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AN INVESTIGATION INTO THE EFFECTS OF BACTERIAL FERMENTATION IN AUTISM

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Abstract

Gastrointestinal symptoms have been observed to be present very often in autistic children. These symptoms are very similar to those observed in Irritable Bowel Syndrome (IBS). An investigation to investigate whether there is a similar cause for autism as well as IBS was done.

Methods for the analysis of breath VOCs and urine organic acids were developed to be applied afterwards in the research of biomarkers of autism and IBS.

Bacterial metabolites in urine and breath were identified by restricting the diets of healthy volunteers to each of two enteral feeds. The way enteral feeds work in the treatment of Crohn's disease (CD) was investigated. Enteral feeds work by reducing bacterial activity in the gut, measurably reducing their metabolic products.

The urine of IBS patients was analysed and bacterial compounds investigated. Some gastrointestinal symptoms are frequently misdiagnosed as IBS. According to the symptoms and treatment that is successful for these different groups of patients three subgroups can be made: "retention and overflow", "musculoskeletal" and "abnormal fermentation" IBS. When the urine of these subgroups was observed, different biomarkers for the various subgroups could be identified. Patients with abnormal fermentation IBS have increased bacterial products in urine, suggesting an increase in bacterial activity in the colon. These biomarkers decreased after an exclusion or fibre-free diet and symptoms improved.

In a further study, potential biomarkers for autism were identified. The origin of the majority of these compounds is unknown, although some of them are of known bacterial origin. A conclusion of this is that autistic children may exhibit abnormal fermentation since GI symptoms manifest at almost the same time as the autistic behavioural symptoms start and may be present from birth. Autistic children share many symptoms with IBS patients, although the biomarkers are different, showing that the GI symptoms observed in autistic children are not IBS in the children studied. More research needs to be done to determine the origin of the autism biomarkers discovered in urine.

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Nomenclature

a.m.u.: atomic mass unit

ASD: autistic spectrum disorder

AUC: Area under the curve

BCA : breath collection apparatus

Capillary column or open tubular column (OTC). This column has small-diameter tubing (0.25-1.0 mm i.d.) in which the inner walls are used to support the stationary phase.

Carrier gas: Mobile or moving phase. This is the phase that transports the sample through the column.

CD: Crohn´s disease

CDF: Common data file

Chromatogram: This is a plot of the detector response (which uses effluent concentration or another quantity used to measure the sample component) versus effluent volume or time.

Column: This is a tube packed or internally coated with the column material through which the sample components and mobile phase (carrier gas) flow and in which the chromatographic separation takes place.

CNS: central nervous system

CSV files: Comma separated variable files

Da: Dalton

GC: gas chromatograph/ gas chromatography

GI: Gastrointestinal

HAN: hydrolysed acid neutral

HB: hydrolysed basic

HPLC: high performance liquid chromatography

HS: headspace

IAG: 3-indolyl-acryloylglycine

IBS: Irritable bowel syndrome

LNH: Lymphoid nodular hyperplasia

MS: mass spectrometer

MSK: musculoskeletal IBS

$NO^{\cdot}$: nitric oxide free-radical

PCA: principal component analysis

ppb: parts per billion, equivalent to μg/liter

ppm: parts per million, equivalent to mg/litre

PDD: Pervasive developmental disorders

PTFE: polytetrafluoroethylene

R+O: retention and overflow patients IBS

RT: Retention time

SCFA: short chain fatty acids

SPME: Solid phase microextraction

SRB: Sulphate reducing bacteria

TD: Thermodesorption

TIC: Total Ion Chromatogram

TMS: Trimethyl silyl

UB: Unhydrolysed basic

UC: Ulcerative colitis

VOC: Volatile Organic Compound

Chapter 1. Introduction and background

The possible involvement of the gastrointestinal tract in the pathogenesis of autism

Autism and causes

Autism or Autistic Spectrum Disorder (ASD) is a lifelong developmental disorder first described by Kanner in 1943: an estimated 0.5% of the population is affected (Shannon McCallum, 2006). ASD is defined as a neuropsychiatric condition, a biological disorder manifesting psychiatric symptoms. Its aetiology is not known and a variety of factors may contribute to its cause.

Immunological, neurodevelopmental, genetic, biochemical and environmental factors seem to be involved (see Table 1). There is a genetic predisposition to the disease (Bailey et al., 1995) (Klauck, 2006), but the number of cases reported is increasing (Herbert et al., 2006), suggesting that environmental factors play an important role.

Table 1. Possible causes of autism.

Possible associated causes
Genetic predisposition (Bailey et al., 1995) (Klauck, 2006)
Thalidomide (Skoyles, 2002)
MMR vaccine (Fudenberg, 1996) (Gupta, 1996) (Wakefield et al., 1998; Fudenberg, 1996)
Mercury (Bernard et al., 2001)
Intra-monocyte pathogens (Binstock, 2001)
Fungal metabolites (Horvath & Perman, 2002)
Autoimmune disease (Comi et al., 1999)
Food intolerance/ increased gut permeability/ opioids (Goodwin et al., 1971) (D'Eufemia et al., 1996) (Panksepp, 1979)
Oxidative stress (Zoroglu et al., 2004) (McGinnis, 2004)

Biological symptoms observed include mental retardation (60% of autistic children), language impairment, epilepsy (1/3 of autistic children are epileptic) (Deykin & McMahon, 1979) and gastrointestinal (GI) problems. These characteristics may play an important role in the development of autism, since people not presenting autistic patterns but presenting some of these symptoms share some biochemical characteristics of autism as well (this will be explained later).

Link between the gut and autism

A striking feature of the clinical presentation of autism is the frequency of symptoms arising from the gut, as reviewed by White (White, 2003). GI symptoms manifest at almost the same time as the behavioural symptoms start and may be present from birth (Horvath & Perman, 2002). This might suggest that GI symptoms are a cause rather than a consequence of the condition. Melmed *et al.* (2000) reported that of the children with pervasive developmental disorders (PDD) in his study, 19% had chronic diarrhoea, 19% chronic constipation and 7% alternated between diarrhoea and constipation. 8% of siblings and 5% of controls reported chronic constipation. In total, 46% of children with PDD had chronic GI symptoms. Wakefield *et al.* (2002) suggest autism has an intestinal origin, because bowel clearance prior to colonoscopy and relief of chronic constipation often improve the behavioural symptoms which are common in autism. They estimated 46% of autistic children compared to a 10% of normal paediatric controls have GI problems, although the percentages of autistic children with GI problems reported have varied (Black et al., 2002) (Taylor et al., 2002) (Wakefield et al., 2002). Furthermore, it is possible that some children have gut abnormalities that are not sufficiently severe to cause symptoms. One-third of parents of autistic children surveyed say their autistic children have daily yellow stools, and 20% a frequency of three or more stools per day (Lightdale et al., 2001). Furthermore, one in four parents surveyed explained that they were unsure if their children were experiencing abdominal pain, because the children could not talk.

Wakefield *et al.* (1998) reported ileal lymphoid nodular hyperplasia (LNH) in 93% of affected children and in 14% of controls. 25% showed a red halo around swollen caecal

lymphoid follicles, which was controversially interpreted as Crohn's disease (CD). Gastric mucosal inflammation has been found in 42% (Horvath et al., 1999). The intestinal epithelium in autistic subjects is more ulcerated and eroded than in controls, and the density of sulphated glycosaminoglycans in the basement membrane and epithelium is greatly reduced, indicating pathological inflammation of the intestine (Furlano et al., 2001). Paneth cell hyperplasia and elevated number per crypt were found, with frequent discharge of granules into the crypt lumen (Horvath et al., 1999), a sign of abnormal bacterial activity in the gut.

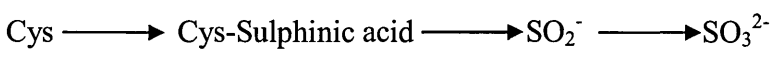
Enzymes in the intestinal mucosa

Decreased activity of one or more intestinal disaccharidases or glucoamylases was found in 58% of autistic children. The most frequent finding was a low lactase level in the small intestine, present in 67% (Horvath et al., 1999; Wakefield et al., 2002). Maltase deficiency was very frequent, as well as sucrase, palatinase and glucoamylase. All the children with low enzyme activities had loose stools and/or flatulence.

Sulphate

A decrease in plasma sulphate was found in 92% of autistic children (Waring et al., 1997). Dietary sulphate intake is mainly through proteins containing cysteine and methionine. Cysteine is transformed into cysteine sulphinic acid by cysteine dioxygenase, and then undergoes transamination and degradation to give sulphite ions, which are oxidized to sulphate by sulphite oxidase. It was found that 46% of autistic children had no oxidation to sulphate compared to 2% in controls (Waring et al., 1997). High levels of cysteine and decreased sulphate concentration in plasma are also found in autism and other autoimmune disorders including ulcerative colitis (UC).

Figure 1. Process of transformation of Cysteine from proteins to sulphate.



Sulphate is involved in the inactivation of catecholamines. If sulphate levels are decreased, increased amounts of neurotransmitter amines produce prolonged effects on the central nervous system (CNS) (Waring et al., 1997). Phenol-sulphotransferase levels, which are needed for the sulphation of catecholamines, are decreased (O'Reilly & Waring, 1993) in autistic children with known food/chemical intolerances.

Sulphation also plays an important role in the digestive tract: gastrin is more active when sulphated on a tyrosine residue. Also, in the acid stomach, proteins are hydrolysed to peptides, and these peptides induce the secretion of secretin, but many autistic children have raised gastric pH which might reduce dietary protein breakdown and secretin production. Cholecystokinin is an active peptide in the brain and the GI tract. When it is sulphated, it induces secretin release. Again, if unsulphated, secretin production could be hampered. Secretin induces the release of digestive enzymes from the pancreas (Waring & Klovrza, 2000).

Reduced sulphate in autism might be leading to a prolonged catecholamine effect on the central nervous system (CNS), and decreased activity of some enzymes, causing biochemical abnormalities in ASD (Waring & Klovrza, 2000).

Increased intestinal permeability

Gut permeability is increased in 43% of autistic children (1996). Horvath *et al.* (2000) measured permeability with lactulose/manitol in 25 participants in a double-blinded randomized placebo-controlled study and 76% showed an abnormal ratio. Reduced sulphation (Waring et al., 1997) and oxidative stress (McGinnis, 2004) are two possible mechanisms that lead to increased intestinal permeability in autism. When mucins are not sulphated their protective role may be disrupted. Autistic patients have high levels of circulating NO·(McClain et al., 1995), which is known to degrade mucin, increasing

intestinal permeability (Xu et al., 2002) and exacerbating inflammation in the bowel (Singh et al., 2004). The increased permeability may allow an increase in peptides passing through the GI mucosa, which might lead to an allergic response.

Food allergy/food intolerance

Autistic children are more susceptible to allergies and food sensitivities than controls. Goodwin *et al.* (1971) suggested a correlation between autism and malabsorption and sensitivity to food. Worsening of neurological symptoms was reported in autistic patients after the consumption of milk and wheat (Lucarelli et al., 1995). An elimination diet was tried (1995) for eight weeks and high levels of IgA antigen specific antibodies for casein, lactalbumin and β-lactoglobulin and IgG and IgM for casein were found although not for gluten. An improvement in behaviour was observed if those foods were eliminated. A relationship between food allergy and infantile autism was suggested (Lucarelli et. Al, 1995). In a separate study, elimination of milk from the child's diet significantly improved the patient's condition (Vojdani et al., 2002).

Neurodysfunction in gut disorders

Autism is not the only neurological condition in which there might be a link with the gut. The relationship between gut and brain has been observed in other diseases such as hepatic encephalopathy (Swapna et al., 2006) and coeliac disease (Benson et al., 1964) (Cooke & Smith, 1996) (Hallert & Derefeldt, 1982) (Wakefield et al., 2002). Coeliac disease is a gluten sensitive inflammatory disease whose clinical manifestations can affect other organs including the central and peripheric nervous system (Mavroudi et al., 2005) (Bushara, 2005). This disease shares biochemical abnormalities with autism, such as opioid related activity changes and amino acid metabolism; and GI symptoms as well as objective improvement when treated with a restriction of dietary protein or therapy against luminal colonic bacteria.

In elderly people, constipation may precipitate confusional states, and people with short bowel syndrome and others undergoing intestinal bypass surgery may suffer from psychiatric and neurological sequelae.

It has been found that 42% of patients with CD and 46% with UC have small white matter lesions on brain scans compared to 16% of controls (Geissler et al., 1995), although their clinical relevance remains uncertain (Hart et al., 1998) . Another link between the gut and the brain is that individuals with CD manifest an increased prevalence of anxiety, depression and panic disorder than control groups (Tarter et al., 1987). This suggests a gut/brain connection although more investigation is needed to confirm this (Sandler et al., 2000).

Chemical links between the gut and the brain

Peptides with opioid activity, such as caseomorphins and gluteomorphins, are produced during the digestion of gluten and casein (Fukudome & Yoshikawa, 1991). Increased levels of these peptides have been found in some children with autism (Shattock et al., 1990). It has been suggested that the behaviour of some autistic individuals may resemble that of people taking heroin or morphine and it is has been observed that learning ability may be reduced in children with high levels of peptides in the urine, especially bovine caseomorphins ingested in the diet (Reichelt et al., 1991).

Some children with autism have elevated levels of specific opioids like β-endorphin in the cerebro-spinal fluid and in peripheral blood mononuclear cells (Cazzullo et al., 1999). An increase in β-endorphin in plasma has been also observed in 53% of mothers of autistic subjects (Leboyer et al., 1999).

The opiate antagonist naltrexone has been used to alleviate the symptoms of autism, and improvement of behaviour has been reported in a subgroup of patients (Panksepp & Lensing, 1991) (Chabane et al., 2000). When naltrexone was administered to 11 subjects with increased β-endorphins, behaviour or functionality was improved in all.

Increased levels of exorphins and endorphins contribute to an increased permeability of the intestinal membrane and blood-brain barrier, allowing more biologically active peptides from the diet to enter the brain (Panksepp & Lensing, 1991) .

Gluten and casein-free diet

A urinary analysis was done by Whiteley *et al.* (Whiteley et al., 1999) and Shattock & Whiteley (Shattock & Whiteley, 2001). The compound 3-Indolyl-Acryloylglycine (IAG) is found in increased levels in urine of people with autism (Shattock & Savery, 1997) although 71% of the normal population excrete it in varying amounts (Mandell & Rubin, 1965) . Its function or origin is not known. IAG may be a metabolic product of the precursor indolyl-3-acrylic acid, which is thought to be produced by the gut flora or by endogenous routes, and it has been linked to defects in tryptophan metabolism (Anderson et al., 2002). However, when a person with no evidence of metabolic defect or dietary abnormalities with high levels of IAG was treated with the antibiotic neomycin, IAG disappeared (Szinberg A., 1965) suggesting this compound has a bacterial origin.

There is an association between cellular immune reactivity to common dietary proteins and a subset of ASD (Jyonouchi et al., 2002). Dietary intervention has been proposed for the improvement of symptoms in ASD in children with raised concentrations of IAG and opioid peptides in the urine (Reichelt et al., 1991). A casein and gluten free diet given to autistic children for a year normalized IAG and peptide levels in urine (Knivsberg et al., 1995) (Knivsberg et al., 2002) and improvement in social, cognitive and communicative skills was seen in 81% of autistic children after three months on the diet (Cade et al., 2000).

GI problems are thus shown to be very common in autistic patients. Symptoms include: an increased intestinal permeability; food intolerance; a decrease in some enzymes and a decreased level of chemical sulphation.

Irritable Bowel Syndrome (IBS)

Gut symptoms in autism are strikingly similar to those of Irritable Bowel Syndrome (IBS). IBS is a poorly understood GI condition (Maxwell et al., 1997) whose symptoms are abdominal pain, excessive flatus and variable bowel habit in which no endoscopic, radiological, histological, biochemical or microbiological cause is apparent. Both conditions may be precipitated by gastroenteritis (Rodriguez LAG. & Ruigomez A., 1999) or antibiotics (Maxwell et al., 2002), both of which exhibit strong changes in bacterial flora.

Although the full pathogenesis of IBS is not yet understood, food intolerance has been reported in IBS as well as in autism. Cereals, dairy products, caffeine, yeast and citrus fruits are the most common foods concerned in both conditions (Jones et al., 1982) and avoiding these relieves the symptoms. In IBS, there is no evidence of Type I allergy, as serum IgE levels are not raised, skin prick and RAST tests are negative and, in contrast to true food allergy a relatively large quantity is required to trigger a reaction. No link has been found with possible toxic chemicals in foods, and although a proportion of sufferers show evidence of hypolactasia, reduction of enzyme activities cannot be the sole cause of food intolerance (Hunter, 1991). Like autism, IBS has been shown in prospective studies to follow attacks of bacterial gastroenteritis and treatment causes of antibiotics. The faecal flora is abnormal; facultative anaerobes were increased (Pimentel et al., 2003) and coliforms, Lactobacilli and Bifidobacteria decreased (Balsari et al., 1982).

The gut of an infant is sterile (Haenel, 1970) (Kolida S. et al., 2000) (Shahani & Ayebo, 1980) until it is colonized by facultative anaerobes and strict anaerobes. The newborn will first come in contact with bacteria in the birth canal and its surroundings (Mountzouris et al., 2002). Infant faecal flora stabilises at four weeks of age and until weaning when facultatives increase.

Intestinal microflora salvage energy from carbohydrates not digested in the upper gut, through fermentation (Pomare et al., 1985). Undigested and/or unabsorbed foodstuffs such as carbohydrates and proteins from the small intestine and various host secretions such as

pancreatic juice, bile, mucus and sloughed epithelial cells firstly become available to bacteria resident in the caecum. Substrate concentrations decrease as bowel contents move distally towards the recto-sigmoid region, giving rise to varying fermentation patterns along the length of the colon.

Fermentation has significant effects on many processes in the colon, including salt and water absorption, pH, epithelial cell metabolism, motility and bowel habit and colonisation resistance, and products of fermentation may be absorbed and reach the liver and peripheral tissues.

Evidence to support the hypothesis that fermentation is abnormal in IBS was provided from studies of the excretion of hydrogen, a gas which in man is entirely of bacterial origin. This was increased in IBS patients, and fell dramatically on an exclusion diet carefully matched with the standard diet for substrates of fermentation. There was a correlation between the decrease of hydrogen excretion and abdominal symptoms, suggesting that abnormal fermentation is causing IBS. It has therefore been suggested that the cause is malfermentation of food residues (Camilleri, 2001) (Hunter, 1991). Increased amounts of abnormal fermentation products as a result of a damaged gut flora might lead to the symptoms which are characteristic of IBS (King et al., 1998). Similarly hydrogen excretion fell, and symptoms improved after treatment of patients suffering from IBS with the antibiotic metronidazole or ingestion of a fibre free diet (Dear et al., 2005).

Malfermentation as a possible factor leading to autism

The possible link between GI and behavioural symptoms has been proposed to be mediated by innate immune abnormalities (Jyonouchi et al., 2005). However, improvement in behaviour is frequently seen in autism after exclusion from the diet of foods to which no specific antibodies are present. In view of the similarities between the gut in autism and in IBS it seems possible that both may share a common pathogenetic mechanism, namely colonic malfermentation.

There are several features to support this suggestion. Abnormal gut flora is known to exist in children with autism (Finegold et al., 2002). Finegold *et al.* found Eubacterium and Lactobacillus were only present in the faeces of one autistic patient each out of 11 but increased counts of Clostridium were found in all, leading to the possibility that various types of autism might be related to variations in the abnormal flora present in colon or small bowel (Finegold et al., 2002). Bolte (2000) suggested that children have an immune-tolerance towards and chronic-infection by a specific pathogen like Clostridium. In the faecal flora of patients with IBS, an increased number of coliforms has been reported (Mättö et al., 2005). Treatment of patients with IBS with probiotics has been proposed (Brigidi et al., 2001), and probiotics might also be of value in autism. Probiotics might also ameliorate reduced levels of enzymes such as lactase, as well as improve the integrity of gut mucosa by modification of allergic inflammation (Isolauri et al., 2000).

The onset of autism also often follows antimicrobial therapy (Finegold et al., 2002), which suggests that it might have an important role in its development as has been shown in IBS (Hunter & Jones, 1985).

In children under 10 years old, 42% of the antibiotics prescribed are for the treatment of otitis media (Sih, 2003). Approximately 75% of children have at least one attack of otitis media before being five, the incidence being greater in males (Bluestone et al., 1996). The frequency of otitis media is increasing. Antimicrobial use disrupts the indigenous intestinal flora and may allow colonization by organisms with virulent properties (Maxwell et al., 2002).

The importance of abnormal gut bacteria is suggested by a report of autism after jejuno-ileal bypass (Lis et al., 1976), since bacterial overgrowth in the bypassed intestine often follows.

Paneth cell hyperplasia and elevated numbers per crypt have been found in most autistic patients studied, with discharge of granules into the crypt lumen (Horvath et al., 1999). Paneth cells regulate ionic composition of the lumen, crypt development; digestion and

intestinal inflammation (Porter et al., 2002). Granules are discharged into the crypt lumen when stimulated by bacteria or lipopolysaccharides which are recognized as foreign. The host is specifically tolerant to antigens of its own enteric flora, but not others (Elson et al., 2001). In autism, overgrowth of extrinsic bacteria could be responsible for the discharge of the Paneth cell granules as well as vitamin B12 malabsorption (Donaldson, 1964) (King & Toskes, 1979), as they bind or metabolise the vitamin. At the same time many autistic children have hypochlorhydria (increasing pH in the GI tract), which would not only hamper protein breakdown, but also reduces colonization by beneficial bacteria such as bifidobacteria.

Bowel clearance prior to colonoscopy, which greatly reduces the number of bacteria in the gut, and relief of chronic constipation both often improve temporarily the behavioral symptoms common in autism. Furthermore, improvement also follows administration of the broad-spectrum antibiotic vancomycin. However, relapse occurs when the therapy is discontinued (Bolte, 2000), possibly because of spore germination in organisms such as Clostridia (Finegold et al., 2002).

Increased delivery of nutrients to the bacterial flora of the lower bowel as a result of enzyme deficiencies will favour the growth of certain bacteria. Anaerobic bacterial fermentation may be increased, causing increased levels of organic acids (Ewaschuk et al., 2002). In a normal intestine, beneficial bacteria are involved in vitamin production, for example folic acid and vitamin B12, in the production of butyric acid, the inhibition of the overgrowth of harmful bacteria, as well as in the breakdown of toxins. If pathogenic bacteria overgrow these properties are lost. In autism there is an increase in the urinary excretion of methylmalonic acid (Wakefield et al., 1998), an indicator of vitamin B12 deficiency, which could lead to impaired myelogenesis in autism (Wakefield et al., 1998) and although folate levels in plasma are not decreased, other pterins like neopterin and monapterin are decreased in urine (Eto et al., 1992) (Messahel et al., 1998) (Moreno-Fuenmayor et al., 1996). Tetrahydrobiopterin administration has led to improvement in the three core symptoms of autism in 54% of cases (Naruse et al., 1986) (Danfors et al., 2005)

although it has been suggested it is useful only for a subgroup of autistic children and more research is needed (Fernel et al., 1997).

The intestinal microflora can influence expression of epithelial glycoconjugates, which may serve as receptors for attachment of pathogenic microorganisms (Salminen et al., 1998), suggesting that increased permeability could be caused by the intestinal microflora.

Disruption of the mucin matrix, impaired sulphation and increased intestinal permeability have also been reported in IBS, and according to Wakefield *et al.* (1998) may be causing intestinal and neuropsychiatric dysfunction.

Some gut bacteria contain lactase, and lactase is decreased in the small intestine of autistic children (Horvath et al., 1999) (Wakefield et al., 2002). Some disaccharidases and oligosaccharidases activities are reduced, which hamper digestion of sugars. Bacteria require glucose for growth, which is obtained from undigested fibre and starch. Normally digestible sugars are absorbed in the small intestine so are not available for bacterial use. However, if these are not broken down, increased quantities might reach the colonic bacteria, leading to an increase in butyrate, acetate and propionate absorption and therefore increasing the non-essential fatty acids. This could contribute to an imbalance between essential and non-essential fatty acids in autistic children.

Abnormal colonization by sulphate reducing bacteria (SRB) could be a factor responsible for increased intestinal permeability. The availability of sulphate in the large intestine is a critical regulatory factor in determining whether sulphate-reducing bacteria or methanogens predominate, since they compete with methanogens for hydrogen in the colon (Willis et al., 1996).

SRB are anaerobes capable of utilizing the sulphate or sulphite ion as an electron acceptor in the dissimilation of organic substrates. However, they are an important source of hydrogen sulphide production within the bowel lumen, which has been implicated in the pathogenesis of ulcerative colitis (Hyspler et al., 2002). It is possible that SRB are

metabolizing sulphate, reducing plasma levels in autistic children, and causing abdominal pain, diarrhoea, bloating or reflux. However, sulphate may be decreased by other means, as 46% of autistic children show null sulphur oxidation.

Following exclusion of milk/dairy products, certain grains, caffeine, food colours and most preservatives from the diet caseomorphin urinary levels remain elevated in two-thirds of all autistic subjects, whereas gliadorphin levels fall (Kniker et al., 2001). This suggests that colonic bacteria are a possible source of opioid peptides in autism. Bacterial synthesis of opioids is not unknown. For example in the skin of the tree frog *Agalychnis callidryas* other opioids have been found (Mignogna et al., 1997), and are thought to be synthesized by bacteria as a defence mechanism against other microorganisms.

IAG could also be produced by bacterial fermentation. IAG is thought to come from tryptophan metabolism, possibly from the metabolism of bacteria. IAG is increased in parents and siblings of people with autism. Bacteria colonize the sterile infant gut through the birth canal, acquiring the maternal colonic bacteria from the mother. Similar bacterial species will synthesize similar metabolites, a possible explanation for the increased IAG in parents, siblings and autistic children and β-endorphin in plasma in 53% of mothers of autistic subjects (Leboyer et al., 1999).

Summary

Abnormal fermentation is suggested by the symptoms observed in autistic patients and its similarities with IBS. This suggests an abnormal colonisation of the gut from bacteria from the mother sometimes followed by ingestion of antibiotics could lead to an imbalance of beneficial and pathogenic bacteria in the GI. Extrinsic bacteria can then colonise the GI tract. Abnormal bacteria can then activate the immune system and give rise to GI problems. SRB might be increased in autistic individuals, causing a decrease of sulphate available. Unsulphated mucins cause an increase of absorption of bacterial metabolites and

undigested food due to the lack of some enzymes. Some of these metabolites might be harmful to the GI tract such as H_2S or biologically active in the brain such as opioids. This added to the prolonged effect of catecholamines in the brain due to the reduced sulphation, could lead to some of the neurological symptoms observed in autism.

Elemental diet (treatment for IBS and mechanism of work)

Inflammatory bowel disease (IBD) is a term for a group of inflammatory conditions of the intestine. The main forms of IBD are CD and UC (Griffiths, 1998), and their difference lies in the location and nature of the inflammatory changes.

CD is a chronic inflammatory disease of unknown aetiology that can involve any part of the GI system. It is characterized by episodes of relapse and remission. Although a standard treatment of CD consists of immunosuppression by corticosteroids and related drugs, it is now recognised that the disease can be successfully controlled by diet (Jones, 1985) (Middleton et al., 1995) (Riordan et al., 1993; Middleton et al., 1995). Foods commonly implicated are similar to those in IBS, with wheat and dairy products being very important (Dear & Hunter, 2002).

UC is a chronic inflammatory disorder of the colon of unknown aetiology, characterized by bloody diarrhoea and abdominal pain interspersed with periods of relative wellbeing. UC patients show inflammation confined to the colonic mucosa. High fibre foods have been also associated with the exacerbation of the symptoms, although patients do not normally respond to dietary treatment.

Enteral feeding has been shown to be the most effective means currently available of inducing long term remission (Knight et al., 2005) in active CD, with success rates of 80 – 90% amongst compliant patients in many trials (Middleton et al., 1995) (O'Morain et al., 1980), especially in children (Canani et al., 2005) (Knight et al., 2005). Nevertheless, the

mode of action of enteral feeds in inducing remission is still poorly understood, and this, together with lack of adequate dietetic support in many British hospitals, is believed to lead to reluctance amongst clinicians to use this treatment, despite its proven efficacy.

There are three types of enteral feeds. Initial remission is induced by the use of elemental diet, that is to say pre-digested enteral feeds in which nitrogen is present as amino acids, starch as maltodextrins, with a single oil as the sole source of fat and added minerals and vitamins. All the constituents are absorbed high in the GI tract, with little if any residue left to pass down to the colonic microflora. Polymeric diets differ from elemental in that they contain a single intact protein, and peptide diets contain nitrogen in the form of peptides of variable length (Verma et al., 2000b).

Suggestions as to the method of action of enteral feeding are many (O'Morain et al., 1980). It was originally thought that they allowed "bowel rest" through decreased gut metabolic activity and altered motility and reduction of intestinal secretions (El-Matary et al., 2003) (Greenberg et al., 1988) (Ruemmele et al., 2000) (Siedman, 1989), but this is now thought to be incorrect, as enteral feeding provides benefit even in subjects concomitantly eating normal foodstuffs (Jonson et al., 2005) (Verma et al., 2000a). As elemental feeds contain no whole protein, it was suggested that they might be hypoallergenic. No evidence exists, however, for food allergy as a factor in the pathogenesis of inflammatory bowel disease, and polymeric diets containing whole proteins are also known to be effective (Middleton et al., 1995). Improvement in nutritional status is a welcome effect of enteral feeding in patients with CD who are frequently poorly nourished (Gassull, 2004) (Siedman, 1989) (Siedman, 1994), but reduction in inflammation can be detected before any improvement in nutritional state occurs, and enteral feeding is ineffective in UC, where malnutrition is also frequent. Early enteral feeds were seen to be highly effective even though they lacked butyrate or glutamine, substances of key importance in the nutrition of the gut mucosa. The failure of enteral feeding in UC also means that a direct anti-inflammatory effect of enteral feeds is also unlikely.

The reason for the effects of enteral feeding in CD could be that, because it contains little or any residue and its nutrients are therefore absorbed, without the need for digestion, high in the jejunum, it supplies the colonic microflora with little in the way of energy substrates, resulting in reduced microbial metabolism, which in turn causes a decline in gut inflammation (Salomon et al., 1990) (Roediger, 1980). In UC, it is possible that the bacteria derive their energy from the breakdown, not of food residues, but of mucus, which is present in large amounts in the colon, and in much smaller quantities in the small bowel, thus accounting for the lack of small bowel involvement, and the failure to respond to diet. Mucus depletion is a characteristic feature of the intestinal mucosa in UC.

It has been shown that it is possible to demonstrate changes in the activity of the colonic microflora in patients with IBS by determination of the excretion of microbial products such as hydrogen (King et al., 1998). This approach, however, is not suitable for those with CD, as they are frequently too ill to tolerate hydrogen collections lasting 24 hours, and also because differences in the extent and severity of the disease mean that one patient cannot fairly be compared with another.

Breath and urine analysis

Metabolic profiling of different conditions can be very useful for the early detection and treatment of different conditions. In order to do this, metabolomics is used. Metabolomics is a powerful tool dedicated to the global study of metabolites, their dynamics, composition, interactions, and responses to interventions or to changes in their environment, in cells, tissues, and biofluids (Katajamaa, M. & Oresic, M. (2007)).

Overview of Potential Diagnostic Media

In order to find a biomarker or group of biomarkers of a disease or condition, we can use different analytical media. Blood, urine and faeces are probably the most used in clinical analysis. They have different characteristics and they are used in different occasions

according to the conditions. Breath is a potential tool that is not widely used, but should be studied further because it is a non invasive and ready available biological medium.

For the purpose of this research it was necessary to decide which medium would be the best in which to search for a biomarker. IBS patients, autistic patients and healthy volunteers were to be involved in the course of this investigation, so the different needs of these groups had to be addressed.

Faeces:

Faeces are a medium that can be used for the diagnosis of different conditions. However, they present several drawbacks. Faeces is a multiphasic solution containing solid matter so measurement of solutes is difficult. Their collection must be done in the presence of a bacteriostatic agent to minimize bacterial overgrowth and alterations of fermentation products. At the same time, the volume of faeces can vary and the comparison of the composition between faeces of two different individuals can become difficult , because the volume excreted by an individual per day is not constant and the concentrations of the analytes cannot be compared. At the same time faeces are quite unpleasant to work with.

The collection of faeces samples in all the patient groups would be possible, although it has too many disadvantages.

Blood:

Blood analysis is one of the diagnostic methodologies used in clinical routine. However, it is invasive and the analysis for organic acids is complex due to the sampling and the sample preparation. Blood plasma and cells contain many enzymes, some of which may affect the levels of some acids.

The collection of blood in the healthy volunteers group and in the IBS patients group would be feasible, however, the collection could become challenging for autistic children, as it is an invasive technique.

Breath:

The most established use of breath analysis is the breathalyser test for breath alcohol analysis. Breath provides a simple and non-invasive alternative to blood or urine. It is a quick method and the medium is readily available. However, breath presents two disadvantages: it only represents the metabolism over a short period, and also getting an appropriate volume of alveolar air from autistic patients can be very difficult.

At the same time, when sampling there is always the problem of compounds in the background air, making a background subtraction necessary.

Urine:

Urine is the most readily available body fluid. The urine specimens used in metabolic studies are random mid-stream urine collections, first urine passed on rising in the morning after an overnight fast, or 24 hour collections.

Urine is advantageous over breath because it collects metabolites throughout a period of time, whereas breath reflects metabolism on a single occasion.

For organic acid analysis urine is best collected without preservative and deep frozen as soon as possible after collection, below -20°C, and kept frozen until analysis.

Urine can be collected more easily from autistic patients as well as healthy volunteers and IBS patients. Urine was the medium which presented the most advantages for the present study. Breath presented advantages that make it ideal for the analysis of volatile organic compounds (VOCs), although it cannot be used with ASD patients.

Breath samples

Breath analysis

Distinctive smells on the breath of patients, such as those with diabetic acidosis, have been detected by physicians. However, breath sampling for clinical applications is not used routinely.

VOCs are present in breath in very low quantities, with the bulk of species in parts per billion (ppb) to parts per trillion (Phillips M. 1992) (Statheropoulos & Gerorgiadou, 2006). Very sensitive techniques are necessary for the detection of these compounds. Breath analysis could provide a simple, non-invasive method for the identification of specific VOC patterns in disease states.

Breath Testing

VOCs produced by metabolic processes are transported by the blood, where they might be transported into the lungs and exhaled in the breath. VOCs diffuse into the lungs, where equilibrium between the alveoli and the alveolar blood is achieved. In this way measurement of VOCs in the end tidal alveolar provides an indirect measure of VOCs produced in the body (Jones, 1985).

However, environmental compounds can also be inhaled and further metabolized, making very difficult the distinction between endogenous and environmentally derived VOCs. Providing subjects with purified air could overcome this problem, although it is not straightforward, may be expensive, requires complex equipment, and background subtraction may still be necessary. An alternative solution is to take breath and background air samples under the same conditions and substract the background sample from the breath sample, giving an alveolar gradient (Phillips, 1997). A positive alveolar gradient suggests

that compounds have been predominantly produced in the body while a negative alveolar gradient suggests that a compound is environmental in origin.

Breath Volatile Organic Compounds and Disease

Phillips et al. (Phillips, 1997) (Phillips et al., 1994) used a breath collection apparatus (BCA) which allows flow and breath volume on tube to be varied (1 and 10 litres were used). The alveolar gradient was calculated from the area under the curve of the breath peak minus the area under the curve of the background air peak. Of the VOCs identified 461 had a positive alveolar gradient while 798 had a negative alveolar gradient. Analysis was standardised by the addition of 1-bromo-4-fluoro-benzene.

Schizophrenic patients could be distinguished from controls with specific VOCs: 2-methylbutane, trichlorofluoromethane, 2-pentanol, pentane, dichloromethane, trichloromethane, benzene, 1-chloro-2-methylbutane, 2,3,3-trimethylpentane, 2,2-dimethylbutane and tetrachloroethane (Phillips et al., 1995). 22 VOCs were found in cancer patients' and control groups' breath samples at different levels distinguishing the two groups (Phillips et al., 1999).

Urine samples

In order to identify the compounds that are bacterial fermentation products, first a screening of the compounds found in urine needed to be done. Making sure that known bacterial products are detectable in urine could help the interpretation of the data.

Some organic acids such as short chain fatty acids (SCFA) are the major end-products of bacterial fermentative reactions in the colon. The most abundant of them are acetic, propionic and butyric acids, although lactate, ethanol, succinate, formate, valerate and caproate are also important. Branched fatty acids originate from amino acids in proteolysis. Other end-products from bacterial metabolism of proteins are ammonia, phenols, indoles and amines (Salminen et al., 1998).

SCFA in urine:

SCFA are produced by bacteria in the gut from dietary compounds. 95% of the SCFA produced in fermentation are absorbed form the gut (Macfarlane & Cummings, 2002) and then metabolised by the gut epithelium, liver and muscle. They are produced primarily from carbohydrates, although some are produced by protein and amino acid fermentation as well. Normal SCFA levels in urine are very low, in the order of ppb.

Acetic acid:

Acetic acid appears in portal blood and passes through the liver to peripheral tissues, and it is metabolised by the muscle. It is the principal SCFA in the muscles, and it comes from the fermentation of resistant carbohydrate by the anaerobic bacteria of the colon.

Propionic acid:

Propionic acid inhibits uptake of acetate into the cholesterol synthesis pathway and reduces cholesterol levels in blood. Dihydroxyphenylpropionic acid (DHPPA) is highly elevated in a significant percentage of individuals with autism (Shaw, 1998), which could be a short chain fatty acid released by abnormal fermentation in the bowel of these individuals.

Butyric acid:

This is an important energy source for the colonic epithelium and regulates cell growth and differentiation. Butyrate metabolism is decreased in UC and may be induced by sulphur compounds generated in the large bowel lumen (Salminen et al., 1998). Wakefield et al. (Wakefield et al., 1998) identified enterocolitis in autistic children, so butyrate could be reduced as well and requires further investigation.

Organic acid determination

The organic acid composition of physiological fluids and especially urine is very complex. No simple screening method for organic acids has been found. Most organic acids are not

volatile or stable enough for direct chromatography, and the detectors are not specific for organic acids. Also they are frequently present as salts. The lack of volatility is easily overcome by derivatisation to form esters of the carboxylic acids, either methyl esters or trimethylsilyl (TMS) esters (Tanaka & Hine, 1982). However, derivatisation is time-consuming and a quick analysis tool is needed, therefore only headspace analyses were attempted.

Headspace analysis (HS):

HS analysis is a relatively simple technique used for the concentration of volatile organic compounds. An aliquot of the gas phase in equilibrium with a liquid or solid phase is analysed. In equilibrium, the distribution of the analytes between the two phases depends upon their partition coefficients; thus, the composition of the original sample can be established from the analytical results of this aliquot.

- ✓ Partition coefficient (K): The equilibrium distribution of an analyte between the sample phase and the gas phase.

$$K = \frac{Cs}{Cg} \quad (1)$$ where Cs= concentration of analyte in sample phase and Cg = concentration of the analyte in gas phase.

Compounds with low K values will tend to partition more readily into the gas phase and have relatively high responses and low limits of detection. K can be lowered by changing the temperature at which the vial is equilibrated or changing the composition of the sample matrix. K can be lowered even further by introducing inorganic salt into the aqueous sample matrix. High salt concentrations decrease the solubility of polar organic volatiles in the sample matrix and promote their transfer into the headspace (*salting out*).

- ✓ Phase ratio (β): relative volume of the headspace compared to volume of the sample in the sample vial.

$$\beta = \frac{Vg}{Vs} \quad (2)$$ where Vs= volume of the sample and Vg= volume of gas phase

Lower values for β will yield higher responses for volatile compounds. However, decreasing the β value will not always yield the increase in response needed to improve sensitivity.

Striving for the lowest values for both K and β will result in higher concentrations of volatile analytes in the gas phase and therefore better sensitivity (Restek corporation, 2003).

Direct headspace analysis:

This technique involves equilibration of the sample with the head-space in the container and direct injection of a portion of this headspace gas for chromatographic analysis. This is done in a static system (*static headspace analysis*).

Wahl, H.G. et al. (Wahl et al., 1999) analyzed volatile organic compounds in human urine by HS-GC-MS with a multipurpose sampler. Aliquots of urine (1 ml) were acidified or alkalinized and sealed with PTFE lined rubber septum caps. Then 1 ml was taken out of the vial and injected into the GC. The GC oven was set at 60°C and the temperature was increased at 5°C per minute up to 100°C and then at 25°C per minute up to 240°C.

Total-trapping analysis:

The sample is heated and flushed with inert helium gas, which sweeps the thermally extracted components from the sample headspace and concentrates them in a trap held at liquid-nitrogen temperatures. Heating the sample and purging it facilitates forcing the equilibrium of compounds in the sample in favour of the headspace.

These volatile components concentrated in the trap are next flashed from the cold trap onto the chromatographic column. One problem associated with this technique is that water vapour in the sample eventually freezes, causing plugging of the trap and the termination of the collection process (Pauling et al., 1971). This technique is not widely used any longer.

Selective trapping head-space analysis techniques:

Organics thermally displaced from the aqueous sample are bound on an adsorbent (Politzer et al., 1976). The advantages of this technique are it does not require any solvents and the tubes are reusable. This technique and the previous one are also known as *dynamic headspace analysis*.

Thermal desorption (TD):

Adsorption (sample collection):

Collection of samples requires passive adsorption followed by active adsorption onto a sorbent bed. First air-sampling tubes are situated above a sample, where volatiles will adsorb onto the sorbent in thermal desorption tube. If the temperature is increased, semi-volatile compounds will at adsorb as well. This is followed by active adsorption, when the TD tube is connected onto a pump, and an inert gas such as nitrogen is pumped through. Variation of temperature will make different compounds adsorb onto the tube.

Desorption:

Primary desorption or air sample transfer.

The tube is heated and the volatile and semi-volatile compounds trapped in the desorption tube are then desorbed, and concentrated on an electrically-cooled, sorbent-packed focusing trap. There are different types of traps, with different types, sizes and number of beds. Traps should be chosen accordingly depending on the affinity of the analytes with the sorbents.

Trap desorption

The cold trap is rapidly heated up to release the analytes into the chromatograph. The rapidity of heating enhances the resolution of the compounds, providing sharper peaks. After each use, the sample tubes should be reconditioned and then may be reused.

The two-stage desorption method using cold trapping offers significant concentration of analytes. Target analytes are typically desorbed from a sorbent tube in approximately 100-200ml of inert gas, after primary desorption onto the cold trap the secondary desorption process (flash heating of the cold trap) is typically achieved in 100-200µl of vapour (Markes Inl.,).

TD tubes:

Tube sorbents must be able to retain specific groups of analytes for a specified sample volume and at the same time be able to release the analytes during the desorption process. It is very important to choose the appropriate desorption tube depending on the application. The choice of sorbent principally depends upon the volatility of the analyte concerned. The more volatile the analyte to be trapped, the stronger the sorbent must be (Markes International, 2005).

In order to get a broader range of compounds, sometimes two different sorbents are necessary. For the purpose of the analysis of VOCs, two sorbents were used: Tenax TA and Carbotrap.

Tenax TA is a weak sorbent which adsorbs analytes with 7 to 30 carbons (boiling point approximately from 100°C to 450°C). Carbotrap is a medium to weak sorbent, used to analyse compounds of approximately five to fourteen carbons. With both beds on a tube, aromatic compounds, apolar components with higher boiling point than 100 °C, polar

components with boiling point higher than 150°C, polyaromatic hydrocarbons, ketones, alcohols, aldehydes and apolar components will be analysed.

Thermal desorption and urine:

Organic acids have been determined by TD in urine, however, high volumes are normally needed (Teranishi R. et al., 1972) (Zlatkis et al., 1973) (Zlatkis et al., 1981) (Service et al. 2001a). A method was developed for the analysis of 2 ml of urine (Cert & Bahima, 1984), where a phosphate buffer was added in a jacketed bottle of 125 ml and saturated with sodium chloride. The gas flow passed through a water cooled condenser and then through the Poropak Q and activated charcoal with quartz tubes. Tenax was the sorbent tube normally used for the determination of organic acids in urine.

Solid phase microextraction (SPME)

This is a relatively new sample extraction technique. It has the advantages that it is fast, reducing sample preparation time by 70%, minimizes the use of solvents and their disposal, economical and reusable and versatile. In general, SPME is used to extract organic analytes from gaseous or aqueous sample matrices and it is not applied to the analysis of organic matrices such as solvent impurities (Hinshaw, 2003).

An SPME unit consists of a length of fused silica fibre coated with a sorptive polymer material, in some cases mixed with a solid adsorbent. The fibre is attached to a stainless steel plunger sheathed by a protective needle (see Figure 2).

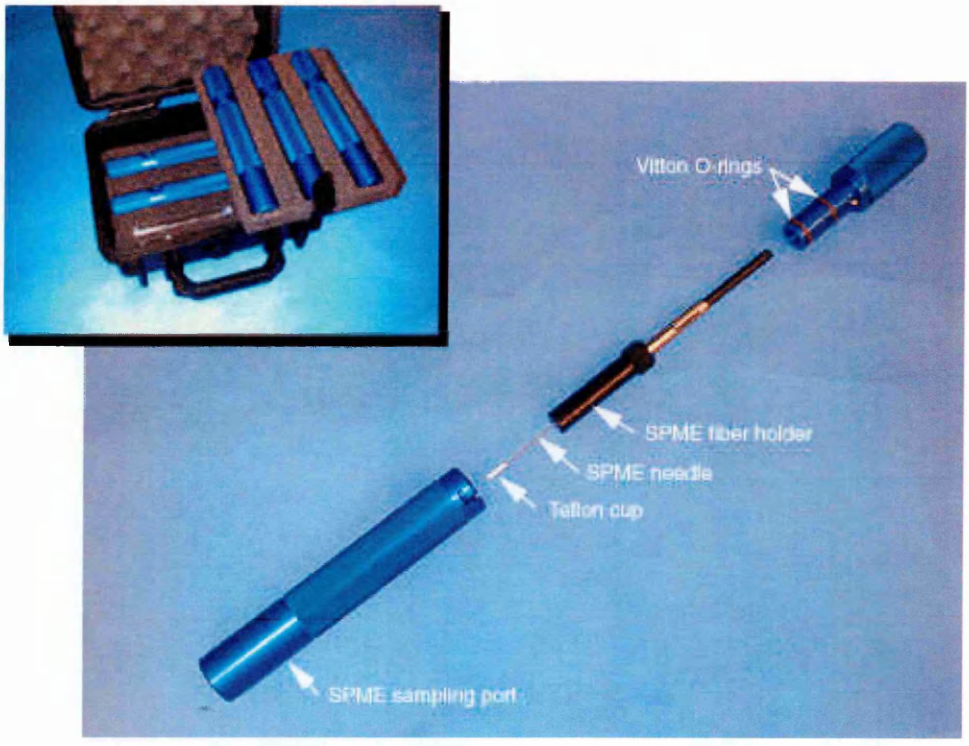


Figure 2. Photograph of a SPME holder (taken from http://www-cms.llnl.gov/s-t/solid_phase.html (Lawrence Livermore))

This technique has two steps: solute absorption from the sample matrix onto the fibre and transfer of the analytes into a chromatography inlet system.

Extraction:

The absorptive layer is exposed to a sample in a liquid or gas phase. The amounts of the solutes in the SPME layer gradually reach an equilibrium level with their surroundings, which represents the maximum solute amounts that can be absorbed and withdrawn under a given set of sampling conditions. The amount of solute in the SPME layer at equilibrium can be approximated by the following equation:

$$M_{i,\,SPME} \approx K_{i\,SPME}\, V_{\,SPME}\, C_i \qquad (3)$$

where $M_{i\ SPME}$ is the amount of solute in the SPME layer at equilibrium, $K_{i,\ SPME}$ is an aggregate solute distribution constant between the SPME absorptive layer and the sample, V_{SPME} is the volume of the SPME layer and C_i is the solute concentration in the sample before SPME sampling.

This equation assumes that the sample volume is much greater than the volume of the SPME layer. SPME coatings typically have thicknesses of approximately 10-100 μm. The volume (V_{SPME}) of a 1 cm long by 100 μm thick annular coating on a 0.56 mm o.d. fibre (24-gauge) is approximately 2 μL:

$$V_{SPME} = \pi L (r_2^2 - r_1^2) = \pi \times 1 \times (0.038^2 - 0.028^2) = 2.07\ \mu L \quad (4)$$

This assumption is valid for sample volumes more than 100-fold the SPME layer volume or more than approximately 0.5 ml for the thickest SPME later of 100 μm. Thinner SPME layers, with smaller volumes, would have smaller minimum sample volumes.

After sampling, the SPME layer and absorbed analytes are transferred into a chromatograph.

SPME and urine

Mills (Anderson et al., 2002) used SPME for the analysis of urine samples for the analysis of compounds such as acetic acid, acetic acid ethyl ester, n-butyric acid, butyric acid butyl ester, formic, isovaleric/2-methylbutyric, n-nonanoic and n-octanoic. The GC conditions used were: no solvent delay, injector 250°C, transfer line 280°C, 40°C for 5 mins, 10°C/min to 220°C, keep for 10mins. The MS had a scan mode for 34 to 300 amu. HS vials were silanised for 1h in a solution of dichlorodimethylsilane. 4 ml of urine were used and three different techniques, salting the sample out, acidifying the sample and alkalising the sample. They found out that under acid conditions sharp, well resolved peaks were obtained for very volatile compounds. Samples were kept at 50°C for 30 mins, then pierced with the Carboxen-PDMS SPME fibre for 30 min.

Solvent extraction:

Diethyl-ether (extracts the more hydrophobic acids), ethyl acetate (improves the extraction of some of the more hydrophilic acids) (or both in succession), acetonitrile, isopropyl ether, tri(n-butyl) orthophosphate and methyl acetate have been used for extraction of organic acids. This is normally carried out in acidified (pH 1-2) and/or salt-saturated urine. Hydrochloric acid and sodium chloride are the most commonly used acid and salt respectively.

However, solvent extraction has limitations imposed by the unfavourable partition coefficients between aqueous solution and organic solvents shown by many organic acids (Anon., 1982). Very pure extracting solvents must be used, evaporation of the extracting solvent is usually accompanied with a loss of compounds of low boiling point, and no one solvent will extract to an equal extent the complete range of volatile compounds present in biological fluids (Zlatkis et al., 1981).

Direct injection of acidified urine onto the GC is regarded as a very useful screening technique for large elevations of SCFA. However, the processing of the urine makes this technique time consuming, and the GCMS when used with direct injection requires high maintenance, since non-volatile salts and protein accumulate on the glass walls. For this reason, this technique was not attempted in our laboratories, but it has been used for a long time (Collin & McCormick, 1974) (Frenkel & Kitchens, 1977) (Harrington et al., 1977) (Maltby & Millington, 1986) (Pettersen & Stokke, 1973) (Pileire, 1978) (Schatowitz & Gercken, 1988) (Zlatkis et al., 1981).

Organic volatile profiles as biomarkers of disease in urine:

VOCs comprise groups of compounds of various polarities, such as alcohols, aldehydes, amines, O- and N-heterocyles, hydrocarbons, isocyanates, ketones and sulphides. The majority contain no more than 12 carbon atoms, have a boiling point of less than 300°C and are likely to be sufficiently stable to survive current analysis processes (Guernion et al., 2001).

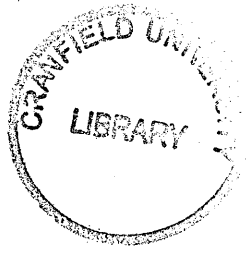
The organic volatile profiles will be affected by factors such as genotype, diet, circadian and seasonal variations, occupation, physical exercise and physiological and clinical status. However, variance of urinary volatiles between individuals is significant, whereas for the same individual the profile from urines collected on different days remains remarkably constant (Zlatkis et al., 1973). The chromatograms of the urinary volatiles can be considered to be characteristic for the individual and little influenced by diet, sex and circadian or seasonal changes.

The organic volatile constituents of biological fluids contain clinically useful diagnostic information for the recognition of metabolic disorders in man (Zlatkis et al., 1981).

Biomarker profiling has been used in *Diabetes Mellitus*: A deficiency in effective insulin alters the metabolism of carbohydrates, lipids and proteins. Therefore, urinary excretion of ethanol, isobutanol, n-butanol and isopentanol is increased (McConnell et al., 1979). According to Wahl (Wahl et al., 1999), 4-heptanone is increased in this condition.

Depending on the form, stage, and severity of the disease, the excretion of one or several of these analytes is changed. This information, combined with the standard glucose tolerance test, provides a positive diagnosis of diabetes. (Zlatkis et al., 1981).

Renal insufficiency: In these cases, less 4-heptanone is excreted (Zlatkis et al., 1981).



Aims and objectives

The initial aim of this study was to identify the compounds in breath and urine that are products of colonic bacterial fermentation. SCFA were initially targeted as known bacterial products and a method was needed for their analysis. There are hundreds of organic volatile compounds in human breath and urine, but their relationship with bacterial fermentation is unknown. In order to do this, subjects were fed an enteral feed that it is thought to deprive bacteria of substrates for their nutrition, so giving a change in the profile of bacterial metabolites. Two different commercial elemental diets were assessed.

The second aim of this study focused on IBS. A biomarker of IBS in urine was looked for through a screening of urine from IBS patients. A study was done to determine if organic compounds found in IBS patients' urine were different to those in controls, and compounds thought to be produced by bacterial fermentation in the bowel were examined.

The third aim of this study focused on the study of Autism, and whether a biomarker of autism could be found in urine. Autistic patients have abnormal bacteria in the gut (Horvath et al., 1999). Concentrations of compounds found to be fermentation products were looked at to determine if autistic patients suffered from abnormal fermentation.

The key objectives of the project were:

- Development of semi quantitative method for breath analysis: different methods have historically been used for breath analysis, although there is no method accepted for routine clinical practice.
- Development of semi quantitative method for the analysis of organic volatile compounds in urine: there are different methods for the analysis of these compounds in urine found in the literature, but they all have disadvantages so a development stage was necessary.

- Identification of the compounds in breath and urine that are produced by bacterial fermentation and the compounds that are metabolised by bacteria in normal circumstances.
- Assessment of method of analysis by comparing enteral feeds that reduce bacterial fermentation in the bowel to clinically normal subjects: There are different commercial diets in the market used to stop fermentation in the GI tract. These diets are made of a mixture of pre-digested compounds which are all absorbed in the small intestine, therefore depriving the bacteria in the gut of substrates. Two different diets were compared.
- To aid the understanding of the way enteral feeds work in the treatment of IBS by examining the spectrum of volatile produced in IBS and non-IBS patients and determining or not their bacterial origin.
- Determination of biomarker(s) for IBS using the above methods.
- Study of bacterial fermentation in IBS patients: the identified compounds in urine involved in bacterial fermentation were studied in IBS patients.
- Determination of biomarker(s) of ASD.
- Study of bacterial fermentation in ASD patients: the identified compounds in urine involved in bacterial fermentation were studied in ASD patients.
- Comparison of IBS and ASD conditions via the patients' metabolic product spectrum.

Chapter 2. Materials and Method Development

Methods for the analysis of urine and breath VOCs were developed for their application in the aforementioned trials. Healthy volunteers were given two different enteral feeds and breath and urine were analysed before and after both diets in order to identify products of bacterial fermentation.

Afterwards, the urine of different types of IBS patients were studied and compared with healthy controls. IBS patients suspected to suffer from abnormal fermentation were put on a fibre free or exclusion diet, and urine was analysed before and after the diet. Biomarkers of the different types of IBS were examined and metabolites produced by colonic bacteria were studied.

Urine of autistic patients and healthy controls was analysed to look for biomarkers of the condition. Bacterial metabolites were investigated in the urine to determine whether there is abnormal fermentation in autism.

Various methods were investigated to use in these trials. The main objective is looking for a method which can be applied in the quick diagnosis of different conditions. For this purpose the method needs to be non-invasive, quick and easily automated.

Materials and Methods

Volunteers

Healthy volunteers were recruited from Cranfield University. All were to be in normal health, and eating a normal diet. Exclusion criteria were; taking any medication other than the oral contraceptive; having received a course of antibiotics in the previous six weeks; taking bacterial products such as pro- or prebiotics, and pregnancy and lactation.

This study was reviewed and approved by the Cranfield University Silsoe Ethics Committee (See Appendix 1).

Breath method development

Materials

Breath and background air samples were collected in a Bio-VOC sampler (Markes International). Tenax TA (100 mg) and Carbotrap (250 mg) desorption tubes (Markes International) were used for the retention of aromatic, apolar and polar compounds with a boiling point higher than 100°C, alkyl benzenes and hydrocarbons. Compounds were identified and detected using a Perkin Elmer Turbomass gas chromatograph mass spectrometer GCMS. Mass spectrometry data was acquired and integrated using Turbomass 4.1 software.

Compounds from the breath and background air were identified from the total ion chromatogram (TIC) using the NIST electronic spectra library.

Method

Volunteers were asked to pass one litre of their breath through the sampler containing a freshly conditioned desorption tube first thing in the morning on different days of the trial, each day at the same time. First a sample of the background air was taken into four 250 ml Bio-VOC sampler (see Figure 3), and then the sample was passed onto an adsorbent tube. The volunteers were then asked to exhaust their lungs into the same syringes. This meant that the syringes contained the air from the final stages of exhalation, much more likely to be that from the lung alveoli.

Figure 3. Bio-VOC sampler (Markes International) attached to a Tenax-Carbotrap tube.

To collect a breath sample the plunger was unscrewed and taken out of the Bio-VOC sampler and replaced by a disposable cardboard mouthpiece. The subjects breathed out through the mouthpiece for as long possible (without taking an initial deep breath). The ATD tube end of the Bio-VOC was left open, without a cartridge, during sampling (Figure 4a), allowing the dead space air to pass through the sampler, only leaving the last 120 ml of end tidal, or alveolar air in the sampler. Immediately after the subject had finished breathing out a desorption tube packed with Tenax and Carbotrap was placed on the open end of the sampler. Then the disposable mouthpiece was removed from the other end, with a one way valve ensuring that no breath escaped from the end of the sampler. The plunger was screwed back into the Bio-VOC sampler and pushed through the whole length of the sampler over a 10 second period (to keep constant flow rate) to transfer the breath onto the ATD tube (Figure 4 b). Four breath samples were collected by the same method and added to the same ATD tube which was then capped for storage until analysis. For the collection of background air, four aliquots of air from the Bio-VOC were added to each ATD tube using the same method as for breath collection. For each volunteer, 1 ATD tube of breath and one of background air were collected and analysed.

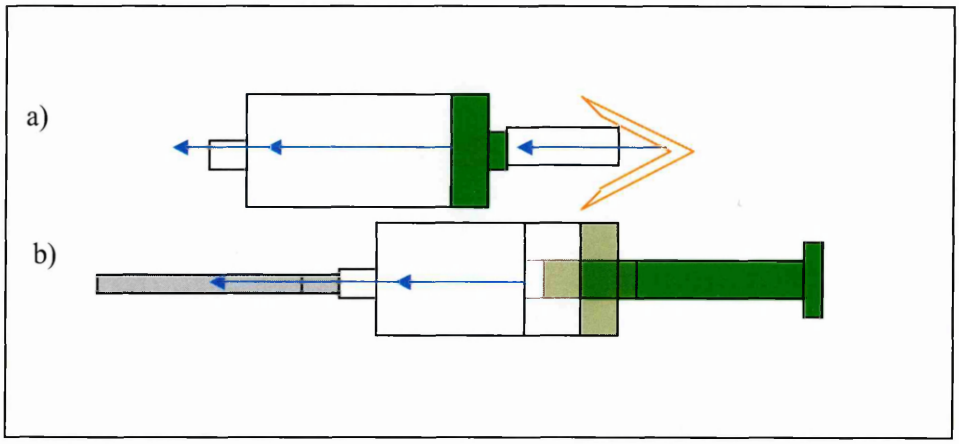


Figure 4. Diagrammatic Representation of Breath Sample Collection Using the Bio-VOC Sampling Equipment. a) the subject breaths out through Bio-VOC for as long as possible. b) the ATD tube is pushed into the open end of sampler, the plunger is screwed into the other end and slowly depressed to pass the breath sample onto the ATD tube (courtesy of Michelle Hall (Hall, 2004))

After the collection of the background and the breath and before analysis, an internal standard was put into the adsorbent tubes.

