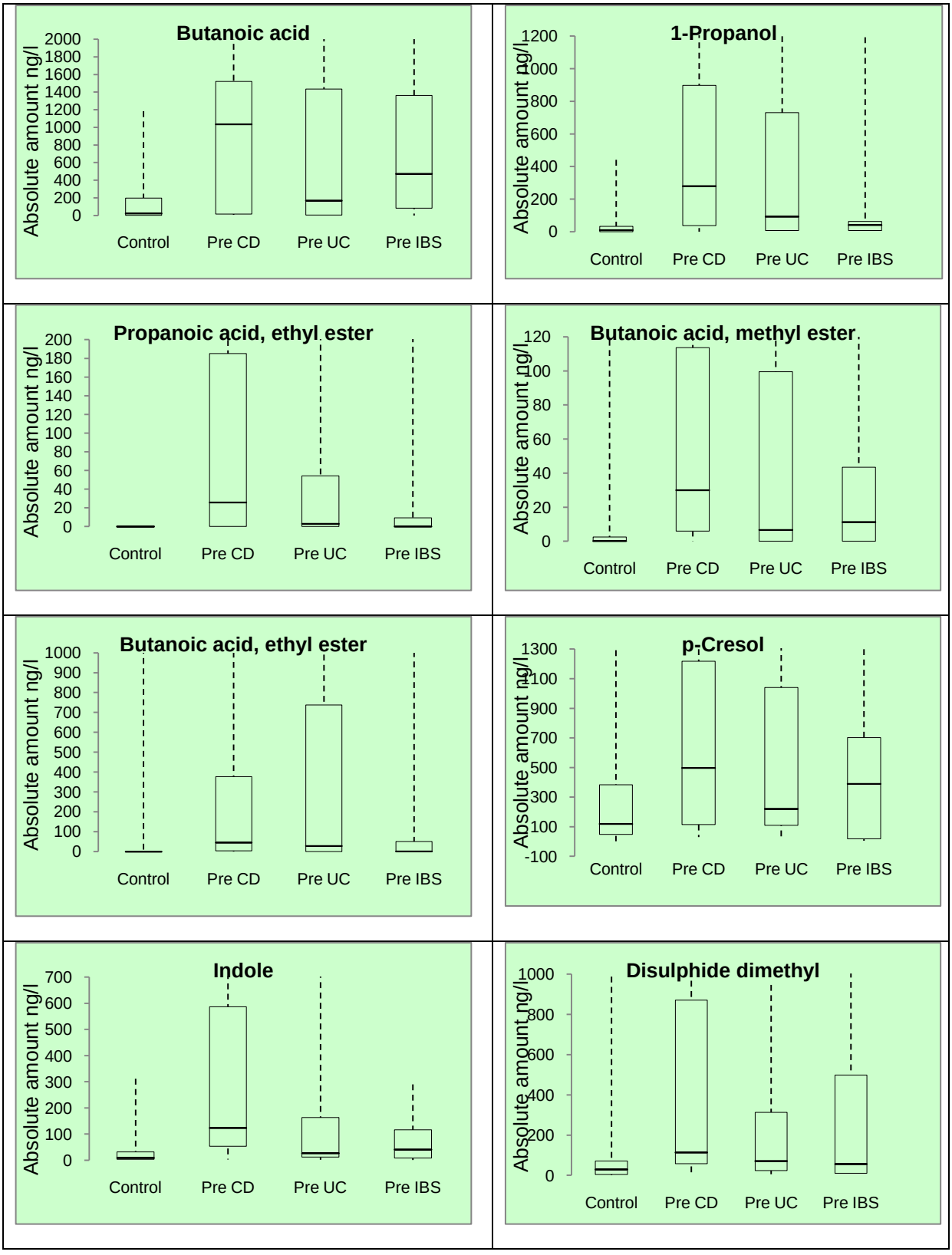

Phenol
Absolute amount ng/l
2000
1500
1000
500
0
Control
Pre CD
Pre UC
Pre IBS

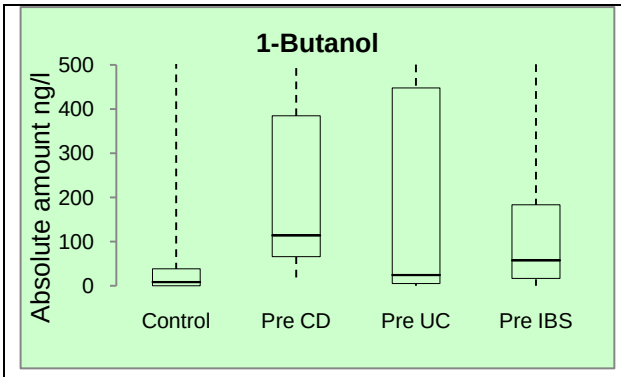
The data is very skewed shown by the outlying lines. Plots have been expanded in Figure 14 to show more clearly the median and quartile values. A high proportion of data from each of the plots had outliers to take into account and the ranges of absolute amounts were highly varied. All control samples had smaller amounts of the compound than the pre disease groups with the exception of acetone; this was expected as it is ubiquitous and a product of the host and bacterial metabolism (Turner, et al., 2006). Non-parametric Kruskal Wallis tests then showed which compounds were significant (Table 6 on page 67). These are marked with an asterisk and the median values of each compound for the disease groups were calculated from the box and whisker plots.

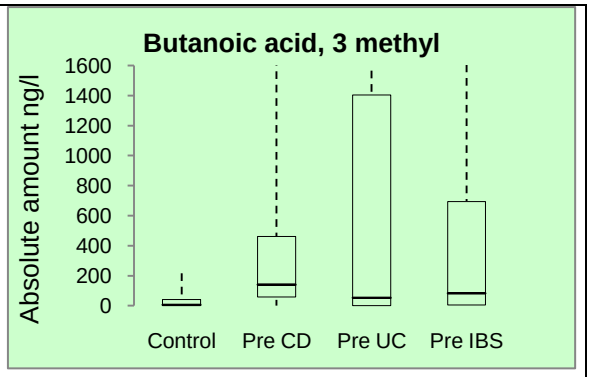
Visually comparing between control and pre treatment disease groups in Figure 14, the vertical scale has been expanded to allow median and upper and lower quartiles to be clearly distinguished. Whiskers indicating outliers are shown as broken lines since many go off the scale.

Figure 14 Box and whisker plots for the candidate compound short-list for faeces




Butanoic acid
Absolute amount ng/l
2000
1800
1600
1400
1200
1000
800
600
400
200
0
Control
Pre CD
Pre UC
Pre IBS
1-Propanol
Absolute amount ng/l
1200
1000
800
600
400
200
0
Control
Pre CD
Pre UC
Pre IBS
Propanoic acid, ethyl ester
Absolute amount ng/l
200
180
160
140
120
100
80
60
40
20
0
Control
Pre CD
Pre UC
Pre IBS
Butanoic acid, methyl ester
Absolute amount ng/l
120
100
80
60
40
20
0
Control
Pre CD
Pre UC
Pre IBS
Butanoic acid, ethyl ester
Absolute amount ng/l
1000
900
800
700
600
500
400
300
200
100
0
Control
Pre CD
Pre UC
Pre IBS
p-Cresol
Absolute amount ng/l
1300
1100
900
700
500
300
100
-100
Control
Pre CD
Pre UC
Pre IBS
Indole
Absolute amount ng/l
700
600
500
400
300
200
100
0
Control
Pre CD
Pre UC
Pre IBS
Disulphide dimethyl
Absolute amount ng/l
1000
800
600
400
200
0
Control
Pre CD
Pre UC
Pre IBS


1-Butanol
Absolute amount ng/l
500
400
300
200
100
0
Control
Pre CD
Pre UC
Pre IBS


Butanoic acid, 3 methyl
Absolute amount ng/l
1600
1400
1200
1000
800
600
400
200
0
Control
Pre CD
Pre UC
Pre IBS

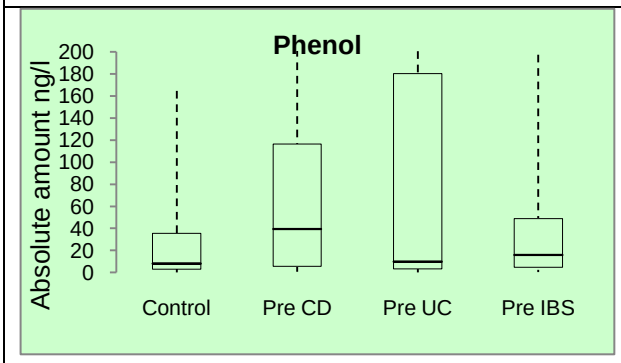

Phenol
Absolute amount ng/l
200
180
160
140
120
100
80
60
40
20
0
Control
Pre CD
Pre UC
Pre IBS

Table 6 Median and p values (Kruskal Wallis one way analysis of variance) for control and pre treatment group candidate compounds. P values that are statistically significant are shown by an asterisk.

Median (concentration ng/l)					**P value**
Compound	**Control**	**CD**	**UC**	**IBS**	
acetone	140	94	121	68	0.700
propanoic acid	5	169	32	80	0.505
butanoic acid	25	1037	169	473	0.047*
1-propanol	10	281	94	43	0.002*
propanoic acid, ethyl ester	0	26	3	0	0.000*
butanoic acid, methyl ester	0.25	30	7	11	0.013*
butanoic acid, ethyl ester	0	46	29	1	0.003*
p-cresol	120	499	221	390	0.177
indole	9	124	27	41	0.001*
disulphide dimethyl	30	114	71	57	0.071
1-butanol	9	115	25	58	0.005*
butanoic acid, 3-methyl	6	141	54	84	0.018*
phenol	8	40	10	16	0.312

The eight significant compounds with a $p < 0.05$ were treated for a *Post hoc* analysis, the Mann Whitney U-test was used to determine significance between pairs, (Table 7).

Table 7 *Post hoc* analysis (Mann Whitney U-test) show differences between pairs within control and pre treatment disease groups, for candidate compounds. Significant values are shown with asterisk.

	Control	Pre CD	Pre UC	Pre IBS
Control		0.009*	0.224	0.018*
Pre CD			0.329	0.708
Pre UC				0.403
Pre IBS				

Butanoic acid

	Control	Pre CD	Pre UC	Pre IBS
Control		0.0002*	0.019*	0.073*
Pre CD			0.211	0.028*
Pre UC				0.309
Pre IBS				

1-Propanol

	Control	Pre CD	Pre UC	Pre IBS
Control		0*	0.015*	0.021*
Pre CD			0.254	0.031*
Pre UC				0.488
Pre IBS				

Propanoic acid, ethyl ester

	Control	Pre CD	Pre UC	Pre IBS
Control		0.001*	0.101	0.088
Pre CD			0.361	0.162
Pre UC				0.828
Pre IBS				

Butanoic acid, methyl ester

	Control	Pre CD	Pre UC	Pre IBS
Control		0.00*	0.015*	0.109
Pre CD			0.871	0.049*
Pre UC				0.25
Pre IBS				

Butanoic acid, ethyl ester

	Control	Pre CD	Pre UC	Pre IBS
Control		0*	0.056	0.045*
Pre CD			0.035*	0.03
Pre UC				0.984
Pre IBS				

Indole

	Control	Pre CD	Pre UC	Pre IBS
Control		0*	0.094	0.059
Pre CD			0.138	0.069
Pre UC				0.825
Pre IBS				

1-Butanol

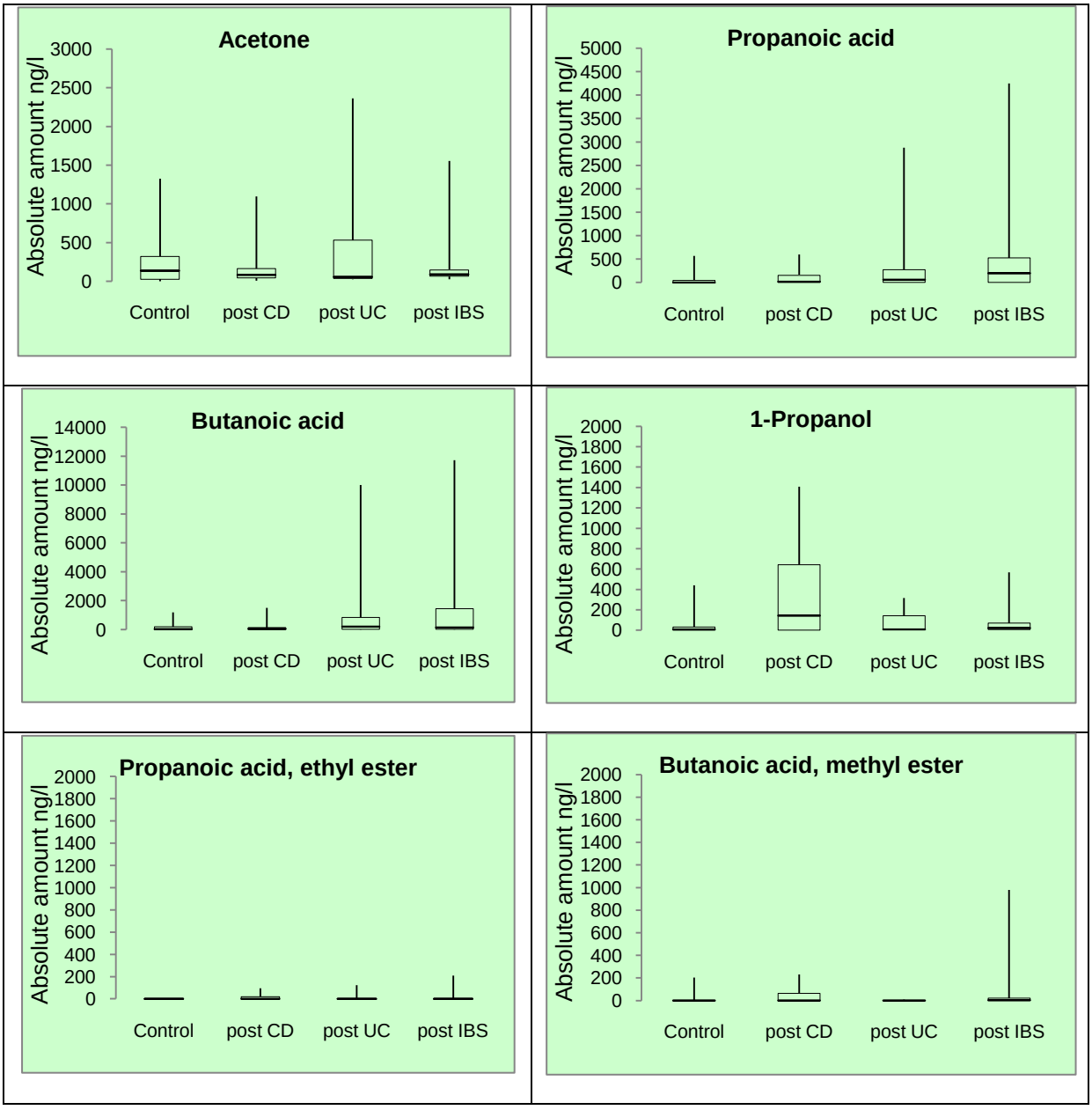
	Control	Pre CD	Pre UC	Pre IBS
Control		0.001*	0.093	0.025*
Pre CD			0.63	0.477
Pre UC				0.925
Pre IBS				

Butanoic acid, 3-methyl

All eight compounds for control and pre CD pairs were significantly different. The differences between control and pre CD are greater than for any of the other pre disease groups when compared with the control samples.

3.2.2 Faecal samples post treatment disease groups and controls

Box and whisker plots in Figure 15 interpret the data from each of the compounds on the short list chosen for potential biomarkers.



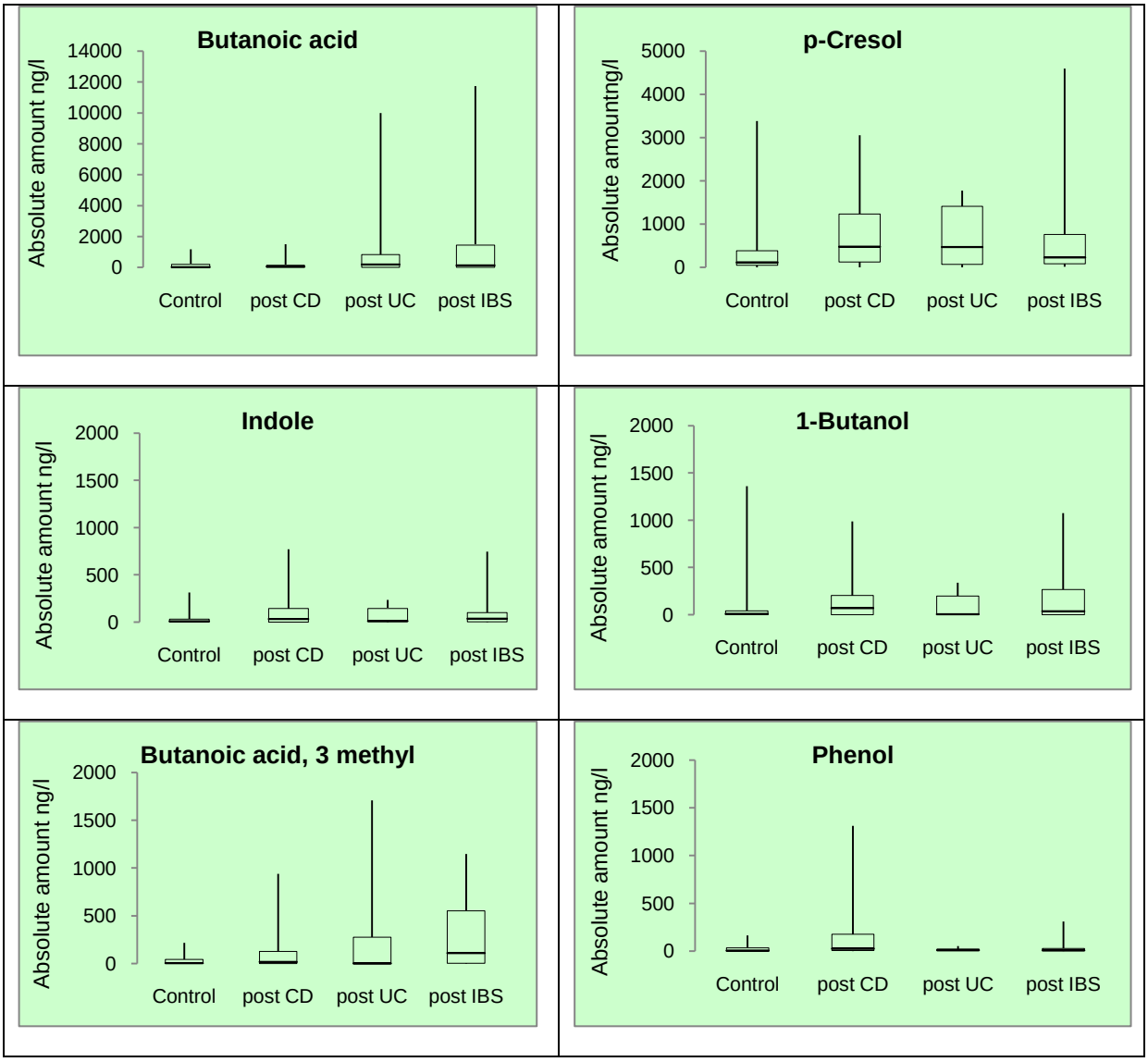
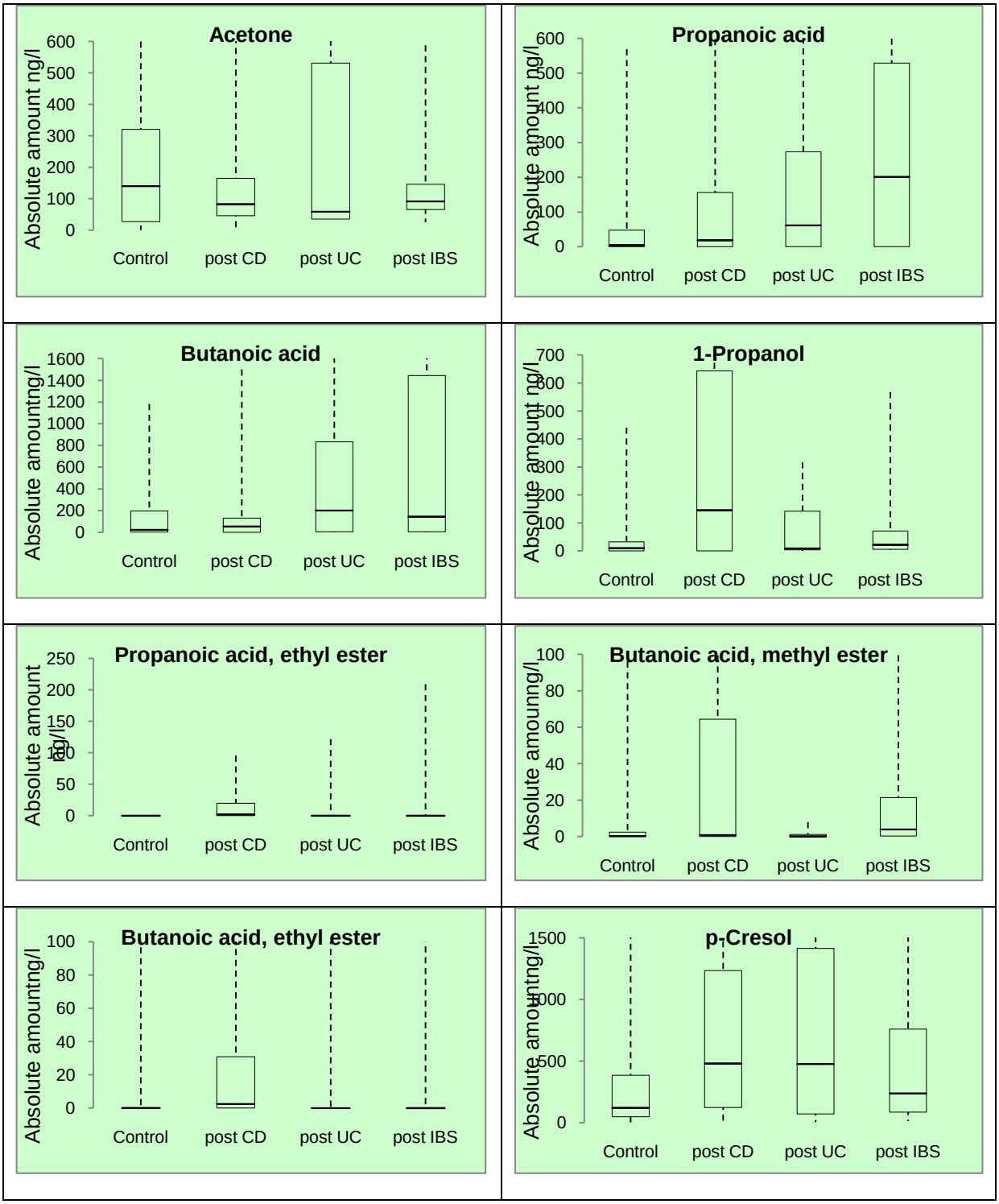


Figure 15 Box and Whisker plots for the candidate compound short-list for faeces. Comparison between control and post treatment disease groups showing outlying values.

Figure 16 shows the median and quartile ranges. Again the data was heavily skewed similar to pre treatment Box and Whisker plots. Post treatment groups showed absolute amounts to be lower than for the pre treatment groups for most of the compounds.


Acetone
Absolute amount ng/l
600
500
400
300
200
100
0
Control
post CD
post UC
post IBS
Propanoic acid
Absolute amount ng/l
600
500
400
300
200
100
0
Control
post CD
post UC
post IBS
Butanoic acid
Absolute amountng/l
1600
1400
1200
1000
800
600
400
200
0
Control
post CD
post UC
post IBS
1-Propanol
Absolute amount ng/l
700
600
500
400
300
200
100
0
Control
post CD
post UC
post IBS
Propanoic acid, ethyl ester
Absolute amount ng/l
250
200
150
100
50
0
Control
post CD
post UC
post IBS
Butanoic acid, methyl ester
Absolute amounng/l
100
80
60
40
20
0
Control
post CD
post UC
post IBS
Butanoic acid, ethyl ester
Absolute amountng/l
100
80
60
40
20
0
Control
post CD
post UC
post IBS
p-Cresol
Absolute amountng/l
1500
1000
500
0
Control
post CD
post UC
post IBS

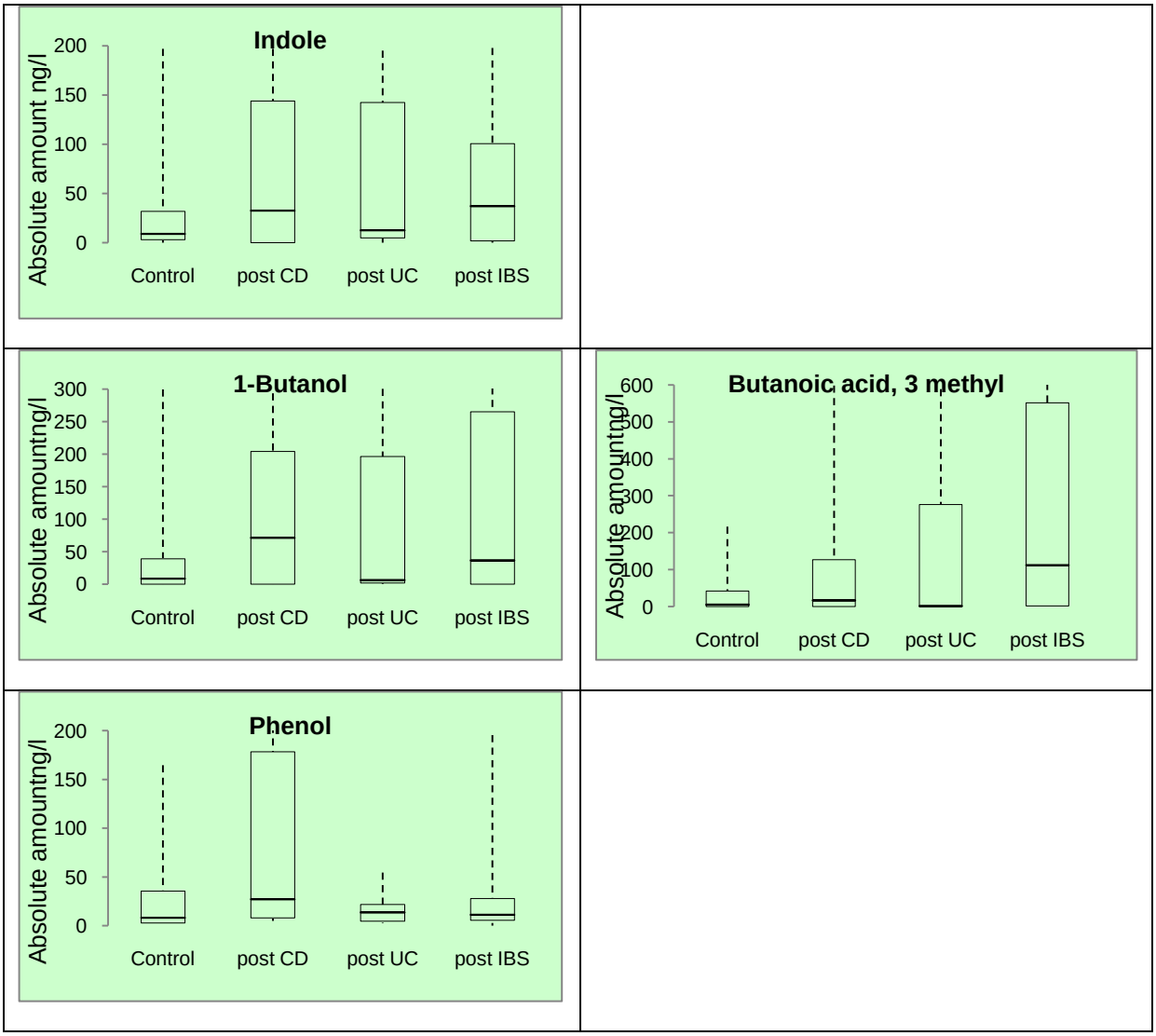


Figure 16 Box and Whisker plots for the candidate compound short-list for faeces, for comparison between control and post treatment disease groups. The vertical scale has been expanded to allow median and upper and lower quartiles to be clearly distinguished. Whiskers indicating outliers are shown as broken lines since many go off the scale.

Table 8 Median and p values (Kruskal Wallis - one way analysis of variance) for control and post treatment group candidate compounds. P values that are statistically significant are marked with an asterisk.

Median (concentration ng/l)					**P value**
Compound	**Control**	**CD**	**UC**	**IBS**	
acetone	140	82	59	91	0.776
propanoic acid	5	19	62	201	0.333
butanoic acid	25	54	202	144	0.348
1-propanol	10	146	9	23	0.203
propanoic acid, ethyl ester	0	2.1	0	0	0.000*
butanoic acid, methyl ester	0.25	0.87	0.21	3.9	0.008*
butanoic acid, ethyl ester	0	2.6	0	0	0.059
p-cresol	120	480	477	237	0.290
indole	9	33	13	37	0.714
disulphide dimethyl					
1-butanol	8.6	72	6	37	0.579
butanoic acid, 3-methyl	6	17	2	112	0.184
phenol	8	27	14	11	0.288

Only two compounds remain significant for post treatment groups (Table 8); significant compounds shown with asterisk. Table 9 shows the differences between pairs.

Table 9 *Post hoc* analysis (Mann Whitney U-test) show differences between pairs within control and post treatment disease groups, for candidate compounds; significant values shown with asterisk.