Phillips et al. (Phillips et al., 1994) standardised the analysis by adding 1-bromo-4-fluoro-benzene. However, this internal standard is a gas, and has several disadvantages: it is expensive and needs an internal standard injection device, which injects the sample onto the back of the tube. However with two-sorbent cartridges, internal standards should be inserted in the same way as samples are, to prevent the internal standard adsorbing onto the stronger bed in the tube, thus avoiding problems of carryover between samples.

Glass wool was inserted into the upstream end of the tube before the injection. Deuterated toluene standard was diluted in methanol and injected onto the glass wool of the tube fitted into the apparatus. Deuterated toluene was chosen for having similar characteristics as the

analytes of interest but without interfering with any of them. Nitrogen was passed over the wool allowing the standard to accumulate on the sorbent after vaporisation through a Markes International Ltd. Calibration solution loading rig (see Figure 5) which flowed nitrogen at a rate of 100 ml/minute. Methanol was the solvent used to dilute the toluene because it does not attach to the adsorbents.

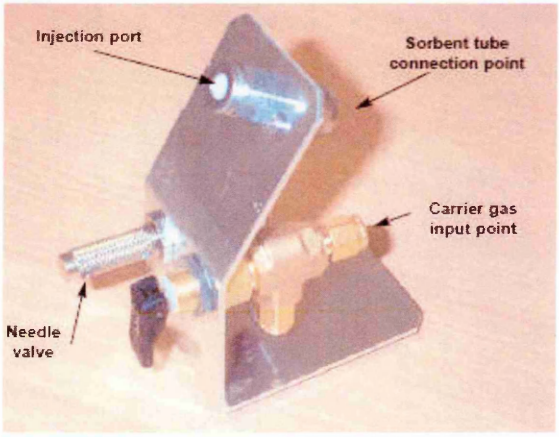


Figure 5. Calibration Solution Loading Rig (courtesy of Markes International).

The tube was then analysed in an automated thermodesorber Perkin Elmer ATD 400 attached to an Autosystem XL GC-MS.

Method development:

In order to analyse the quantities of the various VOCs present in the tubes, it is essential to determine the best conditions for analysis, so different parameters were investigated.

In order to do this the usual technique would be to inject standards of those species for analysis. However, for this particular research, the whole spectrum of compounds attached to the Tenax and Carbotrap adsorbent beds in the tubes were deemed to be important. This spectrum of compounds varies immensely from individual to individual. However it would be impossible to try a long list of potential candidate standards. For this reason, a single individual's breath was used for the purpose of this study instead of a range of standard

chemicals. Breath samples are almost saturated with water and this constrains the optimisation of the analysis. Water can attach to the sorbents in the tube, first stopping volatiles being adsorbed onto the tube (and later the trap) and secondly hampering the chromatographic analysis by acting as co-solvent, and impairing the liquid phase of the GC lining. Water can mask some peaks and change the retention times of the compounds observed. Parameters were optimised especially taking rejection of water into account. Parameters that are beneficial to the chromatography are high carrier gas pressure, long purging times and low initial oven temperatures. The first two of these factors can be detrimental to compound retention on sorbent tubes.

Breath volatiles were collected on three tubes and analysed whilst varying chromatographic and desorption conditions, and an observation was made of the compounds known to be products of the body's metabolism in comparison with those known to be from the environment. The reason for this is even though the breath came from a single person, breath reflects metabolic changes. This meant that even though the breath was taken over a short period of time, the metabolism changes quickly and there could be differences in the compounds observed. The amount of some compounds was observed to increase whereas some decreased.

Something that would reduce this variation would be to insert the expired breath alternately into different tubes. However, it was best to keep testing conditions as close as possible to those applying to the collection of the volunteer's breath would be in the trials.

Thermodesorption parameters:

Different thermodesorption conditions were attempted for the best results. Variables were: outlet split flow, purging times, desorption time, desorption temperature and trap temperature. Experiments were done three times to ensure veracity. In Figure 6 a diagram of the TD system can be observed.

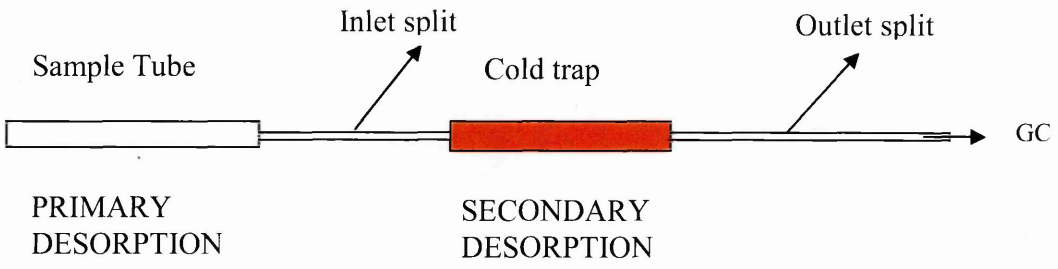


Figure 6. Diagram of a thermodesorption system. The inlet split controls the ratio of flow going into the cold trap. The outlet split controls the ratio of flow going into the GC column.

Different conditions tested:

Split flow: With an inner split flow of 100 ml/min (manufacturer's recommendations), different outer split flows were considered. The inner split is disabled during analysis, but after desorption and cooldown, before being unloaded, sample tubes are depressurized using the inlet split. It also purges the tubing of residual volatiles from previous analysis. The outlet split avoids too much sample arriving to the GC. However, the smaller the outer split flow is the more sample gets to the GC, therefore the more sensitivity the method will have. At the same time superior chromatographic results will generally be obtained if a higher split flow is used, since it considerably increases the gas velocity through the trap during the trap heating. Furthermore, it is important to have a split of more than 5 ml/min, according to the manufacturer's recommendations. However, with high split flows giving less sample arriving in the GC, smaller splits than the recommended were tried. 2 ml/min, 5 ml/min and 10 ml/min outlet split were explored, and three repeats done.

Purging times: The sample tube is purged with carrier gas to remove air prior to desorption. The effect of introducing water to the analysis is very strong, as many peaks

may be masked in the analysis. As a screening of the breath was the main aim of the study, water reduction was very important. The longer the purging time, the more water it is discarded, and the better the analysis is. However, too much purging time could carry a risk of losing sample.

Desorption time: A longer desorption time was thought to give better results, to make sure all the compounds concentrated in the trap were desorbed.

Trap temperature: The trap collects material desorbed for the tube, concentrating the volatiles in a small volume to increase sensitivity. Different temperatures were tested for the trap, ranging from 5°C to 30°C. Temperatures of 0°C or less were not considered because when dealing with a sample with high water content, water would freeze in the trap, damaging the apparatus, at this temperature. Higher trap temperatures would not trap the compounds desorbed from the tubes.

Desorption temperature: For the same reason, the higher desorption temperatures the better the analysis is. The desorption tubes come with recommended temperatures of analysis. 320°C was the manufacturer's advice on VOCs analysis for this particular type of desorption tube, so the temperature was not changed.

GC-MS parameters

The initial oven temperature was decreased in order to improve the analysis of more volatile compounds such as acetone. However, even though the most volatile compounds appeared earlier in the chromatogram, the compounds were not better separated or the resolution was better. If the oven is started at a lower temperature, the time of analysis will be longer if the rate of temperature increase is not compromised; 50 °C was an appropriate starting temperature for the GC.

Standard GC-MS conditions gave good peak separation. 33 Da was the starting point for the MS scan, since it was important to avoid high background interference from helium, oxygen, water and nitrogen, at 4, 16 and 32, 18 and 14 & 28 Da respectively.

Internal standard:

In order to compare different samples analysed by a method it is important to add an internal standard. The internal standard is used to make all areas of the compounds determined comparable. This is used to minimize errors of analysis, because the MS loses sensitivity with each subsequent analysis.

In order to study if the injection of the internal standard method was reproducible, tubes were spiked with 2 µl of a solution of deuterated Toluene (D8-Toluene). This was done in five different tubes first with 1/10000 concentration and then with 1/50000 made in methanol.

A calibration curve was done and the determination of the appropriate concentration of Toluene D8 was calculated.

Results:

Different parameters were tested to improve the sensitivity of the technique.

Split flow:

Rates of 2, 5 and 10 ml/min were tested. The best results observed were with 2 ml/min, although there is a risk of overloading the GC when other samples with more VOCs are analysed. For this reason, the outlet split was kept at 5 ml/min, which gave better results than 10ml/min (see Figure 7).

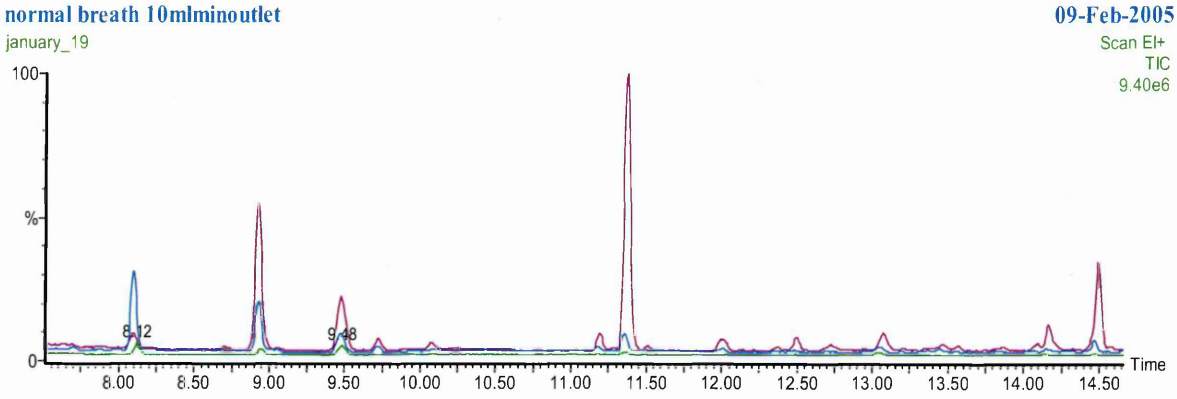


Figure 7 .Chromatograms of breath analysed with different outlet splits: 2 ml/min (purple), 5 ml/min (blue) and 10 ml/min (green). The area under the curve of compounds from breath decreases with higher outlet splits.

Purging times:

1 and 2 minute purge were tested. In Figure 8 the differences between the chromatograms can be observed. 2 minute purge gave a better resolution (in green) than 1 minute (in blue) so 2 minutes was used.

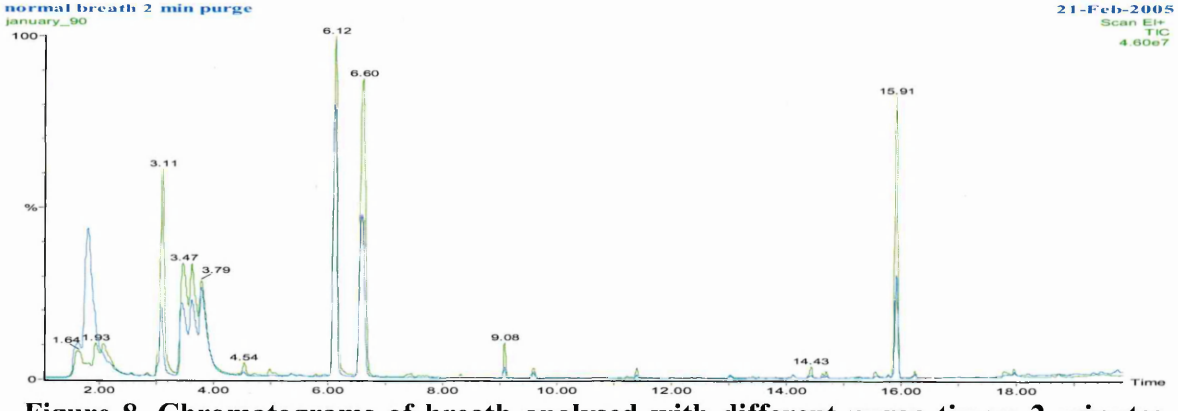


Figure 8. Chromatograms of breath analysed with different purge times: 2 minutes (green) and 1 minute (blue). The area under the curve of compounds from breath increases with higher purge times.

Desorption time:

5, 10, 20 and 30 minutes desorption time were tested. It was observed that the longer the desorption time the worse the analysis was (see Figure 9) and the higher the baseline was.

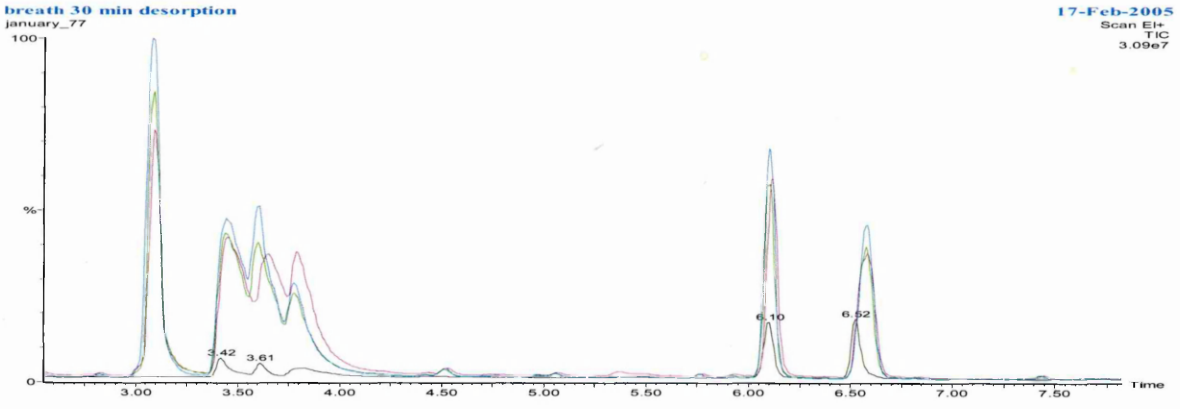


Figure 9 .Chromatograms of breath analysed with different desorption times: 5 minutes (blue), 10 minutes (green), 20 minutes (violet) and 30 minutes (black). The area under the curve of compounds from breath decreases with higher desorption times.

Trap temperature:

The trap temperature was tested at 5, 10, 20 and 30ºC. Up to 30ºC the higher the trap temperature, the better the sensitivity was (see Figure 10). Therefore 30ºC was the chosen temperature for the purpose of this study.

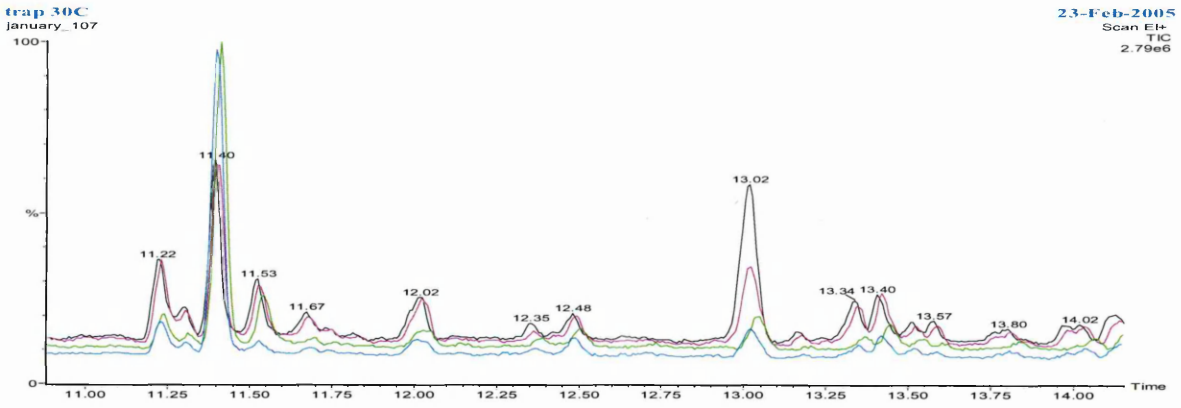


Figure 10. Chromatograms of breath analysed with different trap temperatures: 5°C (blue), 10°C (green), 20°C (violet) and 30°C (black). The area under the curve of compounds from breath increases with higher trap temperatures.

Internal Standard:

Reproducibility:

An empty tube was spiked several times with a 1/10000 by volume of toluene D8. In Table 2 a list of the areas found can be seen.

Table 2. Areas under the curve of D8-toluene, when an empty tube was spiked with 1/10000 concentration of D8-toluene.

Injection	Area	Coeficient of variance
1	52982	
2	53374	
3	61534	
4	64441	
5	60950	
Mean	58656.2	
std dev	5174.586	8.8%
std error	2587.293	

An empty tube was spiked several times with a 1/10000 in volume of toluene D8. In Table 3 a list of the areas found can be seen.

Table 3. Areas under the curve of D8-toluene, when an empty tube was spiked with 1/50000 concentration of D8-toluene.

Injection	Area	Coeficient of variance
1	18154	
2	14415	
3	15363	
4	13149	
5	14388	
Mean	18867.25	
std dev	1882.715	9.98%
std error	941.3575	

The variation of the areas at a given concentration varies slightly, but a coefficient of variance of less than 10% is good enough for this purpose. Therefore this method is reproducible and can be used to spike the samples with an internal standard.

Calibration curve

Tolune D8 in different concentrations was injected in different tubes, where we can see a linear response (see Figure 11).

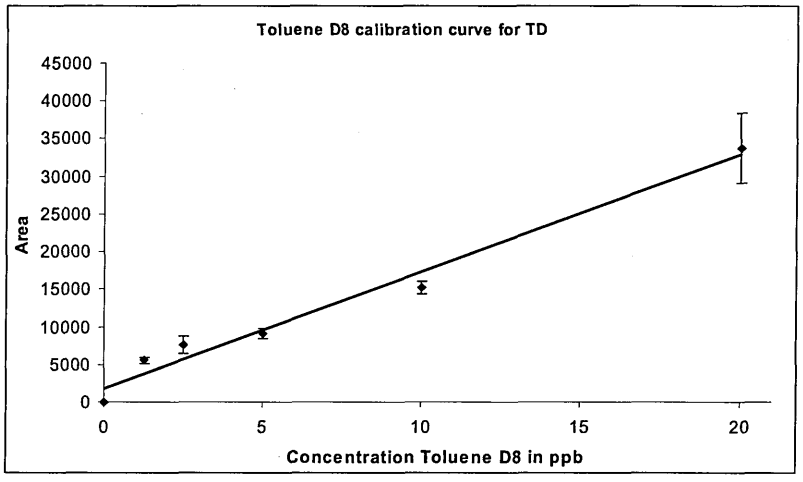


Figure 11. Calibration curve of D8-toluene, when a tube was spiked with different concentrations.

Concentration of Toluene D8

The amount of Toluene to use as internal standard was firstly based on the peak size obtainable. The size had to be similar to the compounds of interest, and the shape small enough for it not to saturate the MS; 2µl of a 20 µg/ml solution was chosen for these two reasons.

Several injections were done to test reproducibility and a calibration curve was drawn. However, some of the chromatograms were ruined in the first 10 minutes of the run. The internal standard could be seen but many of the compounds were masked by a huge peak that seemed to be methanol. The methanol used for the dilution of Toluene-D8 was probably not purged away completely, and increasing purging times when the internal standard was added and in the thermodesorber did not improve this. At the same time, more than 2 minutes purging (added to the purging carried out when the internal standard was introduced) was excessive and compounds of interest could start to desorb.

In order to keep purge time and amount of Toluene-D8 the same, and the amount of methanol used low, the concentration of toluene had to be doubled and injection volume

halved. Therefore 1 µl of 40 µg/ml Toluene-D8 was injected into the tube with a 1 µl syringe.

Final method developed

The tube was thermally desorbed at 300°C for 5 minutes after 2 minute purge. The trap was then heated quickly from 30 to 320°C. No inlet split was used during the analysis but an outlet split of 5 ml/min with 1 ml/min flow rate. It was then separated in the GC with a temperature of 50°C for 4 minutes and then increasing it at 10°C/min until 220°C and was held for 9 minutes.

Data was acquired with an electron energy of 70 eV, to allow library identification, and an emission voltage of 50 eV. The mass spectrometer scan range set for the assay was 33-350 Da.

Urine

Materials

Specimens were collected into a 300 ml urine collection pot (Fisher Scientific). Samples were stored in a -80°C freezer until analysed. Different analysis methods were assessed.

Different techniques for the analysis of urine were assessed, in order to choose a suitable one for the analysis of bacterial metabolites in our laboratories.

A Perkin Elmer Autosystem XL gas chromatograph (GC) and Turbomass mass spectrometer were used. The column used was a ZB-624 (60 m, 0.25 mm ID, 1.40 μm ft) (Phenomenex). A Perkin Elmer headspace autosampler or a Perkin Elmer thermodesorption autosampler was attached to the GC in the cases of headspace or thermodesorption.

Methods

Headspace (HS):

Sample preparation:

A GC vial was filled with 5 ml aliquot of sample and sealed immediately after with a gas tight polytetrafluoroethylene (PTFE) lined rubber septum cap. This vial was inserted in the headspace autosampler at 60 °C for one hour to establish equilibrium of the organic volatiles between the liquid and the headspace. The temperature was not increased to minimize water vapour in the headspace.

1ml of gas was taken out of the headspace of the vial and taken automatically into the GC. The GC oven was kept at 40°C for 1 minute and then the temperature was increased at a rate of 10°C per minute until reaching 200°C, and this temperature was kept for 8 minutes. A full scan of 33 to 350 (EI+) with a 1.50 minutes solvent delay was done with the MS.

Thermodesorption (TD):

Urine (10 ml) was put in a glass Buchner flask fitted with a stopper incorporating one inlet arm. A diagram of the sample collection used can be seen in Figure 12. This method was applied for clinical analysis, and volumes of analysis should be kept to a minimum, although a volume of 10 ml used here or the 20 ml used by Service et al (Service et al., 2001b) is appropriate. A device was set up so that nitrogen (99.9%) was passed through the urine of a healthy subject for 30 minutes. The time was deliberately kept to 30 minutes as one hour is far too long for clinical analysis (Service et al. 2001a). Urine was warmed using a hot plate at 70°C and stirred vigorously. This temperature was chosen to minimise water vapourisation and avoid the use of a 12°C condensation trap (Zlatkis et al., 1981). A pump was installed after the trap to ensure the volatiles carried with the nitrogen passed through it. Due to the high affinity of the trap with compounds that may be in the air, a control experiment was carried out with air as the analyte. In the same way, another experiment with HPLC grade water was made as well as with standards (acetic acid, propionic acid, butyric acid and valeric acid were added). The salting out method was attempted with NaCl to decrease the solubility of polar organic volatiles in the sample matrix and promote their transfer into the headspace. Acidification of the sample was also performed, as a means of hydrolisation of urine. The adsorbent tube used was Tenax TA and Carbotrap.

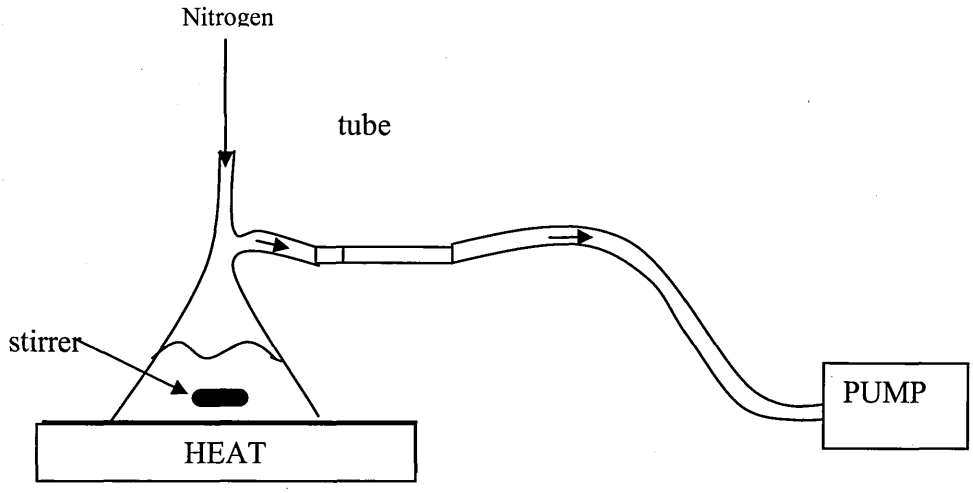


Figure 12 . Diagram of the sample collection for TD. Urine is heated up and stirred while nitrogen is passed onto it and VOCs are collected in an adsorption tube.

Column temperatures were ramped in stages. First the oven was kept at 30°C for two minutes and then the temperature was increased at a rate of 5°C/min until reaching 210°C, and this temperature was kept for 15 minutes.

SPME (solid phase microextraction):

HS vials (20 ml) from Supelco (Poole, UK) capped with PTFE/silicone rubber seals from Perkin Elmer were mounted on a magnetic stirrer submerged in a water bath maintained at 50°C, and the contents stirred continuously so as to release volatile compounds into the HS of the vial. The septum of the sample vial was pierced with the SPME needle and the SPME fibre exposed to the HS vapour for 30 minutes. Polydimethylsiloxane divinylbenzene (PDMS-DVB) (65µm film thickness) SPME fibres were used. A diagram of the set up used for the analysis can be seen in Figure 13. The extracted compounds were then desorbed from the fibre in the GC injector port, split valve closed.

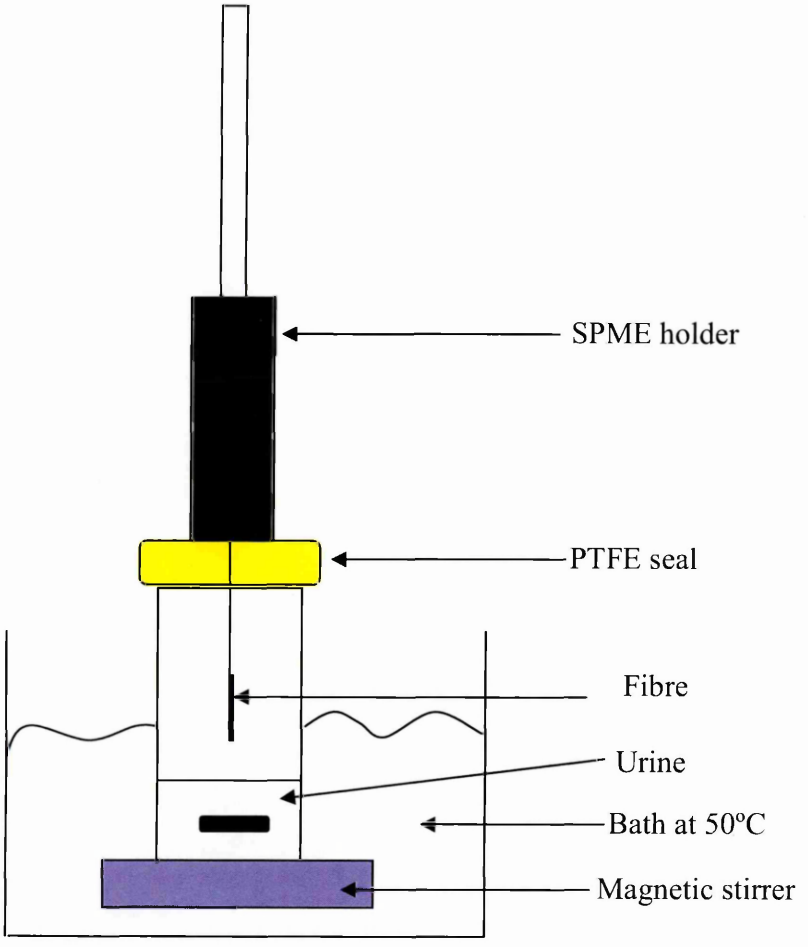


Figure 13 . Diagram of a SPME setup. Urine is heated up and stirred and the SPME fibre is exposed.

Helium was used as the carrier gas at a flow rate of 1 ml/min. The oven is kept at 40°C for 5 minutes, and then the temperature is increased at a rate of 10 °C per minute until it reaches 200°C and is left at this temperature for 5 minutes. The injector was at 250°C. A narrow bore (1mm) splitless injection liner was used (Perkin Elmer).

The method was similar to Mills and Walker (Mills & Walker, 2001) although it varied due to the different instrumentation.

Results

General comparison of HS, TD and SPME

Three techniques were used for the screening of urine to determine which one is most suitable to use in our laboratories. The technique that detected the most number of compounds from in urine was the SPME technique, followed by TD. HS was the worst technique for detecting many compounds, and this is thought to be due to water vapour going into the GCMS disturbing the chromatography. Attempts were made to minimise this by decreasing the sample equilibration temperature and using water-quenching salts, but none of these worked, and the number of compounds identified was very poor. In Figure 14, the chromatograms of 5 ml of urine baked at 60°C and 5ml of urine baked at 30°C. Both chromatograms are similar and only an air peak is detected. Decreasing the temperature did not improve conditions for HS analysis.

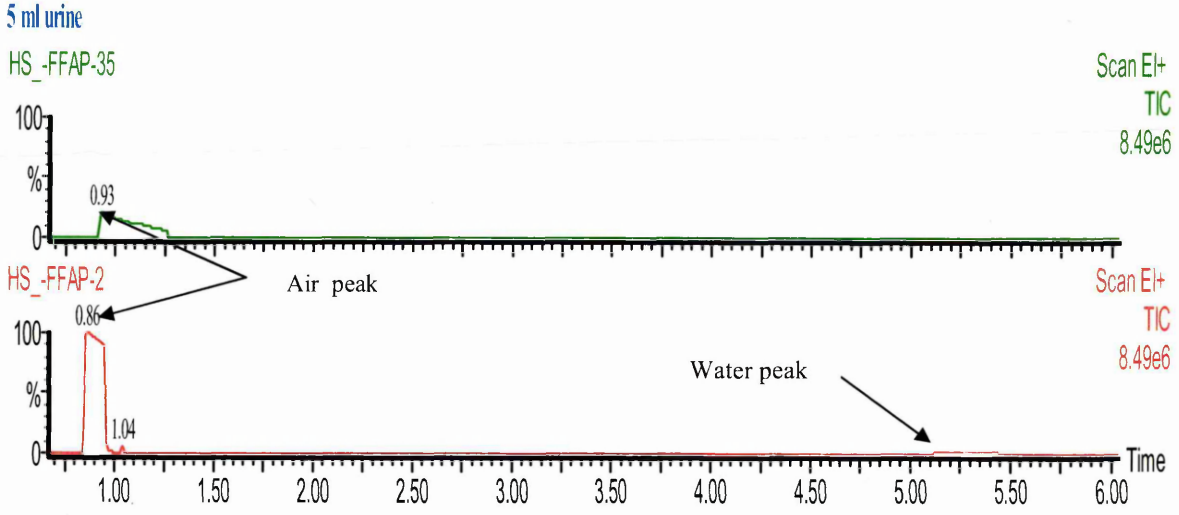


Figure 14. Chromatogram of urine analysed with HS at 60°C (bottom chromatogram) and urine analysed at 30°C (top). Only one air peak and one water peak can be observed.

In both chromatograms, the air peak is the largest peak, followed by a water peak at about 5.25 minutes. When urine was heated up to 30°C, the water peak was smaller than when heated at 60°C, however, no other peaks can be seen.

5g of hydromatrix, Mg_2SO_4 (known drying agents) (Eller & Lehotay, 1997) and silica were added to the sample to see whether they could adsorb the water, therefore allowing a good peak separation. When hydromatrix and Mg_2SO_4 were used, only one peak was observed. See Figure 15, Figure 16 and Figure 17.

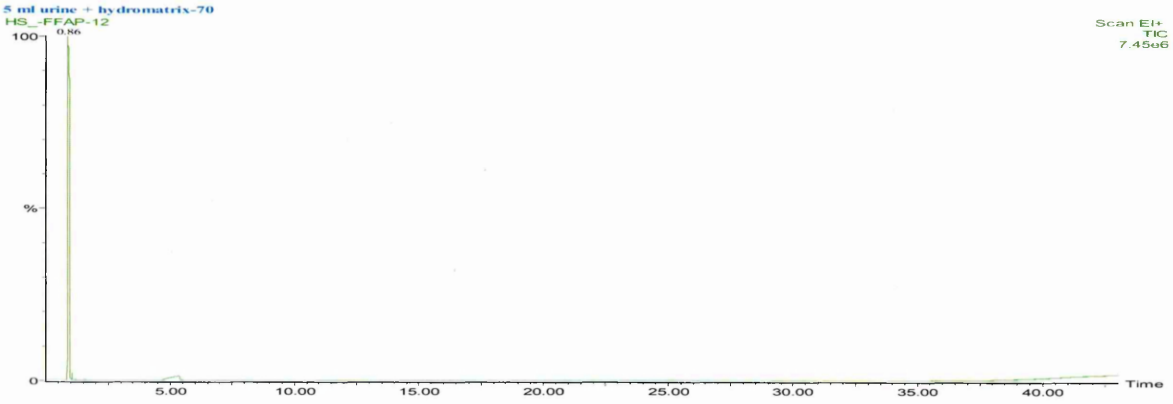


Figure 15 Chromatogram of urine with hydromatrix analysed with HS. Only one air peak and a water peak can be observed.

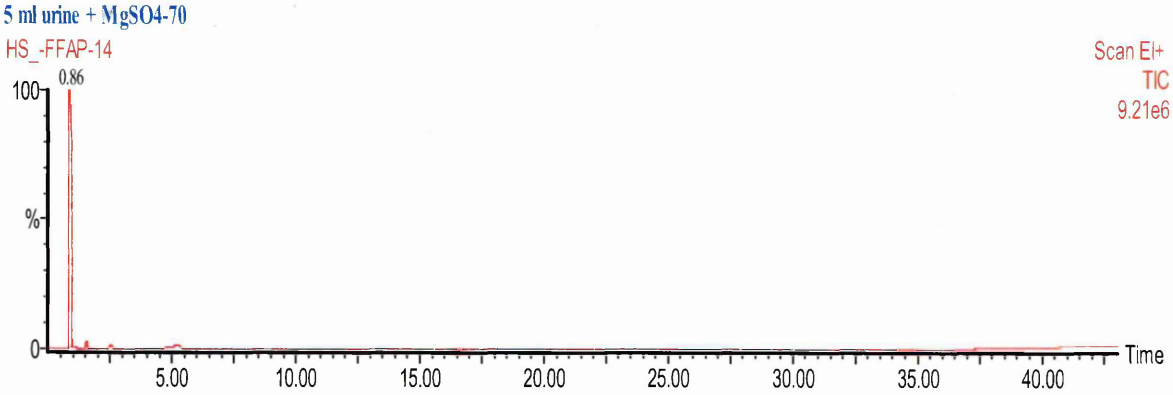


Figure 16. Chromatogram of urine with Mg_2SO_4 analysed with HS. Only one air peak and a water peak can be observed.

When Silica was used, different peaks could be observed (Figure 17).

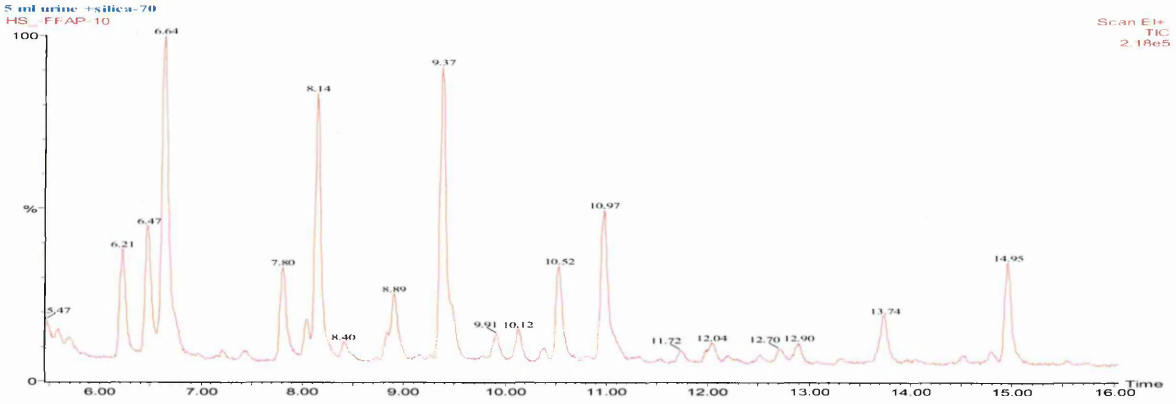


Figure 17 . Chromatogram of urine with Silica analysed with HS. Silica absorbs the water giving a better resolution than only urine.

When plain urine was analysed with the TD technique, different compounds could be observed. In Figure 18 a chromatogram of 10 ml of urine analysed by TD can be seen.

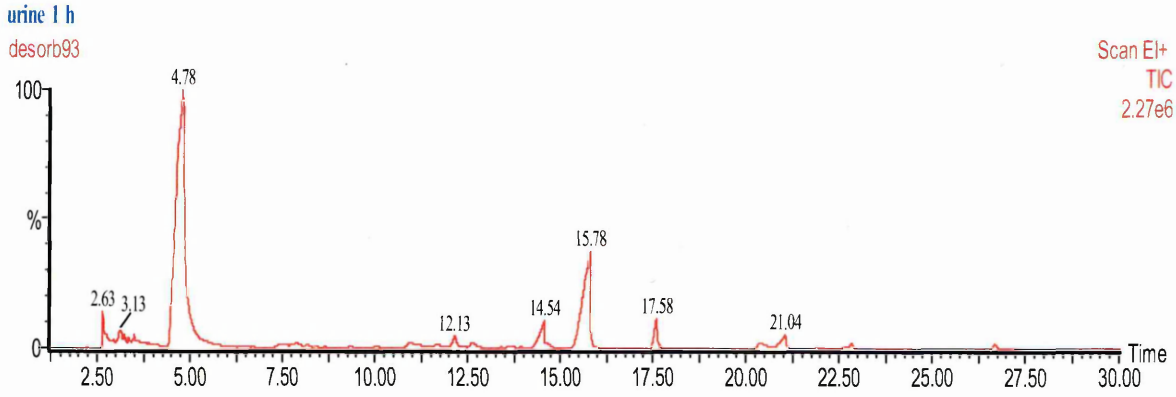


Figure 18. Chromatogram of urine analysed by TD.

When both methods are compared TD is a better technique than HS because more compounds were detected. TD uses a higher volume, but water vapour does not interfere with this technique as much as it does with the HS technique, allowing a better detection.

The fact that a greater volume of urine is needed is a disadvantage, but the major problems encountered were air leaks in the system, water condensation and contamination by volatiles derived from the rubber used to join the desorption tubes to the system. Contamination was minimized by using inert tubing, and water condensation was avoided by heating all the system, but the leaks were very difficult to avoid. This made reproducibility of the results really poor.

When urine was analysed using the SPME fibre, the greatest number of compounds present in urine were detected (see Figure 19) and the best reproducibility was observed. This technique has the disadvantages of using very expensive and delicate fibres, and a time-consuming analysis. However, it is sensitive, and water does not present a problem for volatile analysis. Consumables were disposable and the technique can be automated, making the technique ideal for the purpose.

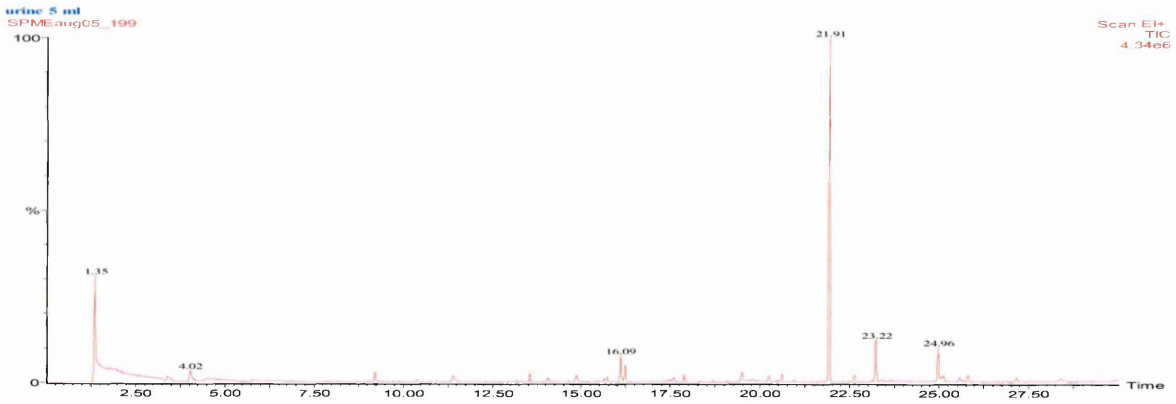


Figure 19 . Chromatogram of urine analysed by SPME analysis.

SCFA detection comparison:

Different concentrations of SCFA were analysed with direct HS, TD and SPME to analyse which technique was best for the detection of this type of metabolites in urine in our

laboratories. SCFA were only chosen because they are known bacterial products found in urine which could assist in the assessment of the efficacy of the different techniques.

Different amounts of SCFA were analysed in urine and in water to see which one gave the best sensitivity and whether there were metabolites in urine that might hamper the detection of these compounds.

Headspace detection of SCFA

A range of concentrations of SCFA in water were analysed by HS (0, 0.075, 0.05, 0.1 ppm) to determine the lowest concentration of SCFA that could be detected with the technique (see Figure 20). The lowest concentration of SCFA used that could be detected in water using the HS technique was 0.02 ppm.

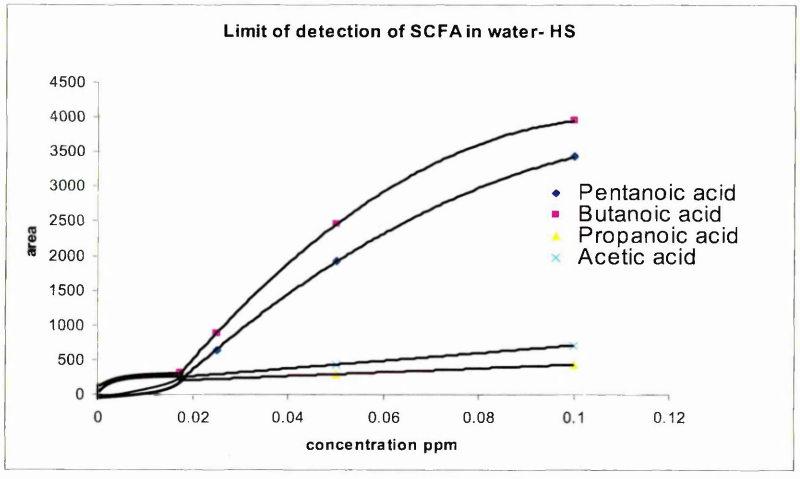


Figure 20. Different concentrations of SCFA in water analysed with headspace analysis.

A variety of experiments were done with HS to investigate the effect of pH, salt and other compounds found in urine in the detection of SCFA.

Urine (2 ml) spiked with 2 ml of SCFA were analysed under a range of acidities. 0, 20, 40, 80 and 160 µl of 0.1 M HCl were added. Increased acidity did not improve the analysis of

propanoic acid and acetic acid, but it did influence the analysis of pentanoic and butanoic acid increasing the detection up to 80µl of 0.1M HCl (see Figure 21). However, increasing the acidity more than 80µl decreased the sensitivity of detection of SCFA in urine.

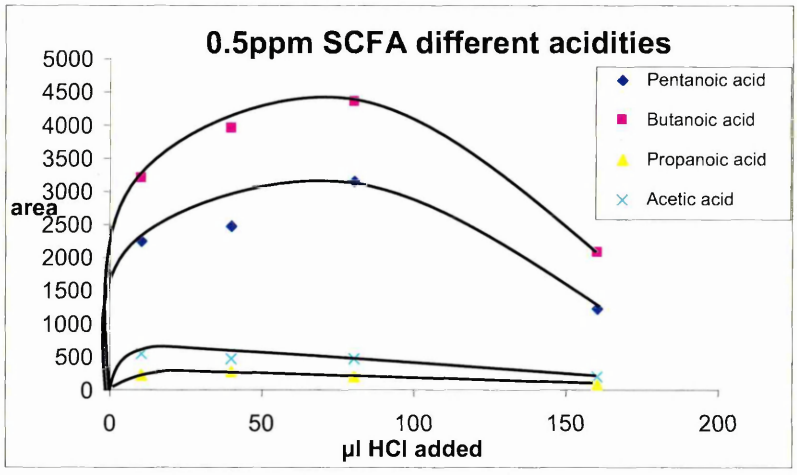


Figure 21. Area of the SCFA peaks found in urine spiked with SCFA at increasing acidities.

When urine was analysed using the HS technique, no peaks other than a water peak were seen. In order to determine whether water is masking the peaks or whether there are compounds that attach to the analytes masking the peaks, different experiments were done.

In order to examine if there is something in urine which is hampering the analysis of SCFA in urine, 4 ml of SCFA solution, each at 1 ppm in water were acidified with 80 µl of 0.1M HCl and increasing volumes of urine were added (see Figure 22).

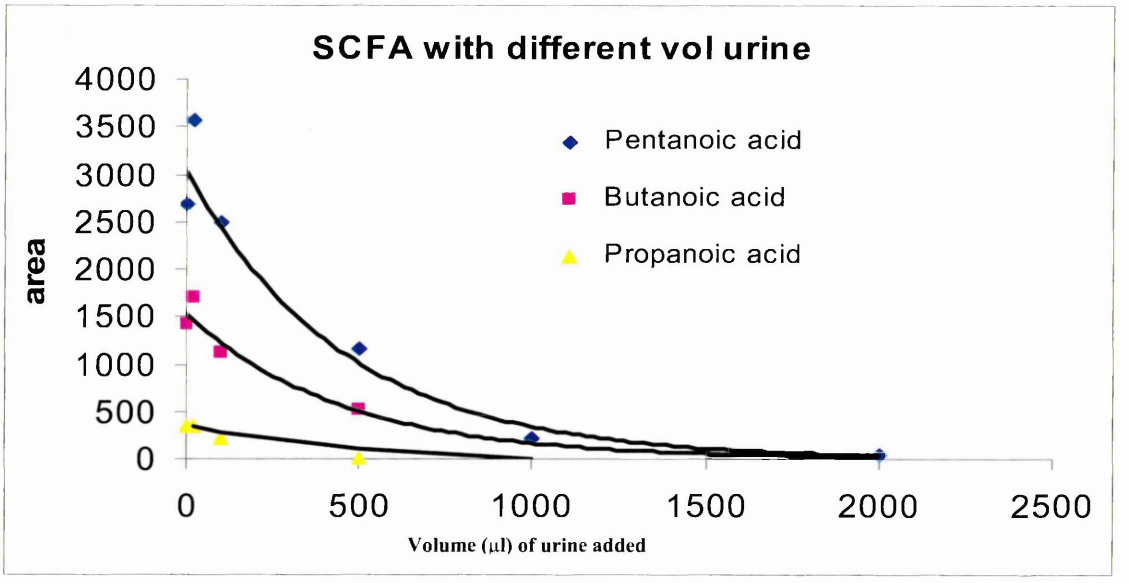


Figure 22. Area of the same volume of SCFA with increasing volumes of urine.

The amount of SCFA detected decreased with increasing volumes of urine added. The decrease of detection of SCFA when urine is added to the medium might be due to compounds in urine hampering their analysis, probably through conjugation. Another possible cause is that increasing volumes of urine added result in the dilution of the SCFAs. In a comparative experiment, to check the effect of sample dilution, a solution of SCFA was diluted this time not with urine but with water, and a decrease in the area of SCFA was once again observed (see Figure 23).

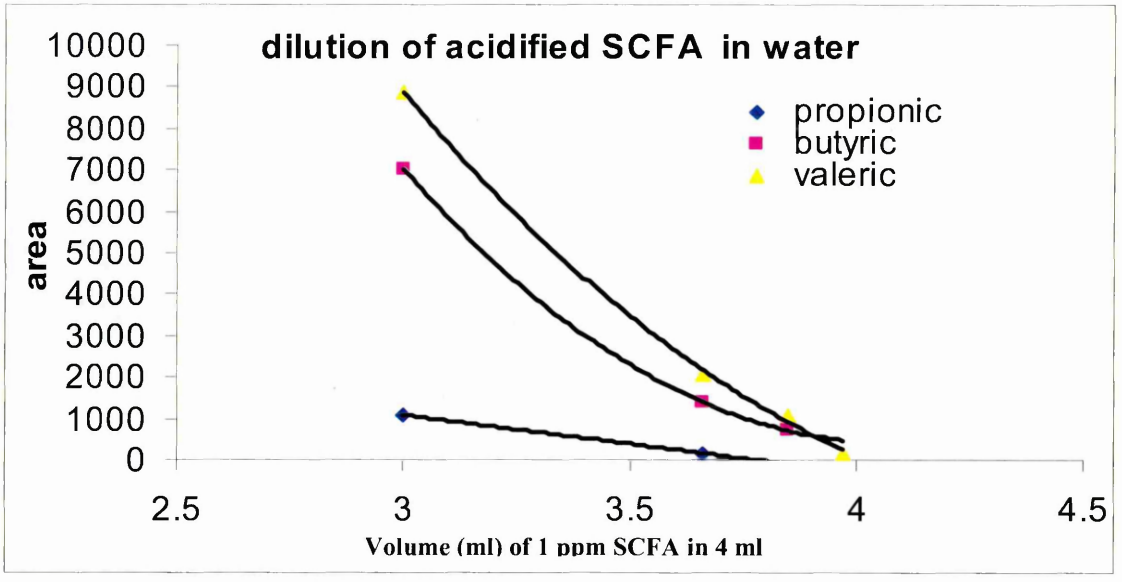


Figure 23. Area of increasing volumes of an acidified SCFA solution diluted in water.

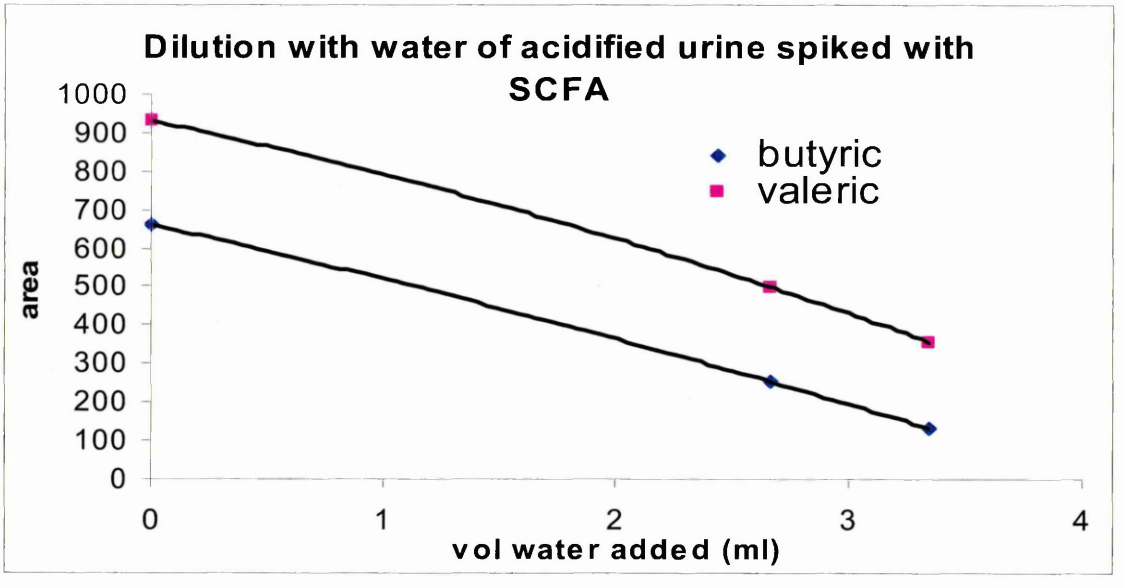


Figure 24. Area of the SCFA found in urine spiked with SCFA when it is diluted with water.

In order to elucidate whether the decrease of the area of SCFA was due to compounds in urine or due to dilution, increasing volumes of urine were added to a solution of SCFA

together with water making up to 2ml. The AUC of the different SCFA analysed can be seen in Figure 25.

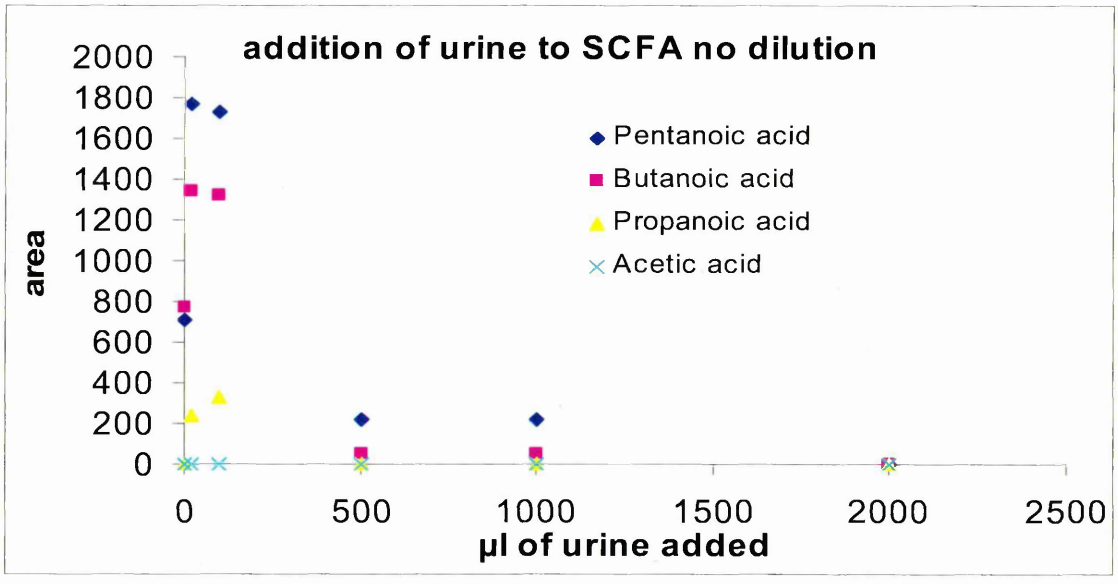


Figure 25. Increasing volumes of urine added to a SCFA solution keeping the final volume constant with water.

When 20 µl of urine are added, there is little effect in comparison with no urine added. However, when more urine is added, SCFA detection decreases. Urine contents probably conjugate with SCFA hampering the analysis, and the decrease of detection of SCFA is not due to the dilution of the sample.

Acidified urine (2 ml) spiked with 1 ml of 1 ppm of SCFA solution was salted out, by adding an amount of salt (0, 0.2, 1, 3 and 6 g were observed) In Figure 26 the various AUCs of the SCFAs can be observed. The AUC increases with increasing amounts of salt, then a point is reached where addition of salt does not improve the detection and after 3 g detection decreases slightly. The addition of 1 to 3 g of salt gave the best results.

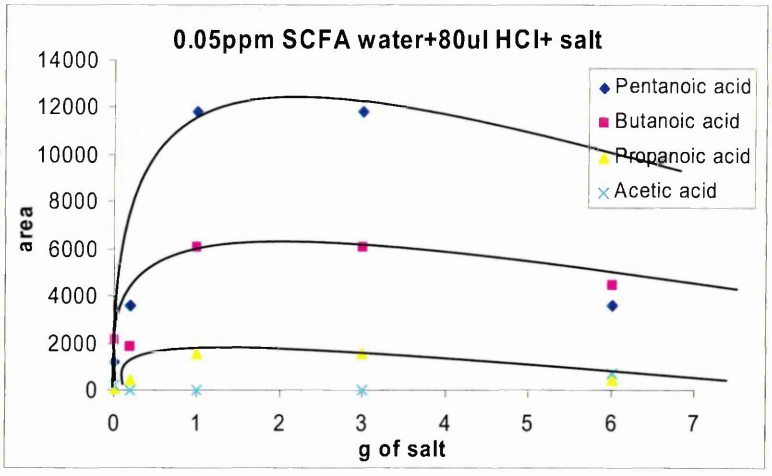


Figure 26. The effect on peak area of varying the amount of salt added to an acidified SCFA solution.

SCFAs detected using TD

Increasing concentrations of SCFA were inserted in the Buchner flask and analysed using TD. In Figure 27 the smallest concentration of SCFA detected with TD can be seen.

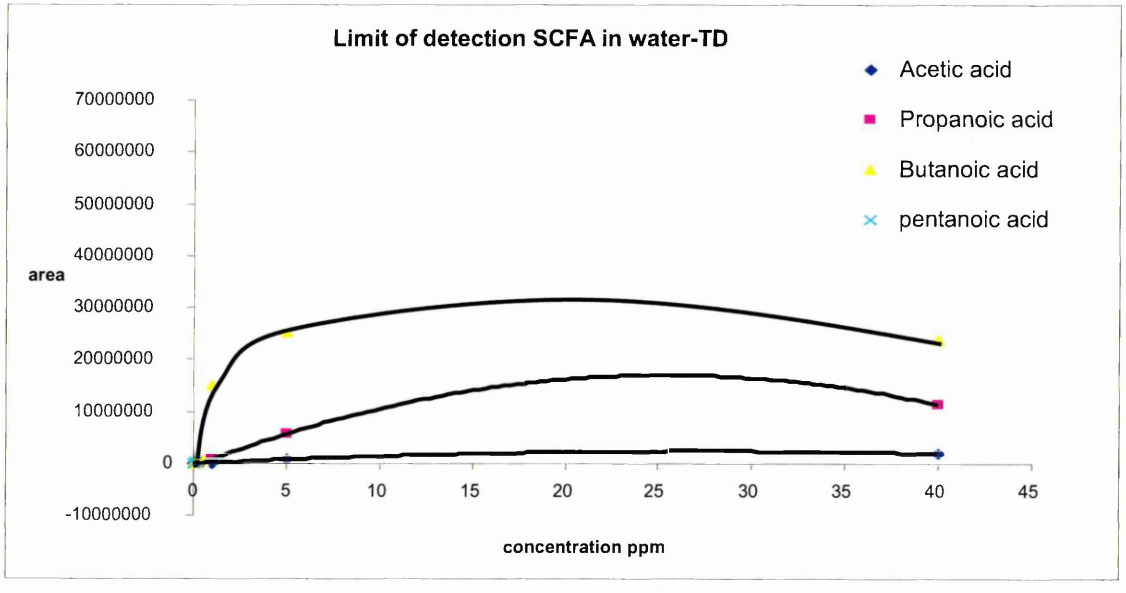


Figure 27. Increasing concentrations of SCFA in water detected using TD.

The smallest concentration of SCFA detected using TD is close to 0.02 ppm of SCFA, which is approximately the same as using HS, but TD has the advantage that when urine is analysed, other compounds present in urine can be seen.

SCFAs detected using SPME

Increasing concentrations of SCFA in water were analysed by SPME to determine the lowest concentration detected using this technique in water. The AUC of the SCFA added in water can be seen in **Figure 28**. The area under the curve of the various concentrations of SCFA added to urine can be seen in **Figure 29**.