	Control	Pre CD	Pre UC	Pre IBS
Control		0*	0.015*	0.021*
Pre CD			0.254	0.031*
Pre UC				0.488
Pre IBS				

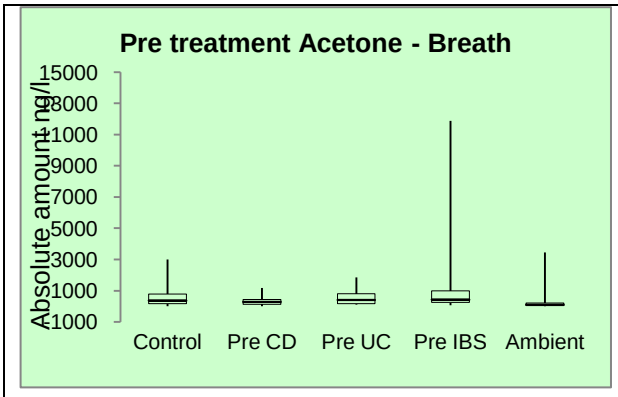
Propanoic acid, ethyl ester

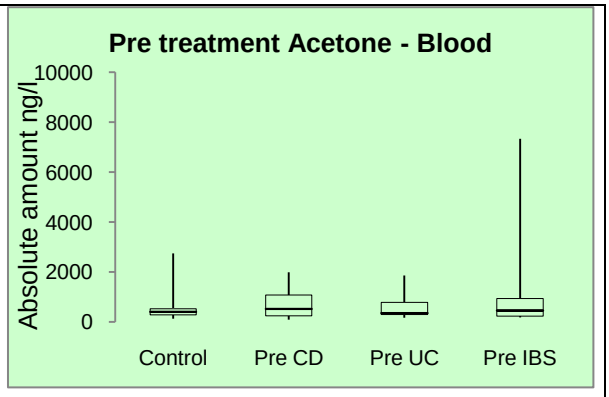
	Control	Pre CD	Pre UC	Pre IBS
Control		0.001*	0.015*	0.109
Pre CD			0.871	0.049*
Pre UC				0.25
Pre IBS				

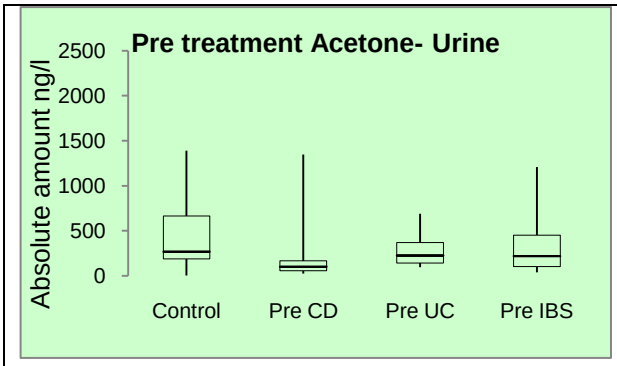
Butanoic acid, methyl ester

3.2.3 Breath, blood and urine samples pre treatment disease groups and controls

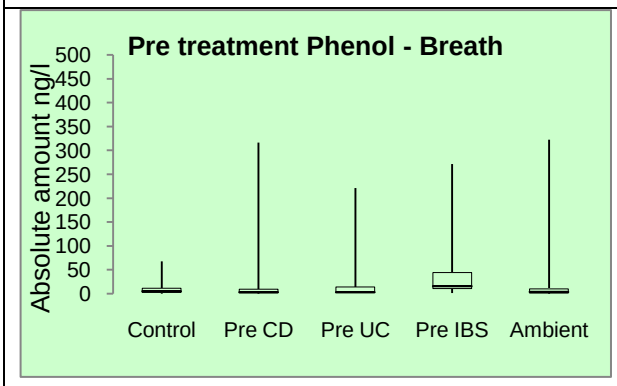
These samples were considered for the same short list of candidate compounds as the faecal samples. Box and Whisker plots in Figure 17 show outliers and Figure 18 shows median values and quartile ranges for acetone and phenol.

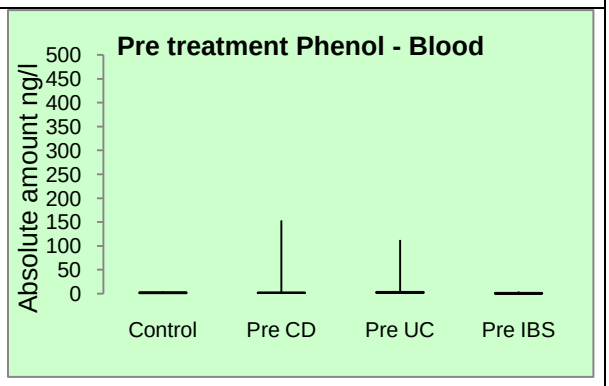


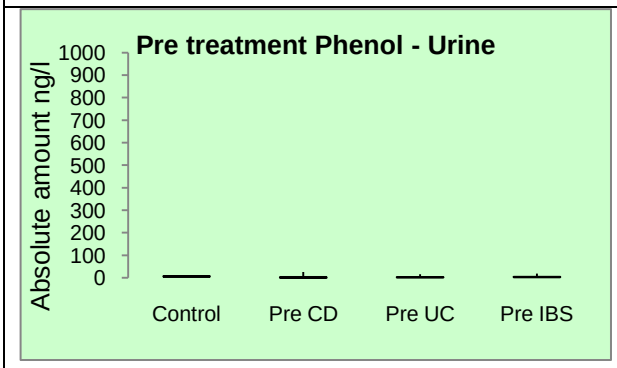




Acetone was significant in urine and breath, not blood.







Phenol found significant in blood and breath.

Figure 17 Box and Whisker plots for pre treatment disease groups breath, blood and urine; for acetone and phenol. Boxes are condensed to see outlier whiskers.

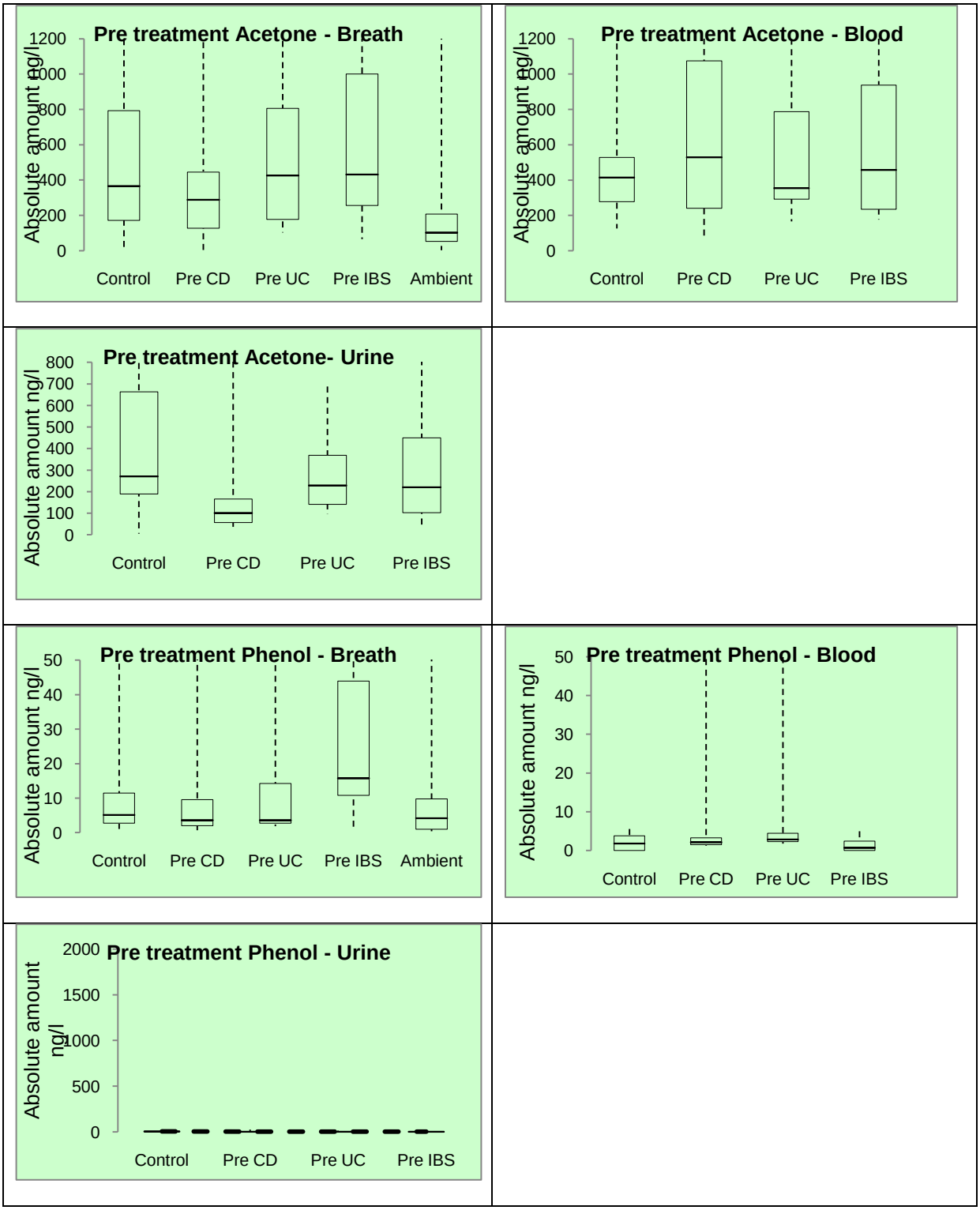


Figure 18 Box and Whisker plots for pre treatment disease groups breath, blood and urine; for acetone and phenol. Plots are expanded to show median, upper and lower quartiles.

Only two compounds from the short-list were found in these samples they were acetone, found in breath and blood but little from urine; and phenol was detected in breath, blood and urine but levels were all very low.

Table 10 shows the median values for acetone and phenol in the sample matrices and ambient air to compare with the breath sample. P values all show to be significant except for blood acetone.

Table 10 Median calculated from box and whisker plots and p values (Kruskal Wallis – one way analysis of variance) for control and pre treatment groups for acetone and phenol. Sample matrices types include breath (breath samples also have an ambient air value for comparison), blood and urine; significant compounds shown with an asterisk

Compound	Sample type	Control	CD	UC	IBS	Ambient	p-value
acetone	Breath	367	289	426	431	103	0.00*
acetone	Blood	415	530	459	356	-	0.944
acetone	Urine	271	92	229	221	-	0.006*
phenol	Breath	5.3	3.6	3.6	15.7	4.17	0.001*
phenol	Blood	1.8	2.2	2.9	0.8	-	0.002*
phenol	Urine	6.6	1.6	3.0	4.1	-	0.167

Comparisons between pairs in groups show a range of significant values as shown in Table 11.

Table 11 Mann Whitney U-test showing significant values* for pairs within the groups for acetone breath and urine and phenol breath and urine.

	Control	Pre CD	Pre UC	Pre IBS	Ambient
Control		0.338	0.89	0.538	0.002*
Pre CD			0.13	0.045*	0.0038
Pre UC				0.762	0*
Pre IBS					0*
Ambient					

Acetone *Post hoc* breath

	Control	Pre CD	Pre UC	Pre IBS
Control		0.003*	0.312	0.398
Pre CD			0.009*	0.02*
Pre UC				1
Pre IBS				

Acetone *Post hoc* urine

	Control	Pre CD	Pre UC	Pre IBS	Ambient
Control		0.473	0.983	0.007*	0.326
Pre CD			0.428	0*	0.741
Pre UC				0.03*	0.309
Pre IBS					0*
Ambient					

Phenol *Post hoc* breath

	Control	Pre CD	Pre UC	Pre IBS
Control		0.526	0.077	0.14
Pre CD			0.049*	0.012*
Pre UC				0*
Pre IBS				

Phenol *Post hoc* urine

3.2.4 Pre and post treatment faecal samples – Compared

Control and Pre and post treatment groups were compared for the significant candidate compounds. Table 12 and Table 13 show the differences.

Table 12 Statistically significant compounds shown by the *. Showing the comparison between pairs for control and pre treatment disease groups.

Compound	Control vs. Pre CD	Control vs. Pre UC	Control vs. Pre IBS	Pre CD vs. Pre UC	Pre CD vs. Pre IBS	Pre IBS vs. Pre UC
1-propanol	*	*	*		*	
butanoic acid, methyl ester	*					
butanoic acid, ethyl ester	*	*			*	
propanoic acid, ethyl ester	*	*	*		*	
indole	*		*	*	*	
butanoic acid	*		*			
butanoic acid, 3 methyl	*		*			
1-butanol	*					

Table 13 Statistically significant compounds shown by the *. Showing the comparison between pairs for control and post treatment disease groups.

Compound	Control vs. Post CD	Control vs. Post UC	Control vs. Post IBS	Post CD vs. Post UC	Post CD vs. Post IBS	Post IBS vs. Post UC
1-propanol						
butanoic acid, methyl ester			*			*
butanoic acid, ethyl ester						
propanoic acid, ethyl ester	*				*	
indole						
butanoic acid						
butanoic acid, 3 methyl						
1-butanol						

There were twenty one instances where significant differences were found between pairs for the control and pre treatment disease groups, compared with just four in the pairs for the control and the post treatment groups, suggesting that post treatment, the disease groups are more similar to the controls.

3.3 Method development results

3.3.1. Compound changes for the different methods

A faecal sample from the original method was compared with the standards using the modified method. Retention times and separation between methyl butyrate and ethyl propionate were different for the modified method as can be seen from comparing faecal sample and standards. Both compounds had a shorter retention time than the original method but separation was slightly longer going from 37 to 62 seconds. Figure 19 and Figure 20 show the differences for each method. Tenax TA tubes as controls with the Sigma-Aldrich compounds were compared with the faecal samples for the same compounds.

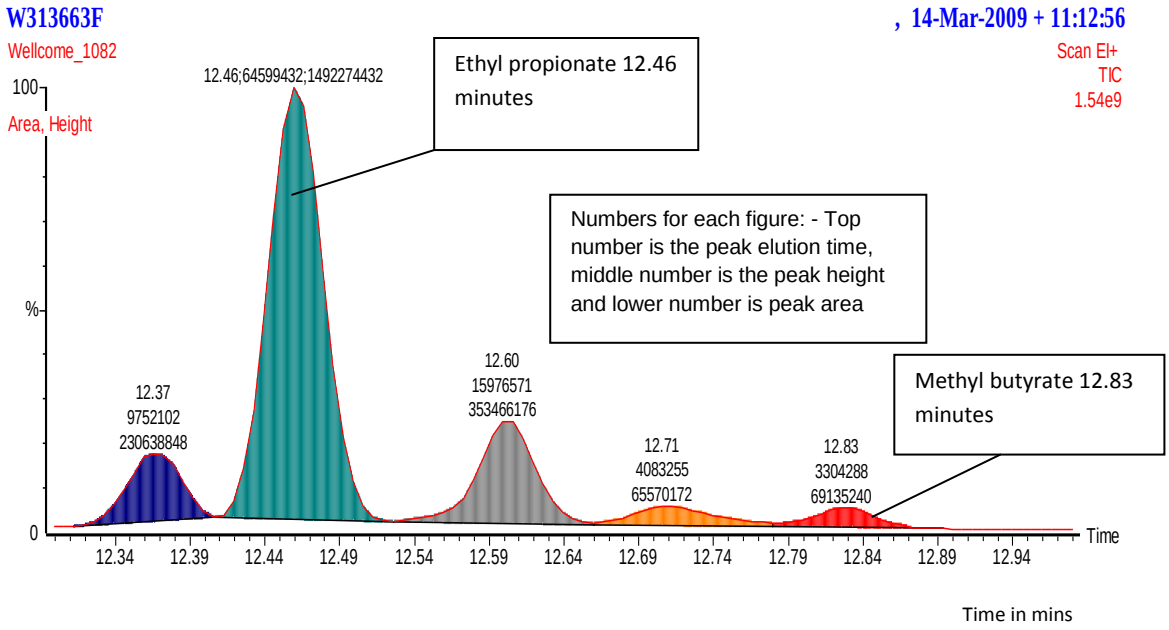


Figure 19 Chromatogram of a faecal sample from the original GC-MS method. Ethyl propionate is shown by the green peak and methyl butyrate is shown by the red peak.

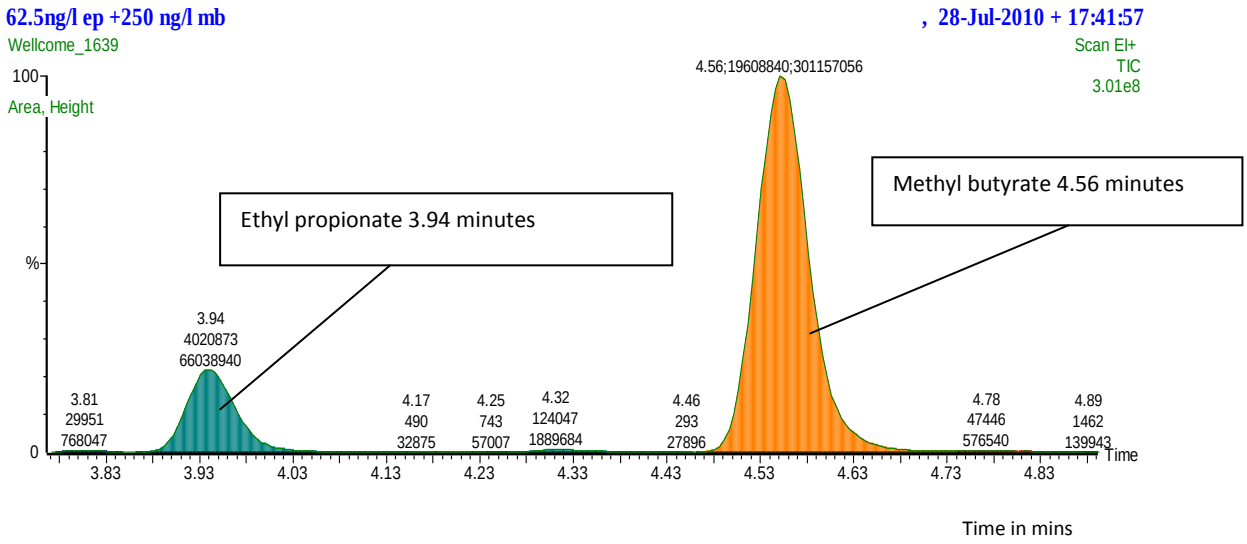


Figure 20 Chromatogram of ethyl propionate (green) and methyl butyrate (orange) using the modified GC-MS method.

A new TD tube containing headspace of a faecal sample was analysed with the modified method alongside TD tubes containing standards. The chromatograms are shown in Figure 21.

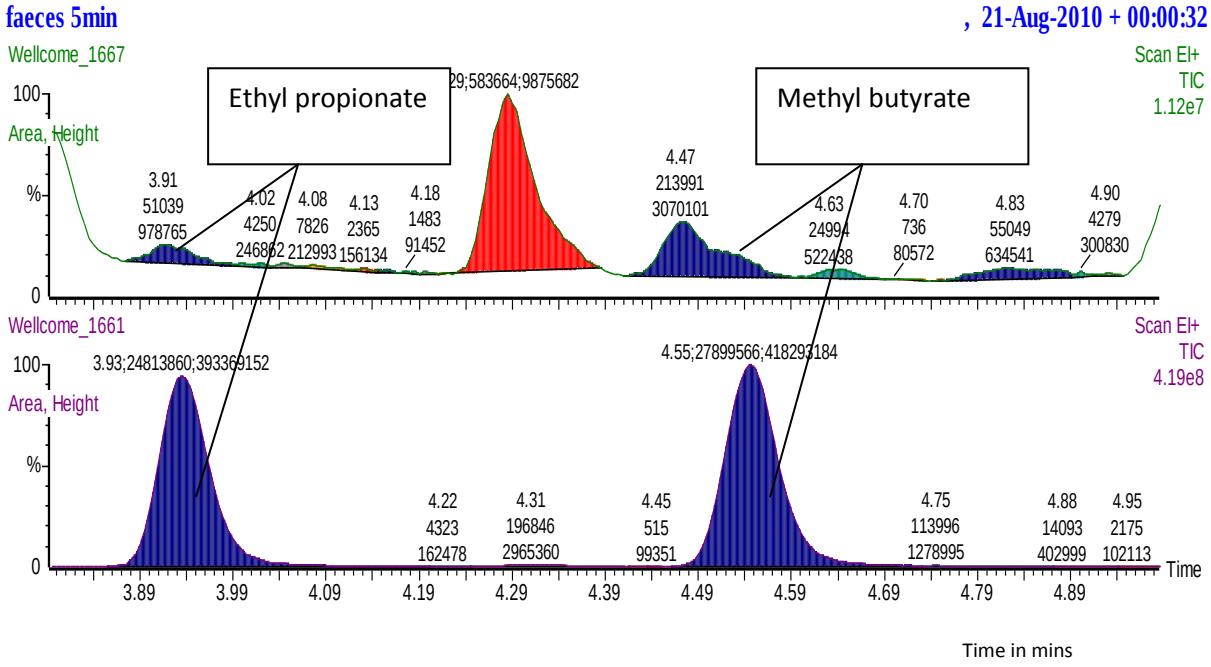


Figure 21 Chromatograms showing faecal sample (top) and ethyl propionate and methyl butyrate (bottom) using the modified GC-MS method.

3.3.2 Regression lines for ethyl propionate and methyl butyrate

Regression lines, Figures 22 and 23 were produced to determine quantification for the chosen standards and their LOD.

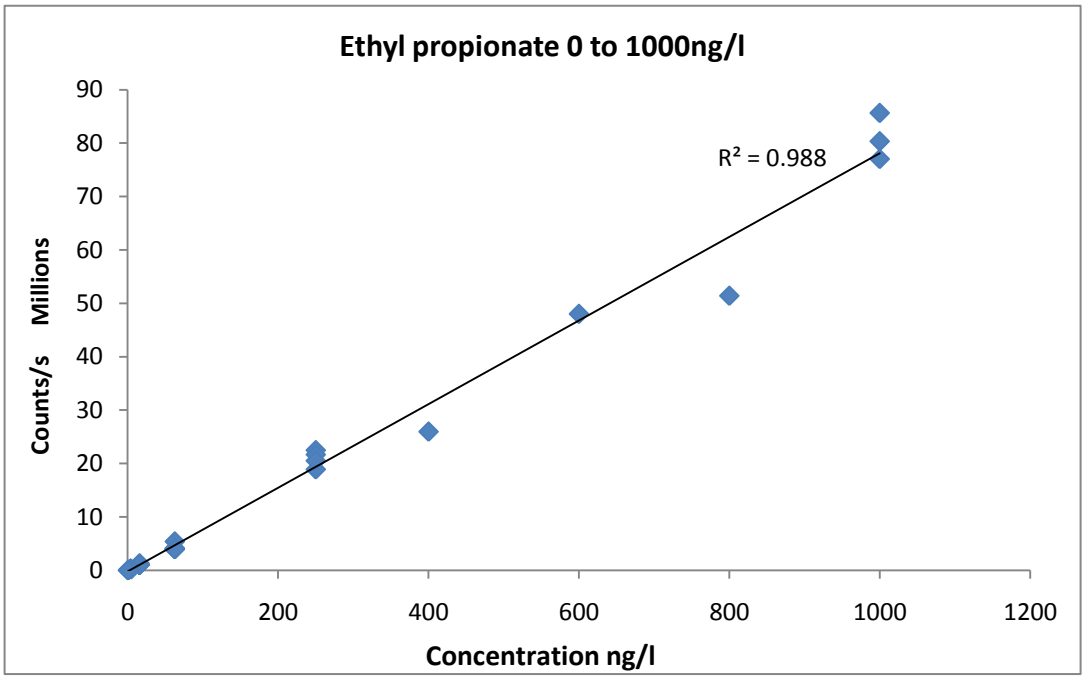


Figure 22 Regression line for ethyl propionate.

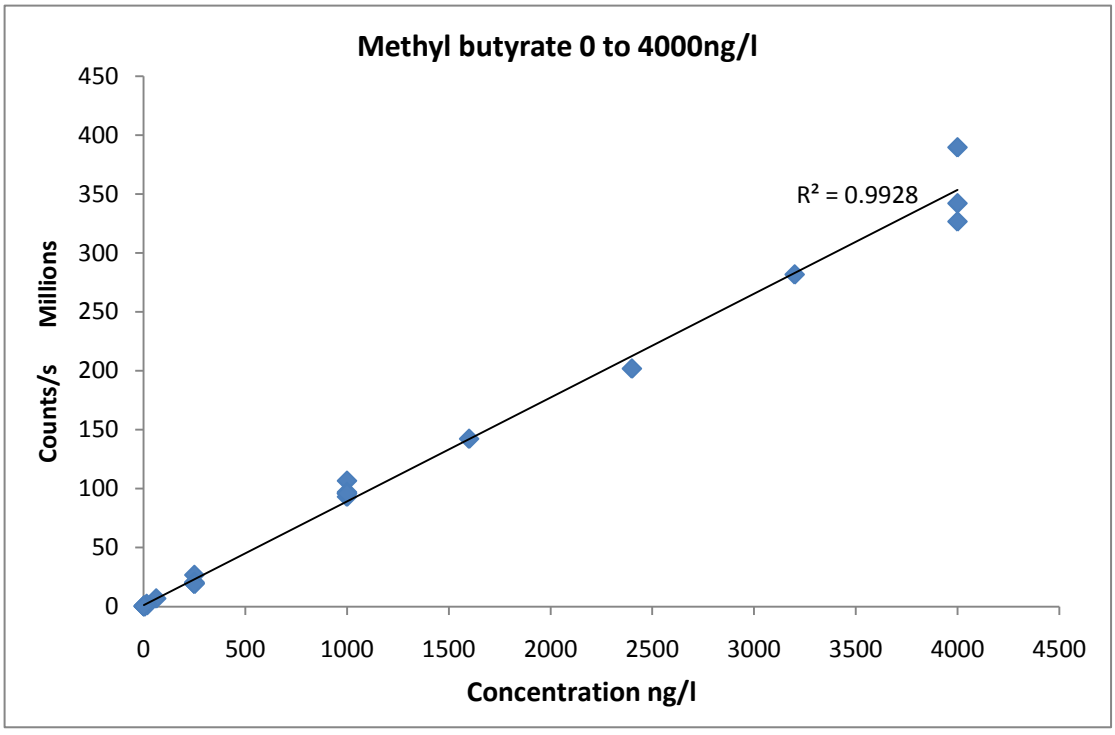


Figure 23 Regression line for methyl butyrate.

Several concentrations of standard were repeated in triplicate to provide sufficient data for plotting the regression line. Linear regression was good for both compounds.

3.3.3 Limit of Detection

Data from the regression lines gave sufficient information to calculate the lower LOD (LLOD) as seen in the graphs below.

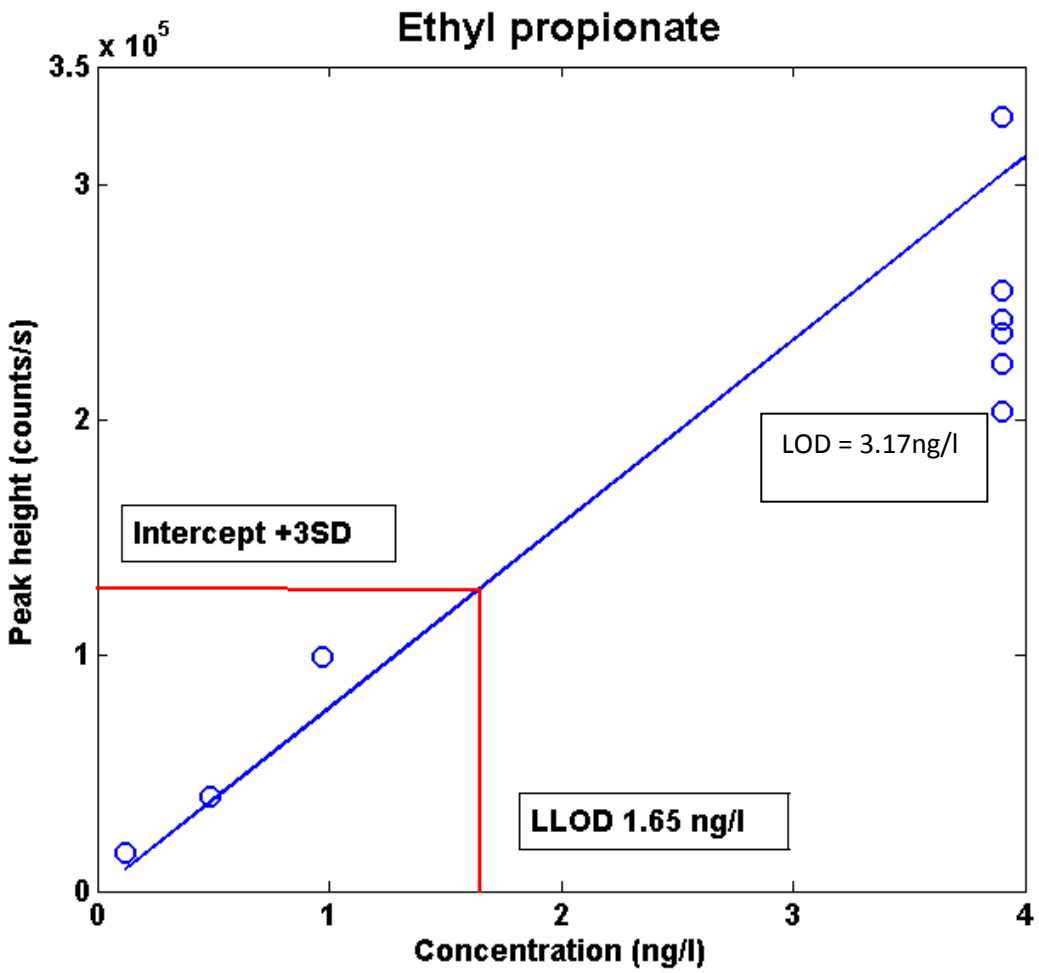


Figure 24 LLOD for ethyl propionate (scale has been expanded from the regression line shown in figure 21to show lower limits).