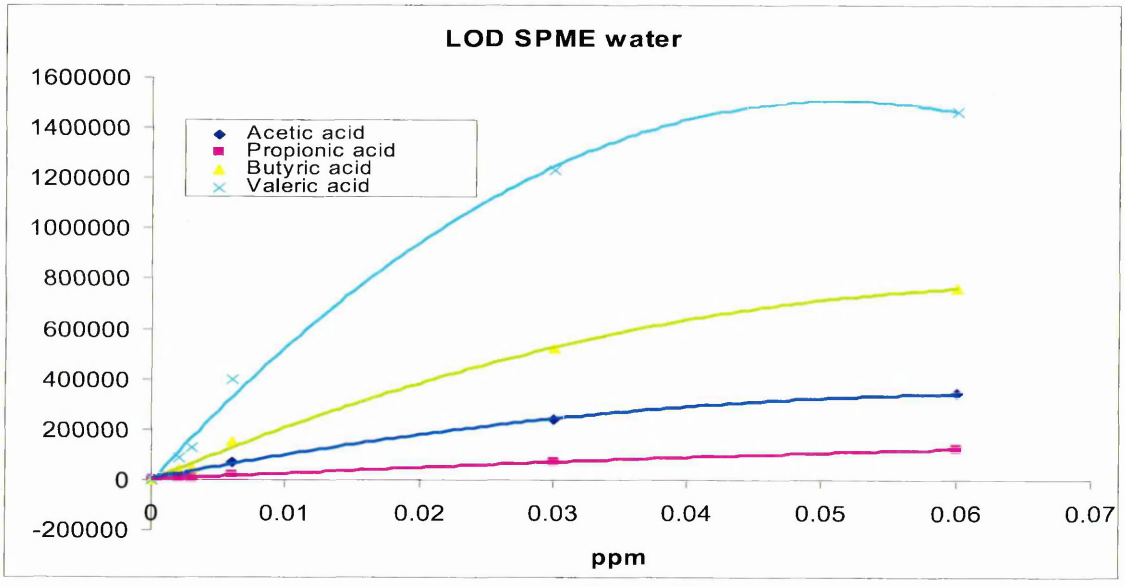


Figure 28. AUC of the SCFAs diluted in water using SPME.

The shape of the AUC of the SCFAs is sigmoideal. The area slowly increases with increasing concentrations of SCFA, but at higher concentrations the AUC get relatively much bigger until it plateaus.

When increasing concentrations of SCFA were analysed in urine a similar pattern was observed.

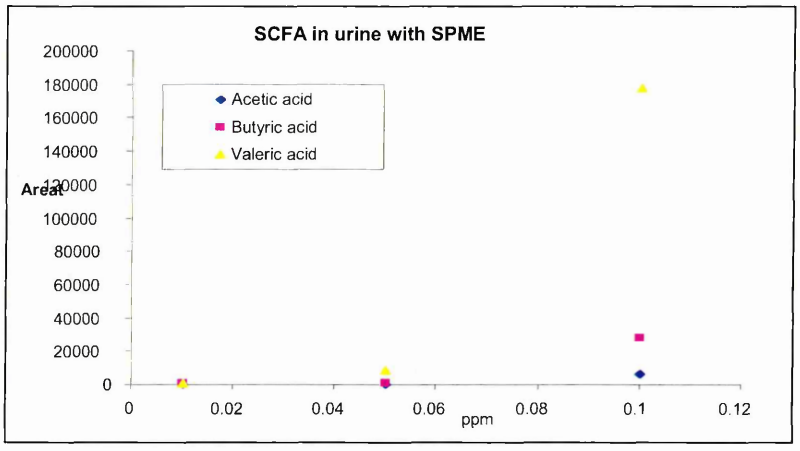


Figure 29. Concentrations of SCFA detected in urine using SPME.

The smallest concentration of SCFAs detected with the SPME technique in water is around 0.0001 ppm, and 0.0075 ppm in urine, whereas using HS and TD is 0.02 ppm.

Urine screening method development:

The best method of those in our laboratories tested was SPME. The lowest concentration detected of SCFA is lower using the SPME technique. At the same time, conditions such as amount of salt added and pH can influence the analysis. Different amounts of salt were added to see the effect on SCFA analysis and the pH of the sample was changed adding different amounts of HCl.

Other conditions such as temperature at which the sample is analysed affect the analysis, however. Mills et al. (Mills & Walker, 2001) assessed this condition and they reached the conclusion that 50°C gives the best compromise between performance and fibre damage. Fibres are very fragile and can be damaged during the course of analysis and conditioning. It is important to note that the use of HCl should be kept to a minimum, as it is also damaging for the fibre.

Salt content

Adding an inorganic salt to a sample (*salting out method*) can improve the resolution of some compounds (Restek corporation, 2003). Different amounts of NaCl were added to the urine and an observation of the best conditions was done.

Five ml of urine were observed with increasing additions of salt: (1) No salt added. (2) 3g of salt added. (3) 3.75 g of salt added. (4) 5 g of salt added. The chromatograms of one of the repeats can be seen in Figure 30.

Experiments were done three times each in a randomised way to detect which one gave the best results. Different organic compounds found in urine were looked at and 3g of NaCl gave the best resolution, since some compounds found in urine appeared when salt was added, and some areas increased (see Figure 30).

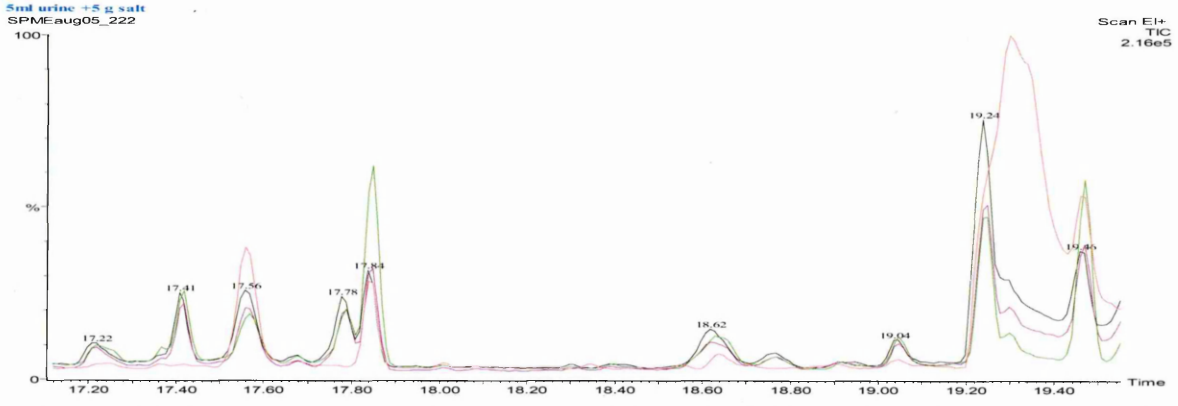


Figure 30 . Chromatograms of urine with different addition of salt. 5 ml of urine with no salt added (in red), 3g of salt (green), 3.75g of salt(purple) and 5 g of salt (black).

Acidification

Urine (5 ml) with 3 g of NaCl were observed with different additions of HCl: (1) No acid added. (2) 15 µl of HCl 37% (3) 25 µl of HCl added. As HCl damages the fibre, the experiment with 25 µl of HCl was done once, and as it did not give better results that the 15 µl addition, it was not repeated. The other two experiments were done three times.

In Figure 31 the chromatogram of one repeat of the urine analysed with ascending volumes of HCl added. The chromatograms show that those where 15 µl of HCl 37% were added gave the biggest peaks due to urine. The pH of this solution was 4.5.

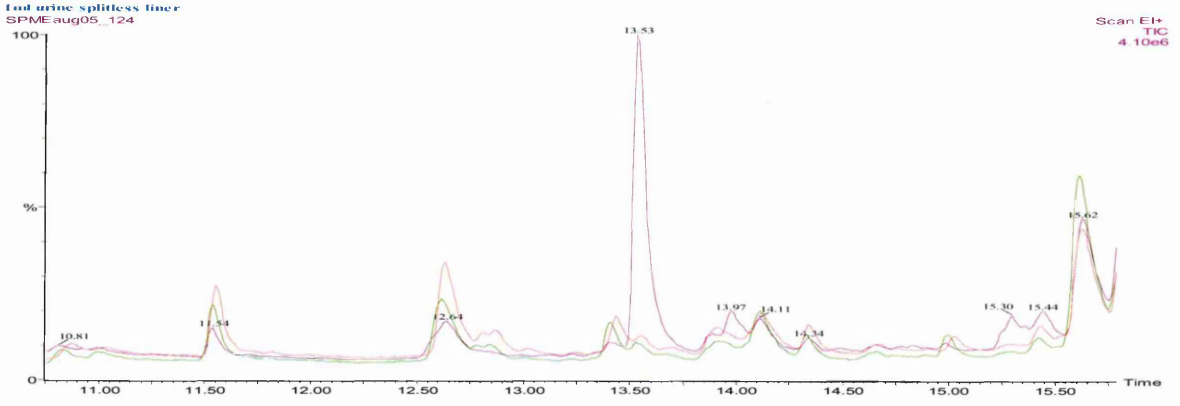


Figure 31. Chromatograms of urine with different addition of acid: 5 ml of urine with no HCl added (in green), 15µl of HCl (red) and 25 µl of HCl (purple).

From these experiments we can conclude that 3 g of NaCl and 15 µl of HCl 37% gave the biggest area of peaks due to organic compounds in urine and should be used in following screening of metabolites in urine.

Internal standard

A quantity of 0.2 µl of 30 µg/ml, giving a total weight of 0.006µg of Toluene D8 was used as an internal standard in urine. This was a good amount to serve as an internal standard, since it was of a similar area to those metabolites to analyse. In Figure 32, the calibration curve of the injected internal standard can be seen.

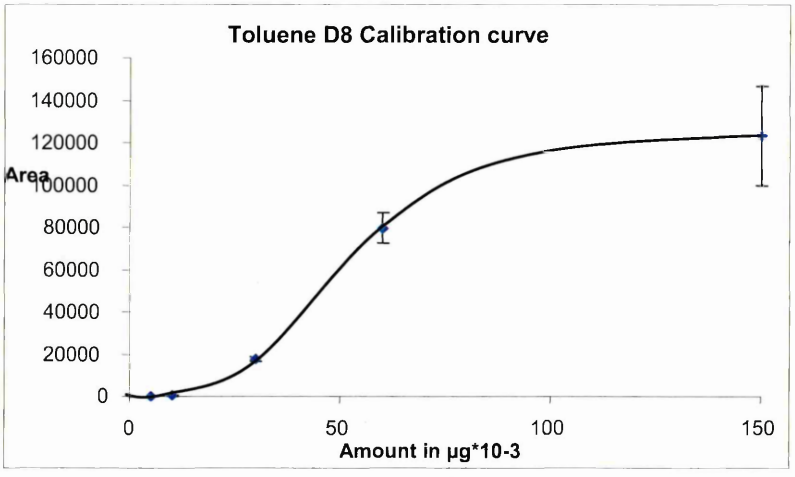


Figure 32. Calibration curve of injected Toluene D8 using SPME.

Summary of the analysis of urine in our laboratories

Instrumentation

A Perkin Elmer Autosystem XL GC and Turbomass MS were used. The column used was a ZB-624 (Cynopropylphenyl-methylpolysiloxane) (60 m, 0.25 mm ID, 1.40 µm ft) (Phenomenex). Helium was used as the carrier gas at a flow rate of 1 ml/min. A narrow bore (1mm) splitless injection liner was used (Perkin Elmer).

The oven is heated at 40°C for 5 minutes, and then the temperature is increased at a rate of 10°C per minute until it reaches 200°C and then left at this temperature for 5 minutes. The injector was at 250°C. The method was similar to Mills and Walker (Mills & Walker, 2001), although it varied due to the different instrumentation.

Sample preparation and SPME procedure:

The sample preparation used was the Mills and Walker (Mills & Walker, 2001) but with some variations. Five ml of urine with 15 µl of HCl 37%, 0.2 µl of 30µg/ml of Toluene-D8 and 3 g of NaCl were inserted in silanised vials. These were mounted on a magnetic stirrer submerged in a water bath maintained at 50°C, and the contents stirred continuously so as

to release volatile compounds into the HS. The septum of the sample vial was pierced with the SPME needle and the SPME fibre exposed to the HS vapour for 30 minutes. The extracted compounds were then desorbed from the fibre in the GC injector port, split valve closed for 15 minutes.

Creatinine

Urine volume excretion into the bladder is highly variable and dependent on a number of uncontrollable variables. This makes quantification of urine solutes almost meaningless without further standardisation. However, the constant rate of creatinine excretion from the body into the urine means that the amounts of solutes in urine do not have to be related to urine volume, rather they can be related to creatinine concentration, which in turn can be standardised according to time period over which the urine is collected. Hence to allow for variation in hydration and urine excretion rates, the areas of the compounds found in urine are related to creatinine concentrations, because the amount of creatinine excreted in 24 hours is constant, whereas the volume is not. Creatinine in house was measured with the Creatinine Test Kit (CinnaGen Inc), based on the Jaffe Method (Cook, 1971) (Bartles et al., 1972) as mentioned in the Diagnostics pack. Creatinine is determined after reaction with picric acid in the presence of alkaline medium. This reaction forms a yellow-red complex dye as indicator which is measured photometrically. The method is linear up to 6 mg/dl according to the manufacturer's specifications. A calibration curve can be seen up to 2 mg/dl in Figure 33. Repeats were done and the same results were given at all times, so the standard error bars are equal to zero. The repeatability of the technique is very good. The measurements were done in a Camspec M350 Double beam UV-Visible Spectrophotometer.

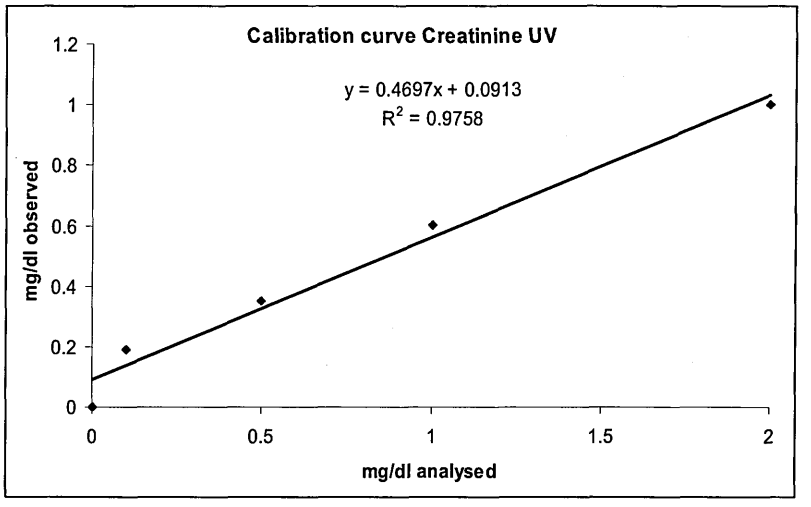


Figure 33. Calibration curve of creatinine using the UV spectrophotometer.

Method analysis used in HFL

Volunteers were asked to provide their first urine samples after rising on the same days that they gave breath samples. Samples were stored in a -80°C freezer until analysed.

These samples were sent to The Horserace Forensic Laboratory (HFL) Newmarket, to be analysed by GCMS. The urine was derivatised in three different ways: hydrolysed acid neutral (HAN), unhydrolysed basic (UB) and hydrolysed basic (HB) before analysis by Simon Hudson.

HAN and HB: 2 ml of water, 3 ml of 1M pH 4.7 acetate buffer containing 115ng/ml D_3-morphine glucuronide, 1.87µg/ml 6-bromo-2-naphthol and 1.333µg/ml D_6-caffeine and 100µl B glucuronidase from *Helix pomatia* were added to 2 ml of urine. The mixture was incubated overnight at 45°C and then extracted with a Varian Nexus after conditioning the cartridge with 1 ml of methanol and 1 ml of water. After loading the sample, the cartridge was washed with 1ml polymeric SPE wash solution (5% methanol in water) and washed with 1ml hexane. Then it was eluted with 1 ml polymeric SPE eluant (30% acetonitrile in methanol) and divided into two aliquots. 600µl was put into a labelled 13x100mm glass

tube (basic fraction) and 200µl into a second labelled 13x100mm glass tube (acid fraction). 5.4 ml 1M pH 9.0 acetate buffer was added to the 600µl aliquot and 1.8ml 1M pH 6.6 acetate buffer to the 200µl aliquot. The sample prepared at pH 9.0 is used for subsequent basic analysis and the sample prepared at pH 6.6 is used for subsequent acidic/neutral analysis.

To prepare the acid sample, a 3ml/150mg Phenomenex Screen C SPE cartridge was conditioned with 1 ml of methanol and 1 ml of water. The 2 ml sample was loaded and then the cartridge was washed with 1ml 10% methanol in pH 9, 0.1 M acetate buffer. The cartridge was washed with 1 ml hexane and then dried with oxygen free nitrogen and then eluted with dried ethyl acetate. 200 µl of sample was evaporated to dryness and reconstituted in 1% MSTFA N-methyl-N-trimethylsilyl-fluoroacetamide (Macherey Nagel)/1% TMAH (trimethyl anilinium hydroxide) (Sigma) and 1% TMSH (trimethyl sulfonium hydroxide) (Macherey Nagel)/ in dichloromethane and 100µl were injected onto a GCMS system using a large volume injection technique.

For analysis a SGE BPX5 column was used. The injector was kept at 35°C for 1.65 min with a 75:1 split and then splitless for 1.5 min. Then it was increased to 320°C at 250°C min^{-1} and it was kept at 320°C for 4 minutes. The column was at 100°C for 2.5 min and then increased to 320°C at a rate of 30°Cmin^{-1} and then kept at that temperature for 5 minutes. The GC was coupled with a MS at 4 scans per second detecting masses of 40 to 550 amu.

To prepare the basic sample a 3ml/150mg Phenomenex Screen CSPE cartridge was conditioned with 1 ml of methanol and 1 ml of water.

The sample was then loaded into the cartridge and then washed with 1 ml of acetic acid 1M followed by 1 ml of methanol followed by diethyl ether. The cartridge was dried and then eluted with 1ml of 10% triethylamine in dried ethyl acetate and evaporated to dryness. The sample was reconstituted with 2%MSTFA/5%BSTFA in dichloromethane and 100µl were injected onto a GCMS system using large volume injection technique.

For analysis a SGE BPX5 column was used. The injector was kept at 35°C for 1.65 min with a 75:1 split and then splitless for 1.5 min. Then it was increased to 320°C at 250°C min^{-1} and it was kept at 320°C for 4 minutes. The column was at 70°C for 3 min and then increased to 320°C at a rate of 25°Cmin^{-1} and then kept at that temperature for 7 minutes. The GC was coupled with a MS at 2 scans per second detecting masses of 40 to 550.

UB: 3 ml of water were added to 2 ml of water and then 0.5 ml of 4M NaOH were added. 1.5 ml diphenylamine (1.5µg/ml) in chloroform were then added and mixed on a rotary mixer for 10 minutes. It was then centrifuged at 3000 rpm for 10 minutes. The organic layer was transferred to a high recovery GC vial and evaporated carefully at room temperature. It was then reconstituted in 30µl 35% MSTFA / MTPAP (4µg/ml) in toluene and then the vial was capped. Then it was injected into a GCMS.

For analysis a SGE BPX5 column was used. The injector was kept at 275°C splitless for a minute. The column was at 80°C for 1 min and then increased to 320°C at a rate of 25°Cmin^{-1} and then kept at that temperature for 7.8 minutes. The GC was coupled with a MS detecting masses of 40 to 500 amu.

Creatinine

Samples sent to HFL for direct injection analysis were related to the area under the curve of creatinine enol N1,N3, O-TMS. This compound was found after the UB analysis in all the urine samples. In order to investigate whether this metabolite is a product of creatinine, some urine samples were spiked with increasing concentrations of creatinine. The AUC of the peak of creatinine enol N1,N3, O-TMS increased with increasing concentrations. This means that it is a product creatinine conjugated through the analysis method. A calibration curve can be seen in Figure 34.

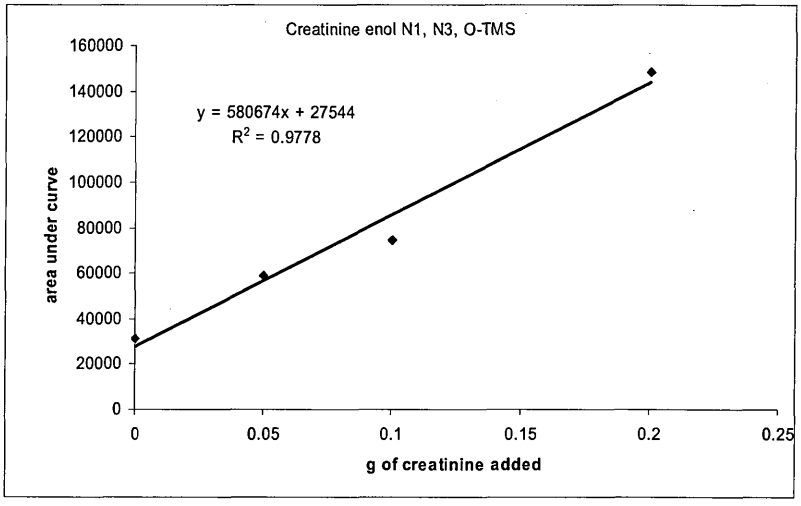


Figure 34. Calibration curve of creatinine (which after conjugation through UB analysis appears in the GCMS as Creatinine enol N1,N3, O-TMS).

Conclusion and discussion

Urine and breath were the media chosen for the study of colonic fermentation in the gut. These media were thought to be the most appropriate for non-invasive measurement of analytes, because of relatively easy collection protocols and sample preparation can be relatively quick. Urine has the advantage over breath of showing an analyte profile over a period of time, whereas breath only shows the metabolites at a particular point in time. However, breath is ready available and simple to collect, which potentially makes it an important tool for medical diagnosis.

A method for the analysis of breath was developed for the study of the VOCs it holds, for application in a number of different medical conditions. Sample collection was not difficult; however, care was needed when injecting the breath sample into the tubes since the rate of injection into the tube is determinant for the adsorption of the VOCs onto the tube. If this technique was to be used as a standard breath collection technique, this process

should be automated and improved so as to diminish error. Also, the volunteers were required to exhale into the sampler in a particular way, which makes this method not applicable to some populations such as children with challenging behaviour.

Background air had to be collected prior to the breath sample, and later substracted from the sample. Background air is normally constant in a ventilated room, but during the sampling, different people were present, making the background air change slightly. Having to collect breath and background air makes the collection and analysis not as simple as a simple exhalation, but overall breath analysis is quick and simple to do.

Urine was the other medium chosen for the purpose. It reflects metabolism over a period of time, making it ideal to study certain conditions. However, some people were reluctant to donate urine for personal reasons, making the recruitment process difficult.

Different methods of urine analysis were observed: HS, TD and SPME. HS did not prove to be a valuable technique with the instrumentation available. Urine analysis through this technique proved a challenge, probably due to the high water content. Only when silica was used, some analytes could be observed, but not enough for the use of the technique as a screening tool.

TD required glassware that is not available in small sizes, which made it require big volumes of urine, not ideal for urine diagnostics. However, there were other problems in relation to this technique: water condensation and air leaks. Urine was heated and stirred to volatilise certain compounds. Water evaporated in this process and condensed before the desorption tube. Heating the all the setup made the water not condense before the tube but inside the tube, giving problems with the VOCs not attaching to the adsorbent and during the analysis. If the tube was also heated, the most volatile compounds were desorbed from the tube. At the same time, very pure nitrogen was needed, which makes the technique more expensive. For these experiments 99.9% purity nitrogen was used, which contains considerable amounts of impurities, therefore including more volatiles in the sample.

The biggest problem of all was the air leaks, since a flexible connection from the flask containing the urine to the tube was needed, and a complete seal was never achieved. At the same time, using connectors like tubing gave rise to contaminants in the analysis that were not accountable for.

SPME is the best technique. Its sensitivity to detect SCFA was much better than the TD technique, it needed small volumes of urine, the setup is disposable and can be automated. This technique allowed us to detect different compounds in urine that are due to fermentation.

The GC-MS technique does not respond linearly to increasing amounts of compound. In some cases this response might look exponential, although in others it looks sigmoidal. From these experiments we can see that response is sigmoidal in general but in some cases the slow response to increasing concentrations is not observed depending on the concentrations observed. In different graphs, whatever it was observed was drawn, but the response is probably sigmoidal.

The methods of analysis used by HFL have the potential to identify a great number of compounds present in urine. However, the compounds are modified for their identification, which the parent compound identification needs to be taken into account. First compounds are liberated from their glucuronic acid or sulphate conjugates through enzyme hydrolysis. Then the compounds are modified to help their chromatographic performance. In the case of HAN, methyl will be added to hydroxyl and amine groups. In the case of UB and HB TMS derivatives will be formed.

Chapter 3. Elemental diet

In order to identify the compounds in urine and breath which are bacterial metabolites in normal circumstances, and to determine whether the enteral feeds decrease bacterial activity, a cross-over trial was done with healthy volunteers of two different enteral feeds. The mechanism of work of these diets was assessed using the methods developed in the previous chaper, looking at the individual compounds and the general spectrum of urine and breath. Number of bowel movements a day in these volunteers as well as change in breath odour were also used as an indication of colonic fermentation activity changes.

Volunteers and methods

Volunteers were recruited from students of either sex of the University of Cranfield at Silsoe. All were to be in normal health, and eating a normal diet. IBS, migraine and pre-menstrual syndrome in particular were excluded by completion of a symptom questionnaire (Appendix 3), as all these conditions are possibly associated with abnormal colonic fermentation. Exclusion criteria were; taking any medication other than the oral contraceptive; having received a course of antibiotics in the previous six weeks; taking bacterial products such as pro- or prebiotics, and pregnancy and lactation.

Diets were supervised by a qualified dietician who confirmed that subjects' normal diets were acceptable, and co-ordinated enteral feeding. Subjects were randomised to receive seven days on either E028 extra or Modulen-IBD, with all other foodstuffs excluded. Water *ad libitum* was the only other item permitted. Nutritional requirements were individually calculated using the Schofield equation (Schofield, 1985). After seven days, subjects returned to their normal diets for 21 days, before commencing the alternative feed for a further seven days.

During enteral feeding, subjects were asked to record how much enteral feed they consumed and to complete symptom score sheets, recording on a daily basis stool frequency, consistency and colour, and any changes in breath odour. Heights and weights

were recorded before the study and after each feeding episode. Breath and urine samples were taken before the diet and at the end of it.

This study was approved by the Cambridge Local Research Ethics Committee (see Appendix 2).

Method

Breath analysis

Volunteers were asked to give 1 litre of breath sample in the morning, preferably after breakfast. First, a sample of the background air was taken into four 250 ml Bio-VOC samplers and then the sample was passed onto a Tenax-Carbotrap adsorbent tube. Afterwards, volunteers were asked to breath into 4 Bio-Voc samplers. The ATD tube end of the Bio-VOC was left open during sampling, allowing the dead space air to pass through the sampler, only leaving the last 120 ml of alveolar, end tidal air in the sampler. Breath was passed onto a Tenax-Carbotrap tube, and deuterated toluene standard was loaded onto the TD tube in the manner described in chapter 2.

The tube was then analysed in an automated thermodesorber Perkin Elmer ATD 400 attached to an Autosystem XL GC-MS.

The tube was thermally desorbed at 300°C for 5 minutes after 2 minute purge. The trap was then heated quickly from 30 to 320°C. The inlet split was set at 100 ml/min, the outlet split at 5 ml/min with 1 ml/min flow rate.

It was then separated in the GC with a temperature of 50°C for 4 minutes and then increasing it at 10°C/min until 220°C and was held for 9 minutes.

Compounds were detected and identified using a Perkin Elmer Turbomass mass-spectrometer. Data was acquired with an electron energy of 70 eV, to allow library identification, and an emission voltage of 50 eV. The mass spectrometer scan range set for

the assay was 33-350 Da, with the lower limit set at 33 to avoid high background interference from helium, oxygen, water and nitrogen. Mass spectrometry data was acquired and integrated using the Turbomass 4.1 software.

Urine analysis

Urine (3 ml) were analysed in house through SPME-GC-MS adding 3 g of NaCl and 15 µl of HCl 37% which showed the best results and in HFL through direct injection GC-MS as explained in Chapter 2.

Data analysis

Data were analysed using the principal component analysis (PCA) functions contained within Matlab (Mathworks, USA), and the individual compounds' abundances were studied.

Principal components analysis (PCA)

PCA is a tool used for simplification, clustering and classification of large data sets, such as a large number of related chromatographic runs (Wold et al., 1987). To enable import into Matlab, the chromatograms for the derivatised urine were transformed into comma separated value (CSV) files using WSearch Pro. Once these files are formed, the 'allgcmsread2' script (written by Jon-Lee Davey) converts them into Matlab files with a suffix of ".m". It is these files that Matlab uses in its operations. PCA was conducted on the data using the "pcagui" function in PLS_Toolbox 2.0 (Eigenvector Research Inc., Manson, USA), which runs as a supplement to Matlab. This Matlab add-on enables reasonably straightforward manipulation of data using a graphical user interface, where scaling and other data preparation and analysis functions may be performed. The variables for the PCA input matrix were intended to be the time coordinates outputted by the TurboMatrix software.

Compound identification and quantification

Quantification was also performed on selected compounds. These were identified from the total ion chromatogram (TIC) using the NIST electronic spectra library and library search routines. For the most significant mass of the ion spectrum of a compound, the selected ion chromatogram (SIC) was obtained from the TIC. The area under the curve of the SIC was determined manually and automatically in order to quantify that particular species. In order to do it automatically, the files were transformed into CDF (common data file) files and they were analysed in Matlab, where all the peaks are deconvoluted and a NIST search was done in all of them. The parameters used for it was a height threshold of 1000, area threshold 1000, with threshold 0.05, minimum resolution, scan window 3, Gaussian Smooth 7, 10% library confidence and 85% library confidence in particular peaks. Then the area under the curve of the compounds was analysed using the function c2s_area (Jon Lee-Davey, Appendix 4) in Matlab.

The areas were then related to the area of the internal standard in the case of breath and internal standard and creatinine in the case of urine.

Creatinine of the urine analysed in house was measured with the Creatinine Test Kit (CinnaGen Inc), based on the Jaffe Method. The measurements were done in a Camspec M350 Double beam UV-Visible Spectrophotometer. The creatinine of the samples sent to HFL was measured as a derivative through the UB analysis: Creatinine enol N1, N3, O-trimethylsilyl (TMS) where the AUC was measured, as explained in Chapter 2.

Statistical analysis

In order to determine statistically whether the probability of a compound's presence before or after the diet was significant a McNemar test was used. When present, in order to see if the compound was present in significantly different quantity, a Wilcoxon Rank Test was used. In order to compare the pH of the urine of the volunteers before and after the diet, a paired t-test was used.

Results

In this trial 12 subjects were recruited, aged 23 – 32, of which eight were female. Two females withdrew before the feeding commenced. During the first feeding period two people withdrew after two days feeding, one (female having E028) because of persisting hunger, and the other (male having Modulen-IBD) because of insomnia caused by an empty stomach. Eight subjects completed the first phase. A further subject (male – having Modulen-IBD) withdrew after four days in the second feeding phase because of malaise and headaches. Thus full data were obtained from seven subjects.

Body weight

The body weight was recorded before and after both diets (Table 4). A student t- test showed a very significant difference between weights of the group as a whole before and after the Modulen diet, ($p<0.01$, $t=3.30$). However, there was no significant change after the Elemental E028 diet ($t=1.77$). Weight loss in this trial is due to a lower consumption of calories than needed, but it is important to note that water retention is decreased as well. Fibre has the property of holding water (McConnell et al., 1974) and short chain fatty acids derived from the breakdown of fibre are associated with water in the colon (Williams & Olmsted, 1936) (Bosaeus, 2004). Wintz attributes it to clearance of bulk from the GI tract (Wintz et al., 1970).

Table 4. Records of the subjects' body weights before and after both diets.

Subject number	Weight beginning week of 1st feeding period	Weight end week of 1st feeding period	Weight beginning week of 2nd feeding period	Weight end of 2nd week of feeding
1	88.5kg Modulen	85.5kg	85.5kg E028	86kg
3	71kg E028	70.5kg	70kg Modulen	69kg
4	80kg E028	80kg	79.6kg Modulen	77.5kg

5	64kg Modulen	64kg	64kg E028	64kg
6	60kg E028	59kg	59kg Modulen	58kg
7	83kg Modulen	79kg	80kg E028	80kg
8	83kg E028	80kg	/	/
9	86kg Modulen	85kg	86kg E028	84kg

There was no significant difference between the calories consumed by the group on either diet. ($t=-0.658$, $p<0.05$). Table 5 clearly demonstrates, however, that none of the volunteers achieved the necessary energy intake on either feed, despite being asked to take slightly more than their calculated requirements to prevent weight loss. The volunteers had fewer kilocalories when they had Modulen than when they were on E028, although it was not statistically significant.

Table 5. Energy required in kilocalories, and the energy consumed in both diets throughout the week.

Volunteer	Kcals required with Modulen/week	Kcals taken with Modulen/week	Volunteer	Kcals required with E028 /week	Kcals taken with E028 /week
1	20020	13250	1	20020	16770
3	17094	12000	3	17094	15480
4	16352	-	4	16352	12255
5	14196	9625	5	14196	10320
6	13181	10875	6	13181	10322
7	16877	14250	7	16877	12685
8	-	-	8	17906	13265
9	17822	11750	9	17822	12255

Stool frequency, consistency and colour.

Volunteers were asked to assess their breath odour, as well as consistency, frequency and stool colour according to Figure 35 in order to assess bacterial activity change. Changes in breath odour are shown in Table 6. Bacteria normally break down some compounds in the GI tract. When the bacterial activity is disrupted, these compounds are no longer metabolised, changing the compounds in breath, therefore changing breath odour.

Table 6. Breath odour during both diets. A score of 1 – 4 indicates increasing odour strength (1 being the weakest and 4 being the strongest), but "?" means that the subject was unsure of whether odour had changed.

Day of feeding E028	Volunteer 1	Volunteer 4	Volunteer 5	Volunteer 6	Volunteer 7	Volunteer 8	Volunteer 9
1	2	?	?	2	3	1	2
2	4	3	3	3	4	1	3
3	4	3	3	3	4	?	4
4	4	3	2	3	4	?	4
5	4	3	2	3	4	?	4
6	4	4	2	3	4	?	4
7	4	4	2	3	4	?	4

Day of feeding Modulen	Volunteer 1	Volunteer 4	Volunteer 5	Volunteer 6	Volunteer 7	Volunteer 8	Volunteer 9
1	1	2	1	1	1	-	1
2	4	2	1	2	1	-	3
3	3	2	2	2	3	-	3
4	3	3	2	2	3	-	3
5	3	4	2	2	3	-	4
6	3	4	1	3	3	-	4
7	3	4	1	3	3	-	4

In seven out of eight subjects there was a consistent change in stool colour from brown towards green, on E028 extra (see Table 7). A Spearman test showed a significant difference ($p<0.05$, $R=0.639$) from before and after E028, and between before and after a week of Modulen ($p<0.05$, $R=0.598$), although less so. No significant change was seen in stool frequency or consistency on either diet.

The change in stool colour is very important to assess bacterial activity in the gut, as bacteria normally metabolise biliverdin. However, when bacterial activity is disrupted, biliverdin is not metabolised, giving faeces a greenish colour.

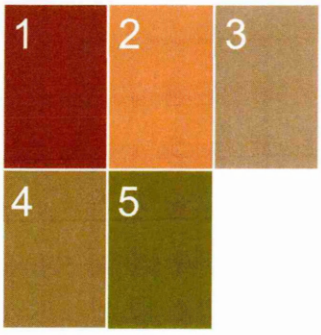


Figure 35. Reference colour of the stools. The more green the colour, the indication is that less biliverdin has been metabolised, therefore the less bacterial activity has occurred. Patients were asked to compare stool colour with this chart.

Table 7. Stool colour on enteral feeds (scale 1-4, "?" means not sure).

Days of feeding E028/ Volunteer number	1	3	4	5	6	7	8	9
1	1	1	1	1	1	1	1	1
2	2	?	2	?	?	3	3	1
3	?	2	1	?	?	3	1	3
4	?	?	1	1	5	5	3	?
5	1	5	1	?	?	5	3	3
6	1	5	1	?	5	5	3	3
7	3	5	5	?	5	5	5	3
Days of feeding Modulen/ Volunteer number	**1**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
1	1	1	2	?	1	1	-	1
2	2	?	2	1	1	1	-	1
3	?	5	?	?	1	5	-	3
4	4	5	?	?	5	5	-	?
5	?	5	2	?	5	5	-	?
6	?	5	?	3	?	5	-	3
7	?	5	5	1	?	2	-	3

Breath odour

Changes in breath odour are shown in Table 6. Six out of seven showed deterioration in odour on E028 extra, and 5 out of 6 on Modulen-IBD. One volunteer did not record the breath odour changes on a daily basis.

With E028, there were 3 volunteers who were not sure of the change in odour. A Spearman test showed a significant difference between the breath of the volunteers before they started the E028 diet and the last day of the diet ($p<0.05$, $R=0.257521$).

With Modulen, there was also a significant change ($p<0.05$, $R=0.573948$) between before and after the diet.

Breath

The breath of the volunteers was analysed and chromatograms were analysed separately, identifying compounds that changed within a pattern after the restrictive diets and as a group, through PCA. PCA enables to see any clustering existing among the samples.

PCA

PCA was performed on the breath chromatograms (see Figure 36) and even though PC 1 versus PC2 did not show any differences between before and after the diets, differentiation could be seen in PC2 versus PC3. The chromatograms before the first diet are clearly separated from those after having had either Modulen or the Elemental E028. At the same time, there is a difference between the chromatograms before they started the first diet and when they started the second diet.

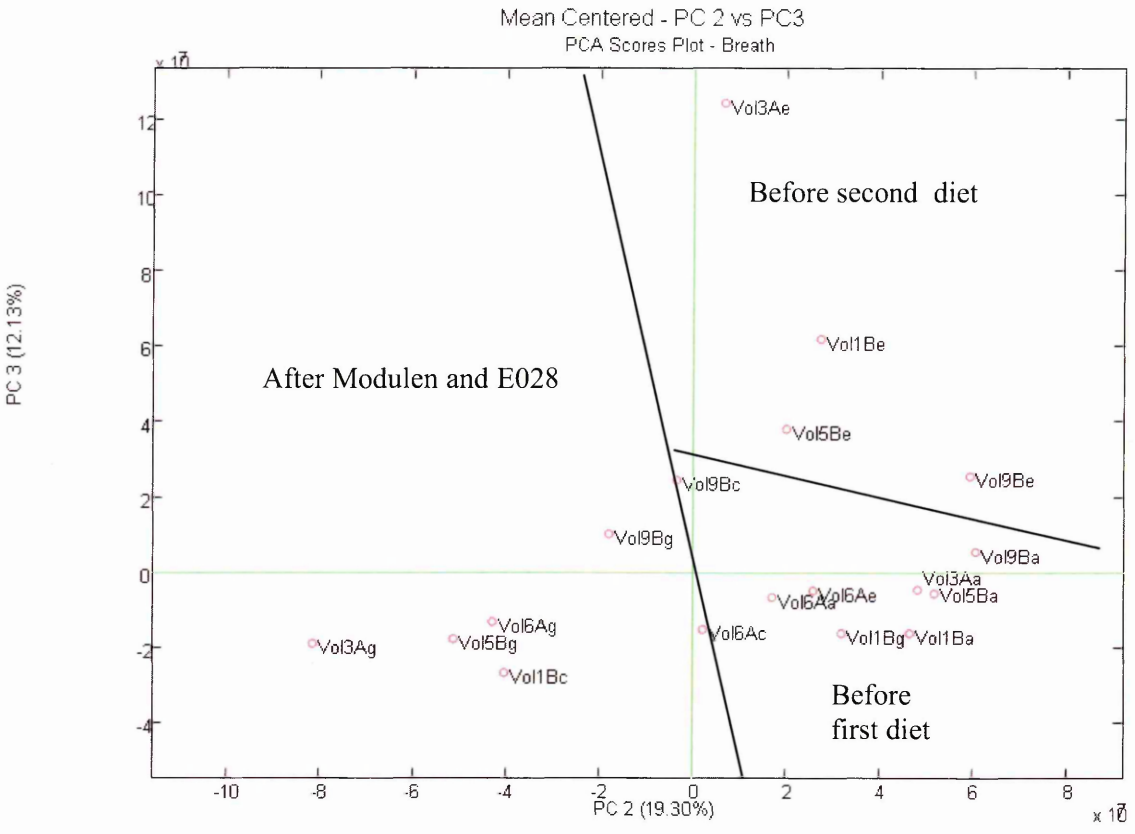


Figure 36. Plot showing PCA of the breath samples taken before and after the diet. The numbers signify the volunteer number allocated, A are the volunteers who started with the Elemental E028 and B the ones who started with Modulen. a) before the first diet, c) after the first diet, e) before the second diet and f) after the second diet.

Compounds observed to change in breath

Over 140 compounds were seen in the breath chromatograms including aldehydes, ketones, saturated and non-saturated hydrocarbons, organic acids, alkenes, alcohols and furans. However, one third were probably of environmental origin. In Table 8 a list of the compounds that were observed that increased or decreased after the diets can be seen. Those that might be of interest in bacterial fermentation can be seen in bold. Table 8 shows the relationship of all the compounds detected, explaining whether there was a difference between the initiation of both diets or not, and if that was statistically significant or not. An

analysis of the effect of those compounds after both diets (E028 and Modulen) was done, also analysing if the change was statistically significant. According to those changes, a judgment on the possible relationship with bacterial fermentation was done.

Table 8. List of compounds and significance of change in breath before both diets started, after Modulen, after E028 and differences between both (x=no, √= yes, *= p<0.05, **= p<0.01, N/A= not applicable, ↑= increase, ↓= decrease).

Compound	Difference before diets		Modulen		E028		Modulen and E028		Bacterial fermentation
	Graph	Statistics	Graph	Statistics	Graph	Statistics	Graph	Statistics	
Pentane	√	x	↓	*	↓	x	x	x	√
Acetone	x	x	↓	**	↓	x	x	x	x
Benzaldehyde	√	*	↑	x	↑	x	x	x	x
2,6-diisopropynaphtalene	x	x	↓	x	↓	x	x	x	√
Benzene, (1-propyloctyl)	√	*	↑	x	↑	x	x	x	√
Phenylmaleic anhydride	x	x	↑	x	↑	x	x	x	x
Hexane	x	x	↑	x	↑	x	x	x	x
Methyl vinyl ketone	x	x	↑	**	↑	x	√	* **(Modulen)**	√
Indole	x	x	↑	*	↑	**	x	x	√
Phenol	x	x	↑	**	↑	*	√	**(E028)	√
2-Butoxyethyl acetate	x	x	↑	x	↑	x	x	x	x
Benzene	x	x	↑	x	↑	x	x	x	√
Cyclohexane	x	x	↑	x	↓	x	x	x	√
Decanal	√	x	↑	x	↑	x	x	x	√
Butyrolactone	x	N/A	↑	x	↑	x	x	x	√
Cyclotetrasiloxane octamethyl	x	N/A	↑	x	↑	x	√	N/A	√
Tetrachloroethylene	√	N/A	↑	x	↓	x	x	x	x
1,4-dioxane	x	N/A	↑	x	↓	x	x	x	x
Ethanol, 2-phenoxy 2-butoxyethyl acetate	x	N/A	↑	x	↑	x	x	N/A	x
Heptane	x	x	↓	x	↓	x	x	N/A	x
Dimethylsulfide	x	N/A	↑	N/A	↑	N/A	√	x	√
Hexanoic acid	x	x	↑	N/A	x	N/A	√	N/A	x
Nonanal	x	N/A	↑	N/A	↑	N/A	√	N/A	√
Ethanol, 2-phenoxy	x	N/A	↑	N/A	↑	N/A	√	N/A	√

With the Modulen diet, methyl vinyl ketone and phenol increased very significantly (see Figure 37 and Figure 38), indole increased significantly (see Figure 39), pentane decreased significantly and acetone decreased very significantly, (Figure 40 and Figure 41). Indole´s alveolar gradient increased very significantly after both diets. Indole´s alveolar gradient changed greatly especially after the E028 elemental diet.

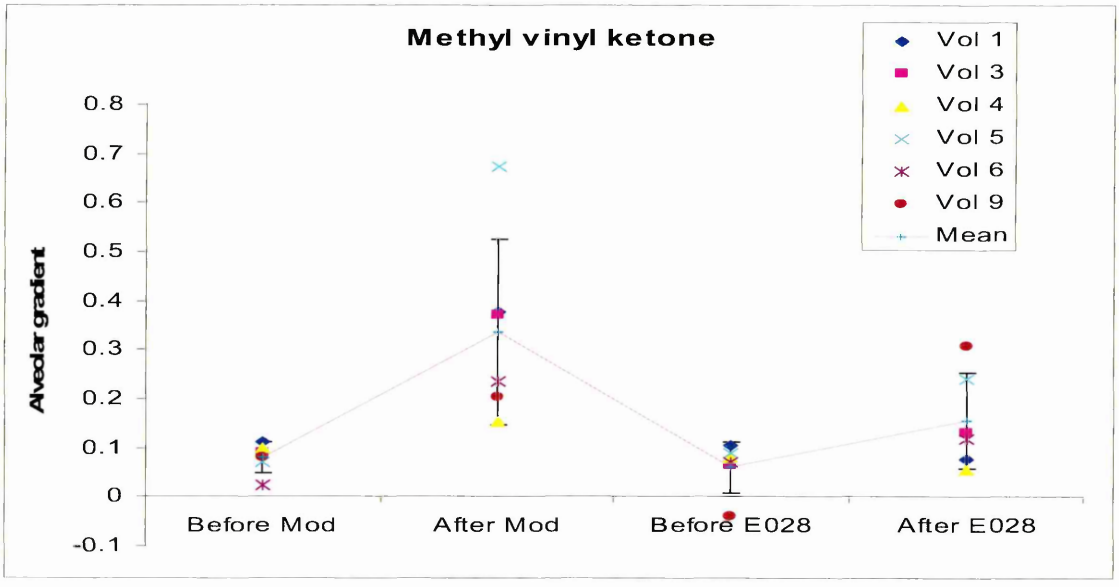


Figure 37. Alveolar gradient of Methyl vinyl ketone in the two diets (vol= volunteer)

Methyl vinyl ketone's alveolar gradient increased very significantly after both diets, being greater after the Modulen diet. The background levels show that the alveolar gradient was very similar in all the individuals before both diets.

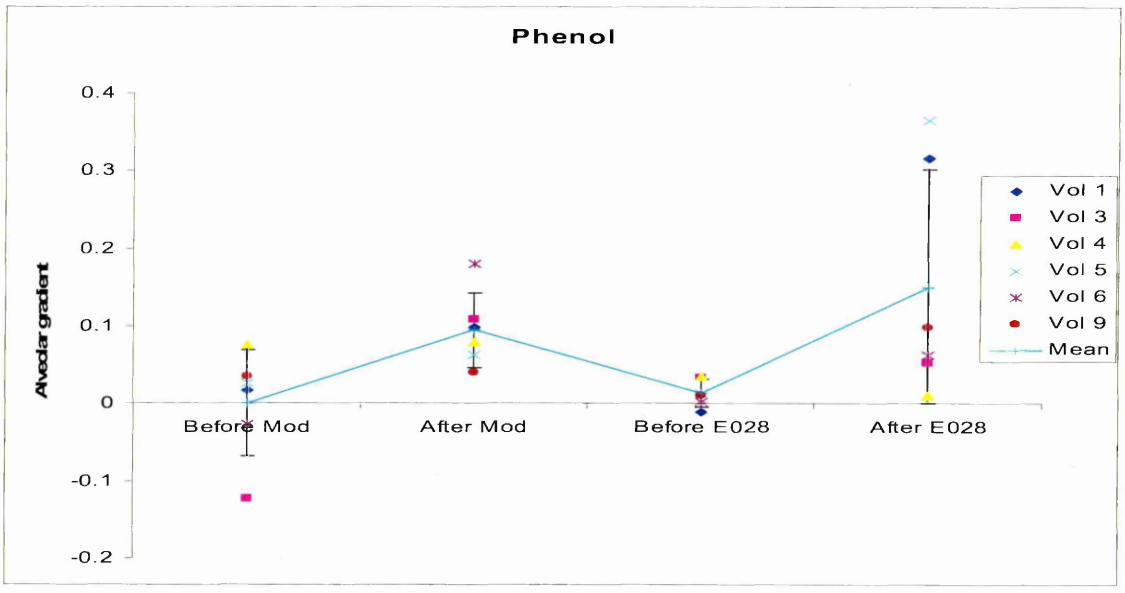


Figure 38. Alveolar gradient of phenol in the two contrasted diets (vol= volunteer).

Phenol's alveolar gradient increased very significantly after both diets.

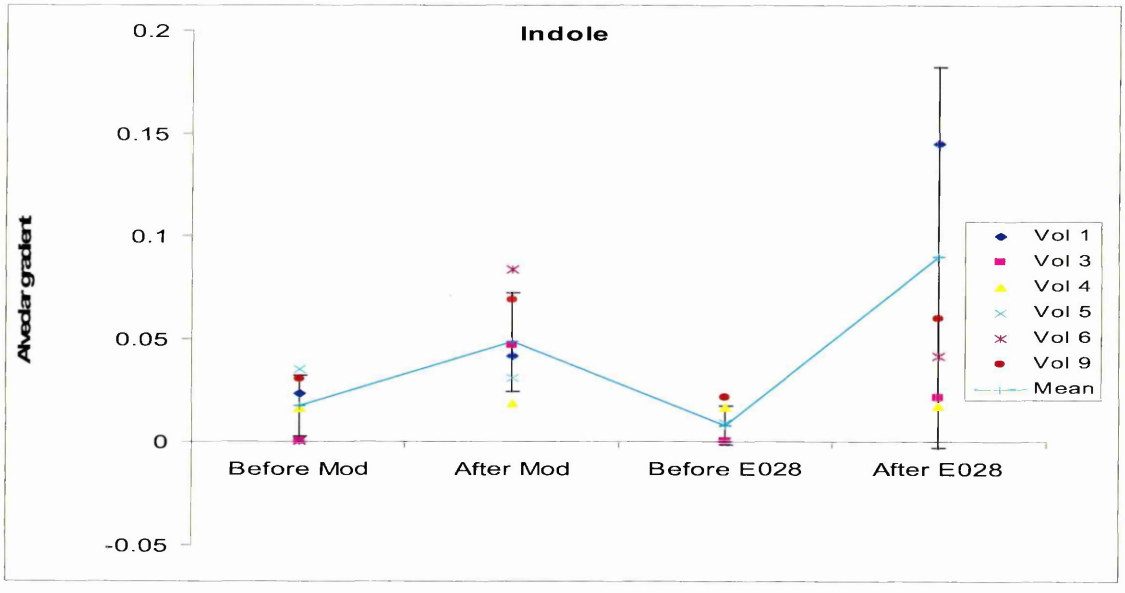


Figure 39. Alveolar gradient of indole in the two contrasted diets (vol= volunteer).

Indole's alveolar gradient increased very significantly after both diets. Indole's alveolar gradient changed greatly especially after the E028 Elemental diet.

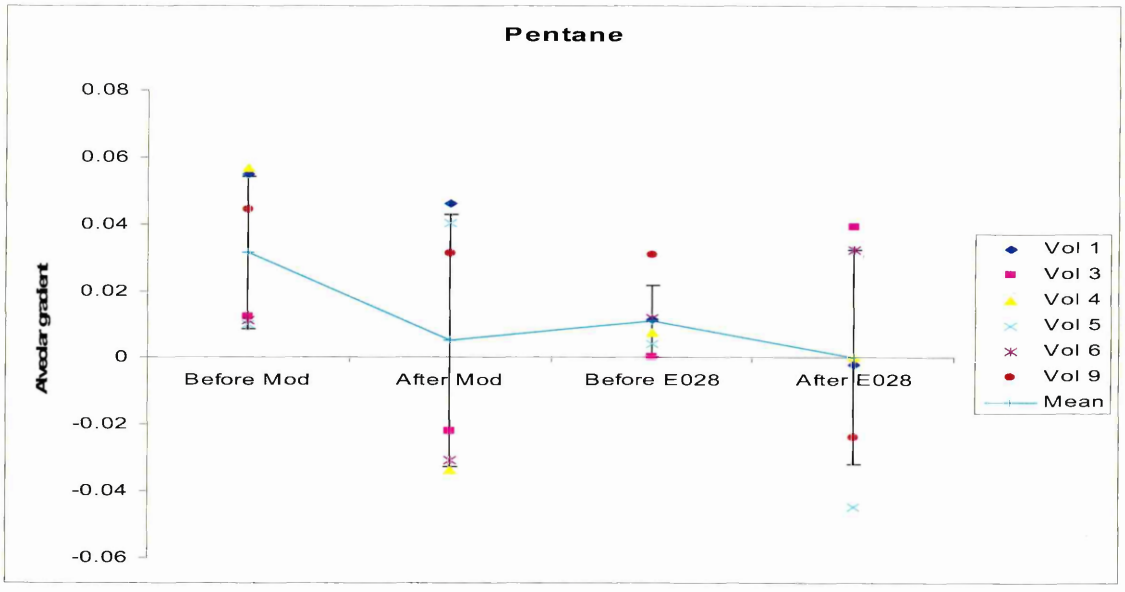


Figure 40. Alveolar gradient of pentane in the two contrasted diets (vol= volunteer).

Pentane is a known metabolite in breath produced by enteral bacteria. Pentane's alveolar gradient decreased significantly after both diets.

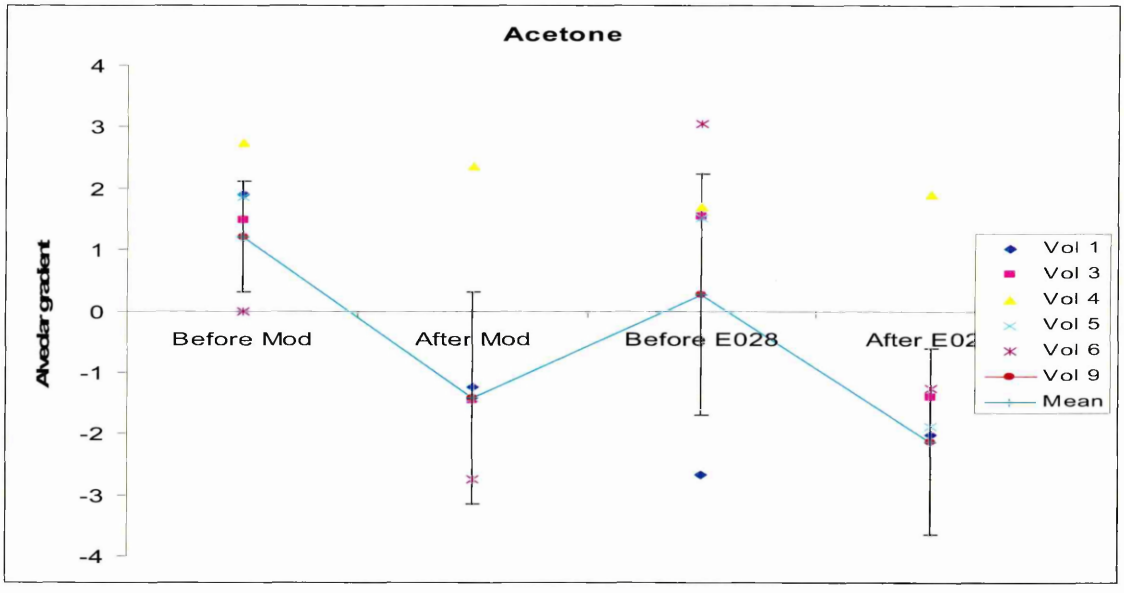


Figure 41. Alveolar gradient of acetone in the two contrasted diets (vol= volunteer).

Acetone's alveolar gradient decreased after both diets, changing from being positive to negative.

With the E028 diet, indole increased very significantly and phenol increased significantly (see Figure 38 and Figure 39), where both compounds can be seen changing throughout the trial.

The increase in methyl vinyl ketone after Modulen was significantly greater ($p>0.025$) than that seen after E028. Likewise, the increase in phenol after E028 was considerably more significant ($p>0.05$) than that seen in Modulen ($p>0.025$). No other significant differences were seen in the concentration in breath VOCs after feeding with the two enteral diets.

Urine samples analysis

PCA done in the chromatograms of the urine samples

PCA was done with the chromatograms of the analysis of urine samples and some separation could be seen with the HAN and with HB analyses (Figure 42 and Figure 43). Figure 43 shows on the bottom left a clustering of all the volunteers before both diets. The urine after the Modulen changed and a new clustering can be seen to the right of this. At the same time, the urine of the volunteers after the E028 diet changed and clustering can be seen at top right, but this group was more dispersed (showed more variation) than either the case for Modulen or the clustering before either diet. PC 1 accounts for 90% of the variability between before they started the diets and after them. However, in contrast to the PCA of the breath analysis, there is no clustering indicating any difference between the analyses at the test points before the start of the first diet and before the start of the second.

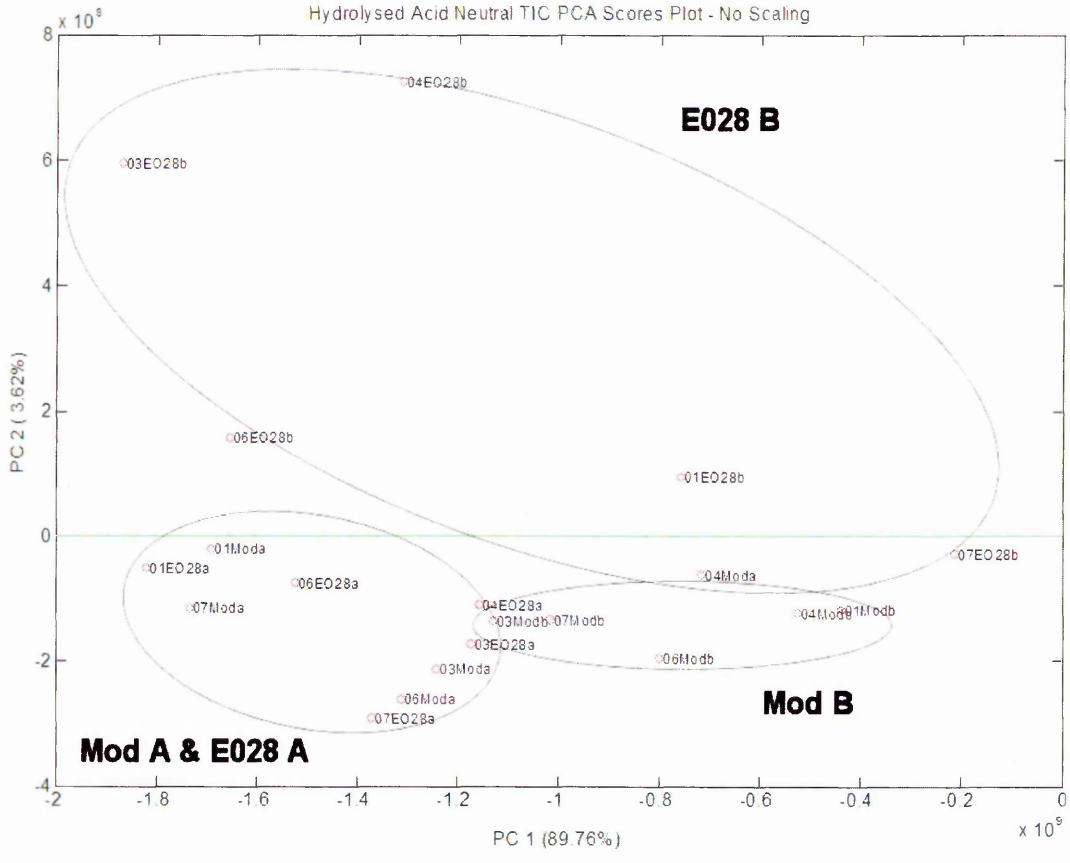


Figure 42. PCA of the chromatograms of the HAN analysis of urine. The number indicates the volunteer number, Mod=Modulen and E028= Elemental E028 diet. a= before the diet, b= after the diet.

Figure 42 and Figure 43 show that urine on a normal diet is different to the urine after having the enteral feeds. In Figure 43 a similar pattern can be seen when urine was analysed using the HB technique. There is clustering of the chromatograms of the urine of all volunteers before both diets and close clustering of those from after the Modulen diet. However, clustering of those chromatograms of urine after E028 is much looser. In this PCA plot PC1 accounts for 87% of the variability between the beginning of the trial and after a week on the diets.