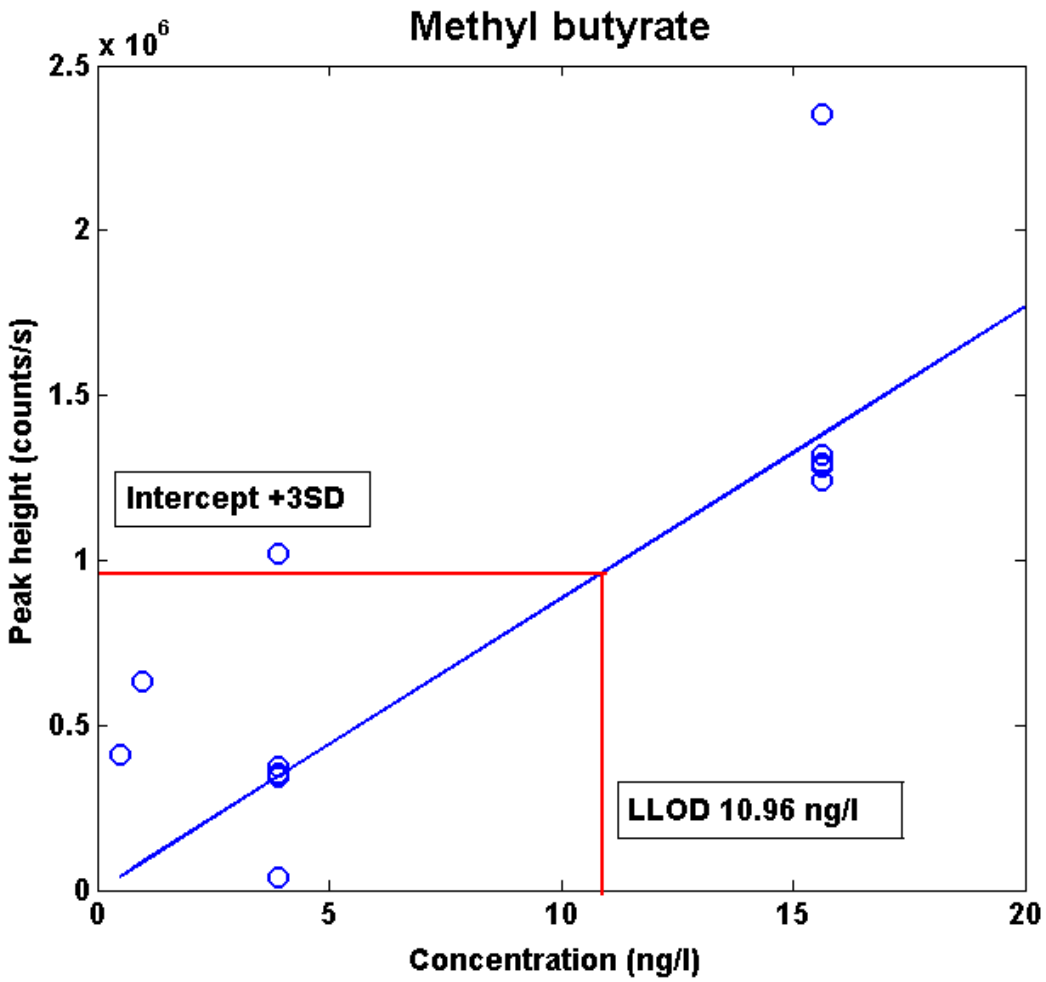


Figure 25 LLOD for methyl butyrate (scale has been expanded from the regression line shown in figure 22 to show lower limits).

The LLOD value was found for both standards. The standard deviation for the lowest concentration of standard used was found and then multiplied by three. This value was extrapolated from the regression line to find the concentration of the standard. The formula for the LLOD is y = mx + c where m is the slope, y is the standard deviation x 3 and x is the LLOD. When the formula is converted to y/m = x the value of x or the LOD can be determined. For ethyl propionate the slope is

78384. When y = 129476 and m = 78384 and the LLOD = 1.65 ng/l. For methyl butyrate when y = 965930 and m = 88084 and the LLOD = 10.97 ng/l.

3.3.4 Faecal samples spiked with methyl butyrate and ethyl propionate

Figure 26 shows the final experiment done to see the effect of spiking TD samples of faecal headspace with standards of methyl butyrate and ethyl propionate. Three Tenax TD tubes containing faecal samples were spiked with methyl butyrate and ethyl propionate each with the following concentrations, 250, 75 and 1.17 ng/l respectively. These were run with controls TD tubes containing standards with concentrations as above.

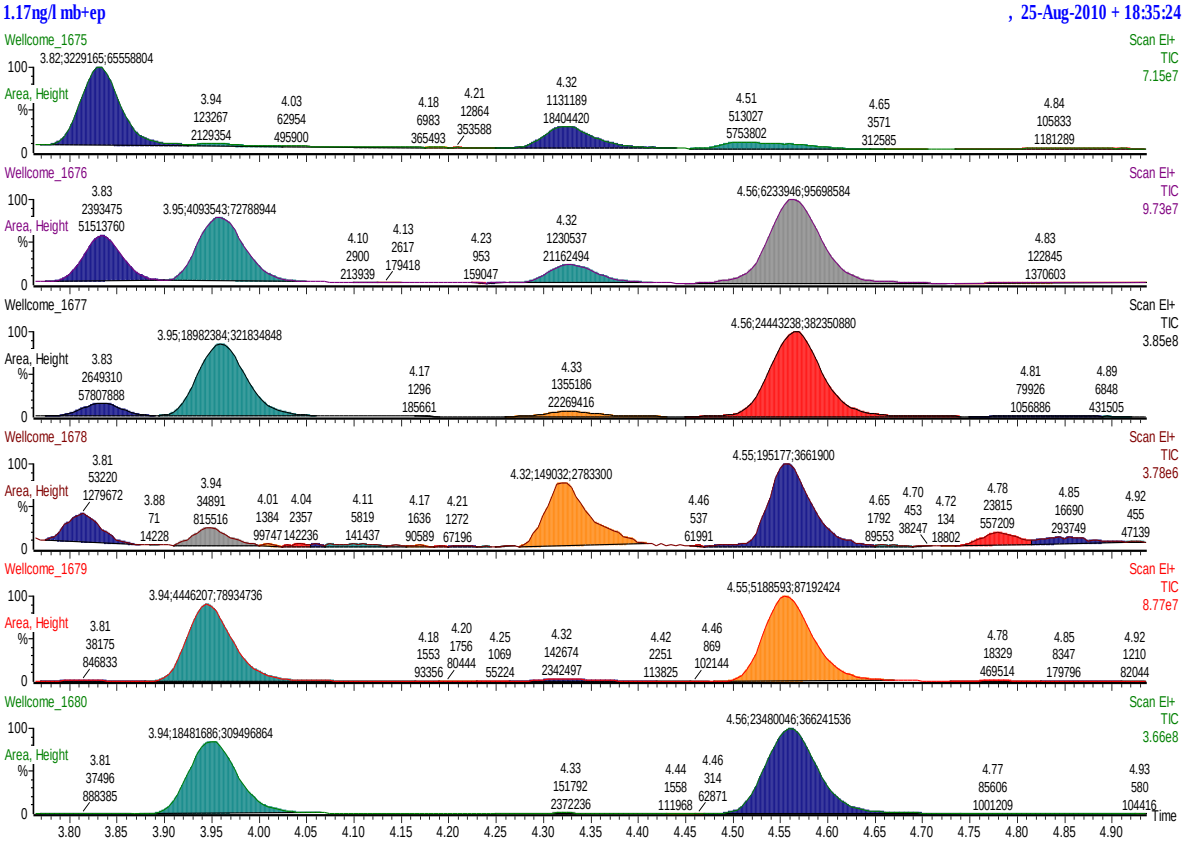


Figure 26 Chromatograms of spiked faeces and compared with ethyl propionate and methyl butyrate see key for explanation.

Key for figure 26 above		
Wellcome number	Faeces	Ethyl propionate and Methyl butyrate(ng/l)
1675	Present	1.17
1676	Present	75
1677	Present	250
1678	Absent	1.17
1679	Absent	75
1680	Absent	250

Graphs were prepared from the chromatographic data. Figures 27 and 28 show these results. Peak height was plotted for control tubes (containing standards) and spiked faeces containing standards.

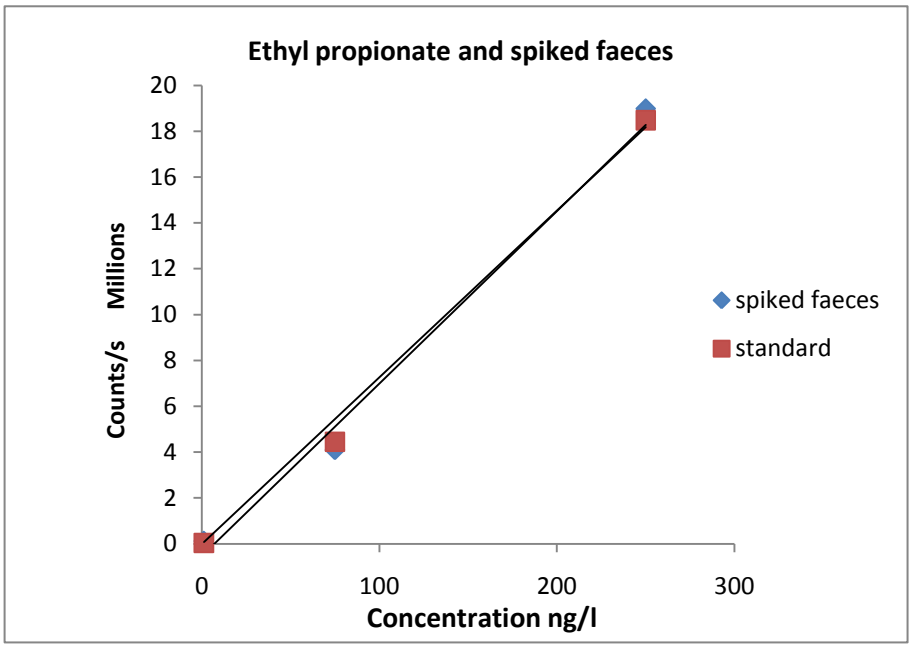


Figure 27 Spiked faeces with ethyl propionate and standard.

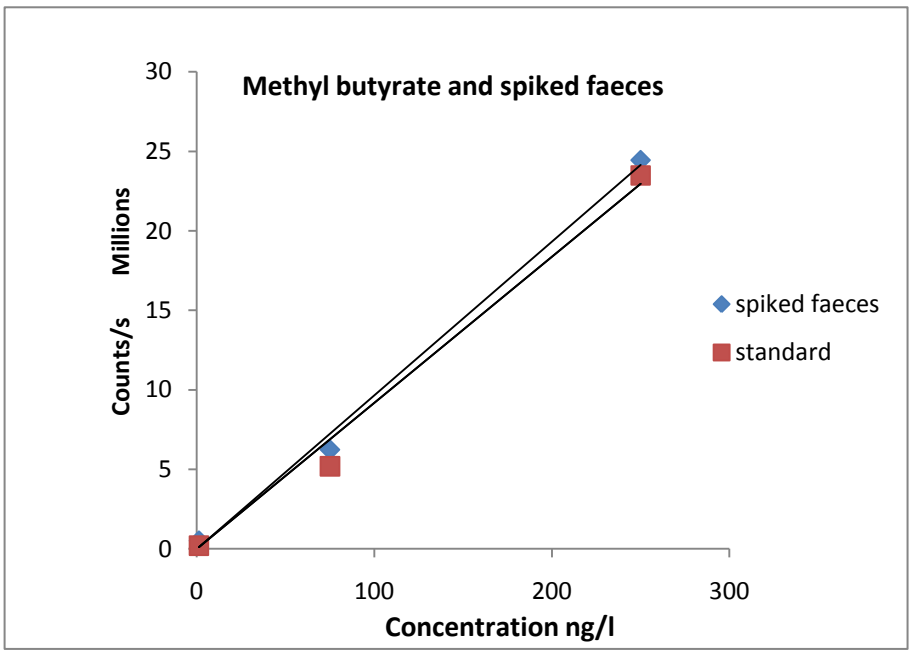


Figure 28 Spiked faeces with methyl butyrate and standard.

The above graphs for both ethyl propionate and methyl butyrate display a very good correlation between spiked faeces and control standards. Indicating these compounds would make suitable for biomarkers with a GC-MS analytical technique.

Chapter 4

4.1 Discussion

ATD GC-MS was a chosen method of analysis for this project as it is the best screening method for the identification of unknown compounds in complex mixtures, and is very sensitive. As a general method for volatile analysis and TD sample collection it provided an efficient way of obtaining breath samples from patients for transport to Cranfield for analysis. The blood, urine and faeces samples also transported to Cranfield were stored before analysis could be carried out and compared with breath. Analysis of all samples provided a great number of identified compounds for data handling.

The faecal samples provided the best set of data, more so than the other clinical samples and were subsequently used for identification of diagnostic biomarkers. Breath, blood and urine were compared with the faecal samples for the same candidate compounds found in faeces, but few were detected. The same procedure could be used again to 'trawl' through data for new sets of candidate compounds for these samples, but is beyond the scope of this project.

Although there is a lot of literature this area of work is still quite new. Continuation of work from this project would involve testing more clinical samples for the chosen compounds to see if they confirm the findings from this study and to improved method development for better analytical resolution. It would be more desirable to obtain the analysis from breath or urine rather than from faeces as both are non-

invasive and avoid the problems of working with faeces, i.e., that such samples are unpleasant for both patient and analyst.

Sample derivatisation may help to find sulphur based compounds, but best avoided as it would require a further step and becomes a laboratory based analysis, a rapid non-invasive technique that can be performed at the bedside or in hospital is preferable.

The sample pool of volunteers was taken without any prerequisite preparation from them, for example overnight fasting. A record of medication, diet and smoking and drinking habits was recorded which could be used in comparison to the data analysed for the clinical samples. Other factors that may have an effect on gut function such as stage of the menstrual cycle although few changes in acetone are noticed (Smith, *et al.,* 2006), lifestyle and occupation many be useful for further investigation into disease (Houghton, *et al*., 2009)

4.1.1 Difficulty in choosing biomarkers

IBD is a diverse condition and aetiology not yet precise, resulting in uncertainty of the origin of a potential marker compound; VOCs are produced from either the commensal bacteria from the gut or from metabolic processes in the host. Disease where markers have traceable origin such as breath isoprene, a biomarker for lipid disorders assist confidence in accurate choice. Isoprene is produced endogenously and thought to be part of the biochemical pathway for cholesterol. (Salerno-Kennedy and Cashman, 2005) IBD is complex by nature and resultant data from sample

analysis produced many hundreds of compounds making choice of markers difficult. A strategy was adopted to ease the task, and 'Candidate Compounds' selected on a number of assumptions, firstly from the greatest abundance as seen from analysis, secondly informed literature sources and SIFT-MS analysis (Turner, 2006) and last from chromatogram inspection using a subset. Thirteen compounds were found from this method from the control and pre-disease groups faecal samples, eight of which were found to be statistically significant (see Table 6). Few of these compounds were however found in blood, urine and breath.

4.1.2 Findings from the candidate compounds

The first of the most abundant compounds was acetone which is ubiquitous and in high concentrations as a marker for diabetes (Probert *et al* 2009). It was not one of the statistically significant compounds but proved the method was reliable. Propanoic and butanoic acid was also found in abundance, these are both products of fermentation in the gut (Garner, *et al.*, 2007). Control groups had less of both acids than disease groups and butanoic acid was greater in disease groups than propanic acid. Post treatment CD had less butanoic acid than pre treatment.