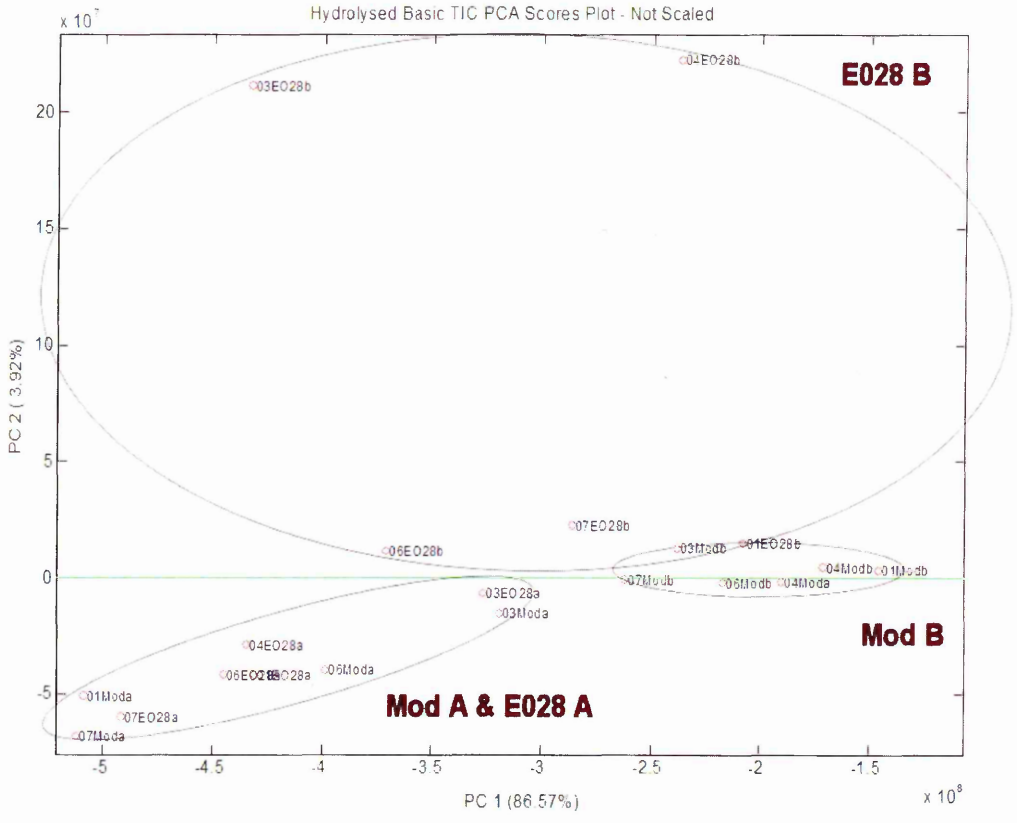


Figure 43. PCA of HB analysis of urine. The number indicates the volunteer number, Mod=Modulen and E028= Elemental E028 diet. a= before the diet, b= after the diet.

Compounds

The individual compounds found in the urine of different volunteers were sought. The AUC of interest in the chromatograms were then made relative to the areas under the curve of the internal standard used and creatinine.

We need to take into account that the urine has been processed for analysis for direct injection into the GCMS, therefore the compounds observed are not likely to be in that form in plain urine. Compounds are conjugated in different forms according to the different analysis (HAN, HB or UB). This should be subject of further work. However, compounds found in the urine analysed through SPME are detected in the form they are present in the urine, without being conjugated or derivatised.

In Table 9, a list of the compounds found in urine of the volunteers before and after the Modulen and the Elemental E028 diets observed through HAN analysis, how they change and likelihood of its involvement in bacterial fermentation can be seen. For instance acetic acid diphenyl ethyl ester was present in similar levels before both diets, although no statistical analysis could be done because in some of the volunteers it was not present and the sample was too small. The amount of this compound in urine decreased after the diet. A statistical analysis on significance of the probability of a compound being present or not after both diets could not be done because of the small sample. At the same time, a statistical analysis on the significance of this compound being decreased after the diets on those volunteers where the compound is present could not be done because of the small sample. However, the compound decreased after both diets, so it is thought to be a bacterial metabolite.

In Table 10 a list of compounds found in the HB and UB urine and their changes with the diets as well as their likelihood to be related to bacterial fermentation can be seen and in Table 11 a list of compounds found in the urine analysed with the SPME technique and their changes with the diets as well as their likelihood to be related to bacterial fermentation can be seen. Those compounds that might be related to bacterial fermentation can be seen highlighted.

Table 9. List of the compounds found in urine of the volunteers before and after the Modulen and the Elemental E028diets observed through HAN analysis, how they change and likelihood of its involvement in bacterial fermentation (x= no change, √= change, ↑= increase, ↓= decrease, - =data not available).

Compound	Difference before diets	Statistics	Modulen	Present/not present Modulen	If present Modulen	E028	Present/not E028	If present E028	Bacterial fermentation?
1,1,3,3-Tetramethyl-1,3-disilaphenalane	√	-	↓	-	-	↓	-	-	√
15-hydroxydehydroabietic acid, methyl ester	√	-	↓	-		↓	x	x	√
1H-Benx(e)inden-3-ol, 2,3,3a,4,5,9b-hexahydro-3a-methyl-, acetate, (3S-(3a, 3aa, 9ba))	x	-	-	-	-	↑	-	-	x
1H, Inden-5-ol,2,3,5,6,7,7a-hexahydro-7a-methyl-1-((tetrahydro-2H-pyran-2yl)oxy)-, (1a,5a,7aa)	x	-	↓	-	-	-	-	-	x
2',4,4',6'-Tetramethoxychalcone	x	x	↓	-	-	↓	-	-	√
2-(1-Pentamethylphenyl)ethyl-3,3-diphenyloxaziridine	√	-	↓	-	-	↓	-	-	√
2(5H)-Furanone,4,5-diphenyl	√	-	↑	-	-	↓	-	-	x
2-(4-Methoxyphenyl)-2(3-methyl-4-methoxyphenyl)propane	√	-	↓	-	-	↓	-	-	√
2-Acetoxy-3-methoxybiphenylene	x	-	↓	-	-	↓	-	-	√
2-hydroperfluoroisobutanoic acid-N,N-di(2-propynyl)amide	x	-	↓	-	-	↓	-	-	√
2-Propenoic acid, 3-(3,4-dimethoxyphenyl)-, methyl ester	x	x	↓	x	-	↓	x	-	√
2H-1-Benzopyran-2-one,7-methoxy-	x	-	↑	x	-	↓	x	-	x
3,5-Dimethyl-1-dimethyldodecylsilyloxybenzene	x	-	↑	x	-	↓	x	-	x
3-carboxy-4-methyl-5-propyl-2-furapropionic acid, dimethyl ester	x	x	↓	-	x	↓	-	x	√
5-Hydroxy-4',7-dimethoxyflavanone	x	-	↓	-	-	↓	-	-	√
3,6-dimethoxy-4-phenanthrol	√	-	↑	-	-	↓	-	-	x
Acetic acid diphenyl ethyl ester	x	-	↓	-	-	↓	-	-	√
Benzenepropanol, 4-methoxy-'a-methyl-	x	-	-	-	-	↑	-	-	x

Benzenamine, 4,4-methylenebis(N,N-dimethyl)	x	x	↓	-	x	↓	x	x	√
Benzoic acid, 3,4-dimethoxy-, methyl ester	x	-	↑	-	-	↑	-	-	√
Benzoic acid, 4-(1-methylethyl)-methyl ester	x	-	↓	-	-	↓	-	-	√
Benzesulfonamide,N-(3-chloropropyl) N methyl	x	-	↑	-	-	↑	-	-	√
Cyclopentaneacetic acid, 3-oxo-2-pentyl-, methyl ester	x	-	↑	-	-	↓	x	-	x
Dimethyl 3,6-epoxytridecanedioate	√	-	-	-	-	↓	-	-	√
Diphenyl sulfone	x	-	↓	-	-	↑	-	-	x
Formamide, N-methyl-N-phenyl	x	-	↑	-	-	↑	-	-	√
Methyl 3(3-methoxycarbonyl) 4-methyl-5-pentyl-2-furanyl propionate	x	x	↓	-	x	↓	x	-	√
Methyl 3-amino-2-thiophenecarboxylate	√	-	↓	-	-	↓	-	-	√
Methyl-4-hydroxybutyl phthalate	√	-	-	-	-	↓	x	-	√
Methyl 2-acetamidoacrylate	√	-	↓	x	-	↓	x	-	√
Methyl 2-methyl-indole-3-carboxylate	x	-	↑	-	-	-	-	-	x
Methyl trans-4-methylcinnamate	x	-	↓	-	-	↑	-	-	x
Methylene chloride	√	-	↑	-	-	↓	-	-	x
Octadecanoic acid, methyl ester	x	x	↑	x	x	↑	x	x	√
Phenol, 2,6,dimethoxy-4-(2-propenyl)	x	-	↓	-	-	↓	-	-	√
Pyrene,1,2,3,3a,4,5,9,10a,10b-decahydro-	x	-	↓	x	-	↓	x	-	√
Tetrahydrofuran-2-one, 5-(1-hydroxyhexyl)	x	x	↓	-	-	↓	-	-	√
p-decylphenoxyacetic acid	x	x	↓	-	x	↓	-	x	√
erythro-8-(1,2-dibromo-2-phenylethyl-)-3,7-dihydro-1,3,7-trimethyl-1H-purine-2,6-dione	x	-	-	-	-	↑	-	-	√
l-Leucine, N-caproyl, methyl ester	√	-	↓	-	-	↓	-	-	√
L-Alanine, N-(M-anisoyl)-, decyl ester	√	x	↓	-	-	-	-	-	√

Table 10. List of compounds found in the HB and UB urine and their changes with the diets as well as their likelihood to be related to bacterial fermentation (x= no change, √= change, ↑= increase, ↓= decrease, - =data not available).

Compound	Difference before diets	Statistics	Modulen	Present/Not present	When present	E028	Present/Not present	When present	Bacterial fermentation?
1-(trimethylsiloxy)-2-methoxy-4-(1-trimethylsiloxyethenyl)-benzene	x	x	↓	x	-	↓	x	-	√
1H-Indole-2-carboxylic acid, 1-trimethyl silyl-trimethylsilyl ester	x	-	↓	x	-	↓	x	-	√
2,2-dichloro-1,1-bis (4-methoxyphenyl)ethane	√	-	↓	-	-	↓	-	-	√
2, (3-benzoxybenzyl)-4-methylphenyl benzoate	√	-	-	-	-	↓	-	-	√
4H-1-Benzopyran-4-one,3,5,6,7,8-pentamethoxy-2-(3,4,5-trimethoxyphenyl)-	x	-	↓	-	-	↓	x	-	√
4-hydroxyanthraquinone-2-carboxylic acid, di-TMS	x	-	-	-	-	↓	-	-	√
7-(2-(Ethoxycarbonyl)-3a,5a-dimethoxycyclopentyl-1)-heptanoic acid, ethyl ester	x	-	↓	x	-	-	-	-	√
2-Propenoic acid, 3-(p tolyl)-trimethyl lilyl ester	x	-	↓	-	-	↑	x	-	x
Cetotiamine	x	-	↑	-	-	↑	-	-	√
Cholest-2-eno(2,3-b)indole, 1'-acetyl-6-methoxy-	x	-	↓	-	-	↓	-	-	√
Chinchonane	x	-	↓	-	-	↓	-	-	√
Anteiso-heptacosanol, trimethylsilyl ether	x	-	↓	-	-	↑	x	x	x
Bis(trimethylsilyl) monostearin	x	x	↓	x	x	↓	x	x	√
Enterolactone(2,3-bis(3-hydroxybenzyl)butyrolactone-di(trimethylsilyl)	x	-	↓	-	-	↓	-	-	√
Hexadecanoic acid, trimethylsilyl ester	x	x	↓	-	x	↓	-	x	√
Octadecanoic acid, trimethylsilyl ester	x	x	↓	-	x	↓	-	x	√
Tritriacontane, 3-methyl	x	-	↓	-	-	↓	x	-	√
3-hydroxycotinine, trimethylsilyl ester	x	-	↓	-	-	↓	-	-	x
Caffeine	x	x	↑			↓			x

3-pyridinecarboxamide, 1,6-dihydro-1-methyl-6-oxo-N-trimethylsilyl	x	x	↓	-	x	↓	-	x	x
Hexadecanoic acid,2,3-bis((trimethylsil)oxy)propyl ester	x		↑			↓			x
1H-Indole-3-acetamide, 1-trimethylsilyl-	**x**	**-**	↑	**-**	**-**	↑	**-**	**-**	√
Arachidonic acid, trimethylsilyl ester	**x**	**-**	↓	**-**	**-**	↓	**-**	**-**	√
Dehydroepiandrosterone,), a-bis(trimethylsilyl)-	x	-	↑	-	-	↓	-	-	x
Palmitelaidic acid, trimethylsilyl ester	**x**	**-**	↓	**-**	**-**	↓	**-**	**-**	√
Urea, N,N-bis(trimethylsilyl)-	**x**	**x**	↓	-	**x**	↓	-	**x**	**x**
Silanol, trimethyl-, phosphate (3:1)	x	-	↓	-	-	↓	-	-	x
Trimethylsilyl ether of glycerol	**x**	**x**	↑	**-**	**x**	↑	**-**	**x**	√
Methyl 3(3-methoxycarbonyl) 4-methyl-5-pentyl-2-furanyl propionate	x	-	↑	-	-	↓	-	-	x
Methylene chloride	x	-	↓	-	-	↓	-	-	x
Thiourea, N,N-bis(1-methylethyl)	**x**	**-**	↓	**-**	**-**	↓	**-**	**-**	**x**
urea, triethyl-	**x**	**-**	↓	**-**	**-**	↓	**-**	**-**	**x**

Table 11. List of compounds found in the urine analysed with the SPME technique and their changes with the diets as well as their likelihood to be related to bacterial fermentation (x= no change, √= change, ↑= increase, ↓= decrease, - =data not available).

Compound	Difference before diets	statistics	Modulen	Present/Not present	When present	E028	Present/Not present	When present	Bacterial fermentation?
Acetone	x	-	↑	-	*	↑	-	x	√
Cyclohexane	x	-	↑	-	x	↑	-	x	√
Hexane, 2,2-dimethyl	x	-	↓	-	x	↓	-	x	x
Butanal, 3-methyl	x	-	↑	-	*	↑	-	x	√
Furan, 2,5-dimethyl	x	-	=	-	x	=	-	x	x
Furan, 2,4-dimethyl	x	-	=	-	x	=	-	x	x
Dimethyldisulfide	x	-	↑	-	x	↑	-	x	√
Hexanal	x	-	↑	-	x	↑	-	x	√
Ethylbenzene	x	-	↑	-	x	↑	-	x	√
Benzene, 1,2-dimethyl	x	-	=	-	x	=	-	x	x
4-Heptanone	x	-	=	-	x	=	-	x	x
Benzene, 1,3-dimethyl	x	-	=	-	x	=	-	x	x
3-Heptanone	x	-	=	-	x	↑	-	x	x
2-Heptanone	x	-	=	-	x	=	-	x	x
Heptanal	x	-	↑	-	x	↑	-	x	√
Pyridine, 2-ethyl-6-methyl	x	-	=	-	-	=	x	-	x
Oxepine, 2,7-dimethyl	x	-	↓	-	x	↓	-	x	√
2H-Pyran, 2-ethenyltetrahydro-2,6,6-trimethyl	x	-	=	-	x	↑	x	x	x
Hexanal, 2-ethyl	x	-	=	-	x	=	x	x	x
Dimethyl trisulfide	x	-	=	-	x	=	-	x	x
1,3,8-p-menthatriene	x	-	↓	-	*	=	-	x	x
Benzaldehyde	x	-	=		x	=		x	x
Cyclohexene, 1-methyl-4-(1-methjylethylidene)	x	-	↓	-	x	=	-	x	x
Limonene	x	-	=	-	x	=	-	x	x

Benzene,1-methyl-2-(1-methylethyl)	x	-	=	-	x	=	-	x	x
1,3,7-Octatriene, 3,7-dimethyl	x	-	=	-	-	↑	x	x	x
1,4-cyclohexadiene, 1-methyl-4-(1-methyl)	**x**	**-**	↓	**-**	*	↓	**-**	**x**	√
1-Hexanol, 2-ethyl	**x**	**-**	↑	**-**	**x**	↑	**-**	**x**	√
Cyclohexene, 4-1-methyl-4-(1-methylethylidene)	x	-	↓	-	*	=	-	x	x
2-butoxyethylacetate	x	-	=		x	=		x	x
Benzene, 1-methyl-4-(1-methylethenyl)	x	-	=	-	x	↓	-	x	x
2-Furanmethanol, 5-ethenyltetrahydro-a-a,5-trimethyl-, cis	x	-	=	-	-	=	x	-	x
1,6-Octadiene-3-ol, 3,7-dimethyl	x	-	↓	-	x	↑	-	*	x
Nonanal	x	-	=	-	x	↑	-	x	x
1,5,7-Octatriene-3-ol, 3,7-dimethyl	x	-	=	-	-	=	x	-	x
2,2-dimethyl-1-oxa-2-silacyclo-3,5-hexadiene	x	-	=	-	x	↑	-	x	x
2,6-dimethyl-1,3,5,7-octatraene, E	**x**	**-**	↑	**-**	**x**	↑	**-**	**x**	√
Pentanoic acid	x	-	=	-	-	↓	x	-	x
Indole	x	-	=	-	x	=	-	x	x
Heptanoic acid	**x**	**-**	↓	**-**	**-**		**x**	**-**	√
Octanoic acid	**x**	**-**	↑	**-**	**-**	↑	*	**-**	√
Nonanoic acid	**x**	**-**	↑	**-**	**-**	↑	**x**	**-**	√
Phenol, 2-methyl	**x**	**-**	↑	**-**	*	↑	**x**	**x**	√

pH of urine change after diet

The pH of the urine obtained before and after both diets was measured, and there was no significant pH change between before and after Modulen ($p>0.05$) or the Elemental E028 diet (Table 12).

Table 12. pH of urine of volunteers before and after both diets.

pH	Before Modulen	After Modulen	Before E028	After E028
Vol 1	7	5	5	6.7
Vol 3	5	5.5	5	5.5
Vol 4	6	7	6	7
Vol 5	7.3	5.5	6.7	6
Vol 6	6.7	6	7	6
Vol 7	5	5	6	5
Vol 9	5.5	5	6.4	6
Mean	**6.1**	**5.6**	**6**	**6**

Discussion

As the concentrations of the chemicals concerned are very low, it is very important to be meticulous in every process, especially cleaning the syringes and keeping all conditions constant at all times. Since the method of concentration of the breath in the adsorbent tube is critical, it is important that a single investigator does the injections into the ATD tubes, keeping the speed of injection constant.

Different compounds excreted on the breath were observed to have positive alveolar gradients. Other compounds had negative alveolar gradients, whereas others showed variation between individuals.

The method of action of enteral feeds is poorly understood. The present investigation was undertaken to examine the possibility that their effectiveness depends on depriving the intestinal microflora of substrates for its metabolism, resulting in the reduced production of

potentially toxic chemicals. We have examined changes which occur in the concentrations of the chemicals excreted on the breath or in the urine as means of attempting to assess whether bacterial activity has been modified after one weeks sole feeding with two enteral feeds – Modulen IBD and E028 Extra. Modulen IBD is a polymeric diet, whereas the E028 one is elemental.

A decrease in bacterial activity could manifest itself in two ways. Its appearance on breath and/or in the urine, implies that a chemical is present in the enteral feed, or possibly that it is normally broken down by intestinal bacteria, but persists, and becomes detectable, when bacterial activity is reduced. Conversely, the disappearance of a chemical may imply that it is present in normal diet, but not in the enteral feed, or alternatively that it is a normal product of bacterial activity which is no longer synthesised when that activity is reduced.

It is important also to consider the effect that some of the compounds produced during this process might also have over the metabolism of other bacteria and their compound production. For instance, the growth of some organisms has been observed to be affected by methanol concentrations, depending on the strain (Caldwell, 1989).

A further aspect to be taken into consideration is that some bacteria may be more sensitive to substrate-deprivation, dying earlier, and allowing competing organisms to overgrow. Furthermore, because the composition of bacterial flora of the gut of different individuals differs, the compounds observed in the breath and urine will also differ.

Despite these problems, some compounds are present in the majority of volunteers, or a pattern can be observed from a preliminary study to discover compounds relevant to bacterial fermentation.

The method used for this trial showed over 140 compounds in breath, and expected trends could be seen in some of the compounds. This could be an indication of the method being a valid one.

The compounds with positive alveolar gradient are thought to be endogenous, because there is more quantity in the breath sample than in the atmosphere. Those with negative alveolar gradients are deemed to be of environmental origin.

Many of the chemicals detected did not change as a result of enteral feeding and need not be considered further. The graphs of all the areas of the compounds not explained further can be found in Appendix 5.

Some compounds, for example caffeine, were seen only after feeding with Modulen IBD and therefore appear likely to be present in the feed itself rather than being produced in the body. There are however a number of compounds which were previously known to be synthesised or metabolised by the gut flora. These include phenols, indoles, enterolactone and octadecanoic acid. It is therefore crucial as a first step to assess the effects of enteral feeding on these marker compounds.

Indole is produced by intestinal bacteria from the essential amino acid tryptophan. Then it is absorbed in the intestine and conjugated further in the liver. An enterohepatic circulation may therefore be set up, with deconjugation in the large bowel (Powel, 2006). Indole was increased in breath after both diets, which suggests that indole was normally broken down by bacteria. However, evidence shows that colonic bacteria produce indole. Cummings et al (Cummings et al., 1979) showed that increasing meat intake increases phenol excretion in urine. However, it falls when further protein is added with wholewheat cereal products. This suggests that bacteria normally produce indole from protein, and other compounds such as short chain fatty acids from carbohydrates. When bacteria are deprived from both, the only source for their metabolism is exfoliated intestinal cells, varying bacterial metabolism, increasing the production of indole, because cells are mainly composed of protein. Changing the pattern of fermentation could result on changes of products of toxic metabolites.

The excretion of indole in the breath was significantly increased in Modulen with a greater effect after E028. Indoles are compounds with an unpleasant smell and it is possible that

they may, at least in part, be responsible for the unpleasant breath odour which is often seen in subjects on enteral feeding. It is perhaps significant that the deterioration in breath odour was more apparent when subjects took E028 than when they took Modulen, possibly reflecting differences in indole production.

As indole is a bacterial product, these changes in its metabolism support the theory that changes in bacterial activity may underlie the effectiveness of enteral feeding in CD.

Phenol may be increased in the same way as indole. Phenol is produced by the conversion of the non-essential amino acid tyrosine by bacteria. Phenols were identified on the breath and showed a significant increase in concentration after feeding with E028 and even greater increase after feeding with Modulen. In the same way, octanoic acid is a carboxylic acid, which is thought to be produced by bacteria, and it increased after both diets, possibly under the same mechanism.

Enterolactone excretion in the urine fell significantly in both enteral feeds. Enterolactone is produced by the bacterial degradation of lignans in the gut and although this reduction may reflect a decreased intake of foods containing lignans, which is derived from cellulose, it may equally the effect of reduced bacterial metabolic activity. Either way, it implies that there has been a significant change in the metabolic activity of the bacteria of the gut.

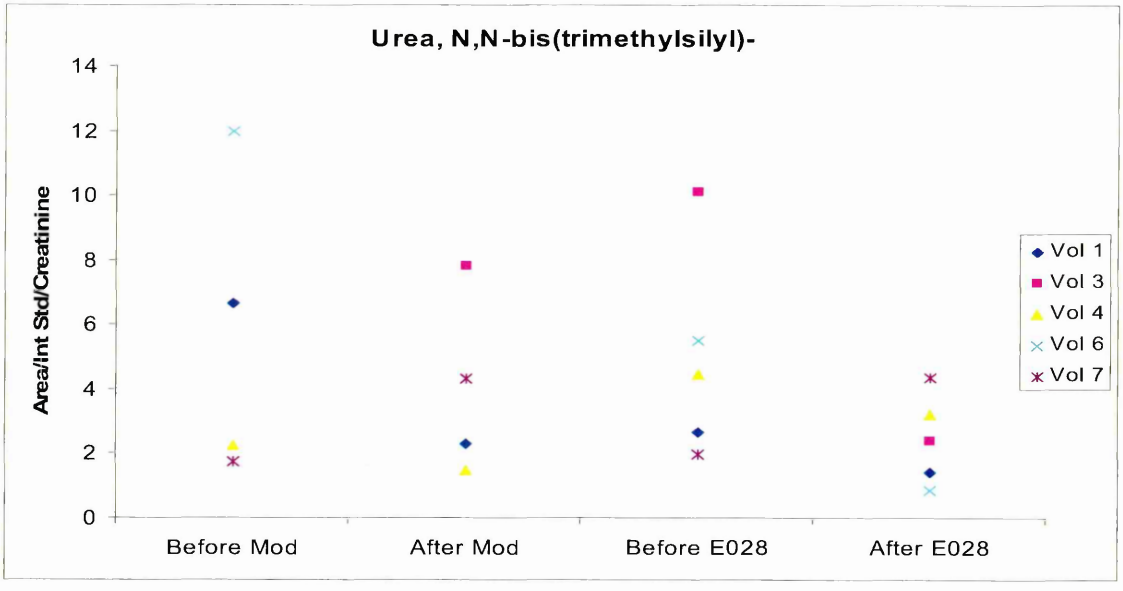


Figure 44. Area under the curve of N,N-bis(trimethylsilyl) urea related to the internal standard and creatinine levels.

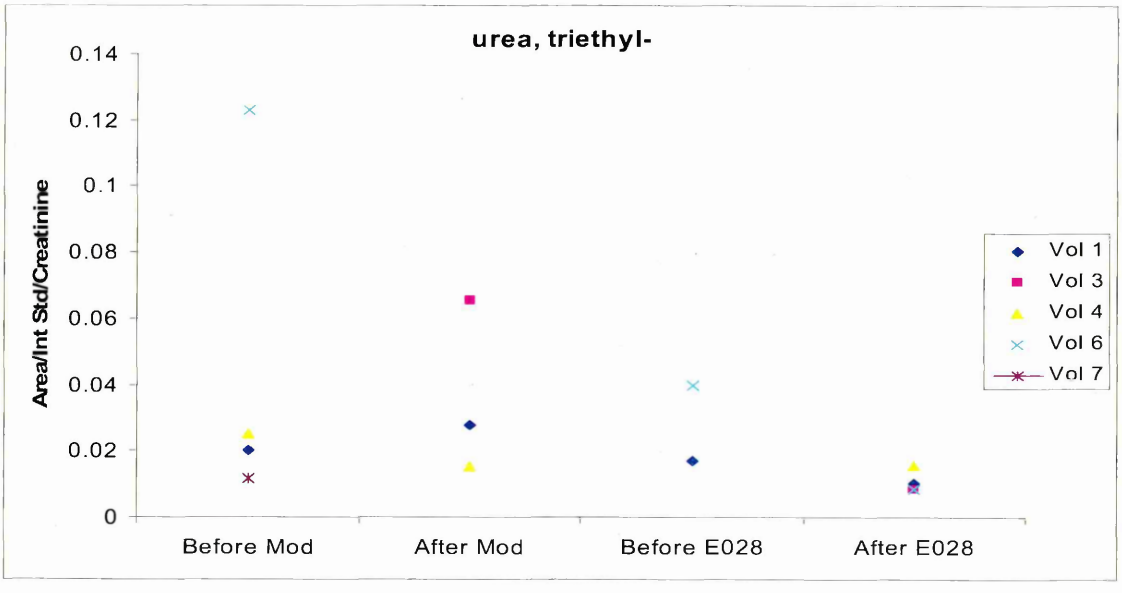


Figure 45. Area under the curve of triethyl urea related to the internal standard and creatinine levels.

Triethyl urea, and N,N-trimethylsilyl urea were found to be decreased in the urine analysis after the diets (see Figure 44). Both compounds are likely to be derivatives of urea, but they are different because they were treated differently in two different urine analyses. Urea is a metabolite derived from protein ingestion, so urea derivatives are expected to decrease after both diets.

Pentane is released in a number of inflammatory conditions, and is believed to be associated with lipo-peroxidation occurring in affected tissues. However, its release is known to be reduced in rats after treatment with the antibiotic clindamycin (7) and so it may also be of bacterial origin. The reductions in breath pentane excretion seen in our volunteers after enteral feeding would be consistent with this.

Thus, the effect of enteral feeding on these chemicals which are known to be produced or destroyed by bacterial activity is consistent with the hypothesis that enteral feeding markedly changes bacterial metabolism. A similar pattern was seen in a number of other chemicals. Whilst, as with enterolactone it is not possible to be entirely certain that the changes seen do not reflect differences in the amounts of the parent compounds in normal food stuffs or enteral feeds, nonetheless the similarity in the response shown after feeding with two different enteral feeds a whole month apart suggests that there is indeed a consistent effect which is likely to be mediated through changes in bacterial activity, and these chemicals are deserving of further study.

It is important to note that acetone in breath decreased after both diets, although it increased in urine after both diets. Acetone is mainly formed by descarboxylation of acetoacetate and dehydrogenation of isopropanol during lipolysis, absorbed in the blood stream and mostly excreted in the expired air (Statheropoulos & Gerorgiadou, 2006). The concentration in breath changes during the day depending on the amount of carbohydrates in the diet (Lord et al., 2002) and is markedly influenced by the period of fasting (Jones, 1987). This finding suggests the difference between the breath and urine analysis, since urine reflects metabolism over several hours, whereas breath reflects metabolism solely at the time of sampling.

Figure 46. Area under the curve of acetone in urine analysed with SPME of healthy volunteers before and after Modulen and E028 diet.

Clinical studies at Addenbrookes Hospital (Cambridge) have demonstrated that E028 extra was significantly more effective than Modulen IBD in the induction of remission in patients with active CD (J. O. Hunter, personal communication). It was therefore possible that this difference may be reflected in differences in the effects of the feeds on the excretion of VOCs. In the event, no significant difference was seen in this study between E028 and Modulen in the excretion of any compound known or suspected to be of bacterial origin. It must be stressed however, that this study was performed in normal healthy individuals and there is no suggestion that any of the chemicals detected were in themselves toxic or of possible importance in the pathogenesis of intestinal disease. Furthermore the number of volunteers involved was small, so that the detection of relatively small differences did not achieve statistical significance. However, the PCA of urine analysis data, both after acidic and basic hydrolysis treatment showed a clear difference between volunteers after a week's feeding with E028 and a week on Modulen, apart from the expected differences which were seen from normal feeding. This suggests that there is indeed an important difference in the potential therapeutic activity of the two feeds, but that the present study was too small to

enable this to be detected. Studies of VOC excretion in patients with active CD may be more helpful in this respect.

The mechanism of action of enteral feeds has been very controversial, (O'Morain et al., 1980) (El-Matary et al., 2003) (Greenberg et al., 1988) (Ruemmele et al., 2000) (Siedman, 1989) (Jonson et al., 2005) (Verma et al., 2000a) (Gassull, 2004) (Siedman, 1994), but this research suggests it works because it contains little or no residue and its nutrients are therefore absorbed, without the need for digestion, high in the jejunum, it supplies the colonic microflora with little in the way of energy substrates, resulting in reduced microbial metabolism. Other studies done in CD patients showed a reduction of anaerobic lactobacilli after a week on an elemental diet, although there was no change on the total bacterial score (Giaffer et al., 1991). This suggests that elemental diet decreases bacterial metabolism, although faecal flora do not change.

Further studies should also include determination of breath and urine metabolites after reduction of the colonic bacterial flora by other measures. Confirmation that the compounds are genuinely the products of bacterial fermentation will depend on the demonstration that they are also influenced by other ways of manipulation such as antibiotic or bowel clear-out. This was not possible in the present study because it was considered that it would be unethical to administer antibiotics to healthy volunteers which could lead to allergic reactions or to the development of antibiotic resistant strains of bacteria in the gut flora.

In retrospect it would also have been an advantage to have separated the two feeding periods by a longer interval. A PCA analysis of breath chemicals suggested that 3 weeks after enteral feeding there was still significant differences in VOCs excreted on the breath and a longer period may be necessary for this to return completely to normal. However, the female menstrual cycle is known to influence the activity of gut bacteria and it was because of this reason that the feed interval was chosen. A delay of two menstrual cycles may have given better results.

Urine analysis reflects body metabolism over a period of several hours, whereas breath only reflects the metabolism over seconds. At the same time, VOCs in urine do not need to be related to the compounds found in the environment, since the background is more consistent, making the data handling easier. However, urine concentrations have to be related to the creatinine concentration to allow for urinary dilution. Furthermore, the compounds analysed were hydrolysed and then reconjugated for analysis, making the analysis indicative of the process, but with the result that the exact compounds involved are not known. This should be the subject of further study. Both methods are non-invasive, but breath is easier to collect, and more comfortable for the volunteer. Breath would be a good method for diagnosis of different diseases if the method was standardised. With both methods we could see a broader range of compounds, making the study more complete.

Conclusion

This trial has confirmed that changes occur in stool colour and in breath consistent with a reduction in colonic bacterial activity. As bacterial activity decreases, stools become green, because the intestinal microflora in the colon do not hydrolyse the bile salts such as biliverdin (green) (Begley et al., 2005) to stercobilin (brown). Breath changed considerably because compounds that are normally broken down by bacteria cannot be broken down any longer, worsening breath in the volunteers. There were also with both feeds changes in the excretion in breath and urine of VOCs known to be of bacterial origin which were consistent with a reduction of bacterial activity, such as phenol and indole in breath and heptanoic and nonanoic acid in urine. Parallel changes after both enteral feeds of the excretion of other VOCs suggested that these compounds too could be of bacterial origin. Further studies are needed to confirm that the changes we have demonstrated also occur after other manipulations such as bowel clearouts or antibiotic treatment which are known to reduce bacterial metabolism. These observations should be extended into patients taking enteral feeds for the treatment of active CD in the hope of detecting chemicals which are involved in the pathogenesis of the disease and enabling the identification of the most effective feed composition.

Chapter 4. IBS

Urine of several patients suffering from gastrointestinal symptoms was analysed and compared with urine of healthy volunteers. Individual compounds detected in the urine as well as the general spectrum of compounds in urine were analysed. Differences in symptoms and successful treatments were correlated with varying concentrations of certain compounds. These compounds may be used as biomarkers of the diseases for early diagnosis and adequate treatment.

Some of the patients suffering from gastrointestinal symptoms improve with an exclusion or fibre free diet. The urine of these patients was analysed before and after the diet and bacterial metabolites were observed to determine if these symptoms might be caused by colonic bacteria.

The study

IBS -bacterial metabolism in IBS

In total 15 patients were recruited from Addenbrookes Hospital. They all suffered from IBS, according to the Rome criteria (Thomson et al., 1989) and were classified into the categories of retention and overflow (R+O) IBS patients, musculoskeletal IBS (MSK) (according to the assessment described by Sparkes) (Sparkes et al., 2003) or IBS patients who suffered from abdominal pain and variable bowel habit. They were all eating a normal diet. Other conditions were excluded by physical examination, rigid sigmoidoscopy, haematological and biochemical blood tests, lactose hydrogen breath test and, if the patient was over 45 years of age, either colonoscopy or barium enema. Other exclusion criteria were: taking any medication other than the oral contraceptive; having received a course of antibiotics in the previous six weeks; taking bacterial products such as pro- or prebiotics, and pregnancy and lactation.

Matched age healthy volunteers were recruited from students of either sex of the University of Cranfield at Silsoe to serve as controls. All were to be in normal health, and eating a

normal diet. IBS, migraine and pre-menstrual syndrome in particular were excluded by completion of a symptom questionnaire, as all these conditions are possibly associated with abnormal colonic fermentation. Exclusion criteria were: taking any medication other than the oral contraceptive; having received a course of antibiotics in the previous six weeks; taking bacterial products such as pro- or prebiotics, and pregnancy and lactation.

Patients on different treatments

Volunteers who were catalogued as IBS patients who suffer from abdominal pain and variable bowel habit were put onto a low fibre diet or an exclusion diet (Dear et al., 2005). A list of the patients that were looked at with the treatments they underwent can be seen in Table 14.

Diets were supervised by a qualified dietician who confirmed that subjects' normal diets were acceptable. Diets were adjusted to achieve the estimated energy balance according to Schofield's equation (Schofield, 1985). Subjects received this diet for two weeks. All food was provided by a metabolic kitchen. Compliance with the diets was assessed by a daily food dairy, which recorded the weight and type of food not consumed. Urine samples were taken before and after the diet.

Those patients suffering from musculoskeletal IBS or retention and overflow were treated appropriately and they were kept on a normal diet. Urine was not analysed after the treatment.

Ethics

This study was approved by the Cambridge Local Research Ethics Committee (see Appendix 6).

Materials and methods

Volunteers were asked to collect urine samples first thing in the morning and in the case of suspected IBS with abnormal fermentation; a morning sample was taken two weeks after a low fibre free diet. Samples were stored in a -80°C freezer until analysed.

These samples were sent to The Horserace Forensic Laboratory (HFL), Newmarket, to be analysed by GCMS by Simon Hudson according to three standard analyses. The urine was analysed in three different ways: hydrolysed acid neutral (HAN), unhydrolysed basic (UB) and hydrolysed basic (HB sample pretreatment). Urine (3 ml) was analysed with these methods as described in Chapter 2 (Materials and Methods). The analysis was done three times for each sample and the mean of the areas of the peaks for each compound was calculated.

Symptom Score Sheets

Volunteers were asked to record a symptom score sheet (SSS) to record objectively how their symptoms were and to assess whether the treatment was successful. The SSS took into account pain severity, pain frequency, abnormal distension, wind, stool frequency and stool urgency according to Table 13.

Table 13. SSS. Patients were asked to fill in a sheet attributing scores according to this table during a week before treatment and during the treatment. The scores are added up to form a score, which is compared before and after the diet to indicate improvement.

	0	1	2	3	4
Pain Severity	no pain	mild	moderate	severe but able to continue	interferes with activity
Pain Frequency	no pain	rare	moderate	very frequent	constant
Abnormal distension	none	mild	moderate	severe but able to continue	interferes with activity
Wind	none	mild	moderate	severe but able to continue	interferes with activity
Stool Frequency	please state number of individual visits				
Stool Urgency	normal able to ignore	increased but able to control	moderate minutes	must run seconds	incontinent

Patients suffering from R+O and MSK filled in the questionnaires. However, only one of the IBS patients filled in the SSS. This patient was put on an exclusion diet and was successful with a 50% improvement; the symptom score was 90 before the diet and 40 after the diet. The improvement was measured by a dietician and was reported as successful or unsuccessful. Three of the other four IBS patients reported to the dietician that they felt much better after the diet. One of them did not fill a SSS or report to the dietician.

The R+O and MSK patients were put on different medication.

Table 14. R+O, MSK and IBS patients SSS before and after treatment. Patients were asked to fill in a form regarding their symptoms (SSS). The table shows the treatment that all the patients suffering from R+O and MSK had, as well as if they felt improvement after treatment. (N/A is not applicable).

	Condition	Treatment	Outcome	SSS before	SSS after	Improvement
No. 1	R+O	Novicol and Normacol	Successful	23	15	35%
No. 2	R+O	Movicol and Low fibre diet	Successful	70	47	33%
No. 3	R+O	Movicol and Normacol	Successful	38	34	11%
No. 4	R+O	Normacol	Successful	N/A	55	N/A
No. 5	R+O	Normacol	Successful	61	31	49%
No. 6	MSK	Physiotherapy	N/A	39	N/A	N/A
No. 7	MSK	Physiotherapy and myofascial nodule injected by GP	Successful	65	-	N/A
No. 8	MSK	Physiotherapy	Successful	103	91	
No. 9	MSK	Physiotherapy	N/A	76	N/A	
No. 10	MSK	Physiotherapy	Felt better but no SSS improvement	56	60	NO
No. 11	IBS	Exclusion diet	Successful	90	40	50%
No. 12	IBS	Low-fibre diet	Successful	N/A	N/A	N/A
No. 13	IBS	Low-fibre diet	Successful	N/A	N/A	N/A
No. 14	IBS	Exclusion diet	Successful	N/A	29	N/A
No. 15	IBS	Low-fibre diet	Successful	N/A	N/A	N/A

Data analysis

Sample chromatograms were analysed using PCA and the individual compounds abundances were studied. The pH of the urine samples was measured with Whatman pH test strips.

PCA

One repeat of all the chromatograms was chosen at random and they were then transformed into comma separated value (CSV) files using WSearch Pro. Once these files are formed, the 'allgcmsread2' script was used to convert them into Matlab files. PCA was conducted on the data using the "pcagui" function in PLS_Toolbox 2.0 (Eigenvector Research Inc., Manson, USA), which runs as a supplement to Matlab. The variables for the PCA input matrix were intended to be the time coordinates outputted by the TurboMatrix software (Jon Lee Davey).

Compound identification and semi-quantification

The compounds found in the urine were identified from the total ion chromatogram (TIC) using the NIST electronic spectra library. The search was done manually and the area under the curve of the selected ion chromatogram was determined. The areas were then divided by the AUC of the internal standard and AUC of the creatinine product of the analysis.

Creatinine of the urine analysed in house was measured with the Creatinine Test Kit (CinnaGen Inc), based on the Jaffe Method. The measurements were done in a Camspec M350 Double beam UV-Visible Spectrophotometer. The creatinine of the samples sent to HFL was measured as a derivative through the UB analysis: Creatinine enol N1,N3, O-TMS (see Chapter 2).

Statistical analysis

In order to determine whether the compound's presence in R+O or MSK and compared control subjects was significant a Chi-squared test with the Yates correction was used. When present, in order to see if the compound was present in significantly different quantities, Mann Whitney was used. ANOVA was used for the statistical analysis of R+O, MSK and IBS.

The probability of a compound being present in these patients before or after the diet was checked for significance using a McNemar test. In order to compare the quantities when these compounds are present, a Wilcoxon Rank Test was used.

The pH of the R+O, MSK and IBS patients was compared with an ANOVA test. ANOVA was used for its ability to compare different parametric samples (pH was considered parametric because of a skew minor to 1 and similar standard deviations). The probability of the pH of the urine of IBS patients before and after the diet being different was measured with a paired t-test.

Results

In total, 15 subjects were recruited, aged 18-45, of which 8 were female. Out of the 15, 5 patients recruited suffered from IBS R+O. Another 5 patients suffered from MSK IBS and another 5 patients suffered from IBS suspected to be related to abnormal fermentation. These last 5 patients were asked to follow a low fibre or an exclusion diet for two weeks. As controls, 5 healthy volunteers were recruited for this trial.

Three sets of samples were done: comparison of R+O, MSK and controls; comparison of R+O, MSK and IBS before and after the diet; and R+O, MSK, IBS before and after the diet with controls. The first two sets were analysed by HFL and the third set in house by SPME. The fact that all the groups could not be analysed and compared through the HFL method is because only 20 samples can be analysed at one time, and different volumes were analysed in the different sets but the same amount of internal standard was added. This made a

comparison between all groups impossible. A quantity of 3 ml were analysed in the first group, 2 ml in the second one and 3 ml in the third group.

Comparison between R+O, MSK and Healthy Volunteers

PCA

PCA was done from the chromatograms obtained through the three GCMS analyses. The PCA obtained from the chromatograms of the HB and HAN urine did not show any clustering of the groups. However, the UB analysis showed clustering of patients suffering from R+O and MSK on one side and controls on the other (see Figure 47 and Figure 48). This might be due to MSK and R+O patients having medication other than real differences in endogenous compounds.

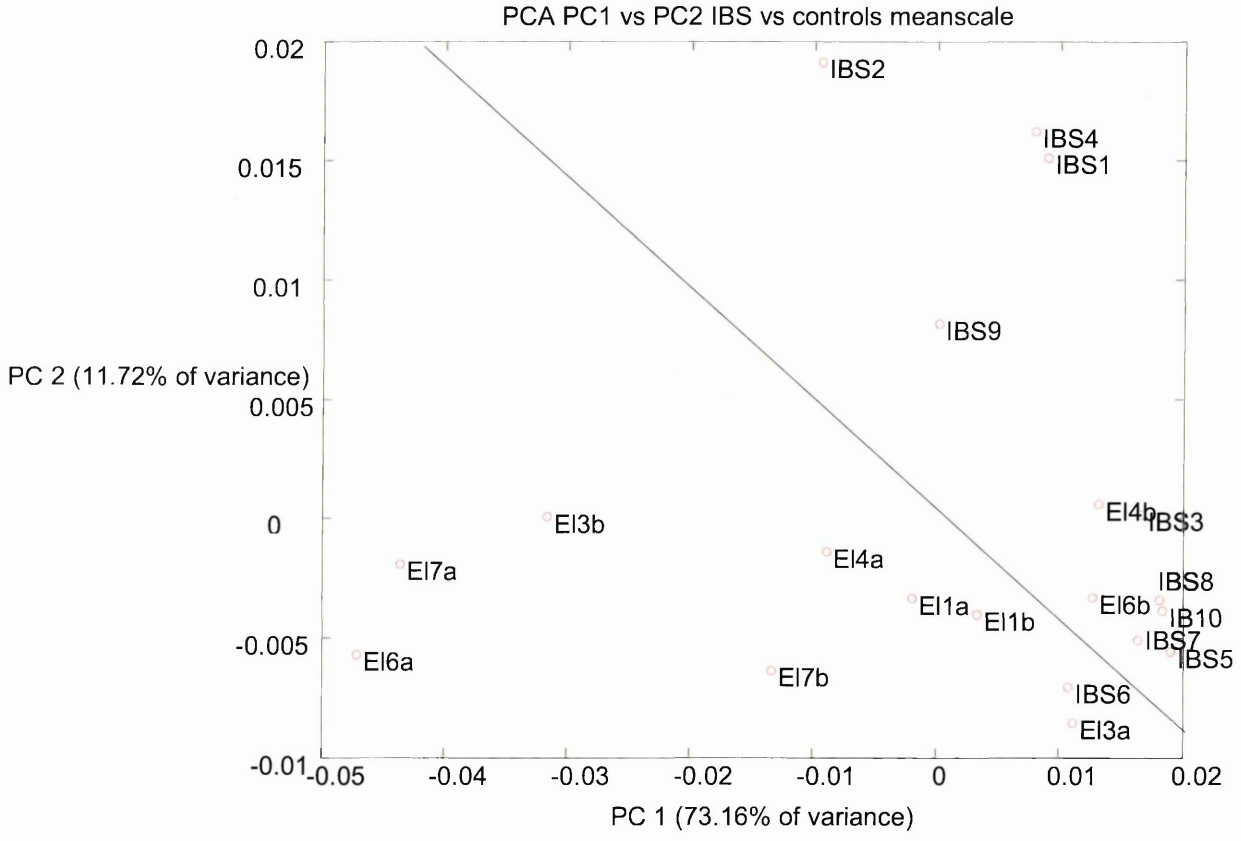


Figure 47. PCA of PC1 versus PC2 of the TIC chromatograms of UB urine obtained from R+O and MSK patients with controls. IBS1-5 are R+O patients, IBS6-10 are MSK patients and The El1-7 are healthy volunteers.

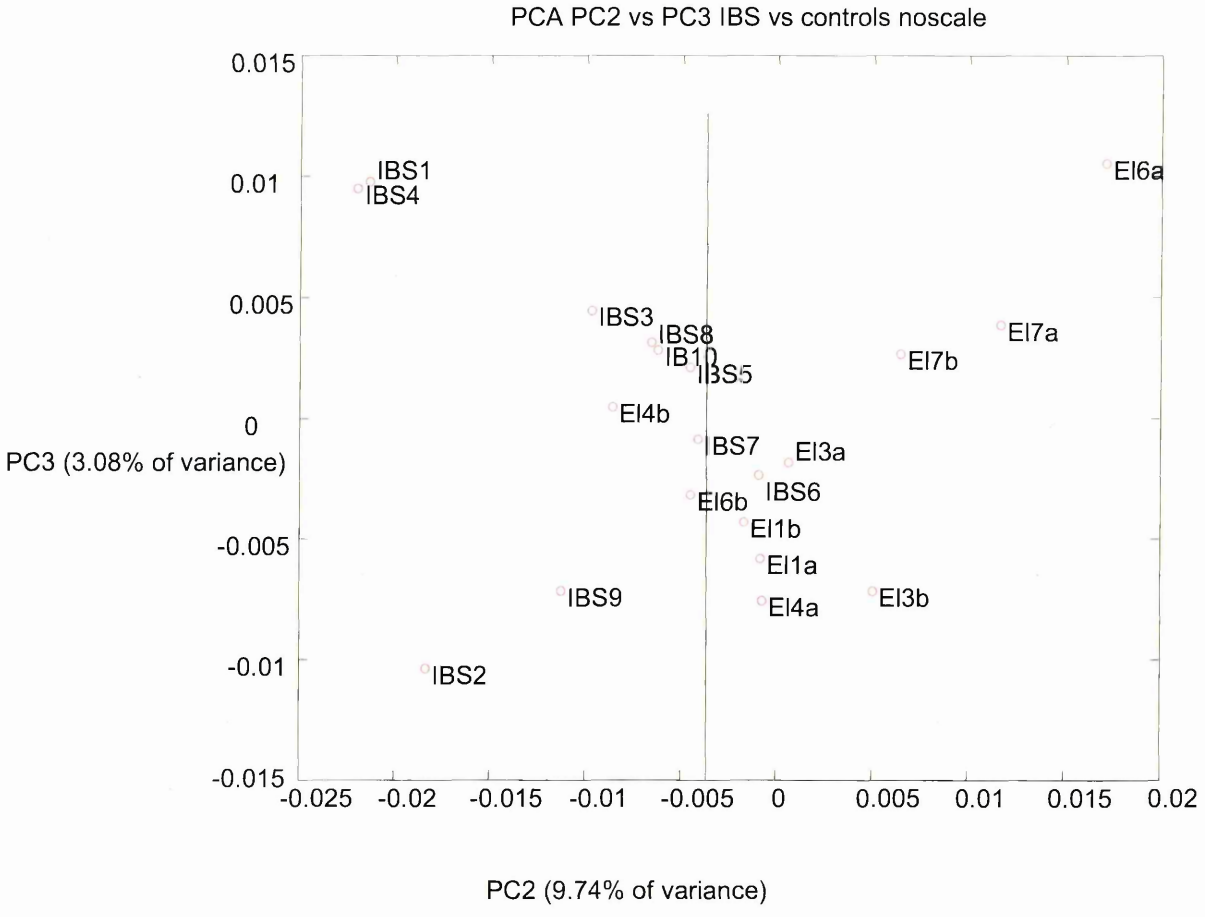


Figure 48. PCA of PC2 versus PC3 of the TIC chromatograms of UB urine obtained of R+O and MSK patients with controls. IBS1-5 are R+O patients, IBS6-10 are MSK patients and The El1-7 are healthy volunteers.

In the PCA of PC1 versus PC2 we can see a clear distinction between patients suffering from R+O and MSK in comparison with controls. However, there is no difference between R+O and MSK groups. In PC2 versus PC3 we can see a separation along PC2.

The PCA of the HB urine and HAN did not show any differences between the groups.

From the PCA of the UB urine we can say that urine from MSK and R+O are similar, but different to healthy controls.

Compounds found in urine

When the individual compounds found in urine were looked at, some compounds were found in different quantities to controls. These compounds were looked at and assessed for further investigation. A list of all the compounds found in the HAN analysed urine can be found in Table 15. Among all these compounds, many might be of interest in aiding early diagnosis of conditions such as R+O and MSK, and some of these are shown below. For instance, a compound similar to 1-iodo-4(2-phenylethenyl) benzene and 1-(3,4-dimethoxyphenyl)- Ethanone (see Figure 49) were found to be increased in healthy controls.

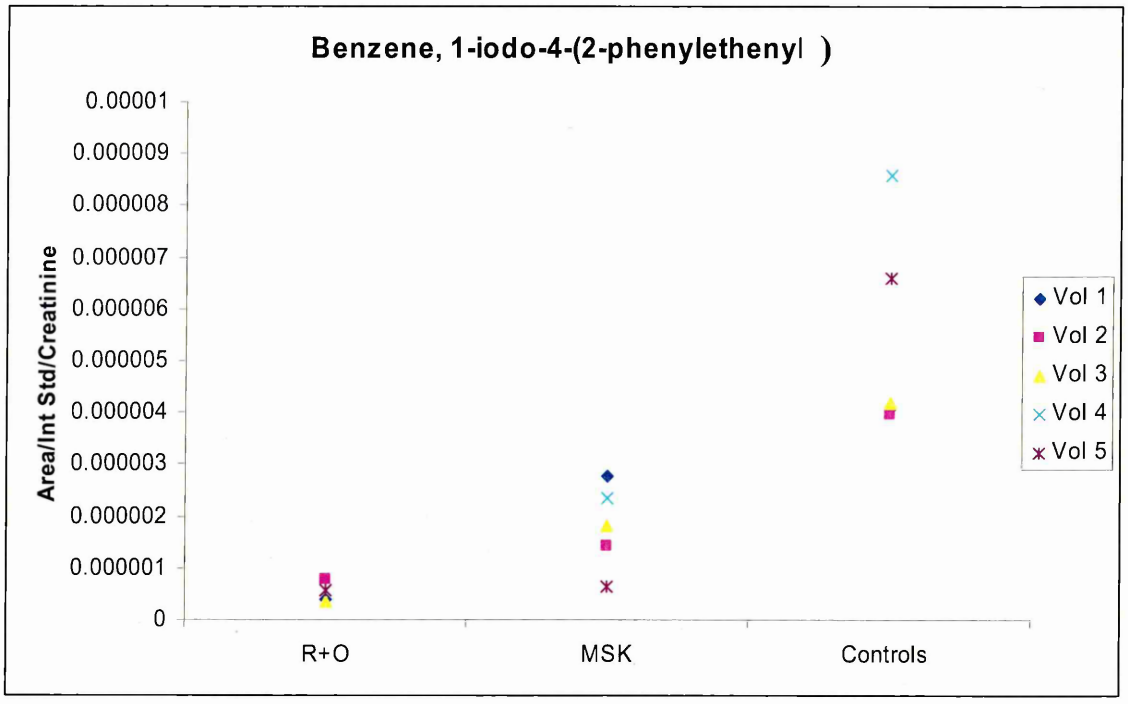


Figure 49. Area of the compound similar to 1-iodo-4(2-phenylethenyl) benzene taking into account internal standard and creatinine in R+O, MSK and controls.

Table 15. Compounds found in urine of patients with MSK GI problems, R+O problems and controls analysed with the HAN method. "=" means they were equal in all conditions, "-"means data not available (due to the small samples), "x" means not significantly different.

Compound	MSK, R+O, Controls	Statistical difference
1-Indole-3-acetic acid, methyl ester	↑ in controls	x
1,3,7,9-Tetramethyluric acid	Present only in controls	-
'2',4,4',6'-Tetramethoxychalcone'	↑ in controls	-
2-(1-Pentamethylphenyl)ethyl-3,3-diphenyloxaziridine	Present only in controls	-
2-Benzoyl-6-hexanelactam	Present only in controls	-
2-Propenoic acid, 3-(3,4-dimethoxyphenyl)-, methyl ester	=	x
2H-1-Benzopyran-2-one, 7-methoxy-	=	x
3,5-Dimethyl-1-dimethyl(tert-butyl)silyloxybenzene	Present only in controls	-
3-Methylnon-1-yn-3-ol	Present only in MSK	-
4-Decanamine, N-ethyl-4-propyl-	Present only in controls	-
4H-1-Benzopyran-4-one, 6,7-dimethoxy-3-phenyl-	Present only in controls	-
5,6-Dimethoxy-7,3',4'-trihydroxyflavone	Present only in controls	-
5-Isopropylidene-3,3-dimethyl-dihydrofuran-2-one	Not present in MSK	-
6H-Benzofuro[3,2-c][1]benzopyran, 3,9-dimethoxy-	Present only in controls	-
8-Phenyl-6-thio-theophylline	Present only in controls	-
Acetamide, N-(4-methoxyphenyl)-	Present only in controls	-
Benzamide, 2,3,4,5-tetrafluoro-N-(3-methylthio-1,2,4-triazol-5-yl)-	=	x
Benzenamine, 4,4'-methylenebis[N,N-dimethyl-	=	x
Benzene-1,4-dicaboxamide, N,N'-diethoxy-	Present only in controls	-
Cinnamoylglycine, methyl ester	=	-
Cyclohexanamine, N-hydroxy-	Not present in controls	-
Decanoic acid, 2-methyl-	Not present in controls	-
Egenine	Present only in controls	-
Ethanone, 1-(3,4-dimethoxyphenyl)-	**↑ in controls**	*
N-Acetyl-S-(2-hydroxybutyl)-L-cysteine methyl ester	=	x
N-Ethyl-4-methyl-4-decanamine	Not present in R+O	-
Phenol, 4-(1-phenylethyl)-	Not present in R+O	-
Pyrolo[3,2-d]pyrimidin-2,4(1H,3H)-dione	=	x

Tetrahydrofuran-2-one, 5-[1-hydroxyhexyl]-	Not present in R+O	-
p-Decylphenoxyacetic acid	Not present in R+O	-
Benzoic acid, 4-methoxy, propyl ester	=	x
Pentadecanoic acid, 14-methyl, methyl ester	=	x
Octadecanoic acid	=	x
N-ethyl-4-methyl-4-decanamine	=	x
Acetic acid, cyclodecyl ester	=	x
Isoquiline, 3,4-dihydro-6,7-dimethoxy-1-methyl	=	x
Isocitronellol	=	x
Octanamide, N-(2-mercaptoethyl)	=	x
2-Propenoic acid, 3-(2-formyl-4-methoxyphenyl)-ethyl ester	Not present in controls	-
Benzene, 1-iodo-4-(2-phenylethenyl)	**↑ in controls**	*
Acetamide, N-(2-4-methyloxylphenyl etoxy)?	=	x

Urine samples were also analysed with the UB method, and some compounds were found in different quantities in MSK and R+O patients in comparison with controls. A list of all the compounds found through this analysis can be seen in Table 16. Graphs can be found in Appendix 7.

Nonanoic acid, trimethyl silyl ester was decreased in healthy volunteers with respect to R+O and MSK patients (see Figure 50). This compound is probably present in urine as nonanoic acid, because in the UB and HB analysis, trimethyl silyl was used to derivatise the compounds for analysis. Nonanoic acid is a carboxylic acid, probably a product of bacterial fermentation and should be investigated further.

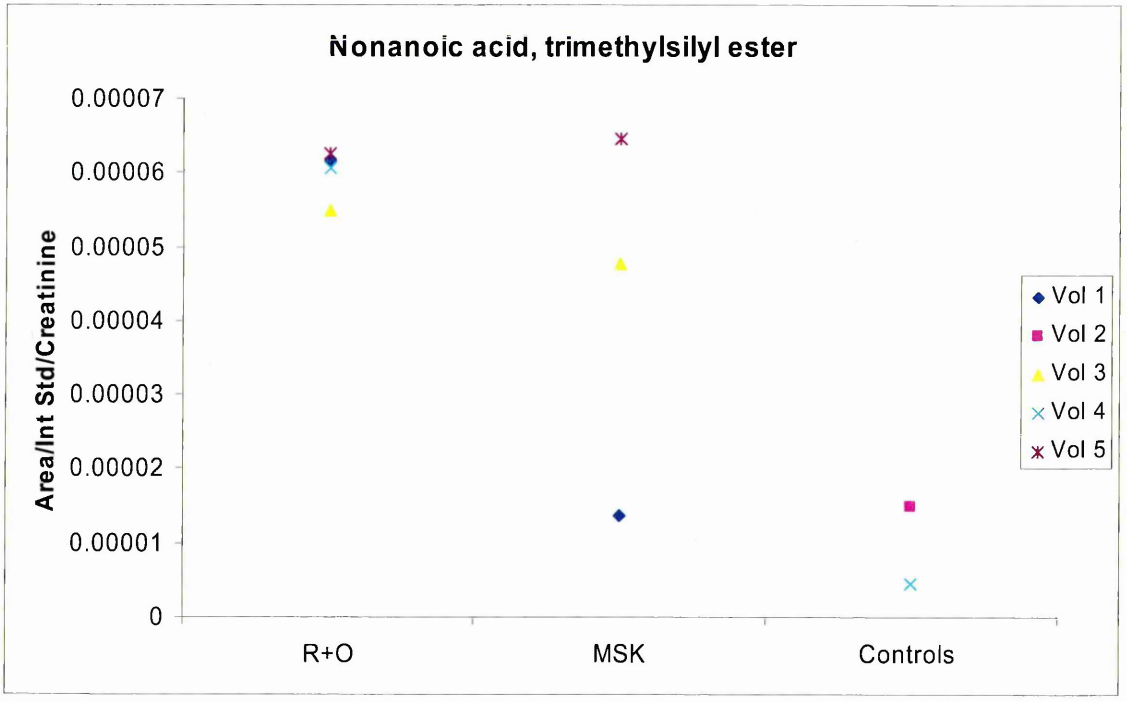


Figure 50. Area of nonanoic acid, trimethylsilyl ester taking into account internal standard and creatinine in R+O, MSK and controls.

From the compounds found in the urine analysed with the HB method, no significant differences were seen in the individual compounds. The list of the compounds can be seen in Table 17.

Table 16. Compounds found in urine of patients with MSK GI problems, R+O problems and controls analysed with the UB method. "=" means they were equal in all conditions, "-"means data not available (due to the small samples), "x" means not significantly different.

Compound	IBS in comparison	Statistical difference
1,6-Dinitro-9-isopropyl-carbazole	**Not present in controls**	-
1-Piperidinyloxy, 2,	**Present only in controls**	-
1-Propanone, 2,2-dimethyl-1-(4-methylphenyl)-3-(1-piperidyl)-	=	-
1-[(Trimethylsilyl)oxy]-2-methylanthraquinone	=	x
1H-Indole-3-acetamide, 1-trimethylsilyl-	=	x
1H-Indole-3-acetamide, N,1-bis(trimethylsilyl)-	=	x
1H-Indole-3-ethanamine, N,1-bis(trimethylsilyl)-	=	x
1H-Phenanthro[9,10-d]imidazol-2-amine	=	-
1H-Purine-2,6-dione, 3,7-dihydro-1,3,7-trimethyl-	=	x
2,2'-Ethylidenebis(6-methoxy-3-methylbenzofuran)	=	x
2,3,4-Trihydroxybutyric acid tetrakis(trimethylsilyl) deriv.	**Present only in controls**	-
2,7-Bis-(2-piperidin-1-yl-ethoxy)-fluoren-9-one oxime	**Present only in MSK**	-
2-Heptanone, 6-methyl-5-methylene-	**Present only in R+O**	-
3-Phenyl-7-(2-amino-4-diethylamino-1,3,5-triazin-6-	=	x
8-Thiabicyclo[3.2.1]octan-3-one, 8-thiabicyclo[3.2.1]oct-3-	=	-
Aniline, N-cyclohexylcarbonyl-4-methoxy-	**Present only in controls**	-
Arachidonic acid, trimethylsilyl ester	=	x
Benzamide, N-(trimethylsilyl)-	=	x
Bis(trimethylsilyl)monostearin	=	x
Cholesterol trimethylsilyl ether	=	x
Dodecanoic acid, trimethylsilyl ester	=	x
Estra-1,3,5(10)-triene-16,17-diol, 2-nitro-3-	=	-
Heptadecanoic acid, glycerine-(1)-monoester, bis-O-	=	x
Hexadecanoic acid, 2,3-bis[(trimethylsilyl)oxy]propyl ester	=	x
Hexadecanoic acid, trimethylsilyl ester	=	x
Myristic acid, 2,3-bis(trimethylsiloxy)propyl ester	=	x
N-Trimethylsilyl-n-heptylamine	**Present only in controls**	-
Nonanoic acid, trimethylsilyl ester	**↓ in controls**	x
Octadecanoic acid, trimethylsilyl ester	=	x
Oleamide, N-trimethylsilyl-	=	x
Oleanitrile	=	x
Oxostephamiersine	=	x
Palmitelaidic acid, trimethylsilyl ester	=	x

Phosphoric acid, tris(2-ethylhexyl) ester	=	x
Phthalic acid, 2-methylallyl tetradecyl ester	=	-
Phthalimide, N-(trimethylsilyl)-	=	x
Silane, [1,4-cyclohexanediylbis(oxy)]bis[trimethyl-, trans-	=	-
Trimethyl(2,6 ditert.-butylphenoxy)silane	=	x
Trimethylsilyl dimethylphosphinate	=	-

When the compounds found in urine MSK and R+O patients and healthy controls analysed with the UB method were compared no significant differences were found.

Table 17. Compounds found in urine of patients with MSK GI problems, R+O problems and controls analysed with the HB method. "=" means they were equal in all conditions, "-"means data not available (due to the small samples), "x" means not significantly different.

Compound	MSK, R+O and controls	Statistical difference when present
Silane, dimethyl(octadecyloxy)propyl-	=	-
Hexasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11-dodecamethyl-	=	x
1-(2-Isopropenyl-3-methoxy-2,3-dihydro-1-benzofuran-5-yl)-7,8-dimethoxy-3a,9b-dihydro-4H-chromeno[4,3-d]isoxazole	=	x
3-Fluoro-5-(trifluoromethyl)benzaldehyde	=	-
Methyltris(trimethylsiloxy)silane	=	x
9-Octadecenamide, (Z)-	=	x
Heptasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11,13,13-tetradecamethyl-	=	x
Benzonitrile, 2-methyl-	=	x
1,2-Benzenedicarboxylic acid, mono(2-ethylhexyl) ester	=	x
Niflumic acid, trimethylsilyl ester	=	x
1,2-Benzenedicarboxylic acid, diisooctyl ester	=	x
1H,7H-Pyrrolo[3,2-H]quinolin-8-one, 6-hydroxy-2,3-dimethyl-6-trifluoromethyl-6,9-dihydro-	=	x
N,O-Bis-(trimethylsilyl)-N-methylleucine	=	-
Octadecanoic acid, methyl ester	=	x
Octadecanoic acid, trimethyl silyl ester	=	x

When the compounds found in urine MSK and R+O patients and healthy controls analysed with the HB method were compared no significant differences were found.

Comparison between R+O, MSK and IBS

PCA

PCA was done of the chromatograms obtained through the three GCMS analyses, although no clustering could be observed (see **Figure 51**). PCA takes into account all the compounds found in urine. No clustering was observed because the compounds responsible for the major differences (those present in major quantities) in the urine did not reflect the differences between R+O, MSK and IBS.

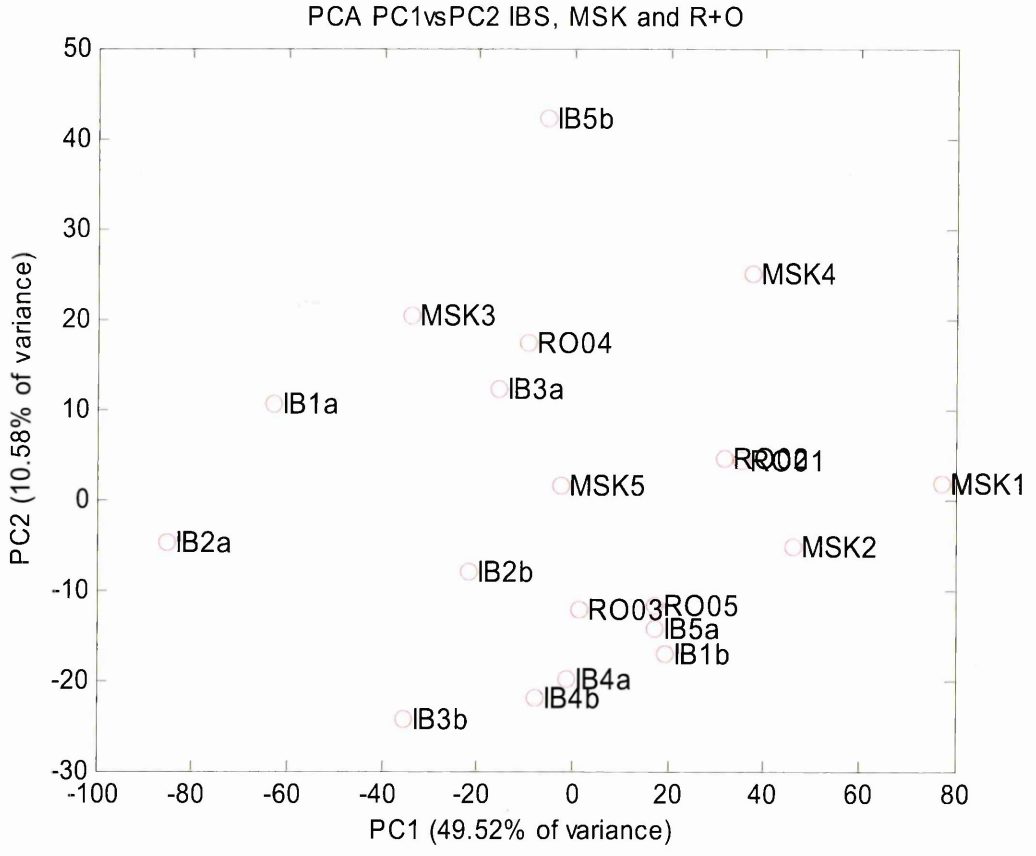


Figure 51. PCA of PC1 versus PC2 of the chromatograms of UB urine obtained of R+O and MSK patients with IBS patients before and after the diet. RO01-05 are R+O

patients, MSK1-5 are MSK patients, IBa patients are IBS patients before the diet and IBb are IBS patients after the diet.

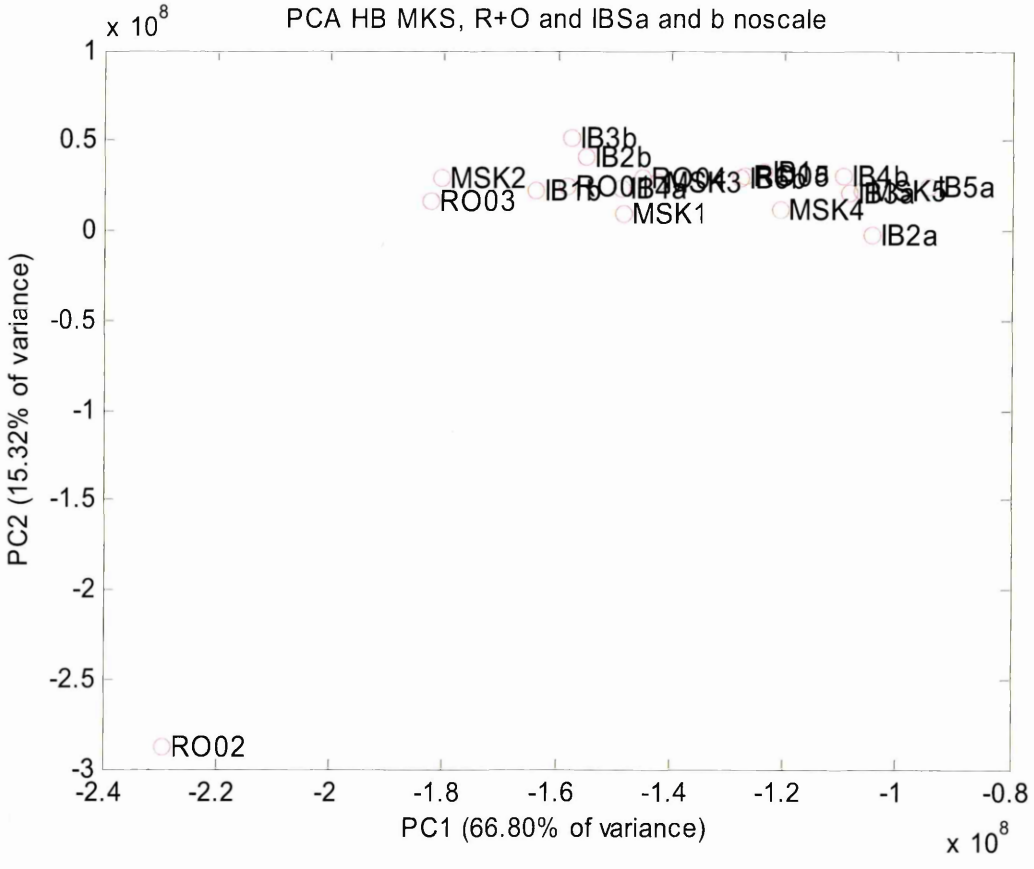


Figure 52. PCA of PC1 versus PC2 of the chromatograms of HB urine obtained of R+O and MSK patients with IBS patients before and after the diet. RO01-05 are R+O patients, MSK1-5 are MSK patients, IBa patients are IBS patients before the diet and IBb are IBS patients after the diet.

No conclusion can be reached from the PCAs of these analyses, since no clustering can be observed. However, there is a R+O patient's urine who had significantly different urine from the rest of the patients. This patient's urine probably had a compound or variety of

compounds different from the rest. This was not looked at in detail because it is not relevant for the course of this study.

Individual Compounds found in urine

When compounds were analysed individually, in the comparison of R+O with MSK and IBS patients suspected to suffer from abnormal colonic fermentation we can observe a trend, where compounds are normally in similar levels in patients with MSK GI problems and with R+O. However, many compounds decrease or increase in the urine of IBS patients when a fibre free or an exclusion diet was undertaken. An example of this trend can be seen in Figure 53 and Figure 54 where 1,2-ethyl methyl benzedicarboxylic acid and 2-nonyl undecanoic acid methyl ester are increased in IBS but decrease after the diet. These compounds are likely to be present in urine as benzedicarboxylic acid and undecanoic acid. Table 18, Table 19 and Table 20 show the different metabolites identified with the three different techniques with the description of their behaviour in the different conditions and the patients with IBS after 2 weeks of an exclusion diet.

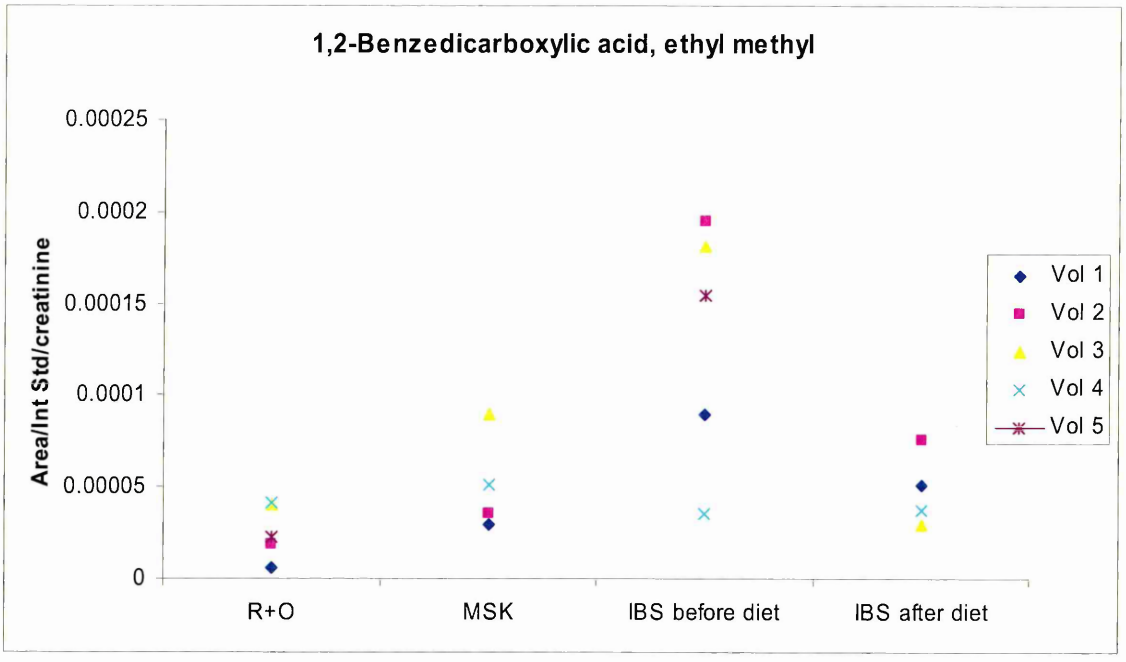


Figure 53. Area of 1,2- ethyl methyl benzedicarboxylic acid, taking into account internal standard and creatinine, of R+O, MSK and IBS patients before and after the diet.

Undecanoic acid, 2-nonyl-methyl ester was also found to be increased in IBS patients, and it decreased after the diet.

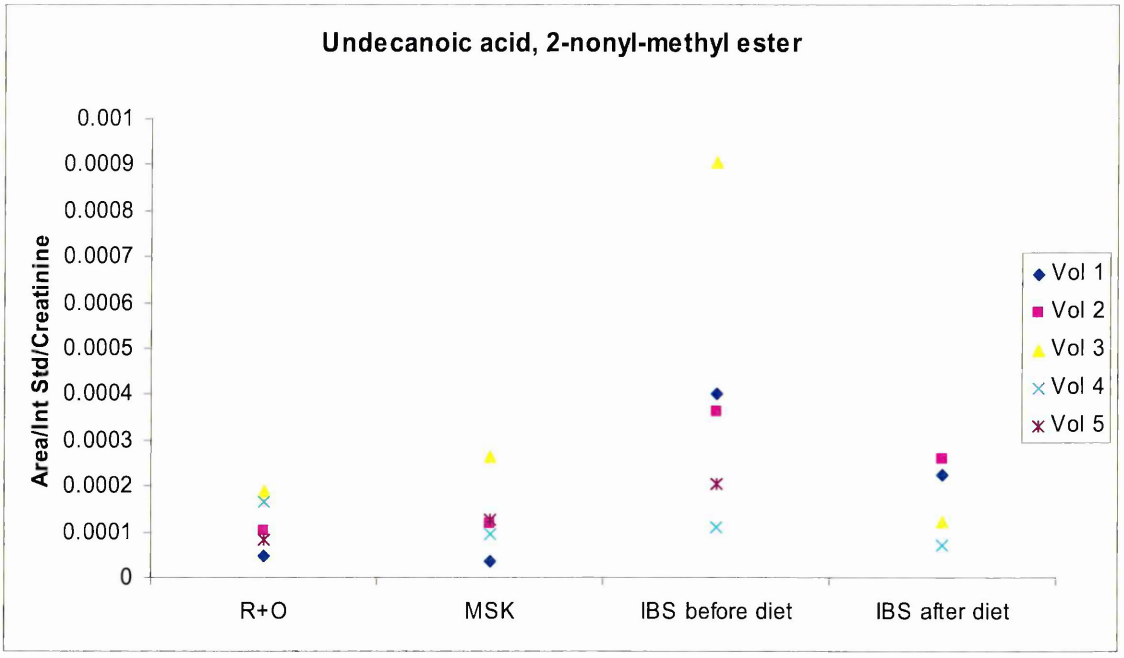


Figure 54. Area of 2-nonyl undecanoic acid methyl ester taking into account internal standard and creatinine, of retention R+O, MSK and IBS patients before and after the diet.

Table 18. Compounds found in urine of patients with MSK GI problems, R+O problems and IBS patients before and after the diet, analysed with the HAN method (↑=increased, ↓=decreased, *=significant (p<0.05), x=not significant, -=not available, √=thought to be related to bacterial fermentation).

Compound	MSK, R+O and IBS comparison	MSK, R+O and IBS statistical difference	MSK and R+O statistical difference Presence/Absence	MSK and R+O statistical difference when present	IBS after the diet	Statistical difference Presence/Absence	Statistical difference when present	Bacterial fermentation?
N-ethyl-4-methyl-4-decanamine	↑ in IBS	*	-	x	**↑ before diet**	-	x	√
N-3-butenyl-N-methyl benzenamine	↑ in R+O	x	x	x	-	-	x	x
4-amino, a-a-dimethyl-, benzeneacetic acid methyl ester	=	x	x	x	-	-	x	√
2,3-dihydro-3,3-dimethylindole-2-one	**Only in R+O**	x	x	-	-	x	*	√
Benzothiazole, 2-methylthio	=	x	-	x	↓	x	x	√
Methyl tetradecanoate	=	x	x	x	=	-	x	x
1,2- ethyl methyl benzedicarboxylic acid,	**↑ in MSK**	*	x	x	↓	x	x	√
2-nonyl- undecanoic acid methyl ester	=	x	-	x	↓	-	x	√
Methyl 3-(3-(3-methoxycarbonyl)-4-methyl-5-pentyl-2-furanyl)propionate	↑ in MSK	x	-	x	↓	-	x	√
Octadecanoic acid, methyl ester	=	x	-	x	↓	-	x	√

3-(2-Iodoetyl)-1,2-dimethoxybenzene	↑ in MSK	*	-	x	=	-	x	x
4,4'-methylenbis(N,N-dimethyl) benzenamine	↑ in IBS	*	-	x	↓	-	x	√
1,2- diisooctyl ester benzenedicarboxylic acid	↑ in IBS	*	-	x	↓	-	x	√
3-carboxy-4-methyl-5-propyl-2-furanpropionic acid, dimethylesther	Present only in R+O	x	x	-	=	-	-	x
4,4-methylenbis NN-dimethyl benzenamine	=	x	-	x	=	-	x	x
N-3-chloropropyl N-methyl benzenesulfonamide	Present more often in R+O	x	x	x	↓	-	x	√

Table 19. Compounds found in urine of patients with MSK GI problems, R+O problems and IBS patients before and after the diet, analysed with the UB method (↑=increased, ↓=decreased, *=significant (p<0.05), x=not significant, -=not available, √=thought to be related to bacterial fermentation)

Compound	MSK, R+O and IBS comparison	MSK, R+O and IBS statistical difference	MSK and R+O statistical difference Presence/Absence	MSK and R+O statistical difference when present	Bacterial fermentation ?	IBS after the diet	Statistical difference Presence/ Absence	Statistical difference when present
Bistrimethylsilylmonostearin	↑ in MSK	x	-	x	√	↓	-	x
trimethylilylester cholesterol	↑ in R+O	x	-	x	x	=	-	x
2,3-bis trimethylsilyloxypropyl ester hexadecanoic acid	**↑ in MSK**	**	-	x	√	↓	-	x
Hexadecanoic acid trimethyl ester	=	x	-	x	√	↓	-	x

Dodecamethyl pentasiloxane	Normally ↑ in MSK	x	x	x	√	↓	-	x
2,3-bistrimethylsilyloxyl-trimethylsilyl ester propanoic acid	↓ in MSK	x		-	x	=	x	-
Silanol, trimethyl-triester with arsenic acid	↓ inMSK	x	x	x	√	↓	-	x
1H-Indole-3-acetamide, 1-trimethylsilyl	↑ in MSK	x	-	x	√	↑	-	x
α-4-bis((TMS)oxy)-benzeneacetic acid, TMS	↓ inMSK	x	x	x	√	↓	-	x
1-Piperidinecarboxaldehyde, 2-(1-formyl)-2-pyrolidinyl)	↑ in MSK	x	-	x	√	↓	-	x
N,N-diphenyl formamide	=	x	-	x	x	=	-	x
Proline	=	x	-	x	√	↑	-	x
Hexadecanoic acid, TMS	=	x	-	x	√	↓	-	x
Heptadecanoic acid, TMS	=	x	-	x	√	↓	-	x
11-cis-octadecanoic acid, TMS	=	x	-	x	√	↓	-	x
Octadecanoic acid, TMS	↓ in MSK	x	-	x	√	↓	-	x
Cyclohexane, (1-hexyltetradecyl)	↓ in MSK	x	-	x	√	↓	-	x
Arachidonic acid, TMS	=	x	-	x	x	=	-	x
Heptacosane	=	x	-	x	√	↓	-	x
Pentacosane	=	x	-	x	√	↓	-	x
Bis(2-ethylhexyl)phtalate	=	x	-	x	√	↓	-	x
2,3-bis(TMS)oxy, hexadecanoic acid propyl ester	**↑ in IBS**	**	-	**x**	√	↓	-	**x**

Table 20. Compounds found in urine of patients with MSK GI problems, R+O problems and IBS patients before and after the diet, analysed with the HB method (↑=increased, ↓=decreased, *=significant (p,0.05), x=not significant, -=not available, √=thought to be related to bacterial fermentation)

Compound	MSK, R+O and IBS comparison	MSK, R+O and IBS statistical difference	MSK and R+O statistical difference Presence/Absence	MSK and R+O statistical difference when present	IBS after the diet	Statistical difference Presence/Absence	Statistical difference when present	Bacterial fermentation?
Octadecenamide	↑ in IBS	x	-	x	↓	-	*	√
1-(2-Isopropenyl-3-methoxy-2,3-(dihydrobenzofuran-5-yl)-7,8-dimethoxy-3a,9B-(4H)-dihydro(1) benzopyrano (4,3)isodiazole (?)	↑ in IBS	x	-	x	↓	-	x	√
Acetic acid, (3,4-dimethoxyphenyl)(TMS)-methyl ester	↑ in IBS	x	-	x	↓	-	*	√
Benzesulfonamide, N-(3-chlorapropyl)-N-methyl	↑ in IBS	x	-	x	↓	-	*	√
Dibutyl phtalate	↑ in IBS	x	-	x	↓	-	*	√
Hexanoic acid, TMS	↑ in IBS	x	-	x	↓	-	*	√
9-Octadecenamide, (Z)	↑ in IBS	x	-	x	↓	-	*	√
TMS ester of glicerol	↑ in IBS	x	-	x	↓	-	*	√
Octadecanoic acid, 2,3-bis((TMS)oxy)propyl ester	↑ in IBS	x	-	x	↓	-	x	√
1H-Indole-3-carboxylic acid, 2-ethoxy-1-(TMS)-5-((TMS)oxy)-ethyl ester	↑ in IBS	x	-	x	↓	-	*	√

Comparison between all groups:

A comparison of the urine obtained from healthy controls, R+O patients, MSK patients and IBS patients before and after the diet was done. The analysis of this urine was done through SPME.

PCA

In Figure 55 the PCA of the chromatograms of the urine of the different groups can be seen. PC 1 accounts for 50% of the variation, and it shows a clear difference of healthy controls from the rest of the volunteers. IBS patients before the diet are also separated from the rest of the volunteers but are more dispersed. All samples were done at different times, so there is a variation due to the differences in the analytical technique. However, all the differences seen in the PCA cannot be attributed to this difference, since there is a clear difference between those samples analysed at the same time.

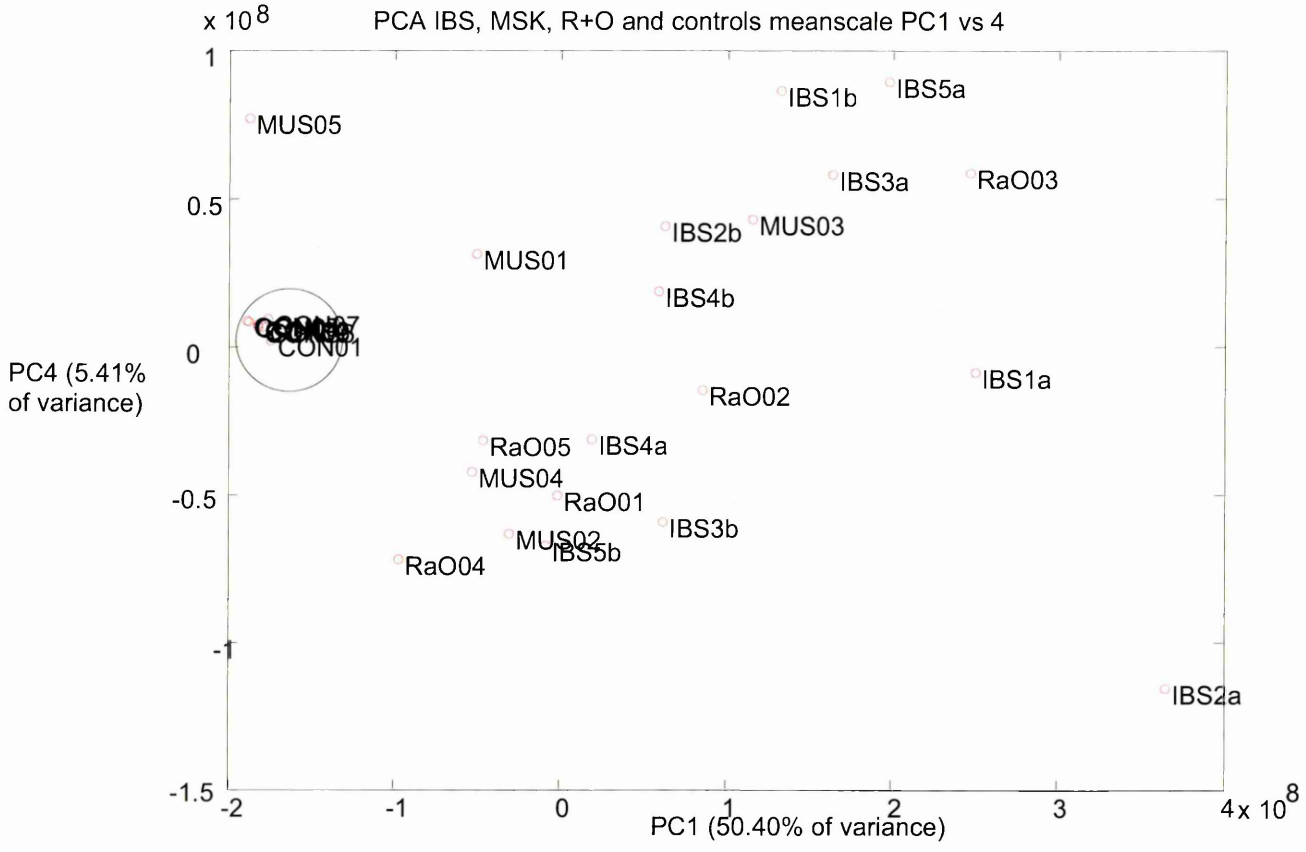


Figure 55. PCA of PC1 versus PC4 of the chromatograms of the urine analysed by SPME. R+O, MSK, IBS patients before and after the diet and healthy controls were compared. IBS1-5a are IBS patients before the diet, IBS1-5b are IBS patients after the diet, CON01-07are healthy controls, RaO01-05 are R+O patients and MUS01-05 are MSK patients

Compounds

The peak area under the curve (AUC) of individual compounds found in the urine of IBS patients analysed using the SPME technique was divided by the AUC of the internal standard and the creatinine derivative and analysed. First, a comparison of these compounds found in R+O, MSK, IBS patients and controls was done. Some compounds were present in similar quantities, although some are shown statistically to be either increased or decreased. Patients suffering from IBS suspected to be due to abnormal

fermentation were put on a fibre free or exclusion diet. There is a trend towards AUCs of IBS patients being increased which return to normal when they are put on a diet. An example can be seen in Figure 57 and Figure 58. In Table 21 a list of all the compounds found in the analysis of IBS, MSK, R+O and controls analysed with the SPME technique can be seen.

Table 21. Table of compounds found in urine of patients with IBS, MSK, R+O and controls analysed with the SPME method. The table shows a comparison between the compounds found in the four groups and also a comparison of these compounds found in the urine of IBS patients before and after the diet. There is also a reflection on the possibility of these compounds due to fermentation.

	R+O/MSK/IBS/Controls	When present	Before and after diet	Before and after the diet presence/Absence	Before and after the diet when present	Fermentation?
Acetone	↑in IBS	x	↓	x	x	√
Ethyl acetate	Not present in controls	-	↓	x	-	√
Cyclohexane	**↑ in IBS**	**	=	**x**	**x**	**x**
Hexane, 2,2-dimethyl	**↑ in IBS**	*	↓	**x**	**x**	√
Butanal, 3-methyl	↑ in R+O, MSK and IBS	x	↓	x	x	√
Furan, 2,5-dimethyl	=	x	↓	x	x	√
Furan, 2,4-dimethyl	=	x	↓	x	x	√
Dimethyldisulfide	=	x	↓	x	x	√
Hexanal	=	x	↓	x	x	√
Ethylbenzene	**↑ in controls**	**	↓	**x**	**x**	√
Benzene, 1,2-dimethyl	**↑ in R+O, ↓ in controls**	**	=	**x**	**x**	**x**
4-Heptanone	=	x	↓	x	x	√
Benzene, 1,3-dimethyl	=	x	↓	x	x	√
3-Heptanone	**↑ in IBS**	*	↓	**x**	**x**	√
2-Heptanone	=	x	↓	x	x	√
Heptanal	↓ in controls	*	↓	**x**	**x**	√
Pyridine, 2-ethyl-6-methyl	=	-	↓	x	x	√
Oxepine, 2,7-dimethyl	=	x	↓	x	x	√
2H-Pyran, 2-ethenyltetrahydro-2,6,6-trimethyl	=	x	↓	x	-	x
Hexanal, 2-ethyl	↓ in controls	x	↓	x	x	√
Dimethyl trisulfide	=	x	↓	x	x	√

1,3,8-p-menthatriene	↓ in controls	*	↓	x	x	√
Benzaldehyde	↓ in controls	*	↓	x	x	√
Cyclohexene, 1-methyl-4-(1-methjylethylidene)	=	x	↓	x	x	√
Limonene	=	x	=	x	-	x
Benzene,1-methyhl-2-(1-methylethyl)	=	x	=	x	x	x
1,3,7-Octatriene, 3,7-dimethyl	=	x	=	x	-	x
1,4-cyclohexadiene, 1-methyl-4-(1-methyl)	=	x	↓	x	x	√
1-Hexanol, 2-ethyl	↑ in IBS	**	↓	x	x	√
Cyclohexene, 4-1-methyl-4-(1-methylethylidene)	=	x	=	x	-	x
2-butoxyethylacetate	=	x	↓	x	x	√
Benzene, 1-methyl-4-(1-methylethenyl)	=	x	↓	x	x	√
2-Furanmethanol, 5-ethenyltetrahydro-a-a,5-trimethyl-, cis	=	x	↓	x	-	√
1,6-Octadiene-3-ol, 3,7-dimethyl	=	x	=	x	x	x
1,3,8-p-menthatriene	=	x	↓	x	x	√
Nonanal	↑ in IBS	*	↓	x	x	√
1,5,7-Octatriene-3-ol, 3,7-dimethyl	=	x	↓	x	-	x
2,2-dimethyl-1-oxa-2-silacyclo-3,5-hexadiene	=	x	↓	x	x	√
2,6-dimethyl-1,3,5,7-octatraene, E	=	x	↓	x	x	√
3-cyclohexen-1-ol, 1-methyl-4-(1-methylethyl)	Present only in IBS	-	↑	x	-	√
Pentanoic acid	↑ in IBS	x	↓	x	x	√
Indole	=	x	=	x	x	√
Hexanoic acid, 2-ethyl	↑ in IBS, no present in controls	-	↓	x	x	√
Acetic acid	Present only in IBS	-	↑	x	-	√
Phenol	↑ in IBS, not present in controls	-	↓	x	-	√
Heptanoic acid	=	x	↓	x	x	√
Octanoic acid	=	x	=	x	-	√
Nonanoic acid	↓ in controls	*	↓	x	x	√
Phenol, 2-methyl	↓ in controls	*	=	x	-	√

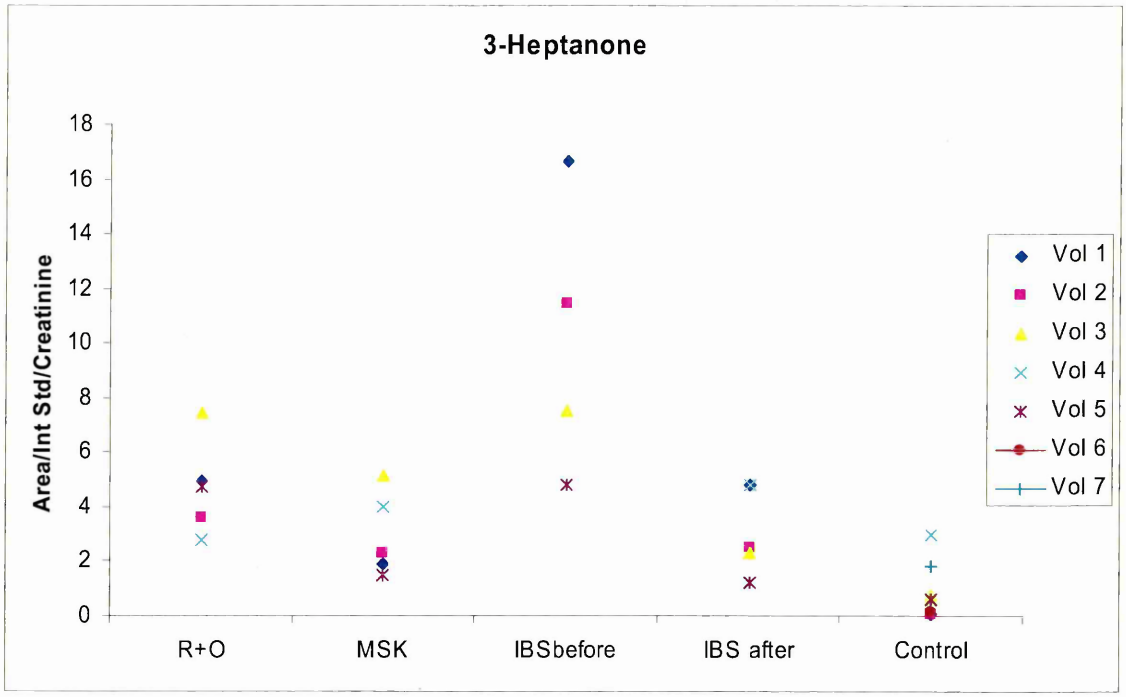


Figure 56. Area under the curve of 3-Heptanone in urine analysed by SPME of R+O, MSK, IBS patients before and after the diet and healthy controls.

2-ethyl, 1-hexanol was found increased in the IBS patients in comparison with controls, R+O and MSK patients. When IBS patients were put on a diet the concentration in urine of this compound decreased.

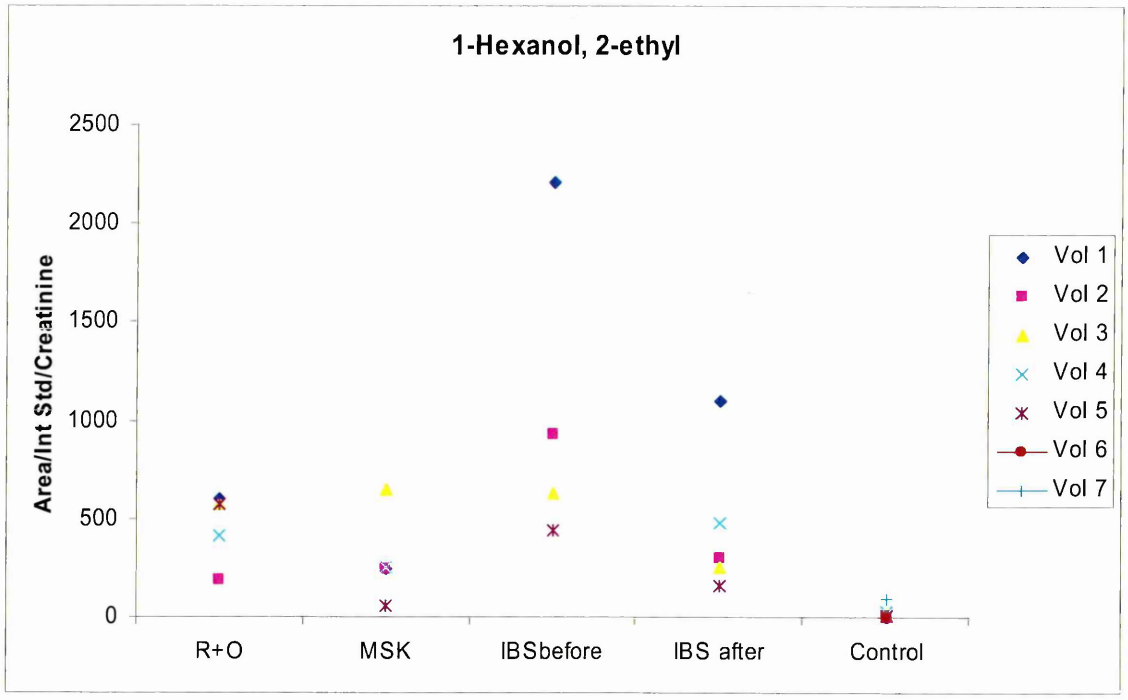


Figure 57. Area under the curve of 2-ethyl 1-Hexanol in urine analysed by SPME of R+O, MSK, IBS patients before and after the diet and healthy controls.

There are other compounds that are increased in IBS, MSK and R+O, in comparison with controls and IBS after the diet, such as butanal, 3-methyl (see Figure 58).

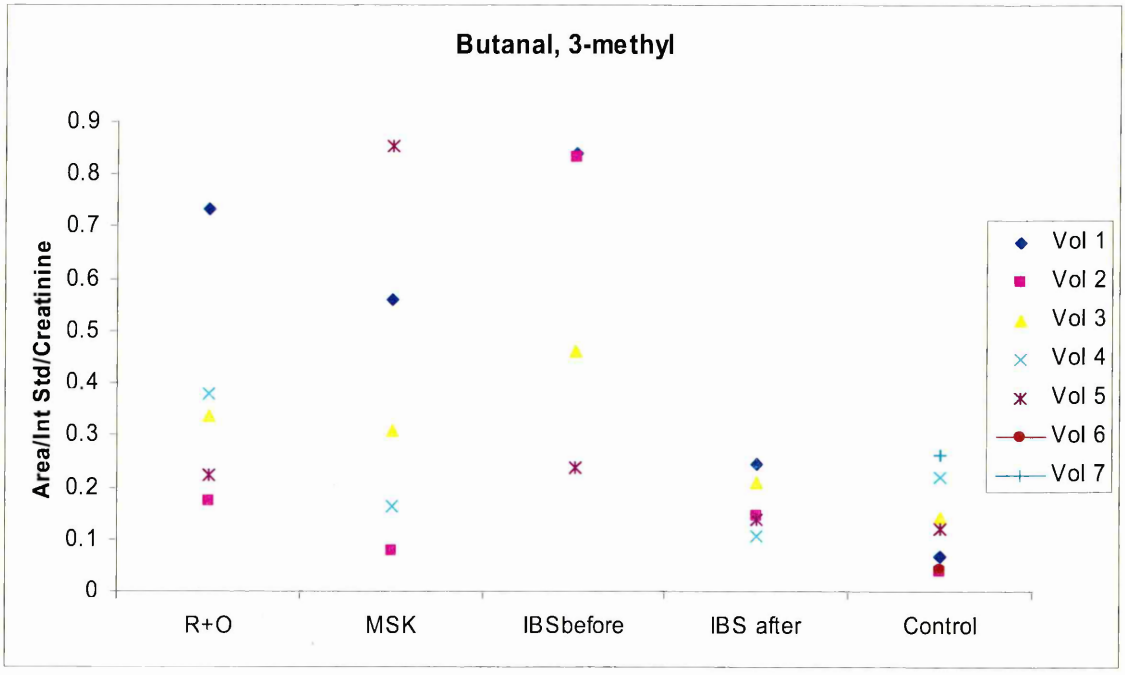


Figure 58. Area under the curve of butanal, 3-methyl in urine analysed by SPME of R+O, MSK, IBS patients before and after the diet and healthy controls.

Measurements of pH

The pH of the samples of urine provided by the MSK, R+O and IBS patients was measured (see Table 22), although no significant differences were seen among the pH of the urine samples Also, there was no significant change between the pH of the urine of IBS patients before and after the diet.

Table 22. pH of urine of R+O, MSK, IBS patients before the diet (IBS1) and IBS patients after the diet (IBS2).

Volunteer	R+O	MSK	IBS1	IBS2
1	7	5	5	5
2	7	5	5	5
3	7.5	7.5	N/A	6
4	6	5.5	7	7
5	6.5	7.5	6	6.5
Mean	**6.80**	**6.10**	**5.75**	**5.90**

Discussion

Patients suffering from GI problems can sometime be misdiagnosed with IBS due to their similarity in symptoms. Food intolerance is a major factor causing IBS (Jones et al., 1982), however, not every case improves on diet, and other factors can also produce IBS, such as hyperventilation and aerophagia, R+O, menstrual disorders, simple constipation, gall bladder problems and MSK (Anon., 2003). Here we studied those IBS patients suffering from food intolerance, MSK and R+O.

The Rome criteria includes as IBS to those patients presenting with R+O and MSK GI problems, but in fact, symptoms are different. R+O patients present a mild constipation, overflowing the bowel approximately every week, leading to painful diarrhoea. MSK GI problems are thought to be abdominal pain caused by pressure on nerves supplying the abdominal wall but there are no bowel symptoms. IBS patients present abdominal pain and variable bowel habit.

The symptoms observed in these conditions are different and they do not respond to treatment in the same way. R+O patients improve when they are given laxatives, MSK patients with painkillers and IBS with food intolerance with exclusion or low fibre diets. For this reason a new criterion should be outlined for the identification of IBS. The importance of the identification of IBS subgroups has already been described by Ragnarsson, G. and Bodemar, G. (Ragnarsson & Bodemar, 1999), Mätö et al. (Mättö et al.,

2005) ,Walter et al. (Walter et al., 2005) and Guilera, M. et al. (Guilera et al., 2005). Better understanding of the IBS clinical profile is fundamental not only from a diagnostic point of view but to be able to plan the type (symptom-directed) and the timing of the therapy (Guilera et al., 2005). Two pain/bloating subgroups and three bowel habit subgroups have been identified (Ragnarsson & Bodemar, 1999), but these subgroups do not really reflect the exact subgroups observed in this trial.

The symptoms observed in R+O, MSK and IBS are different, and the metabolites found in urine are also different as there are compounds that are present in different quantities in IBS patients than in R+O and MSK patients, such as 3-heptanone and 2-ethyl-1-hexanol. The significance of this is immense, because not only reflects a possible different cause of the GI problems but a possible tool for diagnosis of the different conditions. When R+O, MSK and healthy volunteers were compared, the metabolites that were found in different quantities in their urine did not correspond to any of the compounds found to change in IBS patients after the fibre free diet. These compounds that were present only in controls, R+O or MSK patients should be investigated further for possible application, to be applied as a diagnostic tool for these conditions. However, there is no evidence to think these compounds might have a bacterial origin, since they did not change when IBS patients were put on a diet that is thought to decrease bacterial activity.

The PCA done on the chromatograms of urine of MSK and R+O compared with controls show that MSK and R+O are very similar in comparison with controls. However, when other, bacterial fermentation-related IBS patients are taken into account, controls are very different to IBS, and MSK and R+O are in clustered in between these two extremes (see Figure 55). At the same time, IBS patients samples, after an exclusion or fibre free diet are situated where the MSK and R+O patients are, closer to urine normality.

IBS patients suspected to suffer from abnormal bacterial fermentation were put on an exclusion or fibre free diet. The effectiveness of exclusion diets (King et al., 1998) (Parker et al., 1995) and fibre free diets in IBS has been proven (Dear et al., 2005) (Francis &

Whorwell, 1994). Reduced bacterial ativity has been argued by a reduction in the total volume and rate of excretion of hydrogen over 24 hours.

When R+O and MSK were compared with IBS patients before and after a diet, there were several compounds observed to change in IBS patients after the diets, probably due to the change of bacterial fermentation. Some of these compounds were observed to be altered in IBS patients in comparison with the R+O and MSK patients. For instance, octadecanoic acid, methyl ester was increased in IBS patients and when these patients were put on the diet, it decreased. This compound might be a derivative of octadecanoic acid produced through the analysis. As a carboxylic acid, it is thought to be a bacterial product, and this hypothesis is supported by these findings.

4,4-methylenbis N,N-dimethyl benzenamine, is observed to be increased in IBS patients and it decreased after the patients followed either diet. This phenomenon was also observed in compounds such as 1,2-Benzedicarboxylic acid, ethyl methyl; 2-nonyl undecanoic acid, methyl ester and N-ethyl-4-methyl-4-decanamine.

Several compounds that decreased after the diet were observed to be increased in IBS patients on a normal diet. These compounds are thought to be related to bacterial fermentation. This suggests that a possible cause of IBS is increased fermentation. In this study, a semi-quantitative analysis of the compounds was done. Those compounds thought to be related to bacterial fermentation should be studied further. Other studies should be done where bacterial activity is decreased. A quantitative analysis should be done to establish the possible link with bacterial activity as they could be used as biomarkers of the condition in the future. The compounds that might be biomarkers of the abnormal fermentation in IBS are highlighted in Table 23 such as 2,2-dimethyl hexane and 3-heptanone.

The compounds observed in IBS are normally different to those observed in the healthy volunteers, but there are some compounds which are present in both. In Table 23 a relationship of those compounds found in IBS and healthy adults can be seen, as well as the

way these compounds change after enteral feeds in the case of healthy volunteers or fibre free or exclusion diet in the case of IBS.

Table 23. Summary of the compounds which are found in urine of healthy volunteers before and after the Elemental E028 and the Modulen diet and in IBS patients before and after the exclusion or fibre free diet. ↑ means increased, ↓ means decreased, = means no change, MSK means musculoskeletal IBS, R+O means retention and overflow IBS, IBS means IBS patients suspected to have abnormal fermentation and IBS before means IBS patients before the exclusion or fibre free diet.

Compound	Modulen	E028	IBS comparison	IBS on diet
3-carboxy-4-methyl-5-propyl-2-furapropionic acid, dimethyl ester	↓	↓	Present only in R+O	=
4,4-methylenebis(N,N-dimethyl) benzenamine	↑	↓	same in R+O and MSK but ↑ in IBSbefore	↓
N-(3-chloropropyl) N methyl benzesulfonamide	↑	↑	**Present more often in R+O and IBSbefore**	↓
Methyl 3(3-methoxycarbonyl) 4-methyl-5-pentyl-2-furanyl propionate	↓	↓	=	↑
Octadecanoic acid, methyl ester	↑	↑	**same in R+O and MSK but ↑ in IBSbefore**	↓
Bis(trimethylsilyl) monostearin	↑	-	=	↓
Hexadecanoic acid, trimethylsilyl ester	↓	↓	=	↓
Octadecanoic acid, trimethylsilyl ester	↓	↓	=	↓
Hexadecanoic acid,2,3-bis((trimethylsil)oxy)propyl ester	↑	↑	**↑ in MSK and ↑ in IBS**	↓
Acetone	↑	↑	**↑in IBS**	↓
Cyclohexane	↑	↑	↑ in IBS	=
2,2-dimethyl hexane	↓	↓	**↑ in IBS**	↓
3-methyl butanal	↑	↑	↑ in R+O, MSK and IBS	↓
2,5-dimethyl furan	=	=	=	↓
2,4-dimethyl furan	=	=	=	↓
Dimethyldisulfide	↑	↑	=	↓
Hexanal	↑	↑	=	↓
Ethylbenzene	↑	↑	**↑ in controls**	↓
1,2-dimethyl benzene	=	=	↑ in R+O, ↓ in controls	=
4-Heptanone	=	=	=	↓
1,3-dimethyl benzene	=	=	=	↓
3-Heptanone	=	↑	**↑ in IBS**	↓

2-Heptanone	=	=	=	↓
Heptanal	↑	↑	=	↓
2-ethyl-6-methyl pyridine	=	=	=	↓
2,7-dimethyl oxepine	↓	↓	=	↓
2-ethenyltetrahydro-2,6,6-trimethyl 2H-Pyran	=	↑	=	↓
2-ethyl hexanal	=	=	**↓ in controls**	↓
Dimethyl trisulfide	=	=	=	↓
1,3,8-p-menthatriene	↓	=	=	↓
Benzaldehyde	=	=	=	↓
1-methyl-4-(1-methjylethylidene) cyclohexene	↓	=	=	↓
Limonene	=	=	=	=
1-methyl-2-(1-methylethyl) benzene	=	=	=	=
3,7-dimethyl, 1,3,7-Octatriene	=	↑	=	=
1-methyl-4-(1-methyl) 1,4-cyclohexadiene	↓	↓	=	↓
2-ethyl 1-hexanol	↑	↑	**↑ in IBS**	↓
4-1-methyl-4-(1-methylethylidene) cyclohexene	↓	=	=	=
2-butoxyethylacetate	=	=	=	↓
1-methyl-4-(1-methylethenyl) benzene	=	↓	=	↓
5-ethenyltetrahydro-a-a,5-trimethyl-, cis2-furanmethanol	=	=	=	↓
1,6-Octadiene-3-ol, 3,7-dimethyl	↓	↑	=	=
Nonanal	=	↑	**↑ in IBS**	↓
3-ol, 3,7-dimethyl 1,5,7-octatriene	=	=	=	↓
2,2-dimethyl-1-oxa-2-silacyclo-3,5-hexadiene	=	↑	=	↓
2,6-dimethyl-1,3,5,7-octatraene, E	↑	↑	=	↓
Pentanoic acid	=	↓	**↑ in IBS**	↓
Indole	=	=	=	=
Heptanoic acid	↓		=	↓
Octanoic acid	↑	↑	=	=
Nonanoic acid	↑	↑	=	↓
2-methyl phenol	↑	↑	=	=

Octadecanoic acid, methyl ester is increased in IBS patients and decreases after the diet, as well as in healthy volunteers after they have been on both enteral feeds for a week. As discussed above, this compound is very likely to be due to bacterial fermentation.

4,4-methylenebis(N,N-dimethyl) benzenamine, N-(3-chloropropyl) N-methyl benzesulfonamide, hexadecanoic acid,2,3-bis((trimethylsil)oxy)propyl ester, acetone, 2,2-dimethyl hexane, 3-heptanone, 2-ethyl hexanal, 2-ethyl 1-hexanol, nonanal and pentanoic acid followed the same pattern, and they are very likely to be products of bacterial fermentation.

IBS patients' symptoms improved after the exclusion or fibre free diet, and there is a correlation with the decrease of bacterial compounds. A possible cause of IBS is abnormal fermentation, and when these patients are put on an exclusion diet their symptoms improve. We can conclude that a decrease of bacterial metabolism in the colon is an effective therapy for IBS, as suggested by Madden (Madden & Hunter, 2002) and Dear et al. (Dear et al., 2005). Through these experiments we can conclude that several compounds suspected to be bacterial products because they decreased in the urine of IBS patients after the fibre-free or exclusion diet were increased in IBS patients. Since IBS patients'symptoms improved, it is likely that abnormal bacterial fermentation is an important factor causing IBS.

Conclusion

R+O and MSK patients are frequently misdiagnosed as IBS, but many metabolites found in the urine of these patients compared to IBS are different, therefore we can conclude that R+O, MSK and IBS symptoms have different origin.

A possible pathogenesis of IBS is abnormal bacterial fermentation and a decrease in colonic bacterial metabolism with treatments such as exclusion or fibre free diet aids in the treatment of IBS symptoms.

Some compounds have been detected that could be involved in the pathogenesis and diagnosis of R+O and MSK patients, although these should be investigated further.

A bigger trial should be done with IBS patients on a diet which decreases bacterial activity in order to confirm those bacterial compounds. These compounds should be used as a tool for IBS diagnosis with an increase in the ratios of the areas of these compounds in

comparison with the internal standard and the creatinine leading to a possible positive IBS diagnosis.

These compounds have not been studied in other types of GI problems, so tests are needed to confirm no cross reaction with other GI disorders.

This trial only comprised 5 patients in each group, so a bigger study should be done with quantitative measurements before it can be put in place.

Chapter 5. Autism

Urine samples from autistic children were compared to urine from age-mathced healthy volunteers. The differences in concentration or presence of some compounds were looked at searching for biomarkers for autism. Biomarkers could be used in the early detection of the disease. Bacterial compounds in urine were observed to determine whether fermentation is abnormal in the bowel of autistic children.

Autistic children suffer from gastrointestinal symptoms and autistic symptoms often improve with diets. A comparison of autistic urine and urine of irritable bowel syndrome patients whose symptoms improve following a diet was done to determine whether autism and irritable bowel syndrome might have a similar cause.

Materials and Methods

Patients and matched controls

Autistic patients were recruited from The Loddon School, Hampshire aged 8 to 18. They all suffered from ASD, according to the Rome criteria. Those having received a course of antibiotics in the six weeks prior to the trial were excluded, as well as those with any kidney or urine disease, or other abnormality in urine composition such as diabetes melitus or suffering from IBD, coeliac disease or GI infections. Their parents were asked to fill in a questionnaire about their symptoms and onset. The questionnaire was completed by their carer when required.

Matched age healthy volunteers were recruited from the children of either sex of members of staff of the University of Cranfield at Silsoe to serve as controls. All were to be in normal health, and eating a normal diet. IBS, migraine and pre-menstrual syndrome in particular were excluded by completion of a symptom questionnaire, as all these conditions are possibly associated with abnormal colonic fermentation. Other exclusion criteria were: taking any medication other than the oral contraceptive; having received a course of

antibiotics in the previous six weeks; taking bacterial products such as pro- or prebiotics, and pregnancy and lactation.

Ethics

This study was approved by the North and Mid Hampshire Local Research Ethics Committee (see Appendix 8).

Materials and methods

The patients' carers were asked to collect urine samples in urine collection recipients first thing in the morning. Samples were then transferred into 30 ml PTFE bottles and stored in a -80°C freezer until analysed.

These samples were sent to The Horserace Forensic Laboratory (HFL) Newmarket, to be analysed by GCMS (see Chapter 2). Urine (3 ml) was analysed in three different ways: HAN, UB and HB. The areas of the compounds of interest were measured and then related to the areas of internal standard and creatinine.

These samples were also analysed in house looking for VOCs. Urine (3 ml) was analysed with the method described in Chapter 2 (Materials and Methods). The analysis was done three times and the mean of the areas of each compound was used.

Data analysis

Samples were analysed using PCA and the individual compounds abundances were studied using other statistical methods.

PCA

One of the repeat chromatograms was chosen at random for each subject and they were then transformed into comma separated value (CSV) files using WSearch Pro. Once these files are formed, the 'allgcmsread2' (Jon Lee Davey) script convert them into Matlab files.

PCA was conducted on the data using the "pcagui" function in PLS_Toolbox 2.0 (Eigenvector Research Inc., Manson, USA), which runs as a supplement to Matlab. The variables for the PCA input matrix were intended to be the time coordinates outputted by the TurboMatrix software.

Compound identification and quantification

The compounds found in the urine were identified from the total ion chromatogram (TIC) using the NIST electronic spectra library. The search was done manually and the area under the curve of the selected ion chromatogram was determined. The areas were then divided by the AUC of the internal standard and AUC of the creatinine product of the analysis.

Creatinine of the urine analysed in house was measured with the Creatinine Test Kit (CinnaGen Inc), based on the Jaffe Method (Cook, 1971) (Bartles et al., 1972) as mentioned in the Diagnostics pack.. The measurements were done in a Camspec M350 Double beam UV-Visible Spectrophotometer. The creatinine of the samples sent to HFL was measured as a derivative through the UB analysis: Creatinine enol N1,N3, O-TMS (see Chapter 2).

Statistical analysis

In order to determine whether the probability of a compound to be present in the ASD patients or the controls was significant a Chi-squared test with the Yates correction was used. When present, in order to see if the compound was present in different quantities, Mann Whitney was used. In order to analyse the pH of the urine found, an ANOVA test was done.

Results

Eight autistic subjects were recruited, aged 8 to 18 years, of which one was female and seven male. Eight matched age and sex healthy controls were recruited.

Questionnaires

Three of the eight autistic children (38%) recruited presented GI problems. 4 of them (50%) had the ASD onset before the first year, whereas the other 4 started having symptoms in the second year of their lives. Four of them (50%) had a type of food associated with change in their behaviour, principal examples being sugar and chocolate. One of the patients (13% of the autistic children) suffered from something similar to IBS, his parents both suffered from IBS and had found that cheese and wheat worsened the bowel symptoms. He was on a low wheat, low sugar and nut-free diet. He was the only one on a restricted diet, though, 5 patients (62.5%) were regularly given fish oil supplements.

All volunteers were asked about the number of bowel movements they had per week (see Table 24). A very significant difference was found ($p<0.01$), where the average number of movements done by the autistic group was almost 15 whereas the average done by healthy controls was 7.

Table 24. Average number of bowel movements per week done by autistic children and healthy controls.

	Autism	Controls
Volunteer 1	21	5
Volunteer 2	14	10
Volunteer 3	14	10
Volunteer 4	12	6.5
Volunteer 5	7	10
Volunteer 6	14	7
Volunteer 7	14	5
Volunteer 8	21	6
Mean	14.63	7.44

Though this indicates a potentially significant result, the sample population size was small, so a study on the difference in pH of urine samples of autistic children and healthy controls should be done with more subjects to obtain a conclusive result.