Compounds chosen from literature were 1-propanol as it is potentially damaging to the gut (Garner, *et al.*, 2007). Control groups were lower than pre-treatment CD and UC indicating a possibility that this compound is harmful. The butyrate and propionate compounds were higher for all pre-disease groups especially CD and UC although these compounds are essentially beneficial to gut health. Maybe over production of them are harmful (Garner, *et al.,* 2007). Indole was common to all faecal samples and levels were lot higher for the pre treatment CD group indicating a

higher level may be damaging. A study by Garner *et al* 2007 suggested *p*-cresol (4,methylphenol) and dimethyl disulphide were shared by 80% of subjects in a study for GI disease. In the present study they were all found higher in pre disease groups than controls.

Visual inspection compounds 1-butanol and 3-methyl butanoic acid was found to be statistically significant with less in controls than for pre disease groups. Phenol, like acetone, is ubiquitous and was not found to be significant.

In summary all eight candidate compounds that were found significant were all lower in the control group than in the pre-treatment disease groups. Post treatment shows a reduction in the candidate compounds and they appeared more similar to control samples. UC and CD generally had a greater amount of the candidate compounds.

4.1.3 Esters found in faeces

Esters make up a large portion of short-chain fatty acids (SCFAs) in the gut and their presence in colonic fermentation is a reflection on gut wall esterase activity and microbial activity, which may be changed if disease is present (Garner, et al., 2007). Of the eight compounds chosen as potential markers three of them were esters; methyl butyrate, ethyl butyrate and ethyl propionate. All three were statistically significant between the control and pre CD group, ethyl butyrate and ethyl propionate were significant for control and pre UC, ethyl propionate was significant between control and pre IBS and methyl butyrate was significant for control and post IBS. These findings indicate that esters do have a role in gut health.

4.1.4 Sulphur compounds found in faeces

Few sulphur containing compounds were found by GC-MS. These were sulphurous acid, di(cyclohexylmethyl) ester, hydrogen sulphide, dimethyl disulphide and sulphur dioxide. A few samples contained them but generally none was found. Dimethyl disulphide did appear more often than not and was added to the list of candidate compounds. Sulphurous compounds are implicated in inflammatory conditions, faeces from patients with UC contain SRBs and the sulfides produced by them interfere with butyrate-dependent energy metabolism (Christl, 1996).

By their nature the lack of quantifiable samples of sulphides was probably due to their high volatility and other properties such as susceptibility to oxidation, adsorption on to glass and rubber and binding to organic molecules. (Richardson,1999). Further method development on GC-MS would be necessary to quantify these compounds. A method more suitable to measuring sulphides directly from a breath sample has been proven by SIFT-MS. Significant differences were found between all other groups and the CD group. Having said that, the research was to find many sulphur based VOCs that may be indicative of IBD and that could be used as a biomarker. Other studies have found sulphides on breath of patients with disease (Miekisch and Schubert 2006). However, interpretation of this has to be done with care as sulphur is produced by bacteria in the mouth and thus taking breath samples by oral expiration and a nasal sampling regime would overcome this problem.

4.1.5 Method development

The two standards chosen for method development were found to have a shorter retention time and the separation was slightly better than for the original method. This was a favourable result as, if these two compounds were used as biomarkers for disease, the analysis time would be shorter and results from a test would be faster.

The calibration curves made from serial dilutions of the standards show good repeatability and provide a quantification standard for sample analysis and the lower limit of detection for both compounds. These are encouraging results for further work to be carried out on the other significant candidate compounds with the expectation they would provide similar results.

4.1.6 Details from patient volunteers age and gender

The age range 26 to 35 is when UC and IBS appear most commonly. CD affects more people in the age range from 26 to 45 years, concurrent with findings for these diseases mentioned in Sections1.4.3, 1.4.4 and 1.4.5 and all tail off by the 75 age group.

The most striking difference seen from the data is gender for IBS, females outnumber males by 6 times. The indication is that IBS is affected by the biological mechanisms of oestrogen and progesterone during the menstrual cycle, although five of these were over the age of 55 and possibly post menopausal (Houghton, *et al* 2009). Also women are affected by stress more than men and IBS is often brought

on by stress which causes hormonal imbalances (Cain, 2009). The number of volunteers for this was not enough to draw a firm conclusion and this is just an indicator of the possible outcome.

4.1.7 Comments, observations about the project and where it could have been better

- Mid way through analysing breath samples high levels of propan-2-ol was found. It was not discovered why this was occurring until a visit to Addenbrooke's where it was noticed that masks were being cleaned with propan-2-ol in the treatment room where patients were giving breath samples.
- A comprehensive list prepared with all sample numbers and columns for tracking samples could have been drawn up and handed out to all concerned with the analysis and handling of samples before analysis started, to keep up to date with all samples from collection to completion of all laboratory analysis.

4.1.8 Further work

To look at how compounds present ubiquitously in breath and urine vary in concentration in different disease groups.

- To identify markers for breath, blood and urine

- Examine the remaining six candidate compounds for method development and find LOD
- Optimise a method development for compounds of interest and identify sulphur based compounds
- Freeze thaw effect on sample storage
- There is sufficient data still to make a selection of candidate compounds from breath, blood and urine similar to ones found in faeces.
- Further comparisons between pre and post treatment disease groups would be interesting to see the effect of medication.
- Quantification on more of the candidate short-list compounds would increase quantity of data on possible use of them as diagnostic biomarkers.

5 Conclusions

Volatile compounds in headspace of breath, blood, urine and faeces were analysed using ATD-GC-MS to find potential marker compounds for identification of gastrointestinal disease.

- All sample matrices were treated in the same way. From all the clinical matrices the faecal samples provided the most compounds that were used for statistical analysis. Faeces are produced at the 'site' of disease and made a good place for identification of markers where IBD occurs.

- A practical approach to find suitable marker compounds was devised, by looking through literature, listing the most abundant compounds found and by visual inspection of a representative sample of chromatograms. Visual inspection was used to compare a control with each of the disease groups to inspect and identify any differences between them.
- Eight compounds (ethyl esters of propanoic and butanoic acid, butanoic acid, butanioc acid methyl ester, 3-methyl butanoic acid, 1-butanol, 1-propanol and indole) were identified from faeces that were significantly different between disease groups that could potentially be used as marker compounds. They could also discriminate between disease states.
- Breath, blood and urine were treated in the same way as faeces to find suitable markers but only two compounds were found to be significant for disease. Although these compounds were found to be significant the confidence that they could be used as biomarkers remains dubious as the levels found were very low or indeed often absent from many samples.
- Of the eight significant compounds, two (butanoic acid methyl ester (methyl butyrate) and propanoic acid ethyl ester) were chosen to use for method development, which gave a faster retention time, quantification and LLOD.
- Three methods for VOC analysis was used for the Wellcome Project, GC-MS, SIFT-MS and the Breathotron. GC-MS data provided the best results for number and quantity of compounds to choose from.
- At the beginning of this project it was postulated that sulphur compounds may be implicated in disease as they are thought to be damaging to the

sulphomucin layer. Unfortunately GC-MS analysis was not capable of detecting many of them and further method development would be required.

- SIFT-MS was found to be capable of detecting sulphur compounds and time permitting; further handling of data produced from this method may find sulphur markers of interest.
- Of the compounds identified on GC-MS there were many that do not contain sulphur and may have implication with disease, some of which have been identified here. This project has started a process in finding potential markers from faeces, and others, yet to be identified in breath, blood and urine to provide a rapid method for monitoring disease and the detection of disease in its early stages without the need for invasive investigation, preferable for patient and clinician alike.

5 Appendices

5.1 Ethics letter

National Research Ethics Service letter to Professor Hunter dated 4/7/07

National Research Ethics Service

Leeds (West) Research Ethics Committee

A/B Floor, Old Site
Beds General Infirmary
Great George Street
Leeds
LS1 3EX

phone: 0113 392 6788 FE: csimile: 0113 392 2863

04 July 2007

Professor John Hunter
Consultant Physician
Addenbrooke's Hospital
Hills Road

Cambridge

CB2 2QQ

Dear Professor Hunter

Full title of study: Abnormal sulphur metabolism **in the pathogenesis of gastrointestinal disease**

07/Q1205/39

The REC gave a favourable ethical opinion to this study on 24 April 2007.

Further notification(s) have been received from local site assessor(s) following site-specific assessment. On behalf of the Committee, I am pleased to confirm the extension of the favourable opinion to the new site(s). I attach an updated version

of the site lipproval form, listing all sites with a favourable ethical opinion to conduct the research.

R&D approval

[he Chief Investigator or sponsor should inform the local Principal InvestigatL):- at each site of the favourable opinion by sending a copy of this ietter and the attached fair

T

he
research should not commence at any NHS site until approval from ti :e R&D of ice for the relevant NHS care organisation has been confirmed.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with

the Standard Operating Procedures for Research Ethics Committees in the UK.

TO7/Q1205/39 Please quote **this number on all corruspond)nce**

Yours sincerely

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Miss Anna
Fawlk Assistant
Administrator

This Research Ethics Committee is an advisory committee to Y:.,(Kshire and The liurr her Strat,it)i Health Authority

The National Research Ethics Service (NRES) represents the NRES Directorate .-,"t•;in the National Patient Safety Agency and Research Ethics Committees in Eng:.Dn

Form from Leeds (West) Research Ethics Committee for 'List of sites with a favourable ethical opinion'

5.2 Information sheet for volunteers

ABNORMAL SULPHUR METABOLISM IN THE PATHOGENESIS OF GASTROINTESTINAL DISEASE vs 2 26/03/07

'You are being invited to take part in a research study. Before you decide, it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully. Talk to others about the study if you wish.

- *Part 1 tells you the purpose of this study and what will happen to you if you take part.*
- *Part 2 gives you more detailed information about the conduct of the study.*

Ask us if there is anything that is not clear or if you would like more information.

Part 1'

What is the purpose of the study?

Although many people suffer from ulcerative colitis, Crohn's disease and irritable bowel, the causes are still unknown. Mucins are proteins which protect the gastrointestinal tract and contain sulphate. Bacteria in the gut can remove sulphate from mucin, making the gut leakier and more inflamed. The sulphate is converted to hydrogen sulphide which is poisonous. Normally, it is rapidly converted to non-toxic compounds. We think that all three of these diseases are caused by disruption of this process; mucin breakdown may be too fast, hydrogen sulphide may not be removed fast enough or mucin synthesis may be faulty. We aim to find the precise fault points in the three diseases, looking at bacterial strains, individual metabolism and genetic differences in patients and controls. We will use complex computer techniques to integrate all our data. This information will help to identify individuals 'at risk' of suffering from these diseases and may provide improved treatments.

Why have I been chosen?

1. Because the health professionals involved in your care believe that you are

suffering from one of the conditions in which *we* are interested, that is to say, Inflammatory Bowel Disease, or another bowel disorder which produces similar symptoms.

2. We are also seeking healthy volunteers who may provide specimens for the purposes of comparison.

Do I have to take part?

Taking part in this study is entirely voluntary. If you have been approached as a healthy control, be reassured that should you wish not to take part in this study, there are many other people we can ask. If you have been approached as a patient, your future care will not be compromised if you decide not to take part.

What will happen to me if I take part? What do I have to do?

We **need** to ask you a few questions about your health and any medicines you may be taking. Then the nurse will arrange to collect a number of samples. You will be asked to blow into a plastic balloon to fill it up with your breath. She will also take a 20m1 sample of blood and will give you a plastic bottle into which you will be asked to pass a specimen of urine. You will also be given a plastic container in which we would like to you provide a small quantity of your next bowel motion.

The breath and the urine will be analysed to **see** which chemicals are present which contain sulphur. The blood will be used to measure the activity of enzymes involved in sulphur metabolism and also for bNA studies to see if the genetic makeup of people with gastrointestinal diseases, is in any way different. The stools will be analysed to **see** if they contain bacteria which produce a toxic chemical - hydrogen sulphide.

These samples will not reveal any information about you other than that required for this research and all samples will be destroyed after the research has been completed.

What are any possible disadvantages and risks of taking part?

We do not know of any.

What are the possible benefits of taking part?

These tests are completely *new,* and will not be used by the doctors looking after you to help in the management of your case, because we are still investigating their potential value. You personally will receive no benefit from taking part other than of knowing you may have helped in the understanding of these diseases.

'What will happen if I **don't want to carry on with the study?'**

You may choose to withdraw from the study at any stage. Your data will have been identified by an individual number which will also be used to identify the samples. If you decide that you wish your data to be withdrawn from the study, you should inform one of the research team who will ensure that all information collected under your number is destroyed. None of the data will be identified by your name, which is known only to the research team at Addenbrooke's.

Will my taking part in the study be kept confidential?

Yes. All the information about your participation in this study will be kept confidential. (see above for clarification).

Contact details

You may phone Mrs Catherine Price on 01223 586739

Please feel free to ask the research team if you have any further questions.

Part 2

Who has reviewed the Study?

The study has been reviewed and approved by the Leeds (West) Research Ethical Committee.

What happens if new relevant information becomes available during the study?

The study will still be completed in case the further information generated by this study further helps our understanding of the diseases concerned

What if there is a problem?

If you have a complaint, you may address it to the director of the Gastroenterology Department, br Miles Parkes, Box 133, Addenbrooke's Hospital, Cambridge CB2 2QQ.

What if I come to any harm?

It is unlikely that you will come to any harm providing these samples, although sometimes taking a blood sample can leave a small bruise on the arm. However, if anything went wrong, you would be protected by the usual indemnity arrangements of the National Health Service.

Will my taking part in the study be kept confidential?

Yes, no-one other than the team collecting your samples will know that you have taken part. Your GP will not be informed, as at this early stage, the information derived from the tests will not be of any help to him and we shall not in any way change your treatment.

All data will be stored anonymously under the terms and conditions of the Data Protection Act 1998. Data will be stored in locked cupboard and kept in line with Addenbrooke's NHS Trust Data Retention and Destruction Policy' with a retention period of 30yrs in line with the Dept of Health Code of Practice Management

Who is organising and paying for the study?

The study is part of the continuing research program of the Department of Gastroenterology at Addenbrooke's Hospital. The analysis on the specimens which we collect will be performed in the school of Biosciences at the University of Birmingham and at the Dept of Analytical Science and Informatics at the University of Cranfield.

The costs of this study are covered by a grant from the Wellcome Trust - the largest private body funding research in the UK. No individual payments are made to any of the staff taking part.

How will the results of the study be publicised?

In peer-reviewed internationally read medical journals.

THANK YOU FOR YOUR INTEREST

Dawn Fowler
MSc by Research
‘Analysis of volatile marker compounds in body fluid samples from patients with gastrointestinal disease'

Dawn Fowler
MSc by Research
'Analysis of volatile marker compounds in body fluid samples from patients with gastrointestinal disease'

5.3 Volunteer questionnaire

QUESTIONNAIRE Version 1 31/1/07

Project: ABNORMAL SULPHUR METABOLISM IN **THE PATHOGENESIS OF GASTROINTESTINAL DISEASE**

Participant number:

Today's date: Time of sample:

PLEASE ANSWER THE FOLLOWING QUESTIONS:

DOB:

APP

ROX

WEI

GHT

:

APP

RO

X

HEI

GH

T

Do you/have or have you ever had?