PCA

PCA was performed using the chromatograms from all preparation methods. The PCA of the chromatograms obtained in the HAN analysis done by HFL, 2 groups can be seen (see Figure 59), of autistic patients and controls separated by PC1, which accounts for 74% of the variance.

Samples were analysed randomly to avoid the PCA showing a false trend. Therefore the clustering observed in this analysis is purely due to the compounds present in the urine samples.

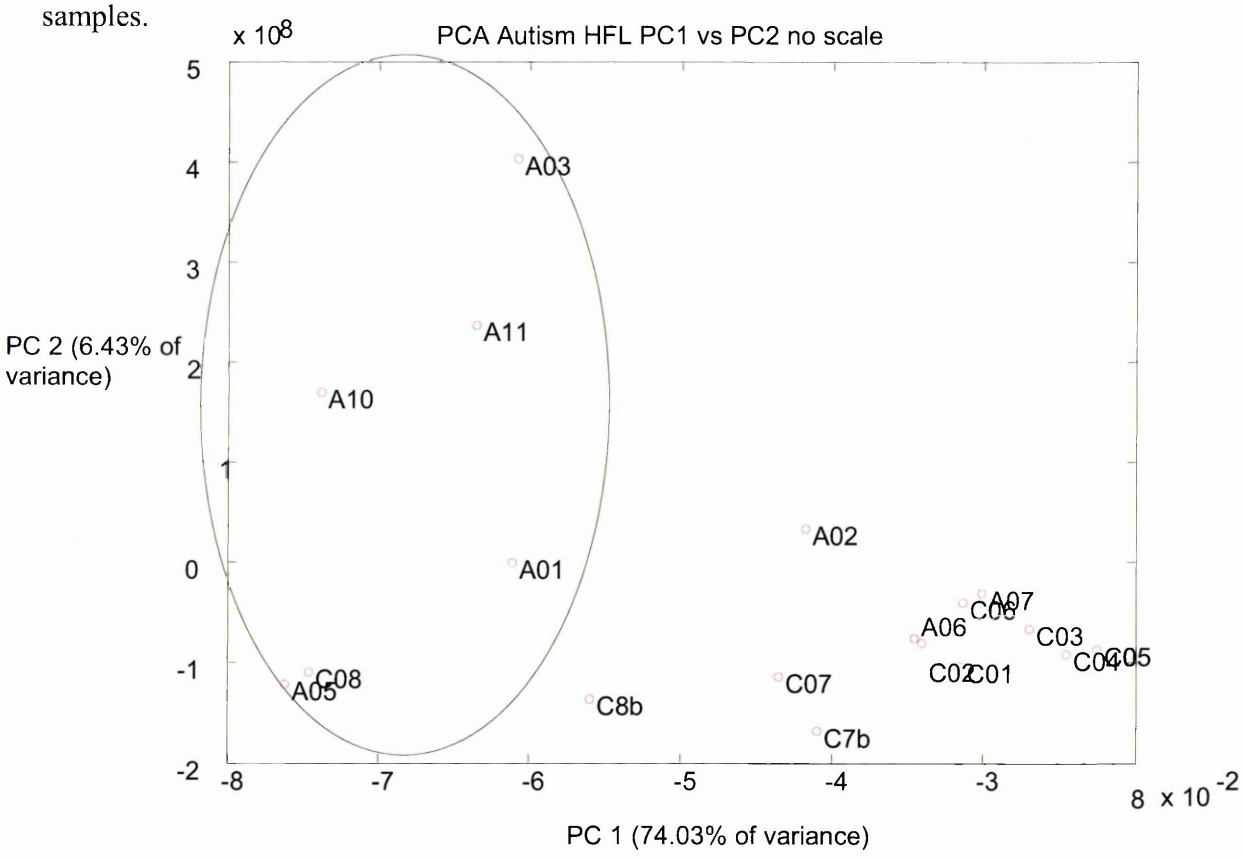


Figure 59. PCA of the chromatograms of the urine samples of autistic children and healthy children analysed by HFL through the HAN method. A01-A11 are autistic patients and C01-C08 are healthy controls.

The PCA done on the chromatograms obtained through the UB and HB analyses gave a very good separation of autistic patients and healthy controls (see Figure 60 and Figure 61).

Clear clusters can be observed in all the PCA plots. In the UB analysis, PC1 clearly separates both groups accounting for 43% of the variance. In HB the PCA created by PC1 and PC3 shows clustering of both groups as well.

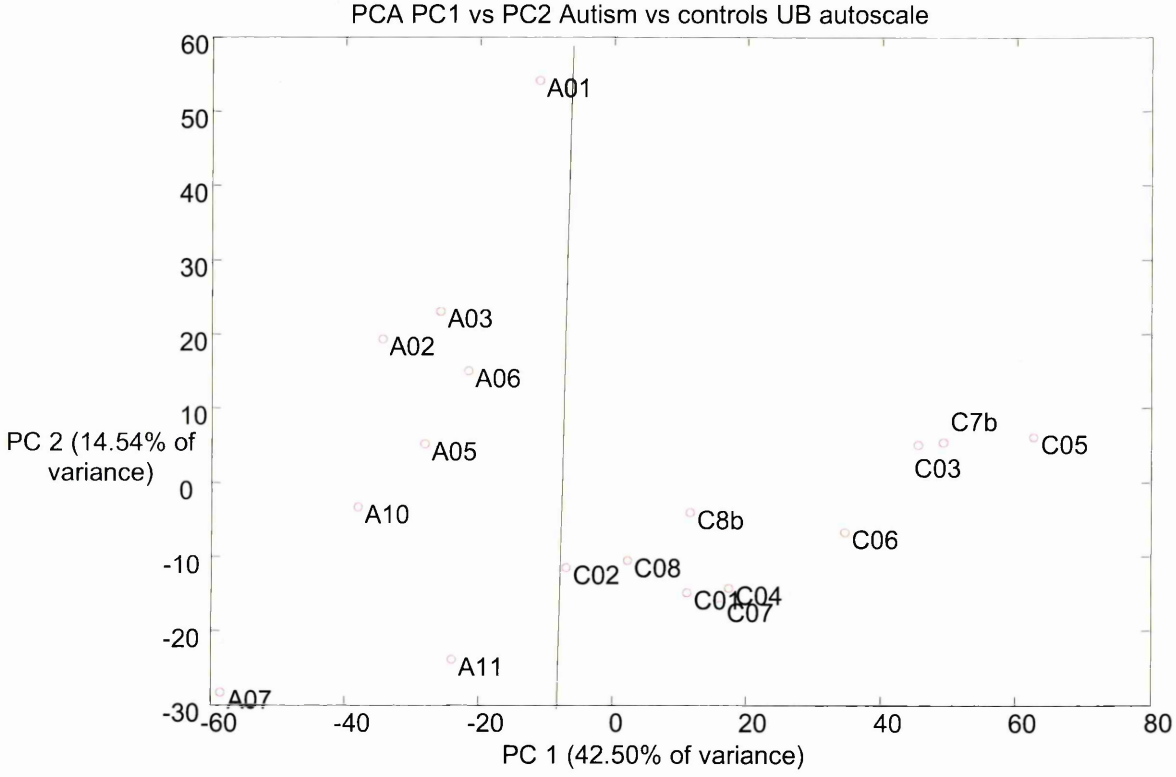


Figure 60. PCA of the chromatograms of the urine samples of autistic children and healthy children analysed by HFL through the UB method. A01-A11 are autistic patients and C01-C08 are healthy controls.

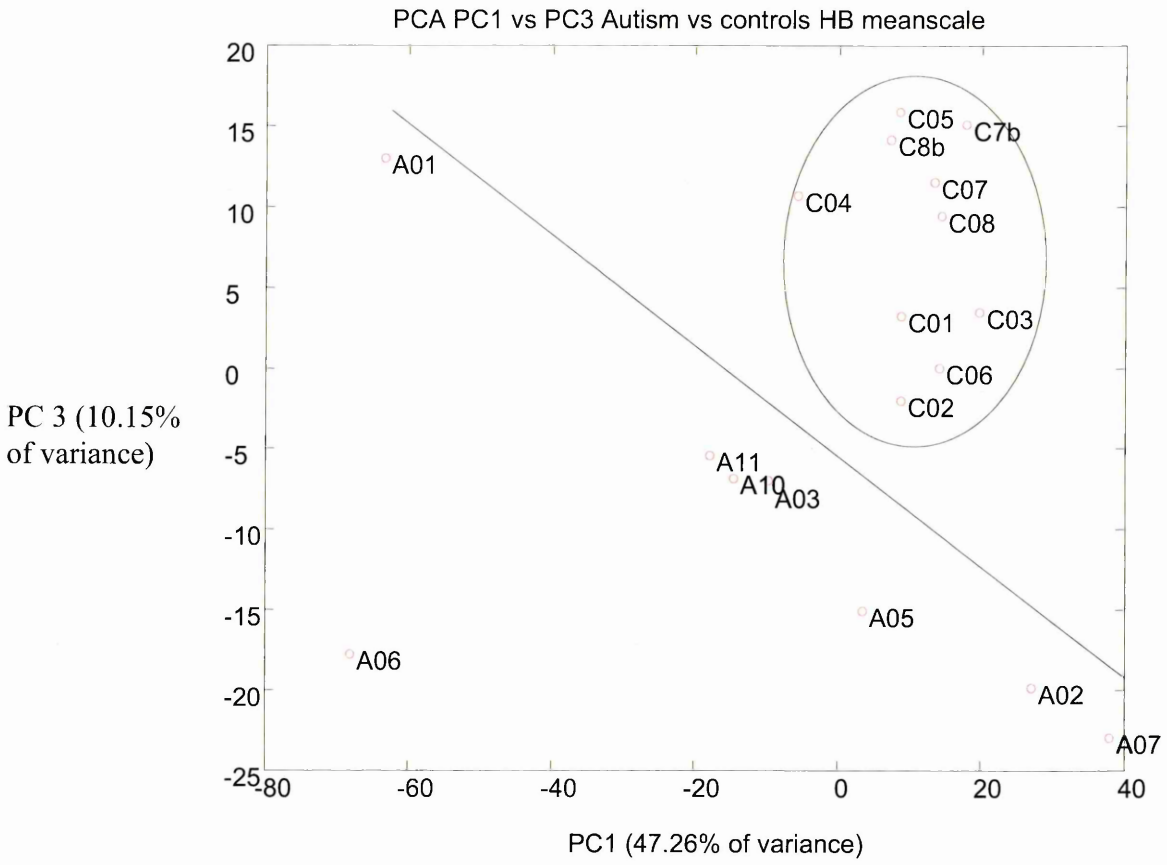


Figure 61. PCA of the chromatograms of the urine samples of autistic children and healthy children analysed by HFL through the HB method. A01-A11 are autistic patients and C01-C08 are healthy controls.

The chromatograms obtained through the SPME technique show a clustering of the healthy volunteers, although the autistic patients are a scattered around the healthy group in the PCA (see Figure 62). All samples were randomised to avoid clustering due to the analysis.

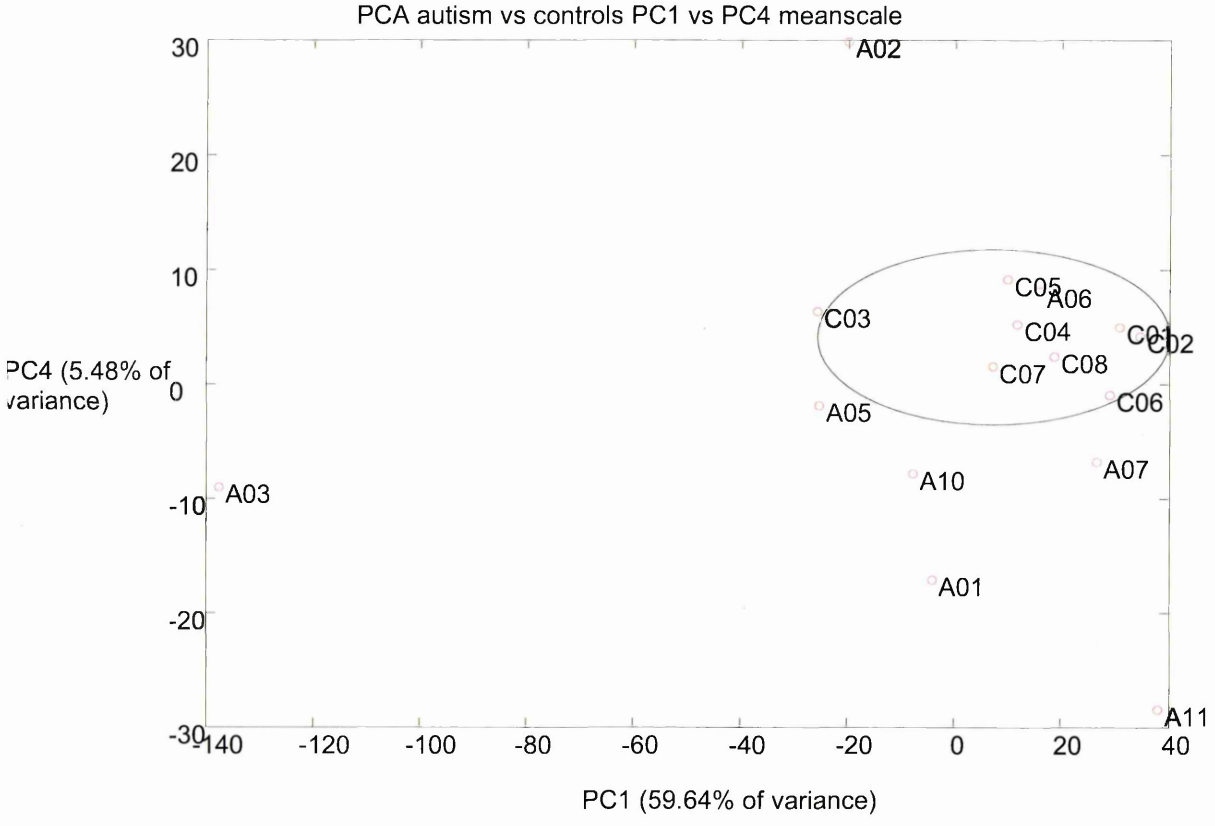


Figure 62. PCA of the chromatograms of the volatile compounds found in urine samples of autistic children and healthy children analysed with the SPME technique. A01-A11 are autistic patients and C01-C08 are healthy controls.

Clustering can be seen with all methods of analysis (see Figure 60, Figure 61 and Figure 62), although the PCAs done on the urine analysed using the HAN, UB and HB methods show a better differentiation of both groups.

Compounds

Different compounds have been observed to be present in different quantities in autistic children in comparison with age matched controls. A list of the compounds detected can be seen in Table 25, Table 26, Table 27 and Table 28. Among all these compounds, many might be of interest aiding early diagnosis of conditions autism. For instance, 1,2,3,4-

tetrahydro-8-methyl,1-naphthalene methanol (see Figure 63) was statistically compared amongst the chromatograms using a Chi-squared test with a Yates correction, and with a probability greater than 99.95% was shown to be present in autistic children, and not in the control group. α,α,δ,8-tetramethyl-, tricyclo(4.4.0.02.7dec)-8ene-3-methanol stereoisomer (see Figure 64), mono-TMS of (pyridoxine-H_20) (see Figure 65) and 3,5-bis (acetyl)-2-(3,8-bis(acetyloxy)-1-octenyl), cyclopentanepropanoic acid, methyl ester (see Figure 66) had a probability greater than 99.99% to be present in autistic children. A Chi-squared test was used. When 3,5-bis (acetyl)-2-(3,8-bis(acetyloxy)-1-octenyl), cyclopentanepropanoic acid, methyl ester was analysed, it was only found to be present in autistic children.

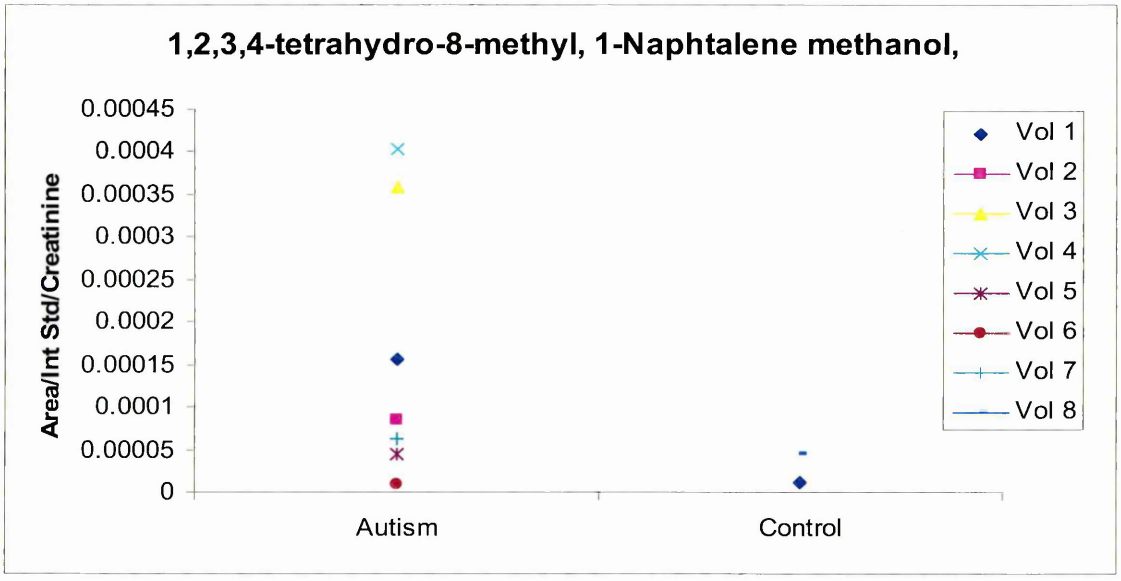


Figure 63. Area under the curve taking into account internal standard and creatinine of 1,2,3,4-tetrahydro-8-methyl, 1-Naphtalene methanol in urine of autistic children and healthy controls. Only two healthy volunteers have this compound present in their urine.

α,α,δ,8-tetramethyl-, tricyclo(4.4.0.02.7dec)-8ene-3-methanol stereoisomer was analysed in all the samples and it was only found to be present in autistic children, not in controls.

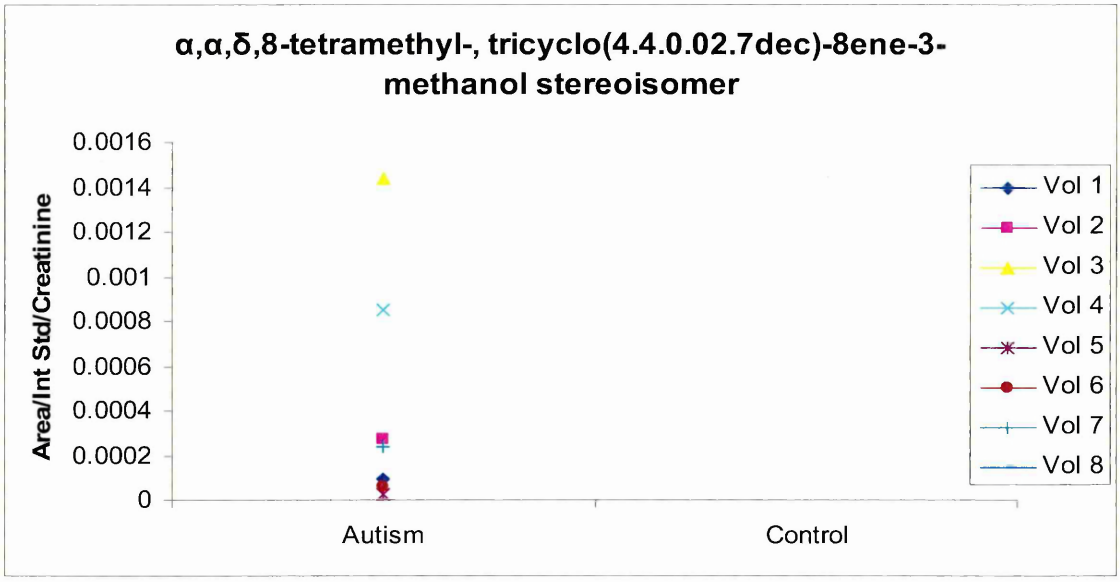


Figure 64 . Area under the curve taking into account internal standard and creatinine of α,α,δ,8-tetramethyl-, tricyclo(4.4.0.02.7dec)-8ene-3-methanol stereoisomer in urine of autistic children and healthy controls. None of the healthy volunteers have this compound present in their urine.

A similar finding was observed with mono-TMS of (pyridoxine- H_20), where it was only found in the urine of autistic children.

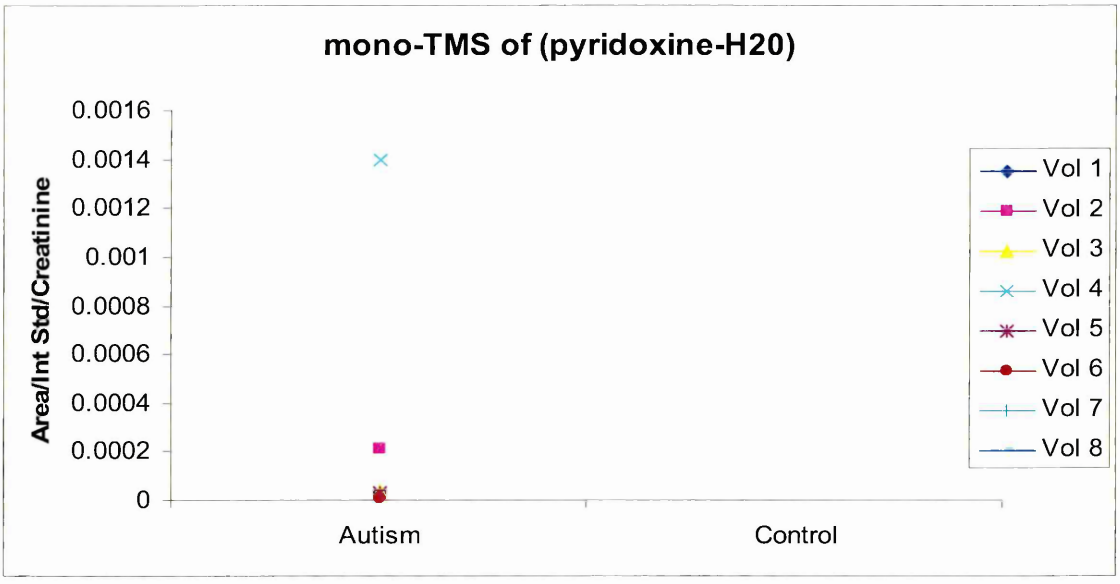


Figure 65. Area under the curve taking into account internal standard and creatinine of mono-TMS of (pyridoxine- H_20) in urine of autistic children and healthy controls. Three of the autistic children had this compound present in their urine, whereas non of the healthy volunteers' urine had it.

When 3,5-bis (acetyl)-2-(3,8-bis(acetyloxy)-1-octenyl), cyclopentanepropanoic acid, methyl ester was analysed, it was only found to be present in autistic children.

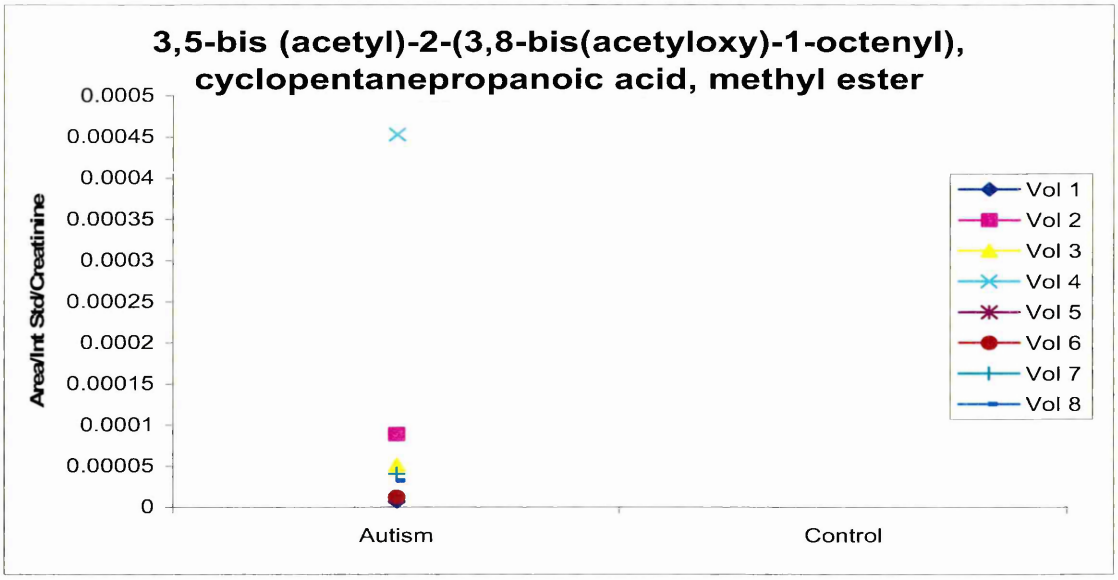


Figure 66. Area under the curve taking into account internal standard and creatinine of 3,5-bis (acetyl)-2-(3,8-bis(acetyloxy)-1-octenyl), cyclopentanepropanoic acid, methyl ester in urine of autistic children and healthy controls. None of the healthy volunteers had this compound present in their urine.

There were only two compounds that were found increased in controls in comparison with autism, implying a lack of these in autistic patients, though there was no statistical significance ($p>0.05$): 2,5-dimethyl furan and 2,4-dimethyl furan (see Figure 67 and Figure 68). These species should be studied further.

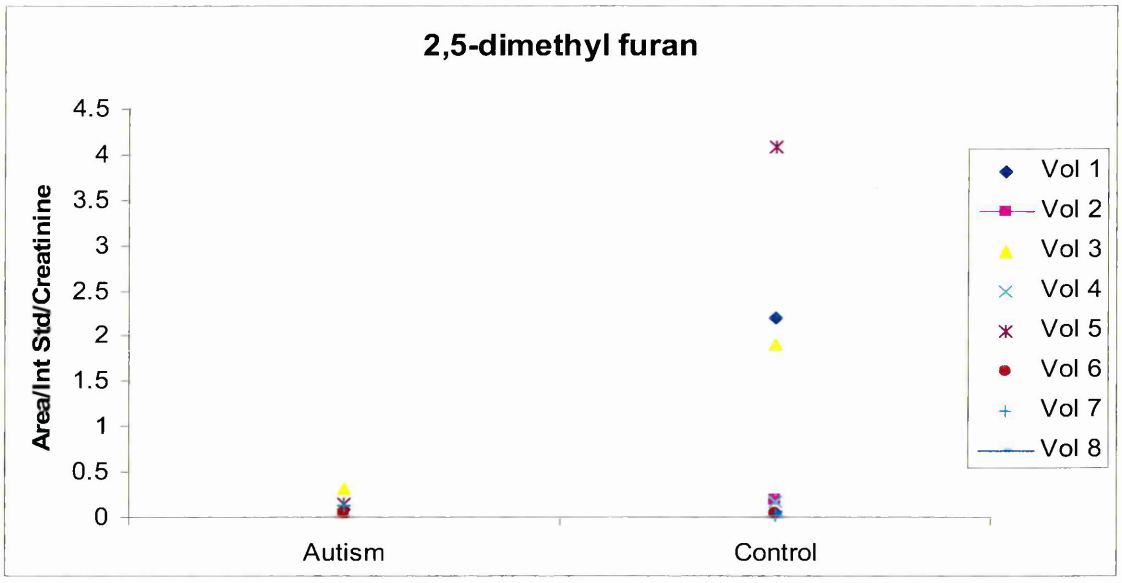


Figure 67. Area under the curve taking into account internal standard and creatinine of 2,5-dimethyfuran in urine of autistic children and healthy controls.

Most controls had higher quantities of 2,4-dimethyl furan than autistic children (see Figure 68).

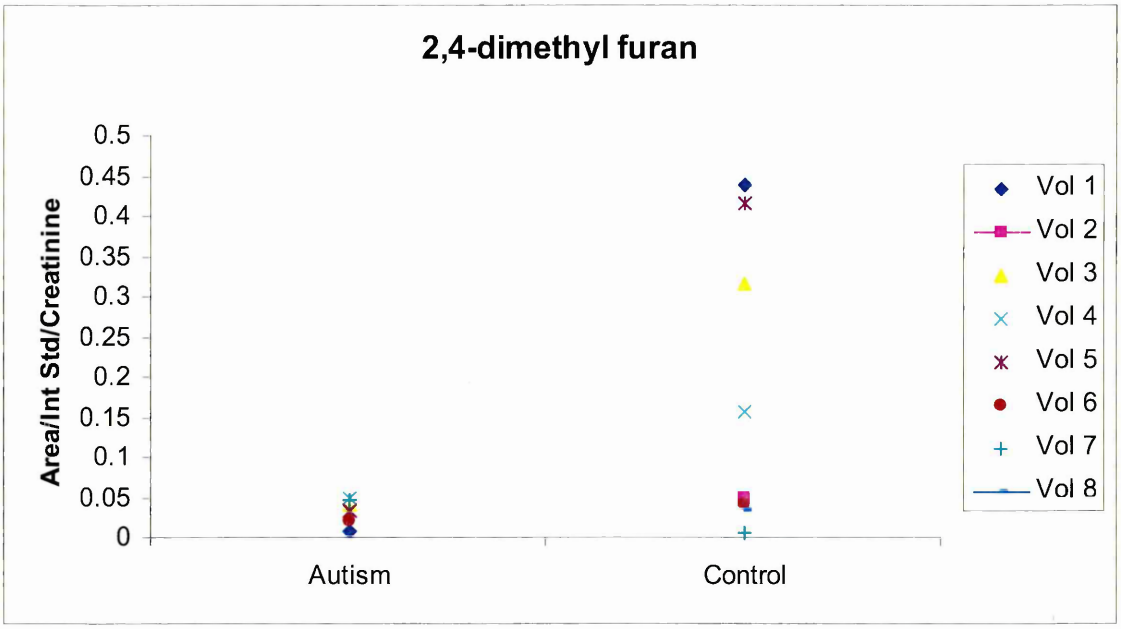


Figure 68. Area under the curve taking into account internal standard and creatinine of 2,4-dimethyfuran in urine of autistic children and healthy controls.

In Table 25, a list of the compounds found in the chromatograms analysed with the SPME technique can be found. Those compounds that could be metabolites used as biomarkers in the future are highlighted, although none were statistically significant.

Table 25. Compounds found in the chromatograms of urine of autistic children and healthy children analysed with the SPME technique. X=no statistical significance, -= data not available, ↑= increased

Compound	Autism vs controls	Statistical difference/Presence/Absence	Statistical difference when present
Acetone	=	-	x
Cyclohexane	=	-	x
Hexane, 2,3-dimethyl	=	-	x
Butanal, 3-methyl	=	x	x
Furan, 2,5-dimethyl	**↑ in controls**	**x**	**x**
Furan,2,4-dimethyl	**↑ in controls**	**x**	**x**
Dimethyldisulfide	=	x	x
Hexanal	=	x	x
Ethylbenzene	=	x	x
Benzene,1,2-dimethyl	=	x	x
4-Heptanone	=	-	x
Benzene,1,3-dimethyl	=	x	x
3-Heptanone	=	-	x
2-Heptanone	=	x	x
Heptanal	=	x	x
Oxepine,2,7-dimethyl	=	-	x
Hexanal, 2-ethyl	=	-	x
Dimethyl trisulfide	=	-	x
1,3,8-p-menthatriene	=	-	x
Benzaldehyde	=	-	x
Cyclohexene, 1-methyl-4-(1-methylethylidene)	=	x	x
Limonene	=	-	x
Benzene, 1-methyl-2-(1-methylethyl)	=	-	x
1,3,7-Octatriene, 3,7-dimethyl	=	x	x
1,4-cyclohexadiene, 1-methyl-4-(1-methyl)	=	-	x
1-Hexanol, 2-ethyl	=	-	x
Cyclohexene, 4-1-methyl-4-(1-methylethylidene)	=	-	x
2-butoxyethylacetate	=	-	x
Benzene, 1-methyl-4-(1-methylethenyl)	=	-	x
2-Furanmethanol, 5-ethenyltetrahydro-a-a,5-trimethyl-, cis	=	x	x
1,6-Octadiene-3-ol, 3,7-dimethyl	=	-	x
1,3,8-p-menthatriene	=	-	x
Nonanal	=	-	x
1,5,7-Octatriene-3-ol, 3,7-dimethyl	=	x	x
2,2-dimethyl-1-oxa-2-silacyclo-3,5-hexadiene	=	x	x
2,6-dimethyl-1,3,5,7-octatraene, E	=	x	x
3-cyclohexen-1-ol, 1-methyl-4-(1-methylethyl)	=	x	x
Pentanoic acid	=	x	x

Indole	=	-	x
Heptanoic acid	=	x	x
Octanoic acid	=	x	x
Nonanoic acid	=	x	x
Phenol, 2-methyl	=	-	x

In Table 26, a list of the compounds found in the chromatograms analysed with the HAN technique can be found. Those compounds that could be metabolites used as biomarkers in the future are highlighted.

Table 26. Compounds found in the chromatograms of urine of autistic children and healthy children analysed with the HAN technique. X=no statistical significance *=p<0.05, **=p<0.01, -= data not available, ↑= increased.

Compound	Autism vs controls	Statistical difference/Presence/Absence	Statistical difference when present
2H-Inden-2-one, 1,4,5,6,7,7a-hexahydro-7a, methyl-(S) (?)	=	-	x
Cyclohexen-1-ol, 1-methyl-4-(1-methylethyl) trans-	=	x	x
Benzoic acid, 4-(1-methylethyl)-, methyl ester	**↑ in autism**	**x**	**x**
Cyclohexene-1-carboxaldehyde, 4-(1-methylethenyl)-(S)-(?)	=	x	x
1H-Indole, 5-methoxy-2-methoxy	**Only present in autism**	*	-
1-Naphtalene methanol, 1,2,3,4-tetrahydro-8-methyl	**↑ in autism**	*	x
Tricyclo(4.4.0.02.7dec)-8ene-3-methanol a,a,d,8-tetramethyl-, stereoisomer	**Only present in autism**	**	-
Ethanone, 1-(3-amino-4-methoxyphenyl)	**↑ in autism**	**x**	**x**
N3-diethyloctanamine	=	-	x
Mono-TMS of (pyridoxine-H2))	**Only present in autism**	**	-
Indole-2-one, 2-3, dihydro-5-hydroxy-1,3-dimethyl	**↑ in autism**	**x**	**x**
1,2-Benzedicarboxylic acid, butyl methyl ester	**↑ in autism**	**x**	**x**
Benzebutyric acid, 2,3-dimethoxy	**↑ in autism**	**x**	**x**
1,3-Adamantanedic carboxylic acid, 4-hydroxy, dimethyl ester	=	-	x
1.4-Fpr,p;-9-hydroxyacteether(?)	=	x	x
a-glucopyranoside, methyl, 2-(acetylamino)-2-deoxy-3-O-Trimethylsilyl)-cyclic methylboronate	=	x	x
Benzeneacetic acid (?)	=	x	x
Propanediamide, 2-ethyl-2-phenyl-N,N′-bis(TMS)	=	-	x
10H-Phenoxaphosphine, 2-chloro-8-ethyl-10-hydroxy-10-oxide	=	x	x
Methyl-3-(3(methoxycarbonyl)-4-methyl-5-pentyl-2-furanyl)propionate	=	-	x
2(1H)-Pyridinone, 1-cyclohexyl-3,4,5,6-tetramethyl	**↑ in autism**	**x**	**x**
Dinordesoxy-9-methyl-7-methoxyeseroline(?)	**Present more often in autism**	**x**	**x**
10,13-Octadecadiynoic acid, methyl ester	**Present more often in autism**	**x**	**x**
1-Heptatriacontanol	**↑ in autism**	**-**	**x**
Octadecanoic acid, methyl ester	=	-	x
Cinnamoylglicine, methyl ester	**Present more often in autism**	*	x
Cyclopentanepropanoic acid, 3,5-bis (acetyl)-2-(3,8-bis(acetyloxy)-1-octenyl)-, methyl ester	**Present only in autism**	**	-

Tetradecanedioic acid, 3,6-epoxy-, dimethyl ester	=	x	x
Cyclopentane-1, carboxylic acid, 4-isopropilidene-2-(2-methoxycarbonyl)ethenyl)-methyl ester	=	x	x
Androstan-3-one, cyclic 1,2-ethanediyl mercaptole (a) (?)	=	-	x
2-Phenanthreneacetic acid, tetradecahydro-7-hydroxy-1, 4b, 8,8-tetramethyl-10-oxo, methyl ester (?)	=	-	x
Acetamide, N-(2-(acetyloxy)-(3,4-bis(acetyloxy) phenyl)ethyl)-N-methyl(?)	=	x	x
1-Cyclopentene-1-propanoic acid, 5-(methoxyimino)-2-(3-(trimethylsilyl)oxy)-1-octyl)-methyl ester	**↑ in autism**	**-**	**x**
Androstan-17-one-3-((trimethylsilyl))oxy)(3a, 5B)	**↑ in autism**	**x**	**x**
Cyclopropaneoctanoic acid, 2-octyl-, methyl ester	=	x	x

In Table 27, a list of the compounds found in the chromatograms analysed with the HB technique can be found. Those compounds that could be metabolites used as biomarkers in the future are highlighted.

Table 27. Compounds found in the chromatograms of urine of autistic children and healthy children analysed with the HB technique. X=no statistical significance *=p<0.05, **=p<0.01, -= data not available, ↑= increased.

Compound	Autism vs controls	Statistical difference/Presence/Absence	Statistical difference when present
Spiro(1.3-dioxolane-2,6'-morphinan),N-2',4,-dihydroxy	=	-	x
2,4-dichlorobenzotrile	=	x	x
Bicyclo(3.3.0)octan-3-one, 6-methyl-6-trimethylsilyloxy	=	-	x
Acetamide, N-(4-_trimethylsilyl)oxy)phenyl)	**↑ in autism**	**x**	**x**
2-Hydroxyamine-6-p-tolylpyrimidin-4-(3H)-one	**↑ in autism**	**-**	**x**
1,3-Benzenediol, 2-(3-methyl-6-(1-methyl ethenyl)-2-cyclohexen-1-yl)5-pentyl	=	x	x
Oxymetazoline	**↑ in autism**	**-**	**x**
Trimethoprim	=	-	x
Enterolactone (2,3-bis(3-hydroxy benzyl)butyrolactone-di-(trimethylsilyl)	**↑ in autism**	**-**	**x**
1-(2-Isopropenyl-3-methoxy-2,3-(dihydrobenzofuran-5-yl)-7,8-dimethoxy-3-α, 9β-(4H)-dihydro(1) benzopyrano (4,3-d)isodiazole (?)	=		x
Hexadecanoic acid, TMS	=	-	x

Table 28 . Compounds found in the chromatograms of urine of autistic children and healthy children analysed with the UB technique. X=no statistical significance *=p<0.05, **=p<0.01, -= data not available, ↑= increased.

Compound	Autism vs controls	Statistical difference/Presence/Absence	Statistical difference when present
1H-Indole-3-acetamide, 1-trimethylsilyl	↑ in autism	-	x
Benzeneacetic acid, a-4-bis 9(TMS)oxy)-TMS	=	-	x
1-Piperidinecarboxaldehyde, 2-(1-formyl)-2-pyrolidinyl)	=	-	x
Formamide, N,N, diphenyl	=	-	x
Proline	=	-	x
Hexadecanoic acid, TMS	=	-	x
Heptadecanoic acid, TMS	**↑ in autism**	-	**x**
11-Cis-Octadecanoic acid, TMS	=	-	x
Octadecanoic acid, TMS	=	-	x
Heptacosane	=	x	x
Pentacosane	=	-	x
Bis(2-ethyl)phtalate	=	-	x
Hexanoic acid, 2,3-bis(T<S)oxy, propyl ester	=	-	x
Octadecanoic acid, 2,3-bis((trimethylsilyl)oxy)propyl ester	=	-	x

pH of urine of autistic children vs healthy children

The pH of the urine obtained from autistic children and healthy controls was measured, although there was not a significant pH difference (p>0.05) (see Table 29).

Table 29. pH of urine of autistic children and healthy children

	Autism	Control
Vol 1	7	6
Vol 2	5.5	6
Vol 3	5.5	6
Vol 4	6.4	6
Vol 5	6	6
Vol 6	6.4	6.4
Vol 7	6	7
Vol 8	6	6
Mean	**6.1**	**6.17**

Discussion

Autistic children and healthy controls were required to supply a morning urine sample and fill in a questionnaire regarding GI problems. From the questionnaire we can conclude there is a very significant difference ($p<0.01$) between the number of bowel movements per week of autistic children and healthy controls. Parents of autistic children often put their children on different treatments, and 10% of them use 15 or more treatments at once (Green et al., 2006). 6 of the 8 autistic children (75%) who took part in this trial were having some kind of treatments (fish oil supplementation, melatonin, probiotics, zinc or diet). 3 of them (38%) were having more than one treatment at the same time, and only 1 (13%) had three treatments at once. These treatments might possibly affect urine analyses.

The results of this study document the subtle differences in urine composition of autistic children and healthy controls. The PCA of the chromatograms obtained by all the techniques used for their analysis show that autistic urine is very different from that of healthy controls. All the techniques of direct injection and SPME showed clustering, although the UB and the HB methods of analysis gave the best clustering.

Different compounds were found to be present in varying concentrations in the urine of autistic patients, influencing this clustering in the PCA. The variation in pH could have influenced the analysis, but no significant difference was observed in both groups. For this reason, these urine analysis methods are tools that could potentially be used for the early detection of autism.

The compounds found to be different in autistic children should be studied further and compared with other groups of children as they of them have a very significant probability of being present only in autistic children (see Table 28), such as 1,2,3,4-tetrahydro-8-methyl,1-naphthalene methanol, α,α,δ,8-tetramethyl-, tricyclo(4.4.0.02.7dec)-8ene-3-methanol stereoisomer, mono-TMS of (pyridoxine-H_20) and 3,5-bis (acetyl)-2-(3,8-bis(acetyloxy)-1-octenyl), cyclopentanepropanoic acid, methyl ester.

To determine the possible bacterial origin of the compounds that are found in altered concentration in autism and the relationship between IBS and autism, the compounds found to be significantly different between patients and controls were investigated.They were also examined in the preceding data concerning IBS patients (including MSK, R+O and IBS) and those IBS patients put on a diet before and after an exclusion or fibre-free diet, as well as healthy volunteers after the enteral feeds. Table 30 shows these compounds and how these changed after the diets.

Table 30. Summary of compounds found in different quantities in the urine of autistic children compared with healthy children with all the analytical techniques (HAN=hydrolysed acid neutral, HB=hydrolysed basic and UB=unhydrolysed basic) and how these are present in IBS patients (MSK=musculoskeletal IBS, R+O=retention and overflow IBS and IBS=Irritable bowel syndrome thought to be due to bacterial fermentation). These were observed on IBS patients before and after an exclusion free or fibre free diet. ↑= increase, ↓=decrease, x=not significant, *=significant difference ($p<0.05$), **= very significant difference (p,0.01), -=not present, N/A= not available.

Technique	Compound	Autism vs controls	Presence absence	Significance when present	IBS after diet	Elemental diet
SPME	Furan, 2,5-dimethyl	↑ in controls	x	x	=	=
	Furan,2,4-dimethyl	↑ in controls	x	x	=	=
HAN	Benzoic acid, 4-(1-methylethyl)-, methyl ester	↑ in autism	x	x	-	↓ after diet
	1H-Indole, 5-methoxy-2-methoxy	Only present in autism	*	N/A	-	-
	1-Naphtalene methanol, 1,2,3,4-tetrahydro-8-methyl	↑ in autism	*	x	-	-
	Tricyclo(4.4.0.02.7dec)-8ene-3-methanol a,a,d,8-tetramethyl-, stereoisomer	Only present in autism	**	N/A	-	-
	Ethanone, 1-(3-amino-4-methoxyphenyl)	↑ in autism	x	x	-	-
	Mono-TMS of (pyridoxine-H_20)	Only present in autism	**	N/A	-	-
	Indole-2-one, 2-3, dihydro-5-hydroxy-1,3-dimethyl	↑ in autism	x	x	2,3-dihydro-3,3-dimethylindole-2-one in R+O only	-
	1,2-Benzedicarboxylic acid, butyl methyl ester	↑ in autism	x	x	↑ in IBS, ↓ after the diet	-
	Benzebutyric acid, 2,3-dimethoxy	↑ in autism	x	x	-	-
	2(1H)-Pyridinone, 1-cyclohexyl-3,4,5,6-tetramethyl	↑ in autism	x	x	-	-

	Dinordesoxy-9-methyl-7-methoxyeseroline(?)	Present more often in autism	x	x	-	-
	10,13-Octadecadiynoic acid, methyl ester	Present more often in autism	x	x	-	-
	1-Heptatriacontanol	↑ in autism	-	x	-	-
	Cinnamoylglicine, methyl ester	Present more often in autism	*	x	-	-
	Cyclopentanepropanoic acid, 3,5-bis (acetyl)-2-(3,8-bis(acetyloxy)-1-octenyl)-, methyl ester	Present only in autism	**	-	-	-
	1-Cyclopentene-1-propanoic acid, 5-(methoxyimino)-2-(3-(trimethylsilyl)oxy)-1-octyl)-methyl ester	Increased in autism	N/A	x	-	-
HB	Acetamide, N-(4-_trimethylsilyl)oxy)phenyl)	↑ in autism	x	x	-	-
	2-Hydroxyamine-6-p-tolylpyrimidin-4-(3H)-one	↑ in autism	N/A	x	-	-
	Oxymetazoline	↑ in autism	N/A	x	-	-
	Enterolactone (2,3-bis(3-hydroxy benzyl)butyrolactone-di-(trimethylsilyl)	↑ in autism	N/A	x	-	↓
	1-(2-Isopropenyl-3-methoxy-2,3-(dihydrobenzofuran-5-yl)-7,8-dimethoxy-3-α, 9β-(4H)-dihydro(1) benzopyrano (4,3-d)isodiazole (?)	↑ in autism	N/A	x	↓ after the diet	-
UB	1H-Indole-3-acetamide, 1-trimethylsilyl	↑ in autism	N/A	x	↑ in MSK and after the diet	↑
	Heptadecanoic acid, TMS	↑ in autism	N/A	x	↓ after diet	-

The methyl ester of 4-(1-methylethyl)-benzoic acid, is shown to be in increased concentration in autistic children, although the probability of this compound being higher in

autistic children than controls it is not significant ($p>0.05$) although it may be caused by the small sample. This compound was not found in IBS patients, but it was present in healthy volunteers and decreased after the elemental diets. Its decreasing after these diets could mean that it is a compound produced by colonic bacteria, and being increased in autism could mean that fermentation is increased in autistic patients. However, there is evidence to support that IBS patients have an increased fermentation in the bowel, and this compound is not found in these patients. These facts suggest the idea that IBS patients have a different bacterial spectrum to healthy volunteers and autistic patients and the predominant bacteria do not produce this compound. However, it could also mean that the compound is not a product of bacterial fermentation and has another origin. Also, the increase of this metabolite is not statistically significant; therefore no conclusion can be drawn about this compound and fermentation in autism.

1,2- butyl benzedicarboxylic acid methyl ester is found to be increased in autism and in IBS. This compound decreases when IBS patients are put on a diet and it is not found in healthy adults. IBS is thought to have an increased fermentation as discussed in the previous chapter, and this compound is increased in the urine of IBS patients and it decreases after the diets, so it is very likely to be of bacterial origin. The fact that this compound is increased in autistic children could suggest that fermentation is increased in autism, although this compound was not significantly increased ($p>0.05$), so no conclusion can be drawn.

A compound similar to 1-(2-Isopropenyl-3-methoxy-2,3-(dihydrobenzofuran-5-yl)-7,8-dimethoxy-3-α, 9β-(4H)-dihydro (1) benzopyrano (4,3-d)isodiazole was found to be increased in autistic children, and it was decreased when IBS patients were put on a diet, although it was not present in healthy adults. It is possible that this compound is normally produced by colonic bacteria, meaning there is an increased fermentation in autistic patients. However, this metabolite was not significantly increased ($p>0.05$) so no conclusion can be drawn.

1H-Indole-3-acetamide, 1-trimethylsilyl was found to be increased in the urine of autistic children, and so it was in MSK and in IBS patients after the diet. If a compound increases after an exclusion or fibre free diet, it is probably a compound that is normally broken down by bacteria. If a compound that is broken down by bacteria is increased in a condition, it could mean bacterial fermentation is decreased, which does not agree with the possibility of the compounds discussed above of being bacterial fermentation. Also, this compound is found to be increased in MSK patients, so it is possible that this compound does not have any relationship with fermentation in the gut.

Heptadecanoic acid, TMS (see Figure 69) was found in urine analysed with the UB technique and it was found to be increased in autistic children and it decreases in IBS patients when they are put on an exclusion or fibre free diet. This is probably a derivative of a compound produced by bacterial fermentation, and if it is increased in autism it could mean fermentation is increased in autistic children. However, this compound has not significantly increased ($p>0.05$) so no conclusion can be drawn. This compound is likely to be heptadecanoic acid in urine, although it conjugates as heptadecanoic acid TMS during the treatment done for its analysis.

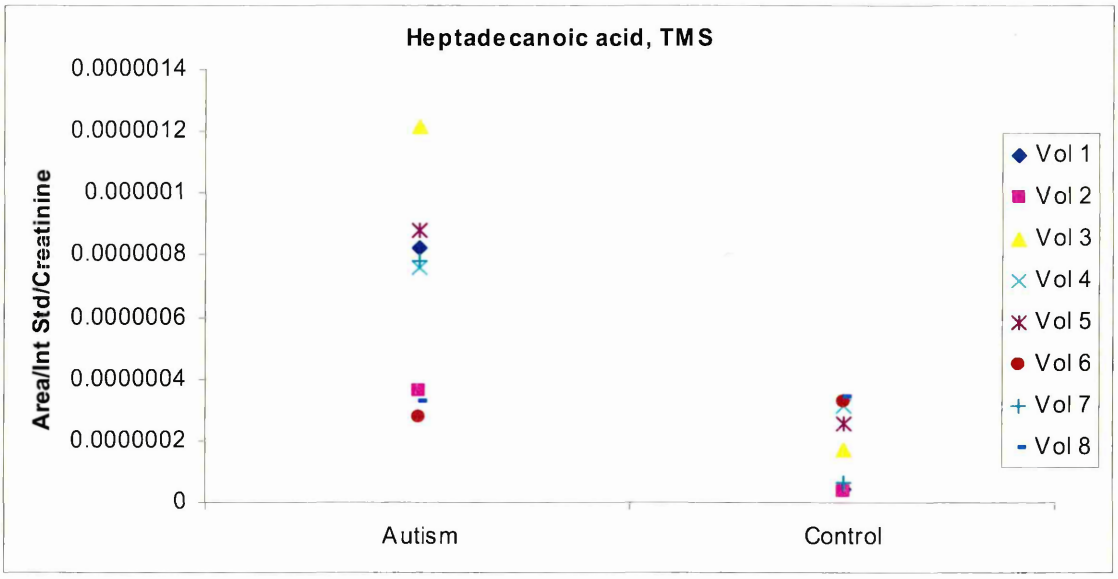


Figure 69. Area of heptadecanoic acid, TMS under the curve taking into account internal standard and creatinine.

If an assumption is done where 4-(1-methylethyl)- benzoic acid, methyl ester; 1,2-benzedicarboxylic acid, butyl methyl ester; the compound found similar to 1-(2-Isopropenyl-3-methoxy-2,3-(dihydrobenzofuran-5-yl)-7,8-dimethoxy-3-α, 9β-(4H)-dihydro(1) benzopyrano (4,3-d)isodiazole and heptadecanoic acid, trymethyl silyl (TMS) are compounds found in urine which are products of fermentation because they decrease after either healthy adults or IBS patients on diets which may decrease bacterial activity, we could conclude that there is a possibility that autistic patients have an increased fermentation in the bowel. However, it is not proved that these compounds are bacterial products, and the increase in autism is not significant, although the sample is rather small, so having a bigger sample could increase the statistical meaning.

Another issue to take into account is the differences between the ages of the volunteers and patients. For instance benzoic acid, 4-(1-methylethyl)-methyl ester is increased in autism but it is present in healthy children as well as adults. However, 1,2-benzedicarboxylic acid, butyl methyl ester; the compound found similar to 1-(2-Isopropenyl-3-methoxy-2,3-(dihydrobenzofuran-5-yl)-7,8-dimethoxy-3-α, 9β-(4H)-dihydro(1) benzopyrano (4,3-

d)isodiazole and heptadecanoic acid, TMS are present in healthy children but not in healthy adults, despite being present in IBS patients. Bacteria are normally stable throughout life, but because of the small sample and the great variability of bacteria among different people, it is not possible to answer why there are compounds found in healthy children and not in healthy adults and vice versa.

Questionnaires were filled in about the number of bowel movements had per week in autistic children and healthy children and the difference found was very significant ($p<0.01$). This reflects the GI problems that autistic children suffer from. A questionnaire with the number of bowel movements obtained per week should be obtained from IBS patients in future research.

The compounds that are increased in the urine of IBS and autism are very different. The bacterial compounds that were found to be increased in IBS are not present or increased in autism, so we can conclude that autistic patients do not suffer from IBS.

Wakefield *et al.*(1998) reported 25% of autistic children showed a red halo around swollen caecal lymphoid follicles, which was controversially interpreted as Crohn's disease (CD). The fact that autistic children do not suffer from UC or CD although they suffer from colitis was discussed by Buie (2006) (Buie, 2006).

Autism and IBS are different conditions. IBS has abnormal fermentation in the bowel, and the compounds which are abnormally increased in the urine of these patients, such as 3-heptanone and 2-ethyl-1-hexanol, are possible causes of the condition, because when these patients are put on a diet which suppresses bacterial activity, the compounds decrease and their symptoms improve.

It is possible that ASD is caused by abnormal fermentation in the bowel as well, but the compounds that were found to be produced or metabolised by bacterial fermentation in healthy adults and in IBS patients were not found or changed in autism and it might be other bacteria/bacterial metabolites which are causing the condition. It is possible that if

different bacteria produce different compounds, a completely different condition is caused. However, the evidence found in this study is not enough and should be studied further.

Bacterial spectra have been observed to be abnormal in autistic children (2002), so it is very possible that the metabolites found in urine in different quantities are from bacterial fermentation. A study with autistic children placed on probiotic supplementation to displace incumbent bacteria has been suggested (Brudnak, 2002), although no trial has been published to date, so their benefits are not known.

Probiotics have been used for the treatment of IBS, as they may ease symptoms by changing gut microflora, reducing mucosal inflammation and exerting antibacterial effects (Niv et al., 2005). However, there was no superior improvement in comparison with placebo (Niv et al., 2005) (Bausserman & Michai, 2005; Sen et al., 2002). Sen et al.(Sen et al., 2002) in an experiment with twelve IBS patients on Lactobacillus plantarum 299v found no difference in hydrogen production after 4 weeks and symptoms did not improve. However, as we have observed in this study, bacterial metabolites produced in autism and IBS are different, therefore the use of probiotics in this condition should be studied.

Chapter 6. Overall conclusions

Method development

Breath and urine were the media chosen for the identification of compounds produced by bacteria. They are non invasive sampling methods each giving the possibility of easy screening of bacterial metabolites. Methods were developed for the analysis of both media.

VOCs were collected from 1 litre of breath using a Tenax Carbotrap tube and stored for analysis in an automated TD-GC-MS. One litre of background air was also collected to determine the compounds that were present in the background. Using this method more than a hundred compounds were detected on breath, establishing that it was a good method for the screening of breath metabolites. Compounds in breath are present in very low levels, approximately in ppbs, so this is a very powerful technique. However, due to rapid gas exchange between blood and outside air, breath analysis may only show the spectrum of compounds produced during metabolism occurring at a specific moment, and background levels of compounds will fluctuate strongly in an air-conditioned laboratory, so collection of the sample had to be done in the same way in all the volunteers by one investigator, so each sample was experimentally controlled using its own technique background air sample. The use of a background sample makes the method more complicated. However, the main drawback of the method is that volunteers had to be clearly instructed on the technique needed to give a representative sample and this was not always easy. It was considered that this drawback made the method not applicable to sample from patients with conditions such as autism.

A method was developed for the screening of urine, specifically to get around some of the issues associated with the collection of breath. HS, TD and SPME were investigated, and SPME gave the best results because the lowest concentration detected of SCFAs of 0.0075 ppm in comparison with 0.01 ppm with HS and TD. Also SPME has the capability of detecting a greater number of compounds.

The lowest possible concentration of four SCFA standards using the SPME technique was around 0.02 ppm in water and 0.0075 ppm in urine with SPME. This showed that it is more difficult to detect SCFAs in urine, possibly due to presence of compounds in urine which complex with or bind to the SCFA.

Hundreds of compounds were observed in urine, and because the SPME technique is very simple and can be automated, it makes it a good technique for the screening of urine.

Elemental diet

Enteral feeding is currently the most effective means available of inducing long term remission in active CD. However, the mode of action in inducing remission is still poorly understood. There are many suggestions about the method of action: bowel rest, altered motility, reduction of intestinal secretions, hypoallergenicity, and improvement in nutritional status.

An elemental diet was used in healthy volunteers to examine the possibility that the effectiveness of enteral feeds depends on depriving the intestinal microflora of substrates for metabolism, resulting in the reduced production of potentially toxic chemical by-products.

Breath and urine of a set of people on each of two enteral feeds were analysed. A number of compounds and groups of compounds are known to be synthesised or metabolised by the gut flora. These include phenols, indoles, enterolactone and octadecanoic acid. Indole increased after the diet, which suggests that indole was normally broken down by bacteria. However indole is a known compound produced by bacteria from the metabolism of protein. At the same time, when bacteria are deprived from exogenous protein, the only other source for their metabolism is from the epithelial cells, and metabolism of these should change the bacterial metabolite spectrum. The production of indole is then increased because cells are mainly composed by protein. Changing the pattern of fermentation could result on changes of products of toxic metabolites. As indole is a bacterial product, changes

in its metabolism support the theory that changes in bacterial activity may underlie the effectiveness of enteral feeding in Crohn's disease.

Enterolactone is also derived from bacteria and their excretion in the urine fell significantly in both enteral feeds, were also decreased after the diets. This suggests that enteral feeding impairs bacterial metabolism.

Elemental diets works reducing bacterial activity in the bowel, as confirmed by changes in stool colour and breath VOC composition, as well changes in bacterially derived compounds found in breath and urine. Further studies are needed to confirm that all the changes we have demonstrated also occur after other manipulations such as bowel clearouts or antibiotic treatment, which are known to reduce bacterial metabolism in healthy volunteers and CD patients.

Two diets, E028 and Modulen, were used, and they showed a similar ability to reduce bacterial activity in the gut.

IBS

Patients suffering from GI problems that suffer from abdominal pain can sometimes be misdiagnosed with IBS due to the similarity in their symptoms. Urine screening and symptoms allowed us to make three different groups of IBS: R+O, MSK and IBS.

R+O patients present a mild constipation, overflowing the bowel approximately every week, leading to painful diarrhoea. MSK GI problems are thought to be pain caused pressure on nerves supplying the abdominal wall but there are no bowel symptoms. We include IBS triggered by food under the general term of IBS in this thesis. IBS patients suffer from abnormal fermentation in the bowel. A new criterion should be outlined for the identification of IBS, fundamental from a diagnostic point of view. Up to date there is no biomarker for any of these conditions. Biomarkers could aid in the early diagnosis of the disease and its treatment.

The compounds observed in the different groups suggest that R+O and MSK are similar, although there were some compounds found to be present in different quantities that should be investigated further for their use as biomarkers of these conditions. N-3-butenyl-N-methyl benzenamine was increased in R+O and 2,3-dihydro-3,3-dimethylindole-2-one and 3-carboxy-4-methyl-5-propyl-2- furanpropionic acid, dimethyl esther were present only in R+O and should be investigated further as biomarkers of R+O. In Table 15 and Table 16 compounds that were present in R+O and MSK patients in different quantities to controls can be found and should be investigated further.

Some compounds were found to be in urine from IBS patients in higher quantities than in controls, R+O and MSK patients such as 2,3-bis(TMS)oxy, hexadecanoic acid propyl ester. These compounds may be used as biomarkers of IBS. These compounds decrease with the diets, suggesting that IBS patients suffer from increased fermentation in the bowel.

When R+O, MSK, IBS before and after the diet and controls were compared, different compounds were observed to be increased in IBS before the diet and decrease after the diet, such as 3-Heptanone; 1-hexanol, 2-ethyl and butanal, 3-methyl. These compounds are likely to be of bacterial origin, as they decrease after the diets. These compounds should be studied in a bigger group of patients as they may be used as biomarkers of IBS in the future.

Autism

Some compounds have been observed to be present in different quantities in urine and blood of autistic children, however, up to date there is not a biomarker for the condition. IAG has been suggested to be a compound which is increased in the urine of autistic children (Shattock & Savery, 1997) although 71% of the normal population excrete it in varying amounts (Mandell & Rubin, 1965).

When urine from autistic children and healthy controls was analysed, PCA of the chromatograms showed that their urine is very different. Compounds were observed and different compounds were found to be present in higher quantities in these patients than in

controls. However, these compounds were not found in the urine of healthy adults that were put on a diet or in IBS patients. We can conclude from this that ASD is not related to IBS, although because the origins of the compounds which are increased in the urine of this condition are not known, no conclusion can be drawn on fermentation in the bowel.

There is evidence to suggest that fermentation in ASD patients is abnormal, but this study cannot conclude that this is the case. It is possible that fermentation is decreased and the compounds found to be increased in IBS patients are low in autistic patients, although these compounds were not found in healthy children either. However, if fermentation is abnormal in ASD, it is possible that the bacteria present in the autistic bowel are different therefore the compounds present in urine would be different. More studies should be done.

There are compounds that are significantly more likely to be present in autistic than in healthy controls, such as 1,2,3,4-tetrahydro-8-methyl, 1-naphtalene methanol; α,α,δ, 8-tetramethyl-, tricycle(4.4.0.02.7dec)-8ene-3-methanol stereoisomer; mono-TMS of (pyridoxine-H2O) and 3,5-bis(acetyl)-2-(3,8-bis(acetyloxy)-1-octenyl), cyclopentanepropanoic acid, methyl ester. In the same way, heptadecanoic acid, TMS was found to be increased in the urine of autistic children. However, this compound is also present in the IBS patients` urine and decreases after the diet, therefore probably being of bacterial origin.

A list of the compounds that were found to be different in autism in comparison with healthy controls can be found in Table 30. These compounds should be studied further in a bigger group as potential biomarkers of autism. At the same time, an investigation on the origin of the compounds should be done, as some of them might be of bacterial origin.

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Appendix 1

Silsoe Ethical approval

Cranfield UNIVERSITY
Silsoe

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Tel +44 (0) 1525 863000
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20th January 2004

Dear Pillar

Re: Silsoe Study: Screening of urine for bacterial metabolites in Autism

I am pleased to confirm that the Cranfield University at Silsoe Clinical Research Ethics committee is satisfied that you have taken into account all the appropriate data protection and ethical considerations which affect Cranfield University as a result of you undertaking the aforementioned study, and are therefore happy for you to proceed subject to the following terms and conditions.

I wish to remind you that should you make any subsequent alterations to the protocol of the aforementioned study which is likely to affect matters concerning data protection and/or the ethical position; the committee must be informed immediately.

Approval to proceed is limited solely to the recruitment of volunteers who are members of staff or currently registered for a higher degree at Cranfield University at Silsoe, Barton Road, Silsoe, MK45 4DT. It is your responsibility to obtain appropriate approval to undertake any research related to this project in or at any external organisation.

It is your responsibility to ensure that the University is indemnified against legal action incurred as a result of you undertaking this research.

I wish you well in the project and you PhD as a whole

Dr Anthony Woodman BSc(Hons), MSc, PhD.
Head, Cranfield BioMedical Centre

Appendix 2:

Elemental diet ethical approval

CAMBRIDGE LOCAL RESEARCH ETHICS COMMITTEE

Box 148
Addenbrooke's NHS Trust
Hills Road
Cambridge CB2 2QQ

Chairman: Dr G E Berrios
Administrator: Elaina M Friend
Telephone: 01223 217983 Internal: (151) 3983
Fax: 01223 216520
Email: elaina.friend@addenbrookes.nhs.uk

Professor John O Hunter
Box No 262
Dept of Gastroenterology
Addenbrooke's NHS Trust
Hills Road
Cambridge
CB2 2QQ

26 February 2003

Dear Professor Hunter

The effects of elemental and polymeric diets on volatile metabolites produced by colonic fermentation and excreted on the breath.

Ethics Reference: LREC 00/090 **(Please quote on ALL correspondence)**

Thank you for completing and returning our recent questionnaire on the above study. We note that you have requested an Ethical Approval extension for a further 2 years.

I am taking Chairman's Action to approve an extension until 11th April 2005. This extension is granted under the following conditions:

- The protocol agreed must be followed and any changes will require prior LREC approval.
- Any serious or unexpected adverse events must be reported to the LREC, study sponsor and other local investigators.
- A progress report must be sent to the LREC every 12 months from the date of approval whilst the study is ongoing, and a final report must be sent within 3 months of the research being completed.

Furthermore, whilst I am sure that every effort is already made to preserve the confidentiality of any patient information used in this study, could you please ensure that the team of investigators and everyone who has access to patient information appreciates the importance of maintaining that confidentiality, particularly in respect of the use of computers and the statutory regulations laid down in the Data Protection Act 1998.

Yours sincerely

Dr GE Berrios MA (Oxon) MD FRCPsych FBPsS FMedSci
Chairman
Local Research Ethics Committee

Appendix 3

Elemental diet questionnaire

1. Are you taking any medication?

2. Have you taken any antibiotics in the last 6 weeks?

3. Are you currently following any special diet? If so please specify

4. Have you used any pre/probiotics, live yogurts or other bacterial products in the previous 2 weeks? If yes please specify

5. Do you suffer from irritable bowel syndrome?

6. Do you suffer from migraine more than once a month triggered by food or menstrually related?

7. Do you suffer from pre menstrual syndrome serious enough to require time off work?

8 What is your current weight in kg?

9. What is your height?

Appendix 4

Script

```
function [Peak_Area,Samples,Compounds,Labels] = c2s_area;

% c2s_allarea investigates which compounds occur in which GCMS samples and
% then determines the abundance of the samples
%
% [Comp_Area,Samples,Compounds,compounds,Files] = c2s_area;
%
% written by Jon Lee-Davey, 2005
% Based on some incomplete Mike M work


[Compounds,Samples,Files,Labels,Data,Headers,Rejects,Reject_Samples] = c2s4;

if isequal(Compounds,[]) == 0;
compounds = char(Compounds);
headers = char(Headers);


Comp = input('Which compound do you want to use? - Enter Number:   ');
Comp = strread(compounds(Comp,:),'%s','whitespace', '')';
comp = char(Comp)


[Filename, pathname] = uigetfile('*.xls','Select a file in the folder ...');
filelist = dir([pathname '\*.xls']);
% Assigning filenames
row = 1;
for filenumber = 1:length(filelist)
Filename = filelist(filenumber).name;
% Removing file extension
Filename = strrep(Filename,'.xls','');
Filename = cellstr(Filename);
labels(row,:) = [Filename];
row = row + 1;
end
Labels = char(labels);
row = 1;
% Access and process files
%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%
h = waitbar(0,'Loading variable data...');
for j = 1:size(filelist,1)
Filename = filelist(j,:).name;
[temp_data,header] = xlsread(Filename,'Summary Report');
header = header(:,15);
headers = char(header);
% creating 'compound list'
```

```
k = 0;
for i = 1:size(header,1);
k = k + 1;
if isequal((findstr(headers(i,:),comp)),1) == 1
matches(k,:) = 1;
else matches(k,:) = 0;
end
end

matches = find(matches);
matches = matches-1;


m = 0;
for p = 1:size(matches,1);
m = m + 1;
peak_area(m,:) = temp_data(matches(p,:),8);
end

if  size(matches,1) > 1;
peak_area = sum(peak_area);
else
if size(matches,1) == 1;
peak_area = peak_area(1,:);
end
if isempty(matches) == 1
peak_area = 0;
end
end

Peak_Area(row,:) = [peak_area];
row = row + 1;

waitbar(j/size(filelist,1));
end

close(h)
else
if isequal(Compounds,[]) == 1;
Peak_Area = [];
sprintf('%s','No area calculated as no compounds available at the selected probability level')
end
end
```

Appendix 5

Compounds in breath and urine from elemental diet

Breath analysis:

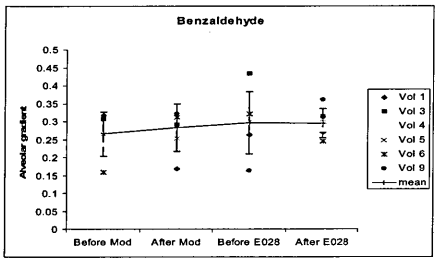

Figure 70. Alveolar gradient of benzaldehyde in the different diets.

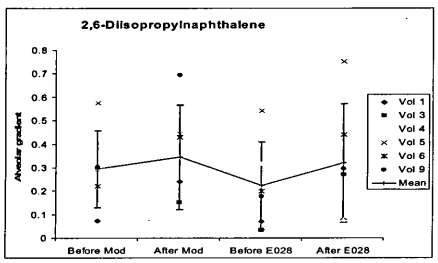

Figure 71. Alveolar gradient of 2,6-diisopropylnaphthalene in the different diets.

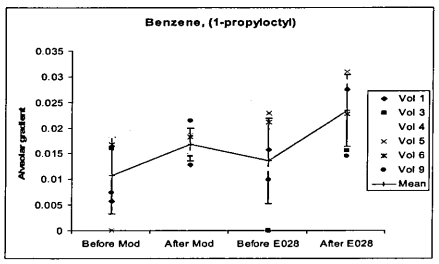

Figure 72. Alveolar gradient of benzene, (1-propyloctyl) in the different diets.

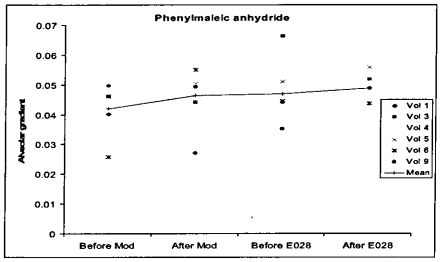

Figure 73. Alveolar gradient of phenylmaleic anhydride in the different diets.

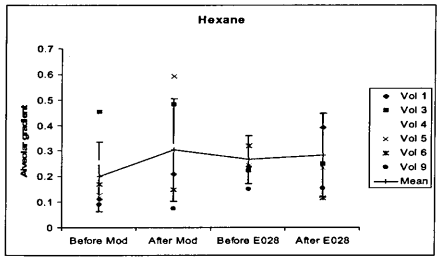

Figure 74. Alveolar gradient of hexane in the different diets.

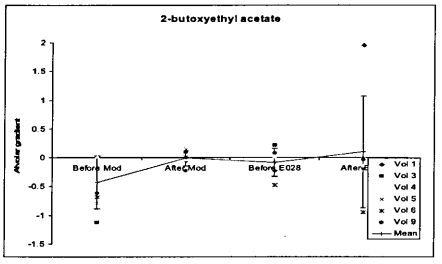

Figure 75. Alveolar gradient of 2-butoxyethyl acetate in the different diets.

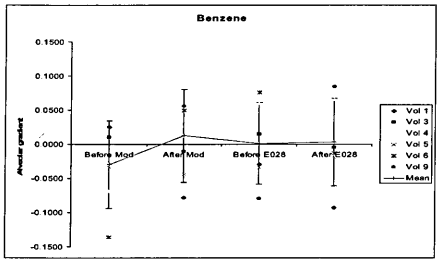

Figure 76. Alveolar gradient of benzene in the different diets.

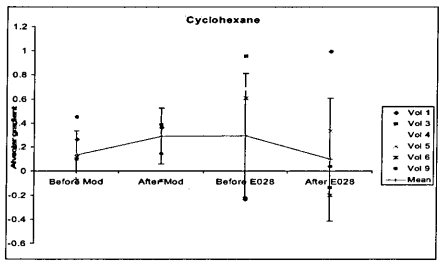

Figure 77. Alveolar gradient of cyclohexane in the different diets.

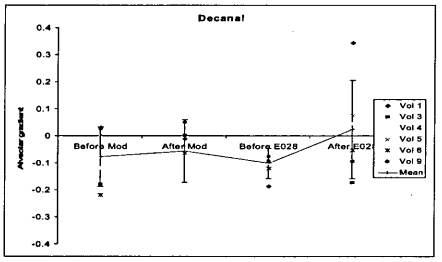

Figure 78. Alveolar gradient of decanal in the different diets.

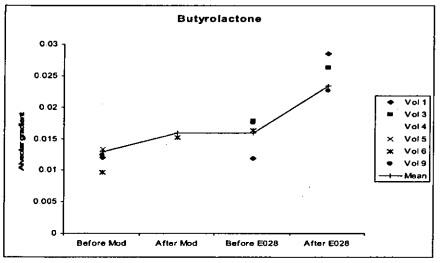

Figure 79. Alveolar gradient of butyrolactone in the different diets.

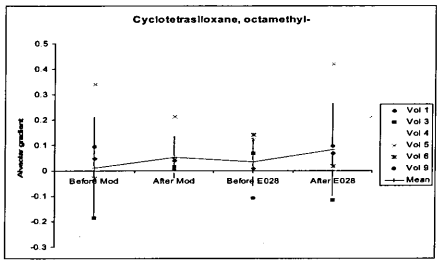

Figure 80. Alveolar gradient of cyclotetrasiloxane, octamethyl in the different diets.

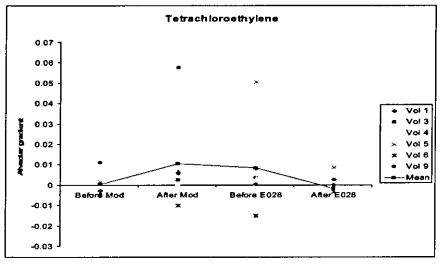

Figure 81. Alveolar gradient of tetrachloroethylene in the different diets.

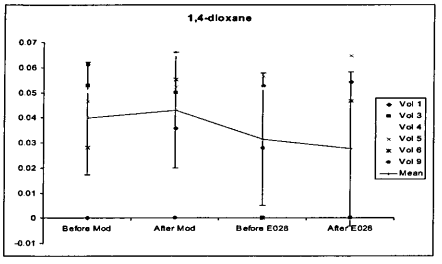

Figure 82. Alveolar gradient of 1,4-dioxane in the different diets.

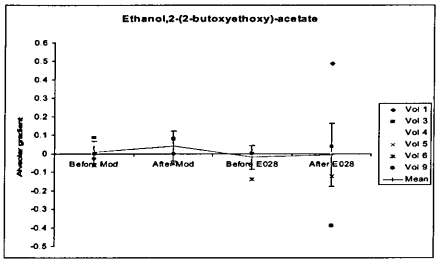

Figure 83. Alveolar gradient of ethanol, 2-(2-butoxyethoxy)-acetate in the different diets.

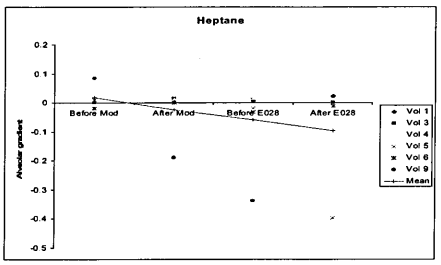

Figure 84. Alveolar gradient of heptane in the different diets.

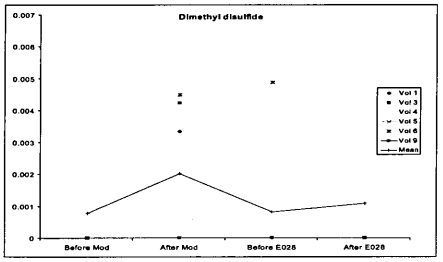

Figure 85. Alveolar gradient of dimethyl disulfide in the different diets.

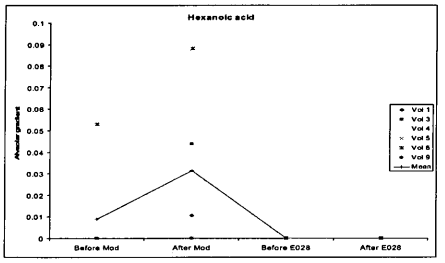

Figure 86. Alveolar gradient of Hexanoic acid in the different diets.

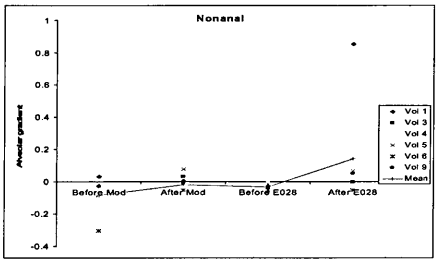

Figure 87. Alveolar gradient of Nonanal in the different diets.

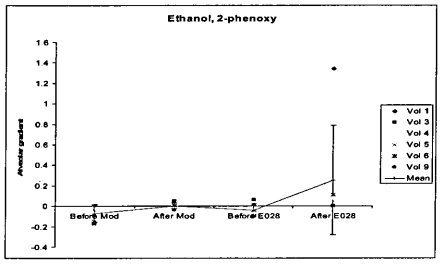

Figure 88. Alveolar gradient of Ethanol, 2-phenoxy in the different diets.

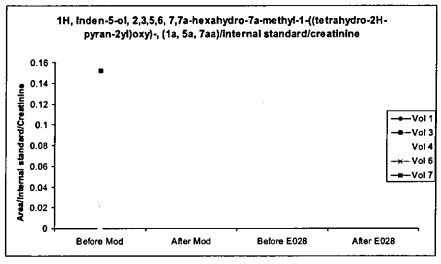

Figure 89. Area under the curve of 1H, Inden-5-ol, 2,3,5,6,7,7a-hexahydro-7a-methyl-1-((tetrahydro-2H-pyran-2yl)oxy)- , (1a, 5a, 7aa) related to the internal standard and creatinine before and after both diets.

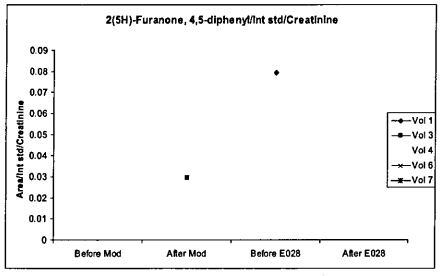

Figure 90. Area under the curve of 2(5H)-Furanone, 4,5-diphenyl related to the internal standard and creatinine before and after both diets.

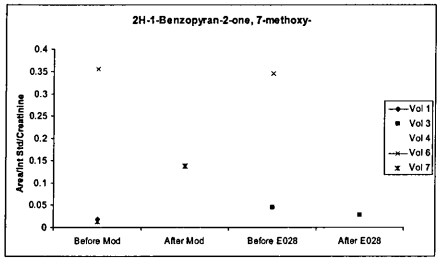

Figure 91. Area under the curve of 2H-1-benzopyran-2-one, 7-methoxy- related to the internal standard and creatinine before and after both diets.

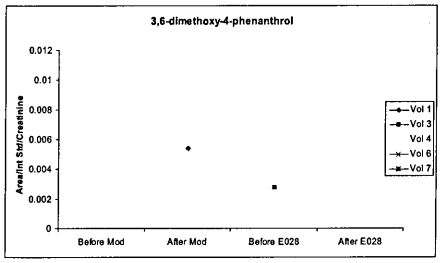

Figure 92. Area under the curve of 3,6-dimethoxy-4-phenanthrol related to the internal standard and creatinine before and after both diets.

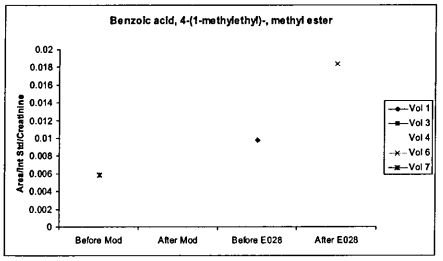

Figure 93. Area under the curve of 4-(1-methylethyl)- benzoic acid, methyl ester related to the internal standard and creatinine before and after both diets.

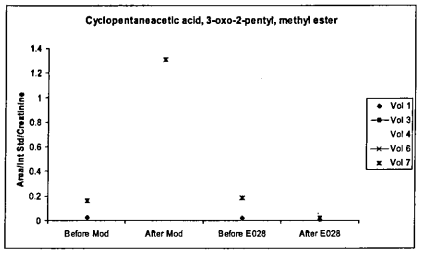

Figure 94. Area under the curve of 3-oxo-2-pentyl, cyclopentaneacetic acid, methyl ester related to the internal standard and creatinine before and after both diets.

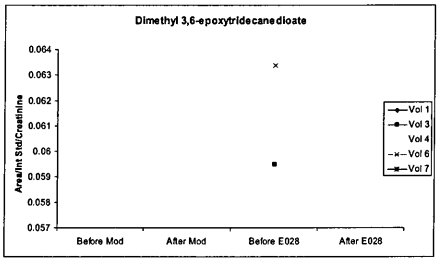

Figure 95. Area under the curve of 3,6-epoxytridecanedioate dimethy related to the internal standard and creatinine before and after both diets.

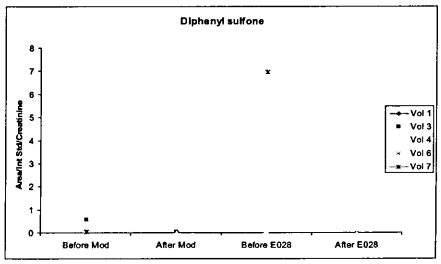

Figure 96. Area under the curve of diphenyl sulfone related to the internal standard and creatinine before and after both diets.

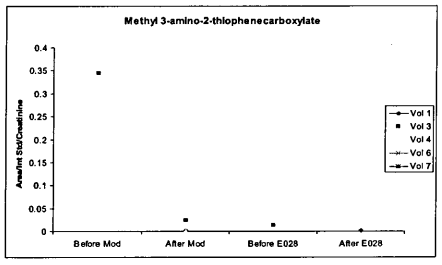

Figure 97. Area under the curve of methyl 3-amino-2-thiophenecarboxylate related to the internal standard and creatinine before and after both diets.

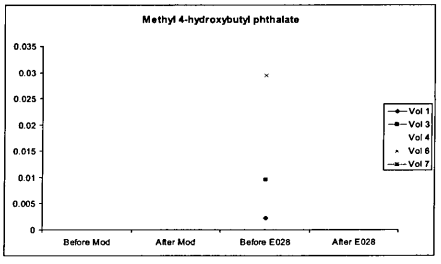

Figure 98. Area under the curve of 4-hydroxybutyl methyl phthalate related to the internal standard and creatinine before and after both diets.

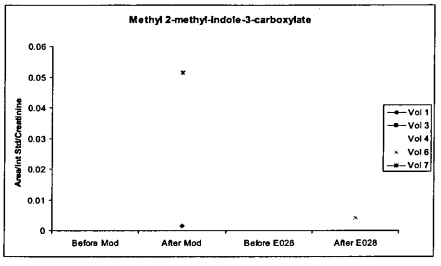

Figure 99. Area under the curve of 2-methyl-indole3-methyl carboxylate related to the internal standard and creatinine before and after both diets.

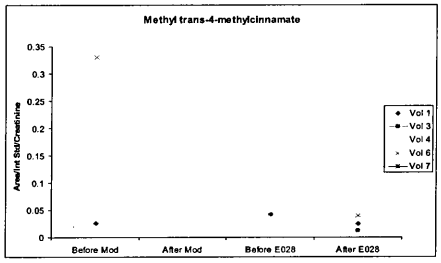

Figure 100. Area under the curve of methyl trans-4-methyh cinnamate related to the internal standard and creatinine before and after both diets.

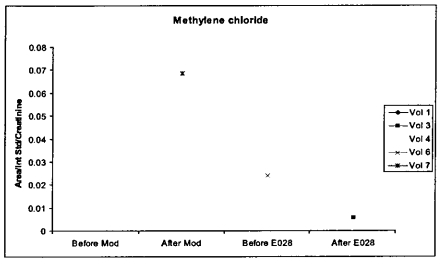

Figure 101. Area under the curve of methylene chloride related to the internal standard and creatinine before and after both diets.

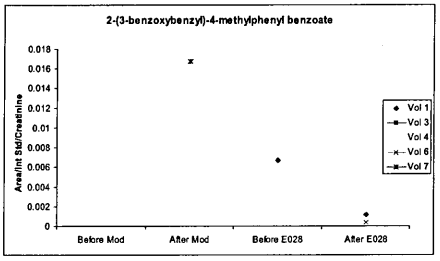

Figure 102. Area under the curve of 2-(3-benzoxybenzyl)-4-methylphenyl benzoate related to the internal standard and creatinine before and after both diets.

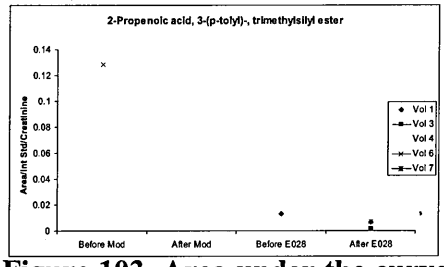

Figure 103. Area under the curve of 2-propenoic acid, 3-(p-tolyl)-, trimethylsilyl ester related to the internal standard and creatinine before and after both diets.

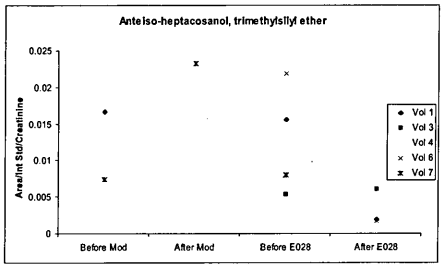

Figure 104. Area under the curve of anteiso-heptacosanol, trimethylsilyl ether related to the internal standard and creatinine before and after both diets.

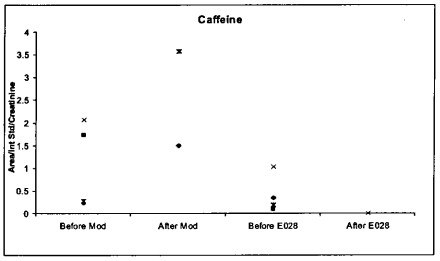

Figure 105. Area under the curve of caffeine related to the internal standard and creatinine before and after both diets.

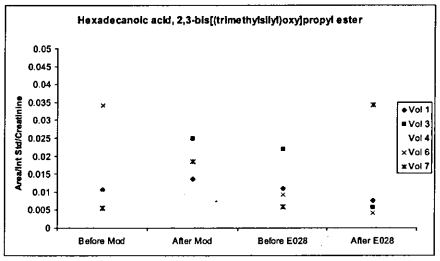

Figure 106. Area under the curve of 2,3-bis ((trimethylsilyl)oxy) hexadecanoic acid, propyl ester related to the internal standard and creatinine before and after both diets.

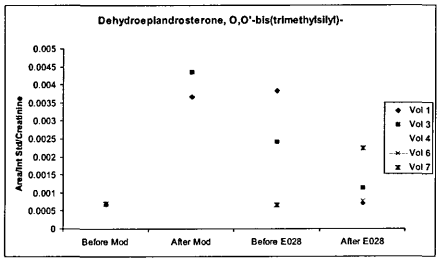

Figure 107. Area under the curve of O,O′-bis(trimethylsilyl) dehydroepiandrosterone related to the internal standard and creatinine before and after both diets.

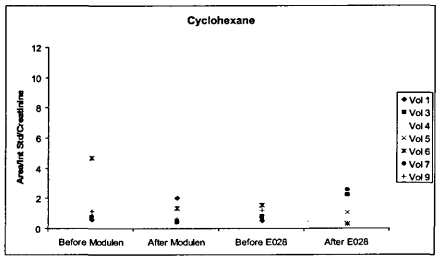

Figure 108. Area under the curve of cyclohexane related to the internal standard and creatinine before and after both diets.

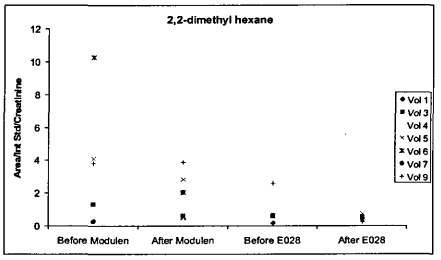

Figure 109. Area under the curve of 2,2-dimethyl hexane related to the internal standard and creatinine before and after both diets.

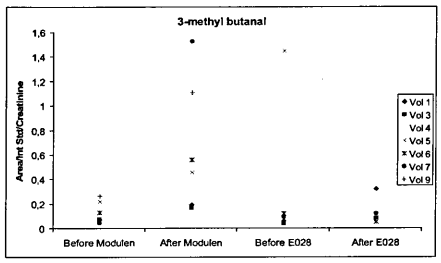

Figure 110. Area under the curve of 3-methyl butanal related to the internal standard and creatinine before and after both diets.

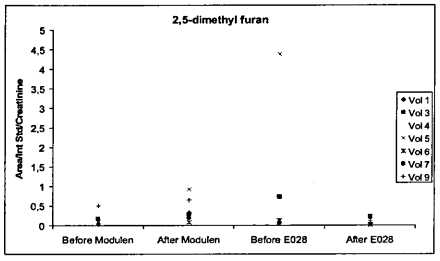

Figure 111. Area under the curve of 2,5-dimethyl furan related to the internal standard and creatinine before and after both diets.

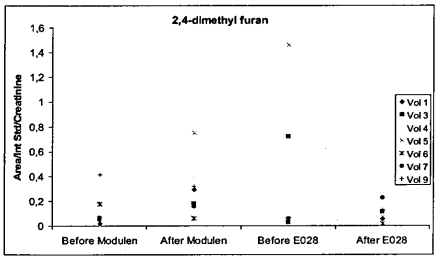

Figure 112. Area under the curve of 2,4-dimethyl furan related to the internal standard and creatinine before and after both diets.

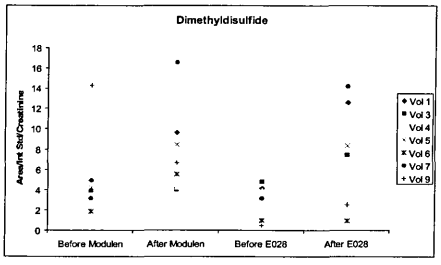

Figure 113. Area under the curve of dimethyldisulfide related to the internal standard and creatinine before and after both diets.

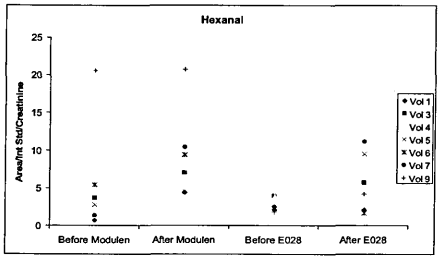

Figure 114. Area under the curve of hexanal related to the internal standard and creatinine before and after both diets.

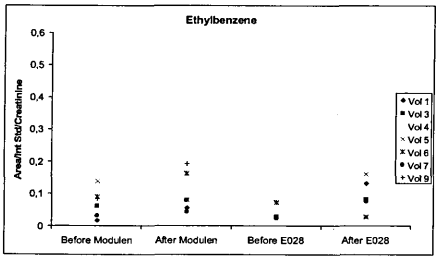

Figure 115. Area under the curve of ethylbenzene related to the internal standard and creatinine before and after both diets.

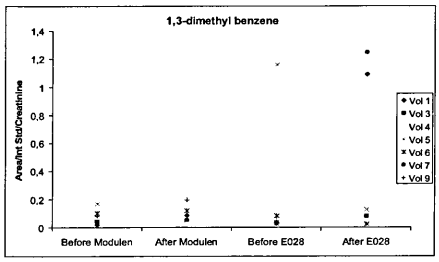

Figure 116. Area under the curve of 1,3-dimethyl benzene related to the internal standard and creatinine before and after both diets.

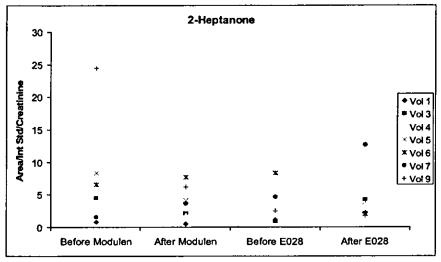

Figure 117. Area under the curve of 2-heptanone related to the internal standard and creatinine before and after both diets.

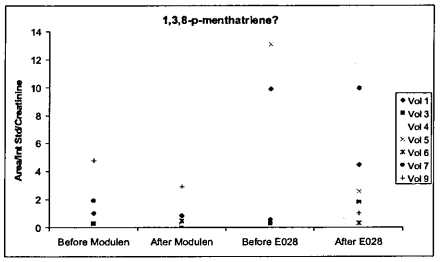

Figure 118. Area under the curve of a compound similar to 1,3,8-menthatriene related to the internal standard and creatinine before and after both diets.

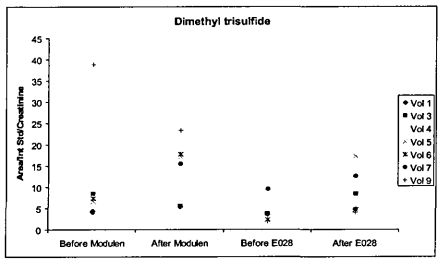

Figure 119. Area under the curve of dimethyl trisulfide related to the internal standard and creatinine before and after both diets.

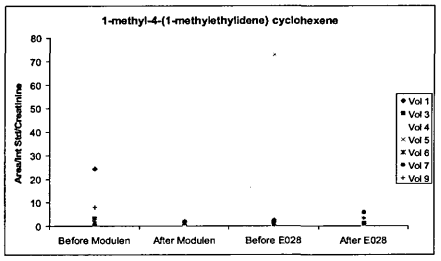

Figure 120. Area under the curve of 1-methyl-4-(1-methylethylidene) cyclohexene related to the internal standard and creatinine before and after both diets.

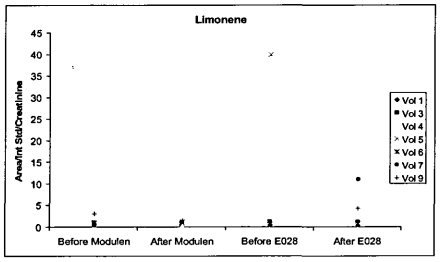

Figure 121. Area under the curve of limonene related to the internal standard and creatinine before and after both diets.

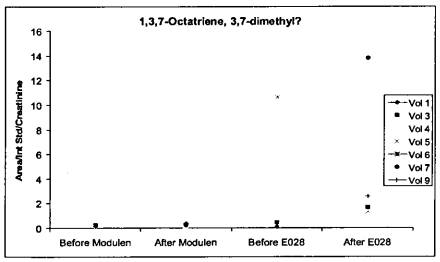

Figure 122. Area under the curve of a compound similar to 1,3,7-octariene, 3,7 dimethyl related to the internal standard and creatinine before and after both diets.

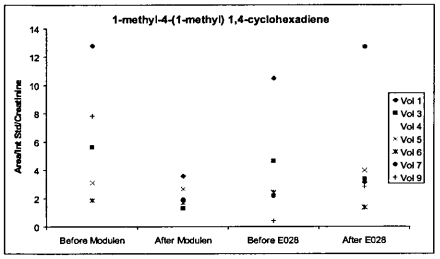

Figure 123. Area under the curve of 1-methyl-4-(1-methyl) 1,4-cyclohexadiene related to the internal standard and creatinine before and after both diets.

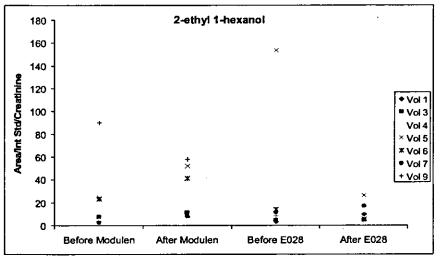

Figure 124. Area under the curve of 2-ethyl-1-hexanol related to the internal standard and creatinine before and after both diets.

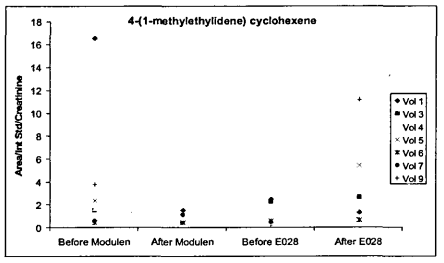

Figure 125. Area under the curve of 4-(1-methylethylidene) cyclohexene related to the internal standard and creatinine before and after both diets.

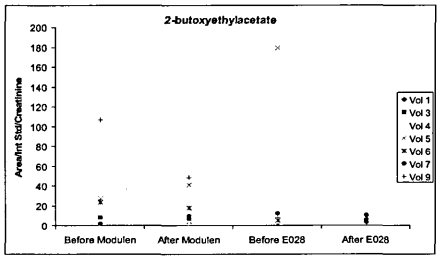

Figure 126. Area under the curve of butoxyethylacetate related to the internal standard and creatinine before and after both diets.

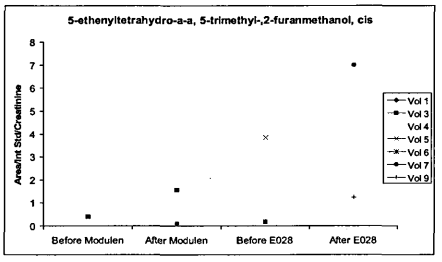

Figure 127. Area under the curve of 5-ethenyltetrahydro-a-a, 5-trimethyl-,2-furanmethanol, cis related to the internal standard and creatinine before and after both diets.

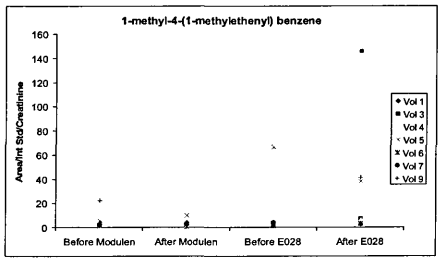

Figure 128. Area under the curve of 1-methyl-4-(1-methylethenyl) benzene related to the internal standard and creatinine before and after both diets.

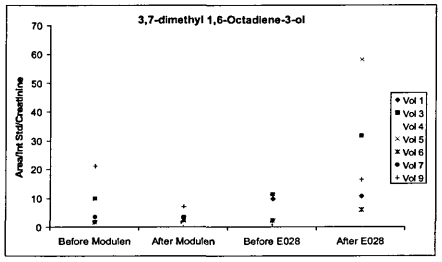

Figure 129. Area under the curve of 3,7-dimethyl 1,6-octadiene-3-ol related to the internal standard and creatinine before and after both diets.

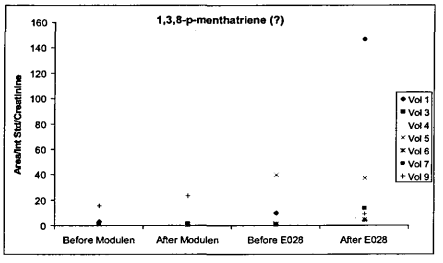

Figure 130. Area under the curve of a compound similar to 1,3,8-p-menthatriene related to the internal standard and creatinine before and after both diets.

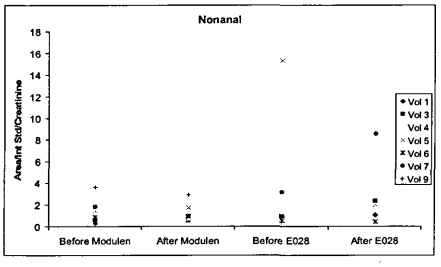

Figure 131. Area under the curve of nonanal related to the internal standard and creatinine before and after both diets.

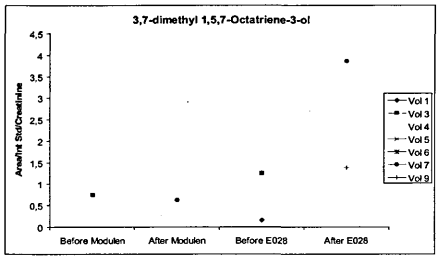

Figure 132. Area under the curve of 3,7-dimethyl 1,5,7-Octatriene-3-ol related to the internal standard and creatinine before and after both diets.

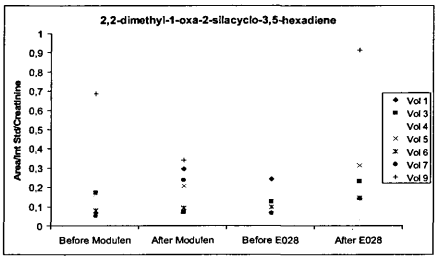

Figure 133. Area under the curve of 2,2-dimethyl-1-oxa-2-silacyclo-3,5-hexadiene related to the internal standard and creatinine before and after both diets.

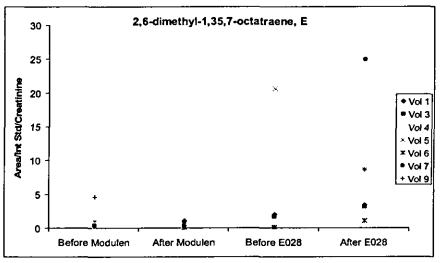

Figure 134. Area under the curve of 2,6-dimethyl-1,35,7-octatraene, E related to the internal standard and creatinine before and after both diets.

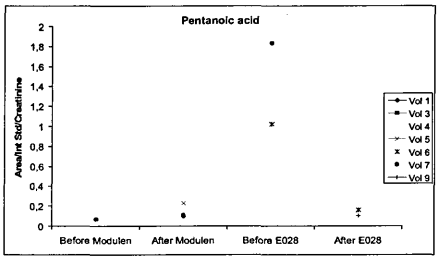

Figure 135. Area under the curve of pentanoic acid related to the internal standard and creatinine before and after both diets.

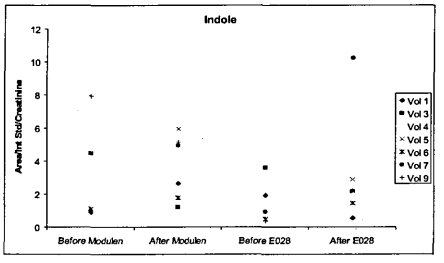

Figure 136. Area under the curve of indole related to the internal standard and creatinine before and after both diets.

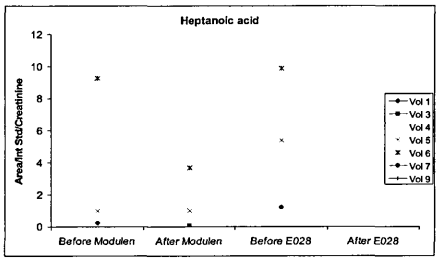

Figure 137. Area under the curve of heptanoic acid related to the internal standard and creatinine before and after both diets.

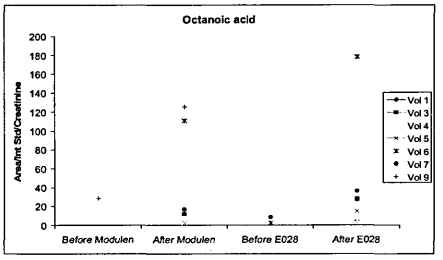

Figure 138. Area under the curve of octanoic acid related to the internal standard and creatinine before and after both diets.

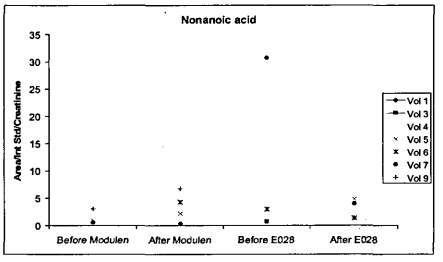

Figure 139. Area under the curve of nonanoic acid related to the internal standard and creatinine before and after both diets.

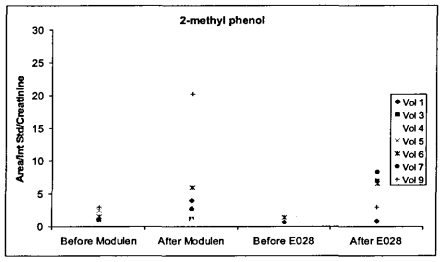

Figure 140. Area under the curve of 2-methyl phenol related to the internal standard and creatinine before and after both diets.

Appendix 6

IBS ethical permission

Appendix 7

Compounds in IBS

R+O, MSK and controls

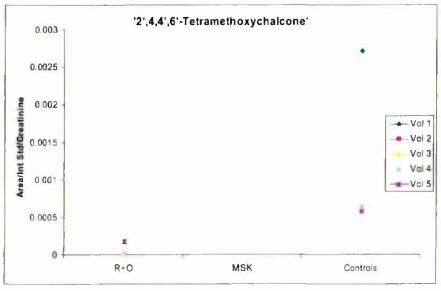

Figure 141. Area of '2,4,4',6'-Tetramethoxychalcone taking into account internal standard and creatinine in R+O, MSK and controls.

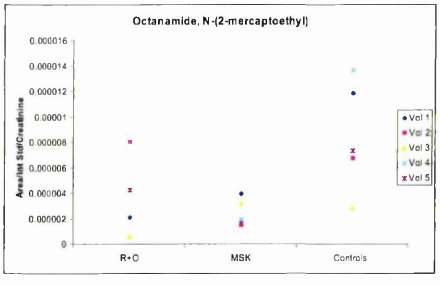

Figure 142. Area of octanamide, N-(2-mercaptoethyl) taking into account internal standard and creatinine in R+O, MSK and controls.

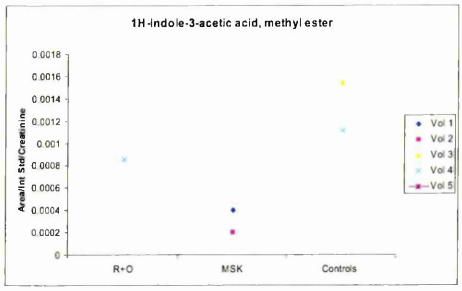

Figure 143. Area of 1H-Indole-3-acetic acid, methyl ester taking into account internal standard and creatinine in R+O, MSK and controls.

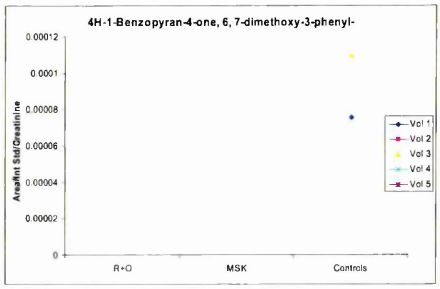

Figure 144. Area of 4H-1-Benzopyran-4-one, 6,7-dimethoxy-3-phenyl taking into account internal standard and creatinine in R+O, MSK and controls.

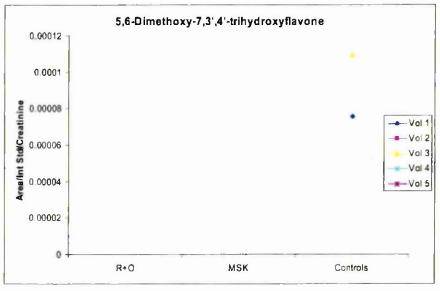

Figure 145. Area of 5,6-Dimethoxy-7,3',4'-trihydroxyflavone taking into account internal standard and creatinine in R+O, MSK and controls.

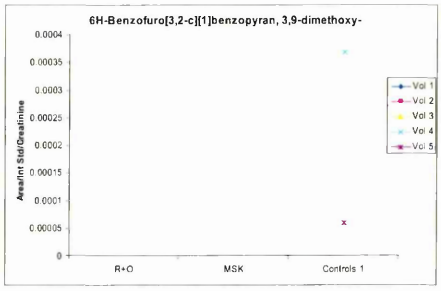

Figure 146. Area of 6H-Benzofuro(3,2-c)(1)benzopyran, 3,9-dimethoxy- taking into account internal standard and creatinine in R+O, MSK and controls.

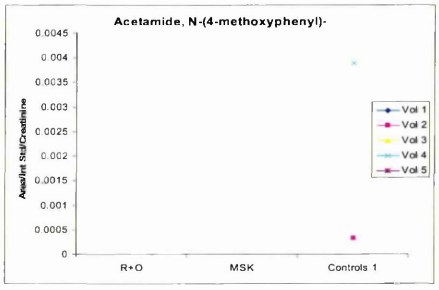

Figure 147. Area of N-(methoxyphenyl) acetamide taking into account internal standard and creatinine in R+O, MSK and controls.

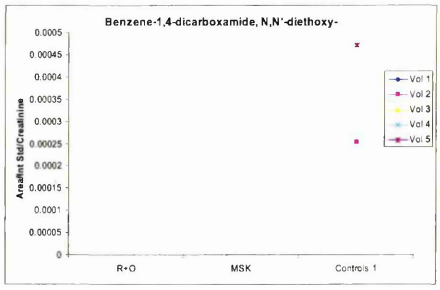

Figure 148. Area of benzene-1,4-dicarboxamide, N,N'-diethoxy- taking into account internal standard and creatinine in R+O, MSK and controls.

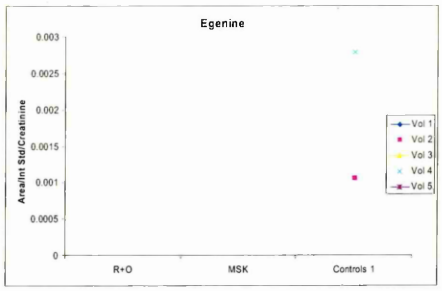

Figure 149. Area of egenine taking into account internal standard and creatinine in R+O, MSK and controls.

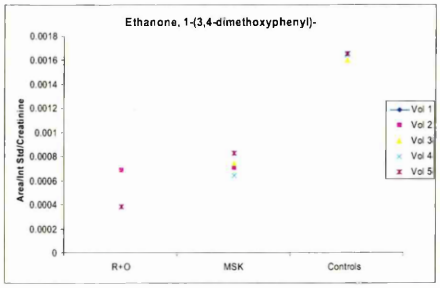

Figure 150. Area of ethanone, 1-(3,4-dimethoxyphenyl)- taking into account internal standard and creatinine in R+O, MSK and controls.

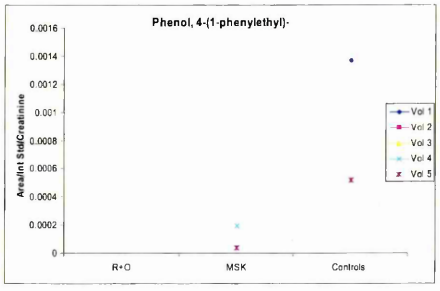

Figure 151. Area of phenol, 4-(1-phenylethyl)- taking into account internal standard and creatinine in R+O, MSK and controls.

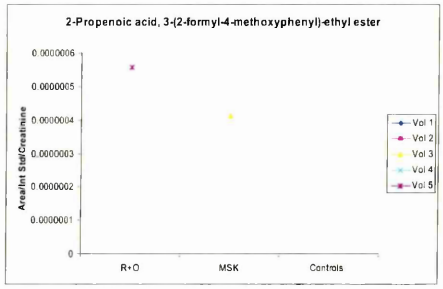

Figure 152. Area of 2-Propenoic acid, 3-(2-formyl-4-methoxyphenyl)-ethyl ester taking into account internal standard and creatinine in R+O, MSK and controls.

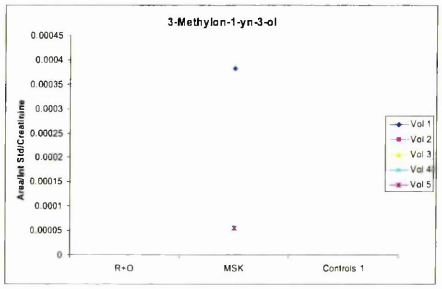

Figure 153. Area of phenol 3-Methylon-1-yn-3-ol taking into account internal standard and creatinine in R+O, MSK and controls.

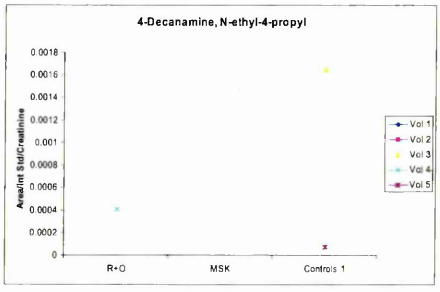

Figure 154. Area of phenol 3-Methylon-1-yn-3-ol taking into account internal standard and creatinine in R+O, MSK and controls.

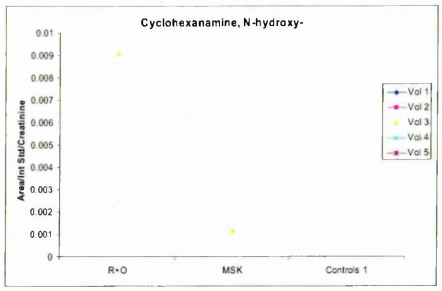

Figure 155. Area of phenol cyclohexanamine, N-hydroxy- taking into account internal standard and creatinine in R+O, MSK and controls.

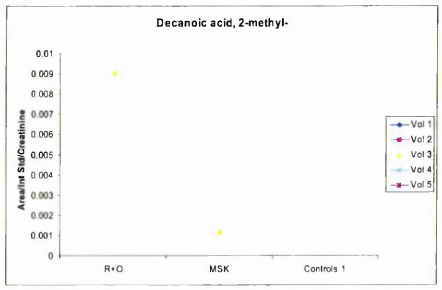

Figure 156. Area of decanoic acid, 2-methyl- taking into account internal standard and creatinine in R+O, MSK and controls.

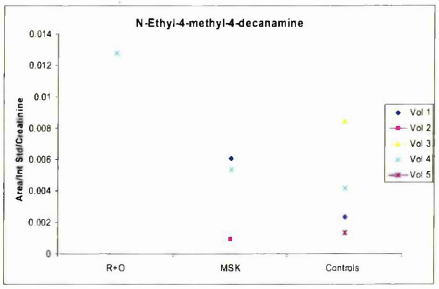

Figure 157. Area of N-ethyl-4-methyl-4-decanamine taking into account internal standard and creatinine in R+O, MSK and controls

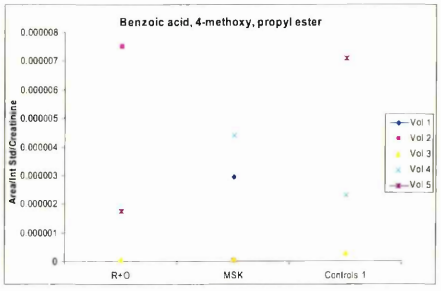

Figure 158. Area of benzoic acid, 4-methoxy, propyl ester taking into account internal standard and creatinine in R+O, MSK and controls

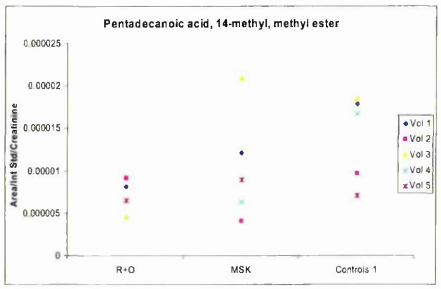

Figure 159. Area of pentadecanoic acid, 14-methyl, methyl ester taking into account internal standard and creatinine in R+O, MSK and controls

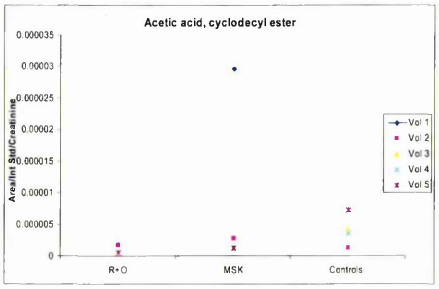

Figure 160. Area of acetic acid, cyclodecylester taking into account internal standard and creatinine in R+O, MSK and controls

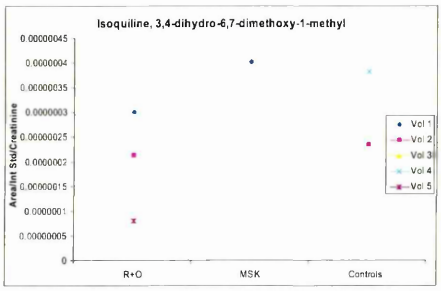

Figure 161. Area of isoquiline, 3,4-dihydro-6,7-dimethoxy-1-methyl taking into account internal standard and creatinine in R+O, MSK and controls

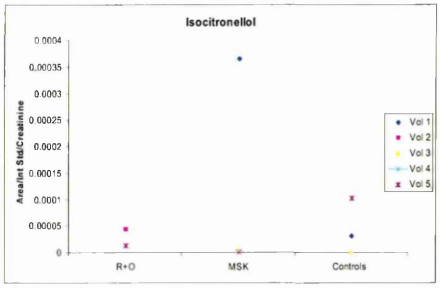

Figure 162. Area of isocitronellol taking into account internal standard and creatinine in R+O, MSK and controls.

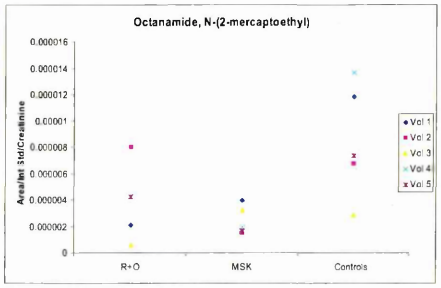

Figure 163. Area of octanamide, N-(2-mercaptoethyl) taking into account internal standard and creatinine in R+O, MSK and controls.

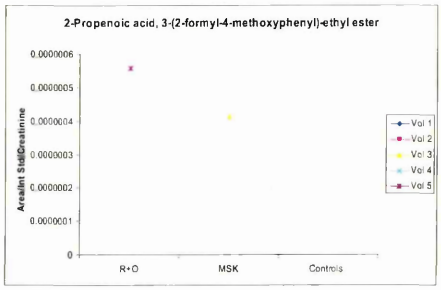

Figure 164. Area of 2-propenoic acid, 3-(2-formyl-4-methoxyphenyl)-ethyl ester taking into account internal standard and creatinine in R+O, MSK and controls.

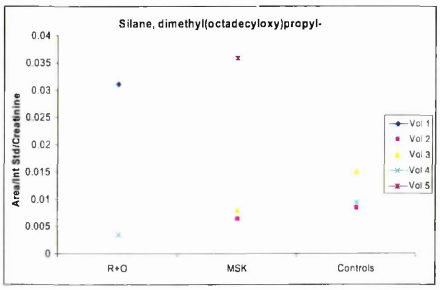

Figure 165. Area of silane, dimethyl(octadecyloxy) propyl taking into account internal standard and creatinine in R+O, MSK and controls.

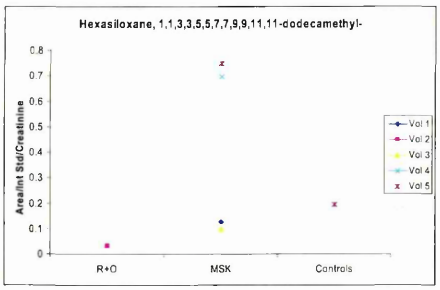

Figure 166. Area of hexasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11-dodecamethyl taking into account internal standard and creatinine in R+O, MSK and controls.

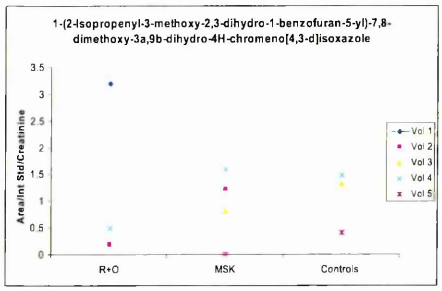

Figure 167. Area of 1-(2-isopropenyl-3-methoxy-2,3-dihydro-1-benzofuran-5-yl)-7,8-dimethoxy-3a,9b-dihydro-4H-chromeno(4,3-d) isoxazole taking into account internal standard and creatinine in R+O, MSK and controls.