Condition	YES	**NO**	If yes, what - details
Asthma			

What did you cat?

Diabetes			
Metabolic Disorder			
Any other long term medical condition			

DO YOU FEEL WELL TODAY?

If not what is wrong (eg: cold, sore throat, headache etc)

BEFORE YOU SUPPLY THIS URINE SAMPLE HOW LONG AGO WAS IT THAT

YOU: I. Ate a standard meal (breakfast, lunch or dinner) — HOURS

What did you eat?

2. At a light snack (sandwich, crisps, sweets etc) -- HOURS

5.4 SOP for GCMS

This method is for the TD tube containing the sample to be analysed and have a brass fitting attached at either end.

1. Make a record of the TD tube number and place in order into a tray, starting with a blank and standard then samples and finish with a standard and blank.
2. Remove both brass fittings and gently pack silanised glass wool into tube end nearest the grooved notch. The tube contains packing material and a small circular gauze near the grooved notch that can be easily dislodged if pushed with forceps. Standards and sample tubes are packed with silanised glass wool but blanks are left empty.
3. Replace with PTFE end caps.
4. Set helium line to flow at 500ml/min.
5. Leave blanks aside then with the remainder of the tubes taking one tube at a time, remove both PTFE end caps, and screw notched end of tube into nut assembly on helium line and secure firmly.
6. With a Hamilton syringe dispense 50ng of standard (d8-Toluene), through septum onto silanised glass wool plug in the tube.
7. Open flow tap and leave for 20 seconds. Switch off flow tap and remove tube from flow line.
8. Remove silanised glass wool and replace both PTFE end caps.
9. Place tubes onto the ATD carousel, starting at number 1 (number sequence displayed on front edge of carousel). Place tube with notched end away from the carousel number display.

Setting up GC/MS for sample analysis

Open 'TurboMasss 4.1 – METHOD 2007022.spl'

1. Find 'Samples' on tool bar, click and then choose 'Add'.
2. Type in the number of tubes you are analysing plus two (this for two cycles for cold trap cleaning)
3. Click 'OK' and the screen will add tube numbers to list in 'File Name' column.
4. The system copies the previous 'Sample ID' and 'File Text' data so highlight the area to delete then press 'delete'.
5. Type in 'trap' into both columns then for each, copy below, see Figure below. Then type in tube codes and sample identification.
6. The 'MS Method' and 'GC Method' columns are both set to add in default methods (PILARTD) and (CWdevel) these are the general methods for most analysis.

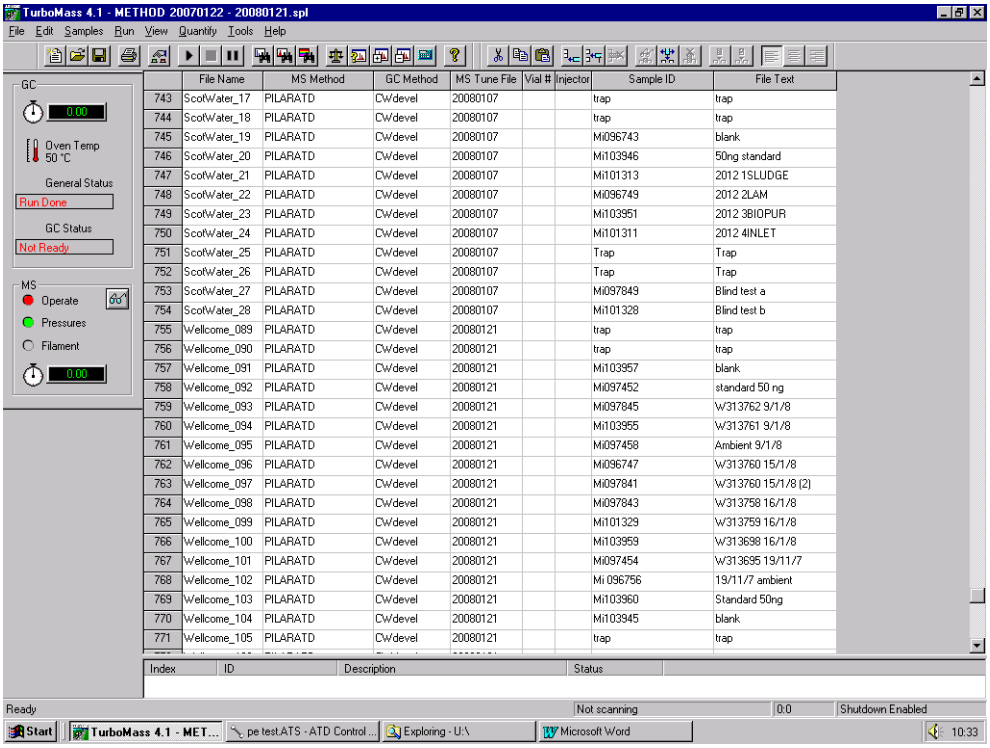

Figure 29 Turbomass sample list

7. Open 'pe test ATS – ATD Control (COM2)' window.
8. Select 'Sequence Editor' tab
9. Select 'Trap condition 00' and drag and drop under 'Method Name' box. Repeat. Then drag and drop 'Tenax Carbotrap desorb 00'. Change 'Last Tube' to the number of tubes for analysis. (use right arrow key to move blue highlight box to next box, this just makes sure the last tube number has been entered)

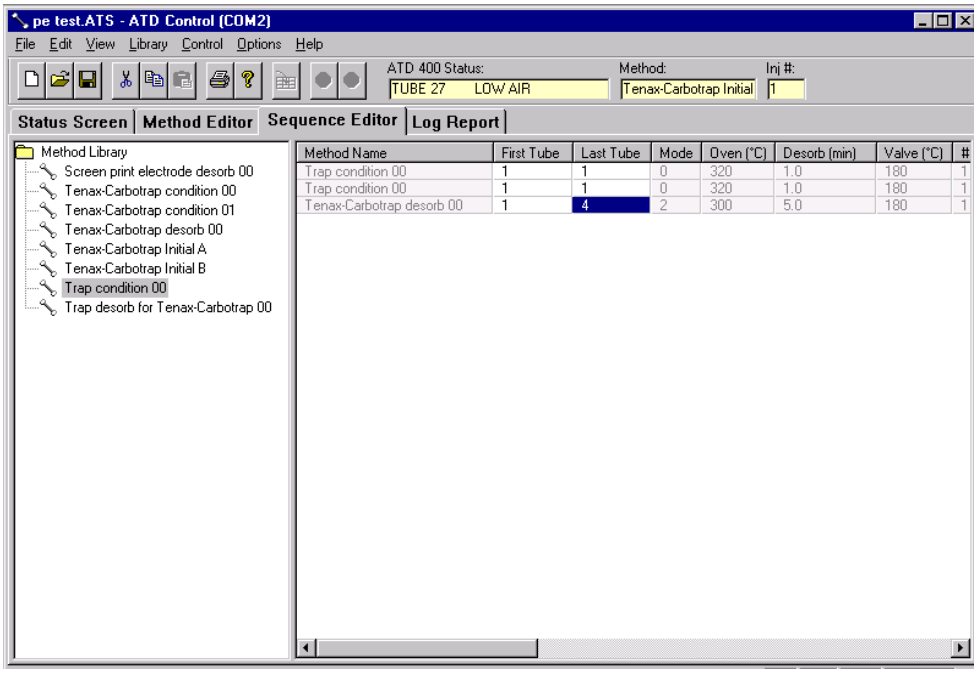

Figure 30 ATD control sequence editor

10. Re-open 'TurboMass 4.1' window and highlight your samples for analysis and press '►'. Observe the 'General Status' box for GC this will indicate when run will start.
11. 'Start sample list run' window will open. Check run sample numbers are correct and press 'OK'.
12. 'TurboMass' window will open, then press 'Yes' if OK.
13. Re-open 'pe test. ATS – ATD Control (COM2)' window and press the green button.
14. Leave to run, check after about 30 minutes to make sure run has initiated.
15. Run progress can be checked by selecting status screen, this shows in diagram what is happening. White boxes under '**Primary Desorb'** and **'Secondary Desorb'** have pre-set conditions and yellow boxes show actual readings

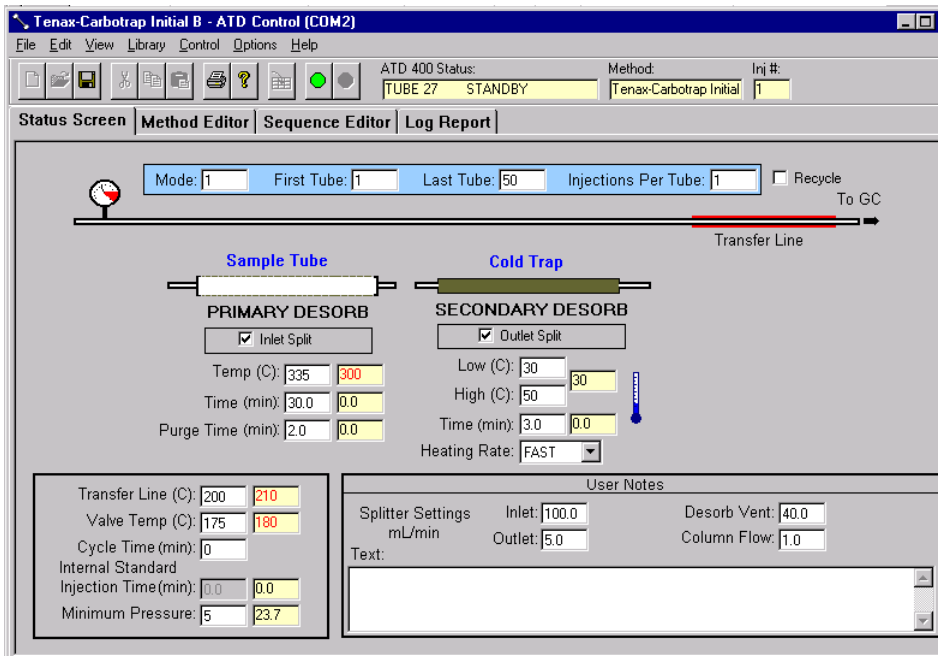

Figure 31 Tenax-Carbotrap Intital B - ATD control (COM2)

16. Fill in log book with run details.
17. Record tubes in TD log book with date and tube contents e.g. Blank, Standard or Sample + code.
18. When run has finished check results and conditions of run and report in log.

Headspace from urine, blood and faecal samples after analysis on the SIFT-MS was drawn from the bag by using a FLEC® constant-flow pump purchased from Markes International using a flow rate of 100ml for 5 minutes and passed onto a TD tube, the tube was then stored at 4° C prior to GC-MS analysis.

Table 14 List of Compounds found in a blank sample balloon (balloon with zero grade air and incubated for 30 mins, 500ml headspace drawn across a thermal desorption tube and internal standard, Toluene D-8, for GC-MS analysis)

Name	R.T.	Amount	Absolute amount
Cyclotrisiloxane, hexamethyl-	1.954	9.33%	16
2-Butanone	2.198	0.41%	1
Ethanol, 2-fluoro-	2.314	0.20%	0
1,3-Dioxolane, 2-methyl-	2.73	1.99%	3
Toluene-D8	4.117	58.70%	100
Pentacarbonyltris(trimethylsilyl)stibinemolybdenum	17.976	0.43%	1
3-(4-Buthoxybenzylthio)-1,2,4-(4H)-triazole	19.166	0.33%	1
(3-Cyclohexyl-5-hydroxy-5-trifluoromethyl-4,5-dihydro-pyrazol-1-yl)-(4-methoxy-phenyl)-methanone	19.172	1.61%	3
p-Anisic acid, 4-nitrophenyl ester	19.178	1.70%	3
3-Ethoxy-1,1,1,7,7,7-hexamethyl-3,5,5-tris(trimethylsiloxy)tetrasiloxane	19.631	0.32%	1
Allophanic acid, phenyl ester	19.798	0.33%	1
Carbamodithioic acid, acetyl-, methyl ester	20.44	0.31%	1

6 Bibliography

Abbott, S. M., Elder, J. B., Spanel, P. and Smith, D. (2003), "Quantification of acetonitrile in exhaled breath and urinary headspace using selected ion flow tube mass spectrometry", *International Journal of Mass Spectrometry,* vol. 228, no. 2-3, pp. 655-665.

Abraham and Nedwell 1978

Allen, A. (1981) "Structure and function of gastrointestinal mucus", *Physiology of the Gastrointestinal Tract*, vol. 1, pp. 617-639

Allen, A. (1989), "Gastrointestinal mucus", *Handbook of Physiology - The Gastrointestinal System,* vol. 3, pp. 359-382.

Aghdassi, E., Allard, J.P., 2000 "Breath alkane as a marker of oxidative stress in different clinical conditions", *Free Radical Biology and Medicine*, vol. 28, issue. 6, pp.880-886

Amselem, C., Puigdollers, A., Azpiroz, F., Sala, C., Videla, S., Fernandes-Fraga, X., Whorwell, P., and Malagelada, J.R., (2010) "Constipation: a potential cause of pelvic floor damage?", *Neurogastroenterol Motil,* vol. 22, pp. 150

Attwood, S. E. A. and Lamb, C. A. (2008), "Eosinophilic oesophagitis and other non-reflux inflammatory conditions of the oesophagus: Diagnostic imaging and management", *Best Practice and Research: Clinical Gastroenterology,* vol. 22, no. 4, pp. 639-660.

Ayele, W.Y., Svastova, P., Rooubal, P.,Bartos. M., Pavlik, I., (2005) "*Mycobacterium avium* Subspecies *paratuberculosis* Cultured from Locally and Commercially Pasturised Cow's Milk in the Czech Republic", *Appl.Environ Microbiol*. vol. 71, no. 3, pp. 1210-1214.

Bassi, A., Dodd, S., Williamson, P., and Bodger, K., "Cost of illness of inflammatory bowel disease in the UK: a single centre retrospective study" *Gut,* vol 53, pp. 1471-1478.

Bowen, R., " The Gastrointestinal Barrier" www.vivo.colostate.edu/hbooks/pathphys/digestion/stomach/gibarrier.html 06/10/2008.

Burstein, E. and Fearon, E. R. (2008), "Colitis and cancer: A tale of inflammatory cells and their cytokines", *Journal of Clinical Investigation,* vol. 118, no. 2, pp. 464-467.

Cain, K.C., Jarrett, M.E.,Burr, R.L., Rosen, S., Hertig, V.L., Heitkemper, M.M.,(2009) "Gender Differences in Gastrointestinal, Psychological, and Somatic Symptoms in Irritable Bowel Syndrome", *Digestive Diseases and Sciences,* vol. 54, pp. 1542-1549.

Cao, W. and Duan, Y. (2006), "Breath analysis: Potential for clinical diagnosis and exposure assessment", *Clinical Chemistry,* vol. 52, no. 5, pp. 800-811.

Christl, S. U., Gibson, G. R. and Cummings, J. H. (1992), "Role of dietary sulphate in the regulation of methanogenesis in the human large intestine", *Gut,* vol. 33, no. 9, pp. 1234-1238.

Collins, S. M., Piche, T. and Rampal, P. (2001), "The putative role of inflammation in the irritable bowel syndrome", *Gut,* vol. 49, no. 6, pp. 743-745.

Creasy, K. E. and Capuano, I. A. (1996), "Novel headspace sampling for process analysis", Vol. 29, pp. 27.

Cummings, J. H. and Macfarlane, G. T. (1991), "The control and consequences of bacterial fermentation in the human colon", *Journal of Applied Bacteriology,* vol. 70, no. 6, pp. 443-459.

Da Silva, F., Nobre, M., Fernandes, A., Antunes, R., Almeida, D., Garcia, G., Mason, N. J. and Limão-Vieira, P. (2008), "Spectroscopic studies of ketones as a marker for patients with diabetes", *Journal of Physics: Conference Series,* vol. 101, no. 1.

De Preter,V., Van Staneyen, G., Esser, D., Rutgeerts, P.,Verbeke, K.,(2009) "Development of a screening method to determine the pattern of fermentation metabolites in faecal samples using on-line purge-and-trap gas chromatographic-mass spectrometric analysis"*Journal of Chromatography A,* vol 9, pp.1476-1483

Di Francesco, F., Fuoco, R., Trivella, M.G. and Ceccarini, A., (2004) " Breath analysis: trends in techniques and clinical applications", *Microchemical Journal,* Vol. 79, Issue 1-2, pp. 405-410

Elson, C. O., Balfour Sartor, R., Targan, S. R., Sandborn, W. J. and Merrick, M. (2003), "Challenges in IBD research: Updating the scientific agendas", *Inflammatory Bowel Diseases,* vol. 9, no. 3, pp. 137-153.

Elson, C. O., Mccabe Jr., R. P., Beagley, K. W., Sharmanov, A., Brandwein, S. L., Ridwan, B. U., Weaver, C., Bucy, R. P., Mcghee, J. R., Sundberg, J. and Birkenmeier, E. (1996), "Regulation of mucosal immune responses - The missing link in IBD?", *Canadian Journal of Gastroenterology,* vol. 10, no. 2, pp. 105-109.

Garner, C. E., Smith, S., De Lacy Costello, B., White, P., Spencer, R., Probert, C. S. J. and Ratcliffe, N. M. (2007), "Volatile organic compounds from feces and their potential for diagnosis of gastrointestinal disease", *FASEB Journal,* vol. 21, no. 8, pp. 1675-1688.

Gibson, G. R., Cummings, J. H. and Macfarlane, G. T. (1993), "Sulphate reducing bacteria and hydrogen metabolism in the human large intestine", *Gut,* Vol. 34, pp. 437-439.

Gibson, G. R., Cummings, J. H. and Macfarlane, G. T. (1991), "Growth and activities of sulphate-reducing bacteria in gut contents of healthy subjects and patients with ulcerative colitis", *FEMS Microbiology Ecology,* vol. 86, no. 2, pp. 103-111.

Gibson, G. R., Cummings, J. H. and Macfarlane, G. T. (1988), "Competition for hydrogen between sulphate-reducing bacteria and methanogenic bacteria from the human large intestine.", *Journal of Applied Bacteriology,* vol. 65, no. 3, pp. 241-247.

Guarner, F. and Malagelada, J. -. (2003), "Gut flora in health and disease", *Lancet,* vol. 361, no. 9356, pp. 512-519.

Gué, M., Bonbonne, C., Fioramonti, J., Moré, J., Del Rio-Lachèze, C., Coméra, C. and Buéno, L. (1997), "Stress-induced enhancement of colitis in rats: CRF and arginine vasopressin are not involved", *American Journal of Physiology - Gastrointestinal and Liver Physiology,* vol. 272, no. 1 35-1.

Hamer, H.M., Jonkers, D.M.A.E., Renes, I.B.,Vanhoutvin, S.A.L.W., Kodde, A., Troost, F.J., Venema, K., Brummer, R.M., (2010) "Butyrate enemas do not

affect human colonic MUC2 and TFF3 expression", *European Journal of Gastroenterology and Hepatology*, vol 22. Issue 9, pp. 1134-1140

Helgeland, L. and Brandtzaeg, P. (2000), "Development and function of intestinal B and T cells", *Microbial Ecology in Health and Disease,* vol. 12, no. SUPPL. 2, pp. 110-127.

Holmes,S., (1987) Nursing standard (Royal College of Nursing (Great Britain) Vol. 24, Issue 35, pp.50-56

Houghton, L.A., Brown, H.,Atkinson, W., Morris.j., Fell,C., Whorwell, P.J., Lockhart, S., and Keevil, B., (2009) "5-hydroxytryptamine Signalling in Irritable Bowel Syndrome with Diarrhoea: Effects of Gender and Menstrual Status", *Alimentary Pharmacology and Therapeutics* Vol. 30 issue 9. pp. 919-929

Hunter, J.O., (1998), "Nutritional factors in inflammatory bowel disease", *European Journal of Gastroenterology and Hepatology,* vol. 10, no. 3, pp. 235-237.

Hvatum, M., Kanerud, L., Hallgren, R., Brandzaeg, P., (2006) "The Gut-joint axis:cross reactive food antibodies in rheumatoid arthritis" *Gut*, vol. 55 pp. 1240-1247

Koronczi, I., Ziegler, K., Krüger, U. and Goschnick, J. (2002), "Medical diagnosis with the gradient microarray of the KAMINA", *IEEE Sensors Journal,* vol. 2, no. 3, pp. 254-259.

Kupari, M., Lommi, J., Ventilä, M. and Karjalainen, U. (1995), "Breath acetone in congestive heart failure", *American Journal of Cardiology,* vol. 76, no. 14, pp. 1076-1078.

Kuzma, J., Nemecek-Marshall, M., Pollock, W. H. and Fall, R. (1995), "Bacteria produce the volatile hydrocarbon isoprene", *Current Microbiology,* vol. 30, no. 2, pp. 97-103.

Lechner, M. and Rieder, J. (2007), "Mass spectrometric profiling of low-molecular-weight volatile compounds - Diagnostic potential and latest applications", *Current Medicinal Chemistry,* vol. 14, no. 9, pp. 987-995.

Manolis, A. (1983), "The diagnostic potential of breath analysis", *Clinical Chemistry,* vol. 29, no. 1, pp. 5-15.

McFarland, L. V. (2008), "State-of-the-art of irritable bowel syndrome and inflammatory bowel disease research in 2008", *World Journal of Gastroenterology,* vol. 14, no. 17, pp. 2625-2629.

Miekisch, W. and Schubert, J. K. (2006), "From highly sophisticated analytical techniques to life-saving diagnostics: Technical developments in breath analysis", *TrAC - Trends in Analytical Chemistry,* vol. 25, no. 7, pp. 665-673.

Miekisch, W., Schubert, J. K. and Noeldge-Schomburg, G. F. E. (2004), "Diagnostic potential of breath analysis - Focus on volatile organic compounds", *Clinica Chimica Acta,* vol. 347, no. 1-2, pp. 25-39.

Morini, S., Zullo, A., Hassan, C., Tomao, S. and Campo, S. M. A. (2008), "Diverticulosis and colorectal cancer: Between lights and shadows", *Journal of clinical gastroenterology,* vol. 42, no. 7, pp. 763-770.

Peakman, T.C., Elliot, P., "The UK Biobank sample handling and storage validation studies" *International journal of epidemiology,* Vol. 37, Suppl. 1,pp. 2-6.

Picton, R., Eggo, M. C., Langman, M. J. S. and Singh, S. (2007), "Impaired detoxication of hydrogen sulfide in ulcerative colitis?", *Digestive diseases and sciences,* vol. 52, no. 2, pp. 373-378.

Pitcher, M. C. L. and Cummings, J. H. (1996), "Hydrogen sulphide: A bacterial toxin in ulcerative colitis?", *Gut,* vol. 39, no. 1, pp. 1-4.

Poli, D., Goldoni, M., Caglieri, A., Ceresa, G., Acampa, O., Carbognani, P., Rusca, M. and Corradi, M. (2008), "Breath analysis in non small cell lung cancer patients after surgical tumour resection", *Acta Biomedica de l'Ateneo Parmense,* vol. 79, no. SUPPL. 1, pp. 64-72.

Probert, C.S.J., Almed, I., Khalid, T., Johnson, E., Smith, S., Ratcliffe, N., (2009) "Volatile Organic Compounds as Diagnostic Biomarkers in Gastrointestinal and Liver Disease" *J Gastrointestin Liver Dis,* Vol. 18, No. 3, pp. 337-343

Pullan, R. D. (1996), "Colonic mucus, smoking and ulcerative colitis", *Annals of the Royal College of Surgeons of England,* vol. 78, no. 2, pp. 85-91.

Pullan, R. D., Thomas, G. A. O., Rhodes, M., Newcombe, R. G., Williams, G. T., Allen, A. and Rhodes, J. (1994), "Thickness of adherent mucus gel on colonic mucosa in humans and its relevance to colitis", *Gut,* vol. 35, no. 3, pp. 353-359.

Reif, S., Klein, I., Lubin, F., Farbstein, M., Hallak, A. and Gilat, T. (1997), "Pre-illness dietary factors in inflammatory bowel disease", *Gut,* vol. 40, no. 6, pp. 754-760.

Rezaie, A., Parker, R. D. and Abdollahi, M. (2007), "Oxidative stress and pathogenesis of inflammatory bowel disease: An epiphenomenon or the cause?", *Digestive Diseases and Sciences,* vol. 52, no. 9, pp. 2015-2021.

Richardson, C.J., (2000) "A New Method for the Determination of Sulphide in Gastrointestinal Contents and Whole Blood by Microdistillation and Ion Chromatography" *Clinica Chimica Acta*, vol. 293, pp.115-125.

Roberts, M.B.V., (1978) "Biology A Functional Approach" Fourth Edition

Roediger, W.E.W., Duncan, A., Kapaninis, O., Millard, S., (1993) "Reducing sulfur compounds of the colon impair colonocyte nutrition: Implications for ulcerative colitis", *Gastroenterology,* vol. 104, Issue 3, pp. 802-809

Salerno-Kennedy, R. and Cashman, K. D. (2005), "Potential applications of breath isoprene as a biomarker in modern medicine: A concise overview", *Wiener Klinische Wochenschrift,* vol. 117, no. 5-6, pp. 180-186.

Salminen, S., Bouley, C., Boutron-Ruault, M. -., Cummings, J. H., Franck, A., Gibson, G. R., Isolauri, E., Moreau, M. -., Roberfroid, M. and Rowland, I. (1998), "Functional food science and gastrointestinal physiology and function", *British Journal of Nutrition,* vol. 80, no. SUPPL. 1.

Sartor, R. B. (2006), "Mechanisms of disease: Pathogenesis of Crohn's disease and ulcerative colitis", *Nature Clinical Practice Gastroenterology and Hepatology,* vol. 3, no. 7, pp. 390-407.

Sato, H., Hirose, T., Kimura, T., Moriyama, Y., and Nakashima, Y., (2001) " Analysis of Malodorous Volatile Substances of Human Waste: Feces and Urine", *Journal of Human Science*, vol. 47, no.5, pp. 483-490.

Shirazi, T Longman, R.J., Corfield, A.P., Probert, C.S.J., (1999) "*Postgraduate Medical Journal*", Vol. 76, pp.473-478.

Simon, G. L. and Gorbach, S. L. (1984), "Intestinal flora in health and disease", *Gastroenterology,* vol. 86, no. 1, pp. 174-193.

Singleton, J. W. (2005), "Progress in inflammatory bowel disease", *Chinese Journal of Digestive Diseases,* vol. 6, no. 2, pp. 59-61.

Smith, D. and Španěl, P. (1996), "The novel selected-ion flow tube approach to trace gas analysis of air and breath", *Rapid Communications in Mass Spectrometry,* vol. 10, no. 10, pp. 1183-1198.

Smith, D. and Španel, P. (2007), "The challenge of breath analysis for clinical diagnosis and therapeutic monitoring", *Analyst,* vol. 132, no. 5, pp. 390-396.

Smith, D. and Španel, P. (2005), "Selected ion flow tube mass spectrometry (SIFT-MS) for on-line trace gas analysis" *Mass Spectrometry Reviews,* Vol. 24, no. 5, pp. 661-700

Smith,D. and Španěl, P. (2010) "Select ion flow tube mass spectrometry, SIFT-MS; new horizons in real time air and breath analysis", *Spectroscopy,* vol. 22, no. 4, pp. 21-24.

Tangerman, A. (2009), "Measurement and biological significance of the volatile sulfur compounds hydrogen sulfide, methanethiol and dimethyl sulfide in various biological matrices", *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences,* vol. 877, no. 28, pp. 3366-3377.

Toso, B., Procida, G. and Stefanon, B. (2002), "Determination of volatile compounds in cows' milk using headspace GC-MS", *Journal of Dairy Research,* vol. 69, no. 4, pp. 569-577.

Turner, C., Spanel, P. and Smith, D. (2006), "A longitudinal study of ammonia, acetone and propanol in the exhaled breath of 30 subjects using selected ion flow tube mass spectrometry, SIFT-MS", *Physiological Measurement,* vol. 27, no. 4, pp. 321-337.

Van den Velde, S., Nevens, F., Van hee, P., van Steenberghe, D. and Quirynen, M. (2008), "GC-MS analysis of breath odor compounds in liver patients", *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences,* vol. 875, no. 2, pp. 344-348.

Villanueva, S., Guadarrama, A., Rodríguez-Mendez, M. L. and de Saja, J. A. (2008), "Use of an array of metal oxide sensors coupled with solid phase microextraction for characterisation of wines. Study of the role of the carrier gas", *Sensors and Actuators, B: Chemical,* vol. 132, no. 1, pp. 125-133.

Walter, S.A., Aardel-Ericksson, E., Thorell, L.H., Bodemar, G., Hallbook,O., (2006) "Pre-experimental stress in patients with irribable bowel syndrome: high cortisol

values already before symptom provacation with rectal distensions", *Neurogastroenterol Motil,* vol 12, pp. 1069-1077

Wang, T., Spanel, P. and Smith, D. (2004), "A selected ion flow tube study of the reactions of H3O +, NO+ and O2+• with some phenols, phenyl alcohols and cyclic carbonyl compounds in support of SIFT-MS and PTR-MS", *International Journal of Mass Spectrometry,* vol. 239, no. 2-3, pp. 139-146.

Web page[1] www.customprobiotics.com/about_probiotics.htm

Date accessed 17/08/2010

Web page[2] www.customprobiotics.com/about_probiotics.htm

Date accessed 17/08/2010

Web page[3] www.dac.neu.edu/cammp/images/intestinal_secretory_mucin.jpg

Date accessed 3/09/2010

Web page[4] www.patient.co.uk/showdoc/23068968/

Date accessed 19/10/2009

*i) Technical Support Note 21: 'Analytical Thermal Desportion-Developing and Optimising Methods' move to references

'*ii) Application note GC for ZB-WAX column for low molecular weight organic acids move to references