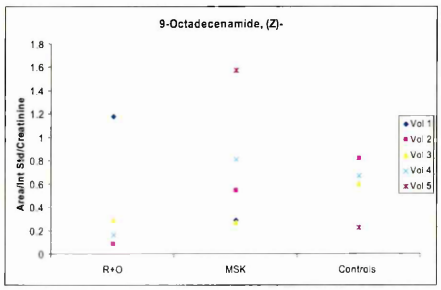

Figure 168. Area of 9-octadecenamide, (Z)-taking into account internal standard and creatinine in R+O, MSK and controls.

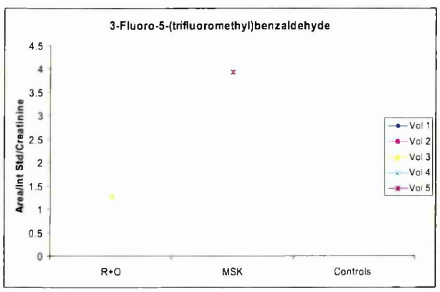

Figure 169. Area of 3-fluoro-5-(trifluoromethyl)benzaldehyde taking into account internal standard and creatinine in R+O, MSK and controls.

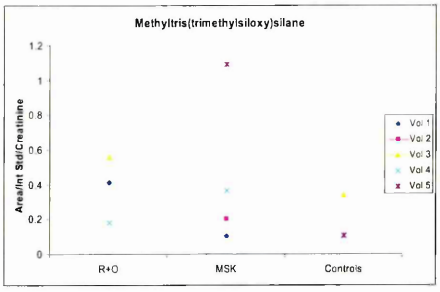

Figure 170. Area of 1-piperidinyloxy, 2,2,6,6-tetramethyl-4-((1-oxooctadecyl)oxy)- taking into account internal standard and creatinine in R+O, MSK and controls.

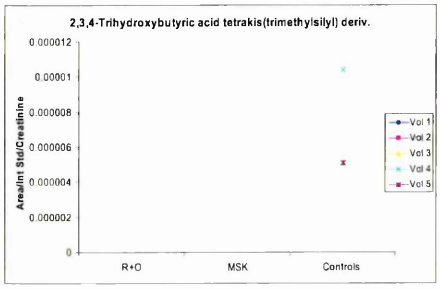

Figure 171. Area of 2,3,4-trihydroxybutyric acid tetrakis(trimethylsilyl) derivative taking into account internal standard and creatinine in R+O, MSK and controls.

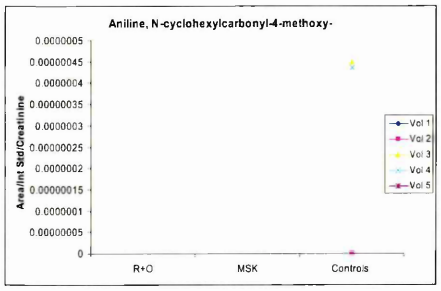

Figure 172. Area of aniline, N-cyclohexylcarbonyl-4-methoxy- taking into account internal standard and creatinine in R+O, MSK and controls.

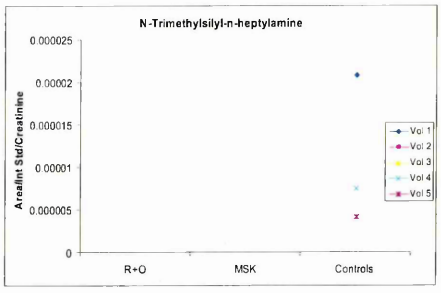

Figure 173. Area of N-trimethylsilyl-n-heptylamine taking into account internal standard and creatinine in R+O, MSK and controls.

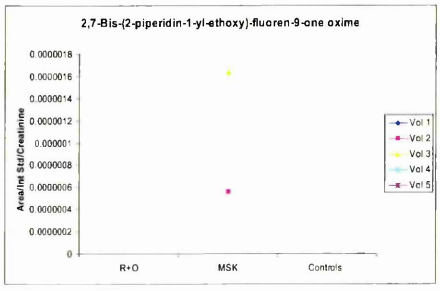

Figure 174. Area of 2,7-bis-(2-piperidin-1yl-ethoxy)-fluoren-9-one oxime taking into account internal standard and creatinine in R+O, MSK and controls.

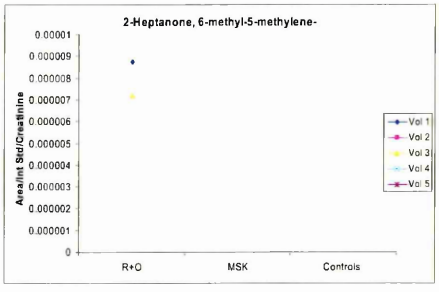

Figure 175 Area of 22-heptanone, 6-methyl-5-methylene taking into account internal standard and creatinine in R+O, MSK and controls.

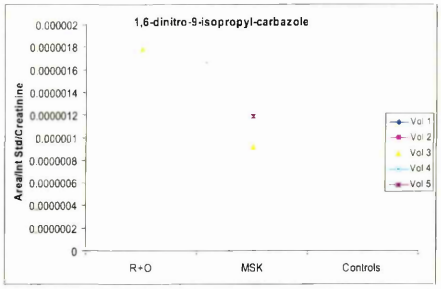

Figure 176. Area of 1,6-dinitro-9-isopropyl-carbazole taking into account internal standard and creatinine in R+O, MSK and controls.

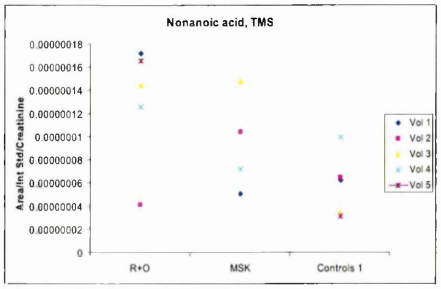

Figure 177. Area of nonanoic acid, TMS taking into account internal standard and creatinine in R+O, MSK and controls.

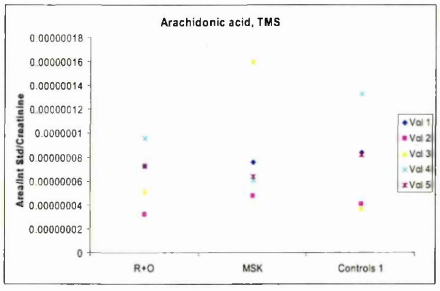

Figure 178. Area of arachidonic acid, TMS taking into account internal standard and creatinine in R+O, MSK and controls.

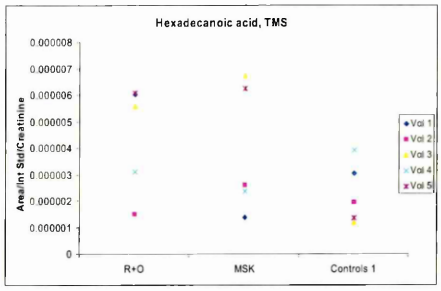

Figure 179. Area of hexadecanoic acid, TMS taking into account internal standard and creatinine in R+O, MSK and controls.

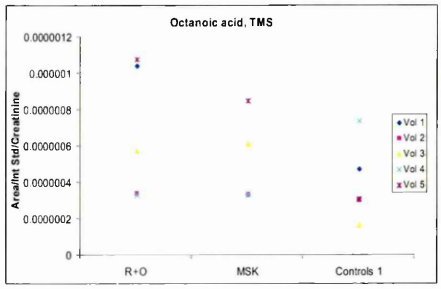

Figure 180. Area of octanoic acid TMS taking into account internal standard and creatinine in R+O, MSK and controls.

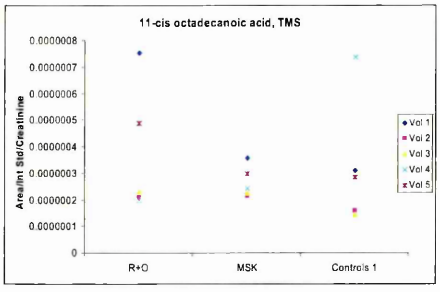

Figure 181. Area of 11-cis octadecanoic acid, TMS taking into account internal standard and creatinine in R+O, MSK and controls.

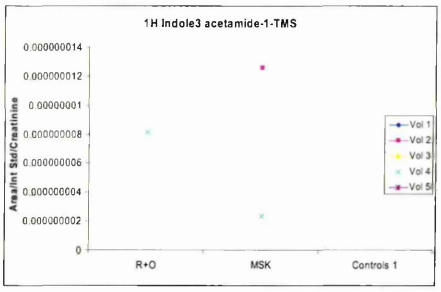

Figure 182. Area of 1H Indole 3 acetamide-1-TMS taking into account internal standard and creatinine in R+O, MSK and controls.

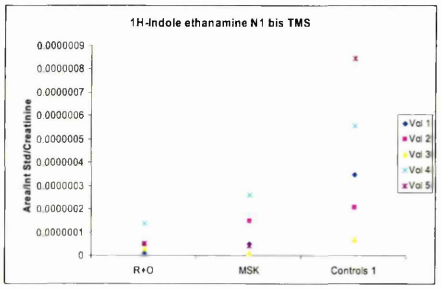

Figure 183. Area of 1H-Indole ethanamine N1 bis TMS taking into account internal standard and creatinine in R+O, MSK and controls.

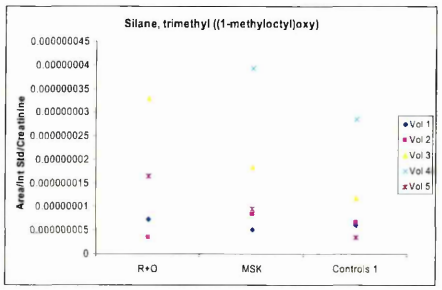

Figure 184. Area of trimethyl (1-methyloctyloxy) silane taking into account internal standard and creatinine in R+O, MSK and controls.

IBS, MSK, IBS 1 and IBS2

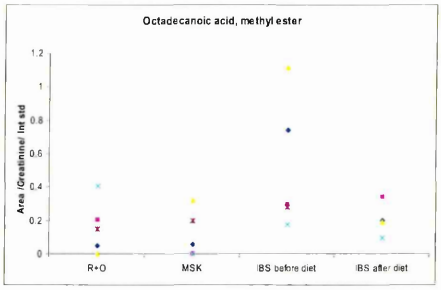

Figure 185. AUC of Octadecanoic acid methyl ester related to the internal standard and creatinine in R+O, MSK and IBS before and after both diets analysed using the HAN method.

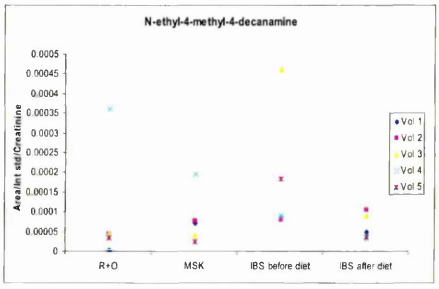

Figure 186. AUC of N-etyl-4 methyl-4-decanamine related to the internal standard and creatinine in R+O, MSK and IBS before and after both diets found in urine analysed using the HAN method.

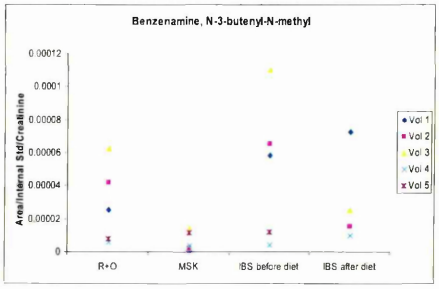

Figure 187. AUC of N-3-butenyl-N-methyl benzenamine related to the internal standard and creatinine in R+O, MSK and IBS before and after both diets found in urine analysed using the HAN method.

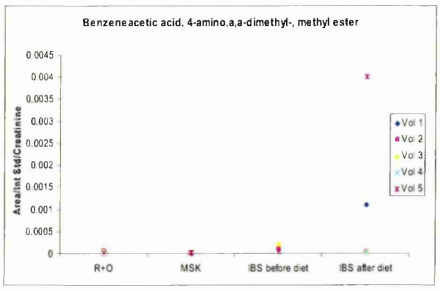

Figure 188. AUC of 4-amino,α, α-dimethyl-, benzeneacetic acid, methyl ester related to the internal standard and creatinine in R+O, MSK and IBS before and after both diets found in urine analysed using the HAN method.

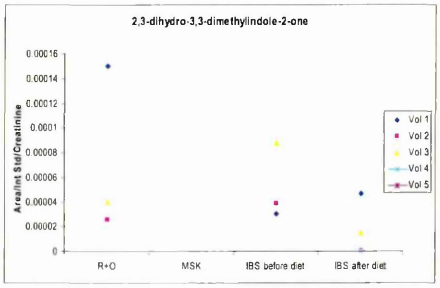

Figure 189. AUC of 2,3-dihydro-3,3-dimethylindole-2-one related to the internal standard and creatinine in R+O, MSK and IBS before and after both diets found in urine analysed using the HAN method.

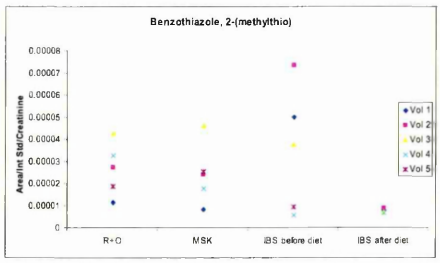

Figure 190. AUC of 2-(methylthio) benzothiazole related to the internal standard and creatinine in R+O, MSK and IBS before and after both diets found in urine analysed using the HAN method.

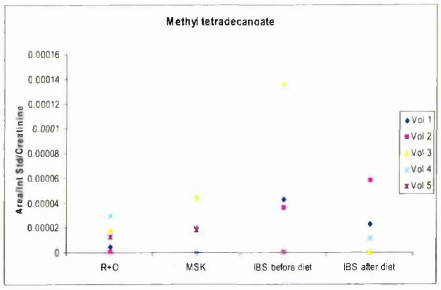

Figure 191. AUC of 2-(methylthio) benzothiazole related to the internal standard and creatinine in R+O, MSK and IBS before and after both diets found in urine analysed using the HAN method.

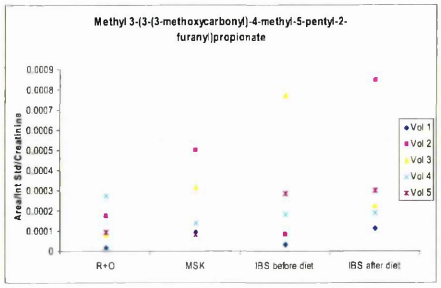

Figure 192. AUC of methyl 3-(3-(3-methoxycarbonyl)-4-methyl-5-pentyl-2-furanyl) propionate related to the internal standard and creatinine in R+O, MSK and IBS before and after both diets found in urine analysed using the HAN method.

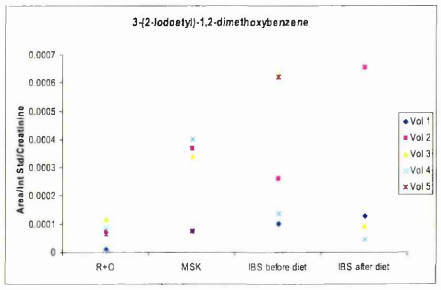

Figure 193. AUC of 3-(2-iodoethyl)-1,2-dimethoxybenzene related to the internal standard and creatinine in R+O, MSK and IBS before and after both diets found in urine analysed using the HAN method.

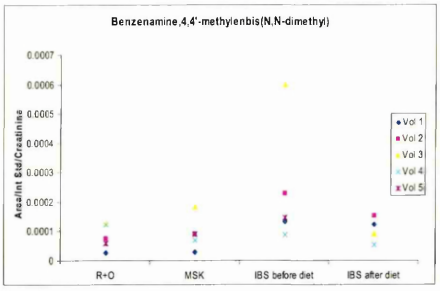

Figure 194. AUC of 4,4′-methylenbis (N,N-dimethyl) benzenamine related to the internal standard and creatinine in R+O, MSK and IBS before and after both diets found in urine analysed using the HAN method.

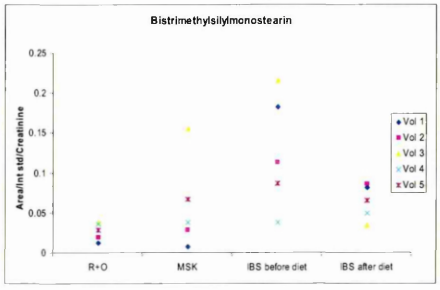

Figure 195. AUC of bistrimethylsilylmonostearin related to the internal standard and creatinine in R+O, MSK and IBS before and after both diets found in urine analysed using the UB method.

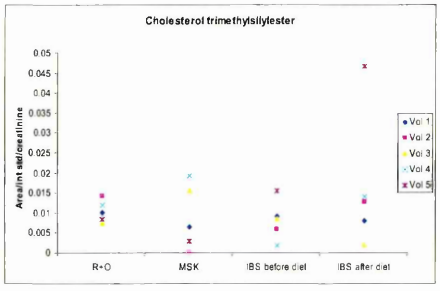

Figure 196. AUC of cholesterol trimethylsilylester related to the internal standard and creatinine in R+O, MSK and IBS before and after both diets found in urine analysed using the UB method.

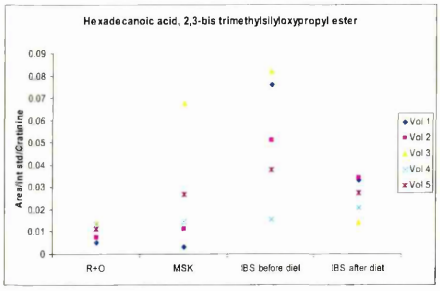

Figure 197. AUC of 2,3-bis trimethylsilyloxypropyl ester hexadecanoic acid related to the internal standard and creatinine in R+O, MSK and IBS before and after both diets found in urine analysed using the UB method.

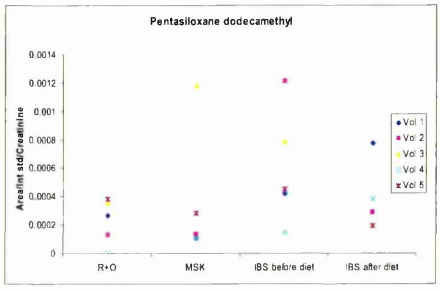

Figure 198. AUC of pentaxiloxane dodecamethyl related to the internal standard and creatinine in R+O, MSK and IBS before and after both diets found in urine analysed using the UB method.

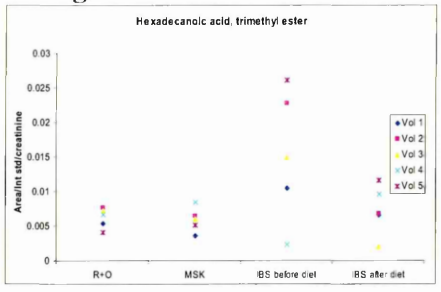

Figure 199. AUC of hexadecanoic acid trimethyl ester related to the internal standard and creatinine in R+O, MSK and IBS before and after both diets found in urine analysed using the UB method.

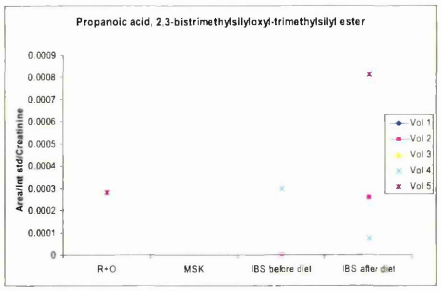

Figure 200. AUC of 2,3-bis trimethylsilyloxyl-trimethylsilyl ester propanoic acid related to the internal standard and creatinine in R+O, MSK and IBS before and after both diets found in urine analysed using the UB method.

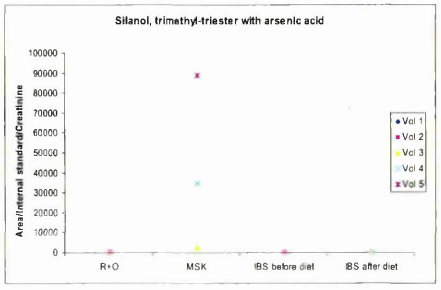

Figure 201. AUC of trimethyl silanol trimester with arsenic acid related to the internal standard and creatinine in R+O, MSK and IBS before and after both diets found in urine analysed using the UB method.

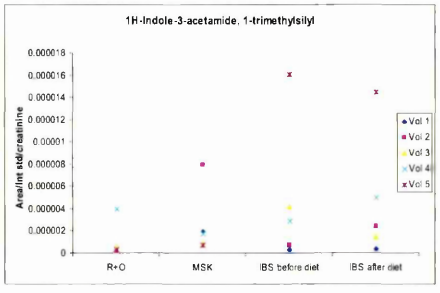

Figure 202. AUC of 1H-indole-3-acetamide, 1-trimethylsilyl related to the internal standard and creatinine in R+O, MSK and IBS before and after both diets found in urine analysed using the UB method.

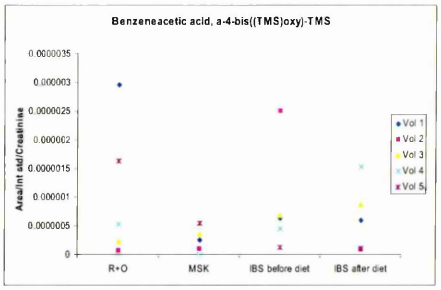

Figure 203. AUC of α-4-bis((TMS)oxy)-TMS benzeneacetic acid related to the internal standard and creatinine in R+O, MSK and IBS before and after both diets found in urine analysed using the UB method.

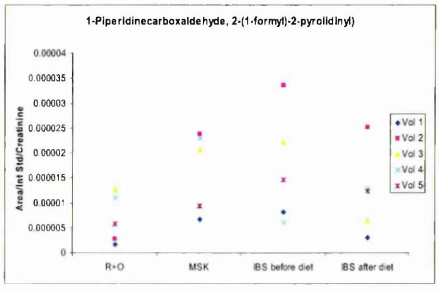

Figure 204. AUC of 1-piperidinecarboxaldehyde, 2-(1-formyl)-2-pyrolidinyl) related to the internal standard and creatinine in R+O, MSK and IBS before and after both diets found in urine analysed using the UB method.

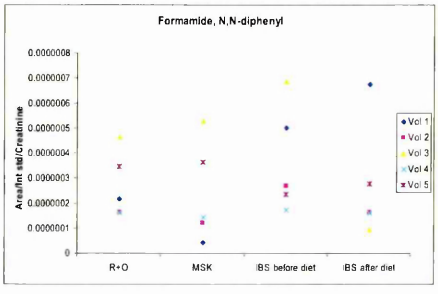

Figure 205. AUC of N,N-diphenyl formamide related to the internal standard and creatinine in R+O, MSK and IBS before and after both diets found in urine analysed using the UB method.

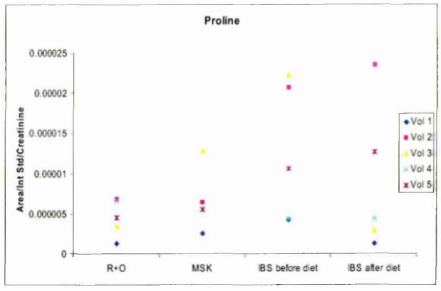

Figure 206. AUC of proline related to the internal standard and creatinine in R+O, MSK and IBS before and after both diets found in urine analysed using the UB method.

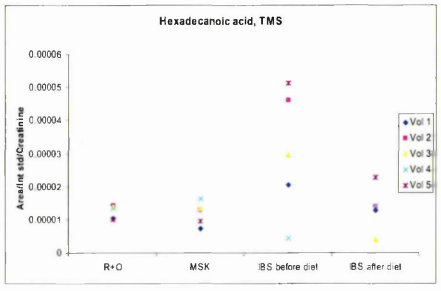

Figure 207. AUC of hexadecanoic acid TMS related to the internal standard and creatinine in R+O, MSK and IBS before and after both diets found in urine analysed using the UB method.

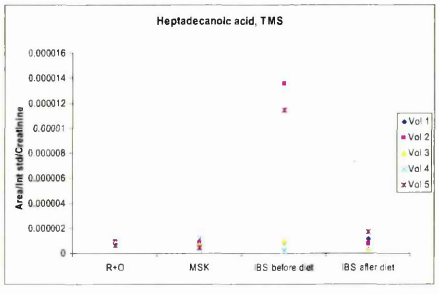

Figure 208. AUC of heptadecanoic acid TMS related to the internal standard and creatinine in R+O, MSK and IBS before and after both diets found in urine analysed using the UB method.

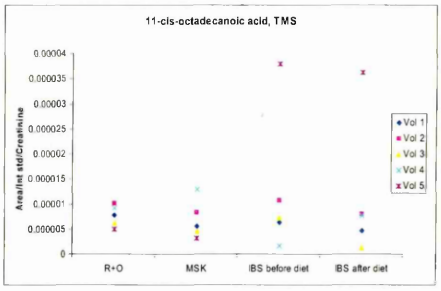

Figure 209. AUC of 11-cis-octadecanoic acid, TMS related to the internal standard and creatinine in R+O, MSK and IBS before and after both diets found in urine analysed using the UB method.

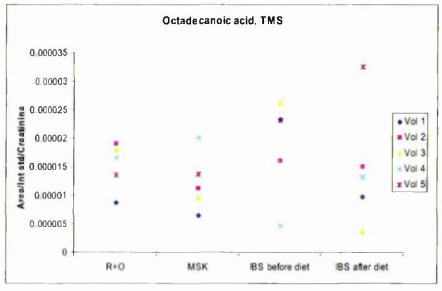

Figure 210. AUC of octadecanoic acid, TMS related to the internal standard and creatinine in R+O, MSK and IBS before and after both diets found in urine analysed using the UB method.

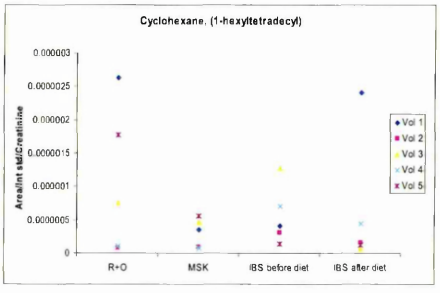

Figure 211. AUC of (1-hexyltetradecyl) cyclohexane related to the internal standard and creatinine in R+O, MSK and IBS before and after both diets found in urine analysed using the UB method.

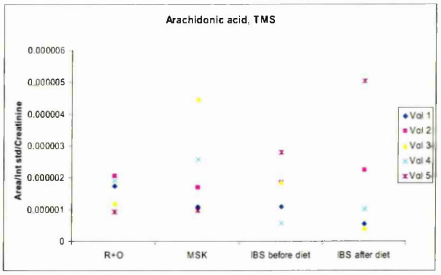

Figure 212. AUC of arachidonic acid, TMS related to the internal standard and creatinine in R+O, MSK and IBS before and after both diets found in urine analysed using the UB method.

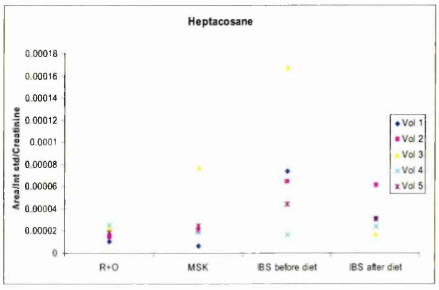

Figure 213. AUC of heptacosane related to the internal standard and creatinine in R+O, MSK and IBS before and after both diets found in urine analysed using the UB method.

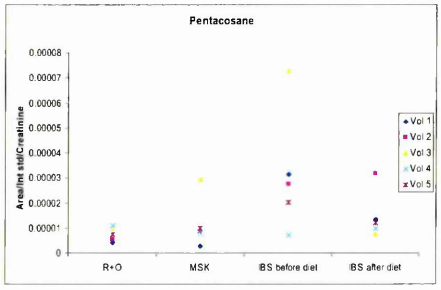

Figure 214. AUC of pentacosane related to the internal standard and creatinine in R+O, MSK and IBS before and after both diets found in urine analysed using the UB method.

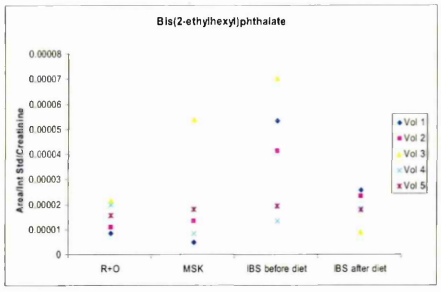

Figure 215. AUC of bis(2-ethylhexyl)phthalate related to the internal standard and creatinine in R+O, MSK and IBS before and after both diets found in urine analysed using the UB method.

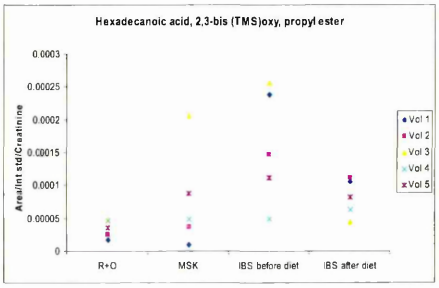

Figure 216. AUC of 2,3-bis(TMS)oxy hexadecanoic acid, propyl ester related to the internal standard and creatinine in R+O, MSK and IBS before and after both diets found in urine analysed using the UB method.

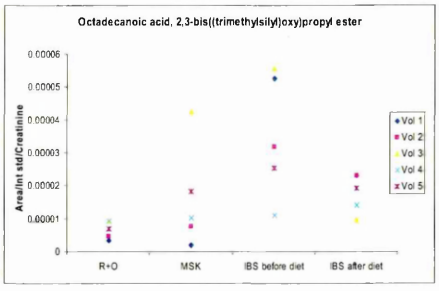

Figure 217. AUC of 2,3-bis((trimethylsilyl)oxy) octadecanoic acid propyl ester related to the internal standard and creatinine in R+O, MSK and IBS before and after both diets found in urine analysed using the UB method.

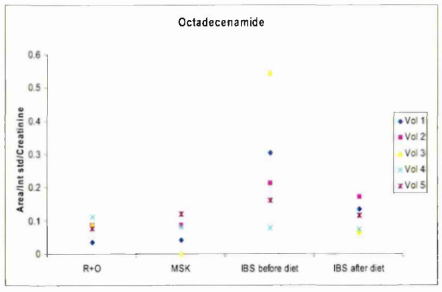

Figure 218. AUC of octadecenamide related to the internal standard and creatinine in R+O, MSK and IBS before and after both diets found in urine analysed using the HB method.

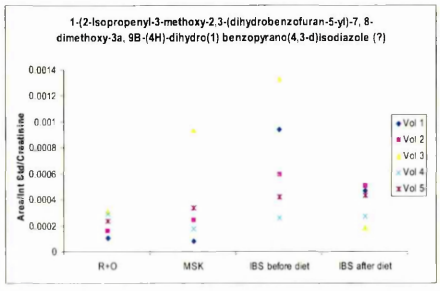

Figure 219. AUC of 1-(2-isopropenyl-3-methoxy-2,3-(dihydrobenzofuran-5-yl)-7,8-dimethoxy-3a,9B-(4H)-dihydro(1) benzopyrano (4,3-d) isodiazole related to the internal standard and creatinine in R+O, MSK and IBS before and after both diets found in urine analysed using the HB method.

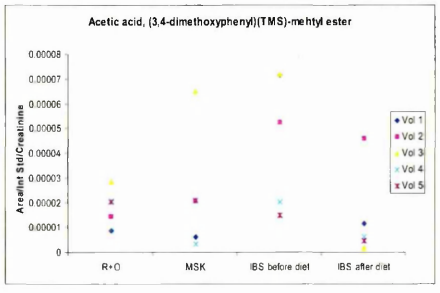

Figure 220. AUC of (3,4-dimethoxyphenyl)(TMS)-acetic acid methyl ester related to the internal standard and creatinine in R+O, MSK and IBS before and after both diets found in urine analysed using the HB method.

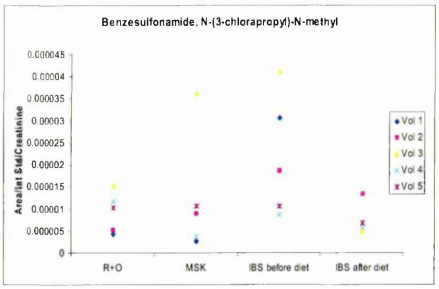

Figure 221. AUC of (N-(3-chloropropyl) benzesulfonamide-N-methyl related to the internal standard and creatinine in R+O, MSK and IBS before and after both diets found in urine analysed using the HB method.

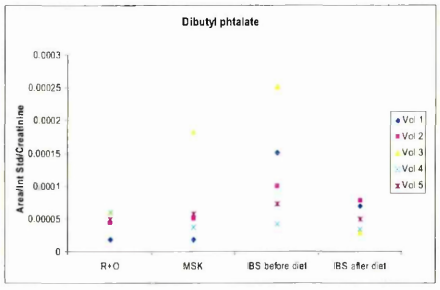

Figure 222. AUC of dibutyl phtalate related to the internal standard and creatinine in R+O, MSK and IBS before and after both diets found in urine analysed using the HB method.

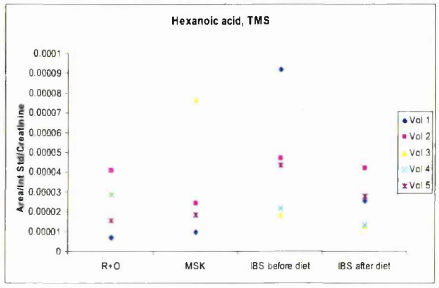

Figure 223. AUC of hexanoic acid, TMS related to the internal standard and creatinine in R+O, MSK and IBS before and after both diets found in urine analysed using the HB method.

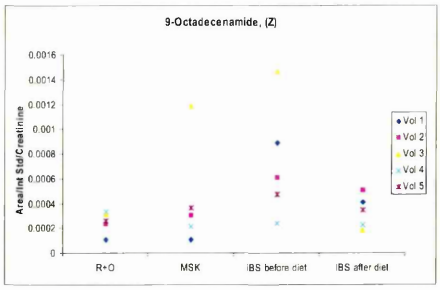

Figure 224. AUC of 9-octadecenamide, (Z) related to the internal standard and creatinine in R+O, MSK and IBS before and after both diets found in urine analysed using the HB method.

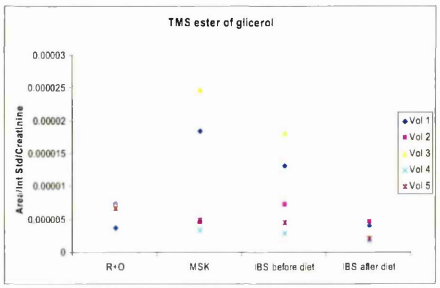

Figure 225. AUC of TMS ester of glicerol related to the internal standard and creatinine in R+O, MSK and IBS before and after both diets found in urine analysed using the HB method.

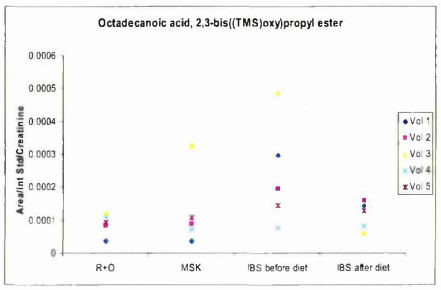

Figure 226. AUC of 2,3-bis((TMS)oxy)propyl ester octadecanoic acid related to the internal standard and creatinine in R+O, MSK and IBS before and after both diets found in urine analysed using the HB method.

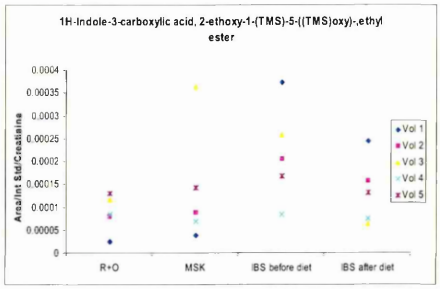

Figure 227. AUC of 1H-Indole-3-carboxylic aicd, 2-ethoxy-1-(TMS)-5-((TMS)oxy)-ethyl ester octadecanoic acid related to the internal standard and creatinine in R+O, MSK and IBS before and after both diets found in urine analysed using the HB method.

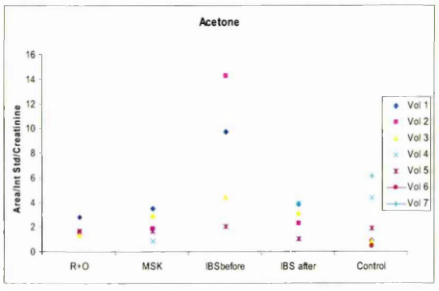

Figure 228. AUC of acetone related to the internal standard and creatinine in R+O, MSK, IBS before and after both diets and controls urine analysed using the SPME method.

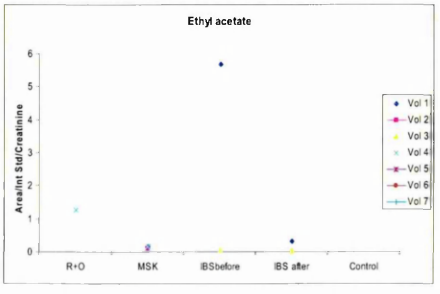

Figure 229. AUC of ethylacetate related to the internal standard and creatinine in R+O, MSK, IBS before and after both diets and controls urine analysed using the SPME method.

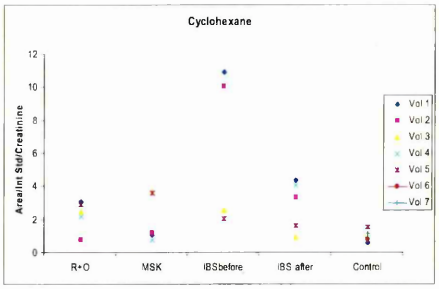

Figure 230. AUC of cyclohexane related to the internal standard and creatinine in R+O, MSK, IBS before and after both diets and controls urine analysed using the SPME method.

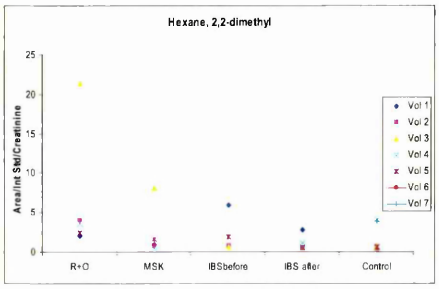

Figure 231. AUC of 2,2-dimethyl hexane related to the internal standard and creatinine in R+O, MSK, IBS before and after both diets and controls urine analysed using the SPME method.

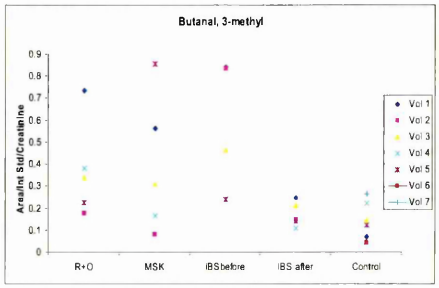

Figure 232. AUC of 3-methyl butanal related to the internal standard and creatinine in R+O, MSK, IBS before and after both diets and controls urine analysed using the SPME method.

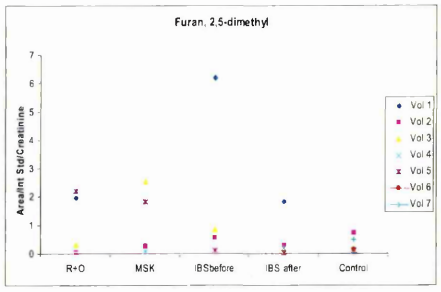

Figure 233. AUC of 2,5-dimethyl furan related to the internal standard and creatinine in R+O, MSK, IBS before and after both diets and controls urine analysed using the SPME method.

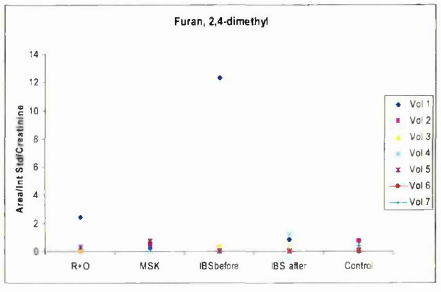

Figure 234 . AUC of 2,4-dimethyl furan related to the internal standard and creatinine in R+O, MSK, IBS before and after both diets and controls urine analysed using the SPME method.

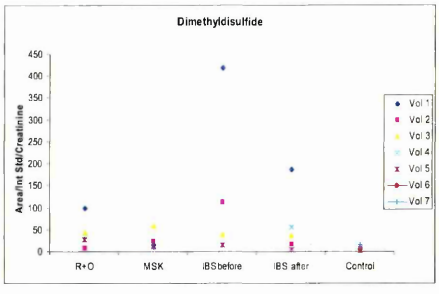

Figure 235. AUC of dimethyldisulfide related to the internal standard and creatinine in R+O, MSK, IBS before and after both diets and controls urine analysed using the SPME method.

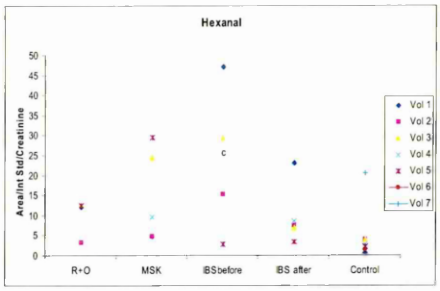

Figure 236. AUC of hexanal related to the internal standard and creatinine in R+O, MSK, IBS before and after both diets and controls urine analysed using the SPME method.

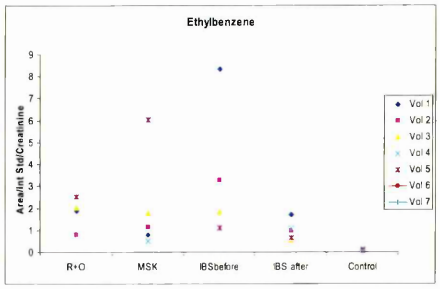

Figure 237. AUC of ethylbenzene related to the internal standard and creatinine in R+O, MSK, IBS before and after both diets and controls urine analysed using the SPME method.

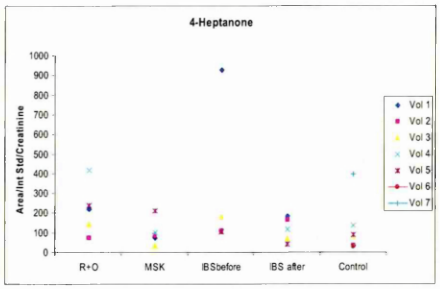

Figure 238. AUC of 4-heptanone related to the internal standard and creatinine in R+O, MSK, IBS before and after both diets and controls urine analysed using the SPME method.

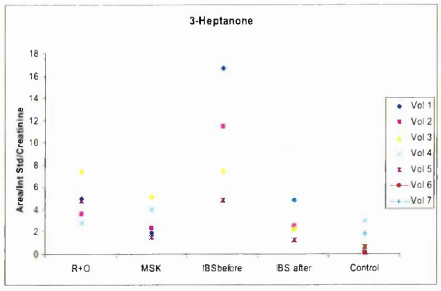

Figure 239. AUC of 3-heptanone related to the internal standard and creatinine in R+O, MSK, IBS before and after both diets and controls urine analysed using the SPME method.

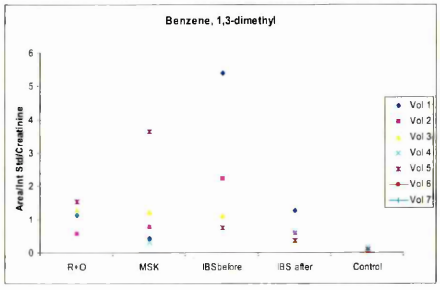

Figure 240. AUC of 1,3-dimethyl benzene related to the internal standard and creatinine in R+O, MSK, IBS before and after both diets and controls urine analysed using the SPME method.

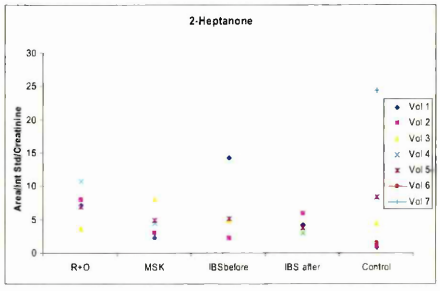

Figure 241. AUC of 2-heptanone related to the internal standard and creatinine in R+O, MSK, IBS before and after both diets and controls urine analysed using the SPME method.

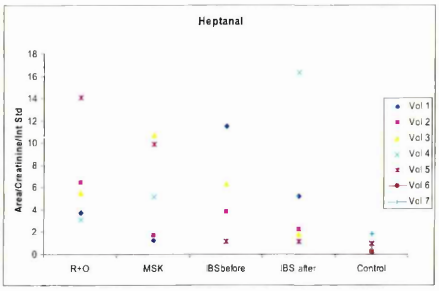

Figure 242. AUC of heptanal related to the internal standard and creatinine in R+O, MSK, IBS before and after both diets and controls urine analysed using the SPME method.

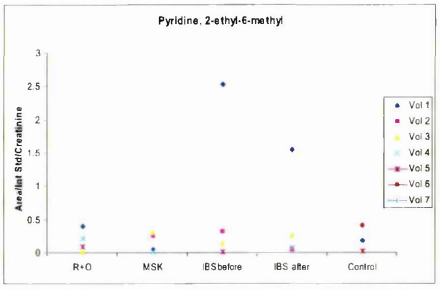

Figure 243. AUC of pyridine, 2-ethyl-6-methyl related to the internal standard and creatinine in R+O, MSK, IBS before and after both diets and controls urine analysed using the SPME method.

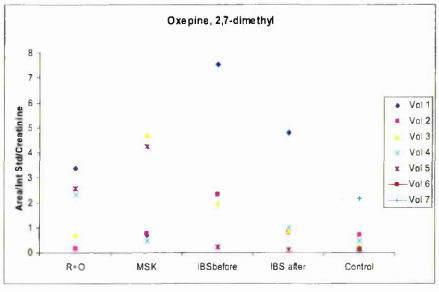

Figure 244. AUC of 2,7-dimethyl oxepine related to the internal standard and creatinine in R+O, MSK, IBS before and after both diets and controls urine analysed using the SPME method.

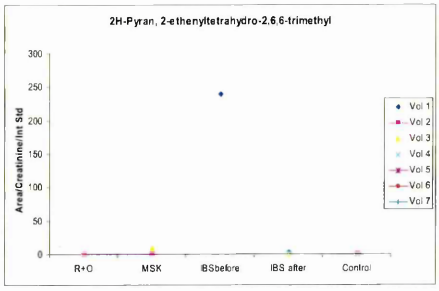

Figure 245. AUC of 2H-pyran, 2-ethenyltetrahydro-2,6,6-trimethyl related to the internal standard and creatinine in R+O, MSK, IBS before and after both diets and controls urine analysed using the SPME method.

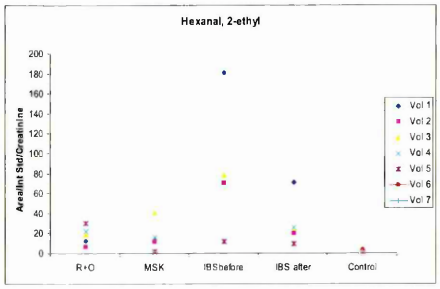

Figure 246. AUC of 2H-ethyl hexanal related to the internal standard and creatinine in R+O, MSK, IBS before and after both diets and controls urine analysed using the SPME method.

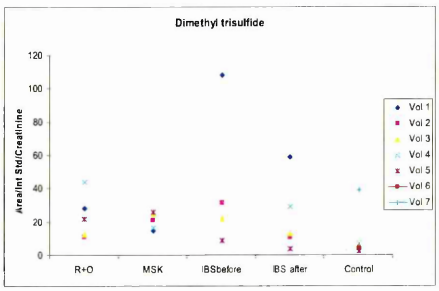

Figure 247. AUC of dimethyltrisulfide related to the internal standard and creatinine in R+O, MSK, IBS before and after both diets and controls urine analysed using the SPME method.

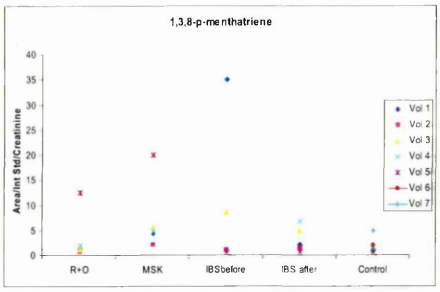

Figure 248. AUC of 1,3,8-p-menthatriene related to the internal standard and creatinine in R+O, MSK, IBS before and after both diets and controls urine analysed using the SPME method.

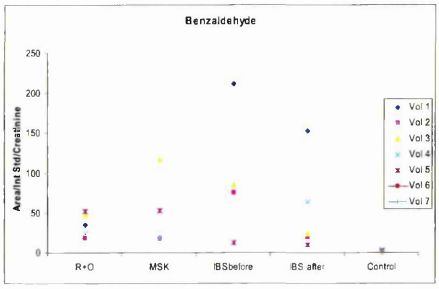

Figure 249. AUC of benzaldehyde related to the internal standard and creatinine in R+O, MSK, IBS before and after both diets and controls urine analysed using the SPME method.

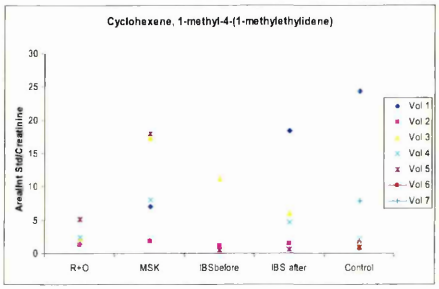

Figure 250. AUC of 1-methyl-4-(1-methylethylidene) cyclohexane related to the internal standard and creatinine in R+O, MSK, IBS before and after both diets and controls urine analysed using the SPME method.

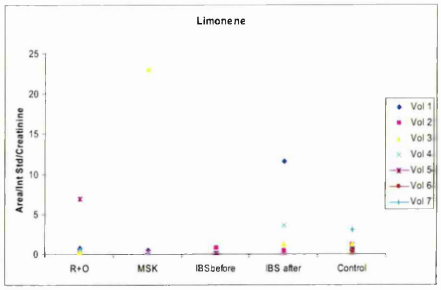

Figure 251. AUC of limonene related to the internal standard and creatinine in R+O, MSK, IBS before and after both diets and controls urine analysed using the SPME method.

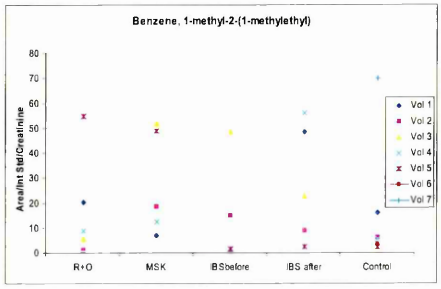

Figure 252. AUC 1-methyl-2-(1-methylethyl) benzene related to the internal standard and creatinine in R+O, MSK, IBS before and after both diets and controls urine analysed using the SPME method.

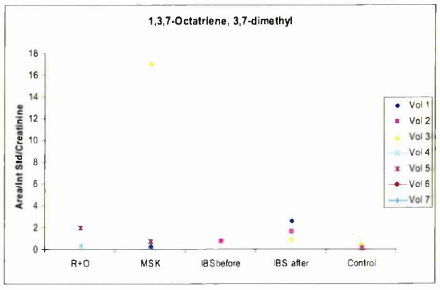

Figure 253. AUC 1,3,7-octatriene, 3,7-dimethyl related to the internal standard and creatinine in R+O, MSK, IBS before and after both diets and controls urine analysed using the SPME method.

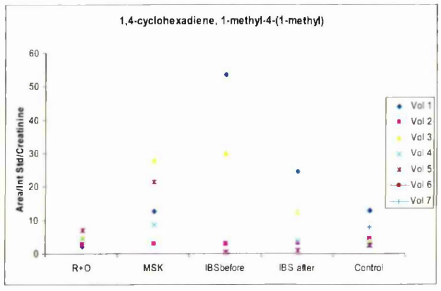

Figure 254. AUC 1,4-cyclohexadiene, 1-methyl-4-(1-methyl) related to the internal standard and creatinine in R+O, MSK, IBS before and after both diets and controls urine analysed using the SPME method.

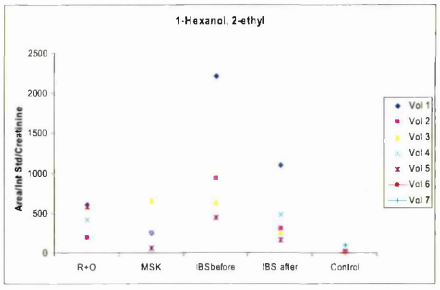

Figure 255. AUC of 2-ethyl 1-hexanol related to the internal standard and creatinine in R+O, MSK, IBS before and after both diets and controls urine analysed using the

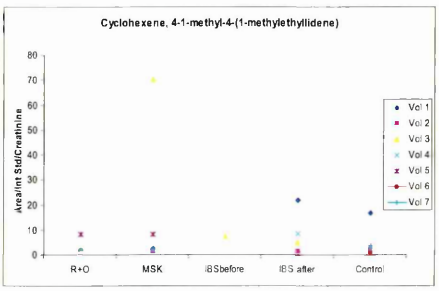

Figure 256. AUC of 4-1-methyl-4-(1-methylethylidene) cyclohexene related to the internal standard and creatinine in R+O, MSK, IBS before and after both diets and controls urine analysed using the SPME method.

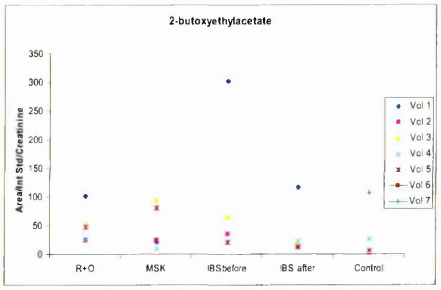

Figure 257. AUC of 2-butoxyethylacetate related to the internal standard and creatinine in R+O, MSK, IBS before and after both diets and controls urine analysed using the SPME method.

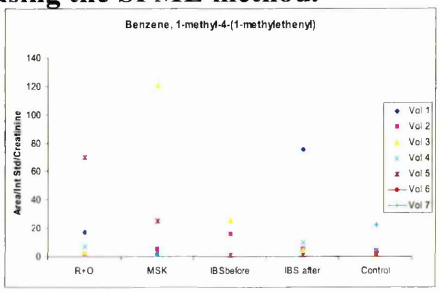

Figure 258. AUC of 1-methyl-4-(1-methylethenyl) benzene related to the internal standard and creatinine in R+O, MSK, IBS before and after both diets and controls urine analysed using the SPME method.

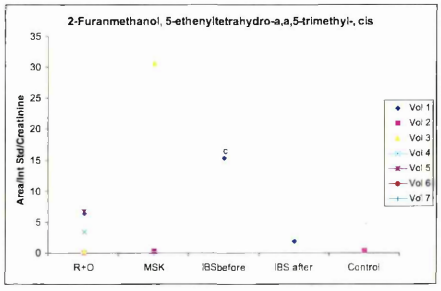

Figure 259. AUC of 2-furanmethanol, 5-ethenyltetrahydro-a-a-trimethyl-, cis related to the internal standard and creatinine in R+O, MSK, IBS before and after both diets and controls urine analysed using the SPME method.

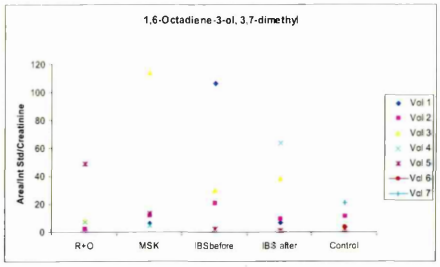

Figure 260. AUC of 1,6-octadiene-3-ol, 3,7-dimethyl related to the internal standard and creatinine in R+O, MSK, IBS before and after both diets and controls urine analysed using the SPME method.

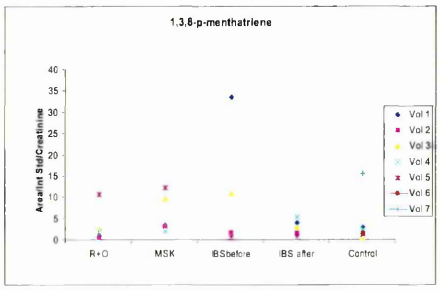

Figure 261. AUC of 1,3,8-menthatriene related to the internal standard and creatinine in R+O, MSK, IBS before and after both diets and controls urine analysed using the SPME method.

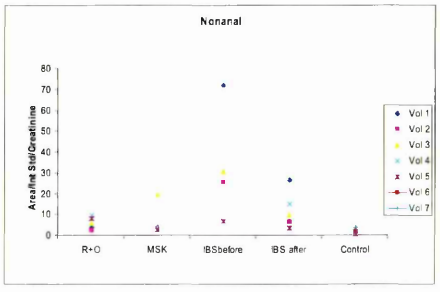

Figure 262. AUC of nonanal related to the internal standard and creatinine in R+O, MSK, IBS before and after both diets and controls urine analysed using the SPME method.

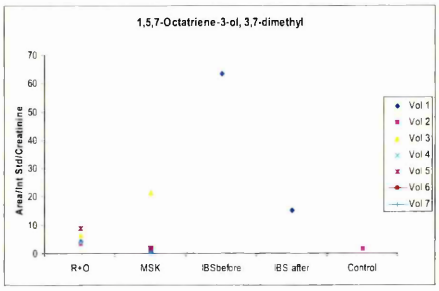

Figure 263. AUC of 1,5,7-octatriene-3-ol, 3,7-dimethyl related to the internal standard and creatinine in R+O, MSK, IBS before and after both diets and controls urine analysed using the SPME method.

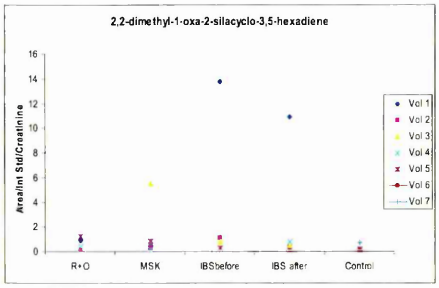

Figure 264. AUC of 1,2-dimethyl-1-oxa-2-silacyclo-3,5-hexadiene related to the internal standard and creatinine in R+O, MSK, IBS before and after both diets and controls urine analysed using the SPME method.

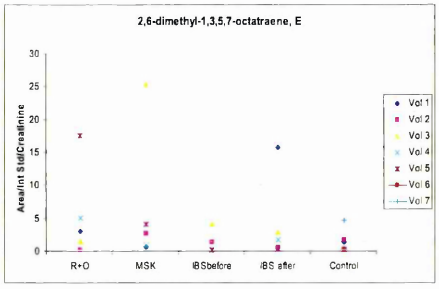

Figure 265. AUC of 2,6-dimethyl-1,3,5,7-octatraene, E related to the internal standard and creatinine in R+O, MSK, IBS before and after both diets and controls urine analysed using the SPME method.

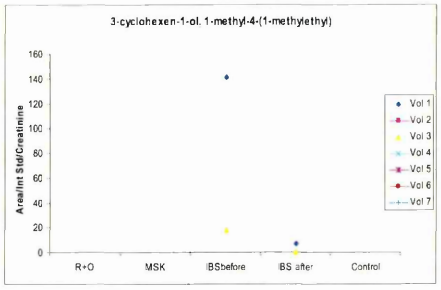

Figure 266. AUC of 3-cyclohene-1-ol, 1-methyl-4-(1-methylethyl) related to the internal standard and creatinine in R+O, MSK, IBS before and after both diets and controls urine analysed using the SPME method.

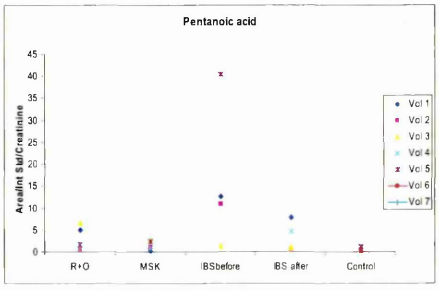

Figure 267. AUC of pentanoic acid related to the internal standard and creatinine in R+O, MSK, IBS before and after both diets and controls urine analysed using the SPME method.

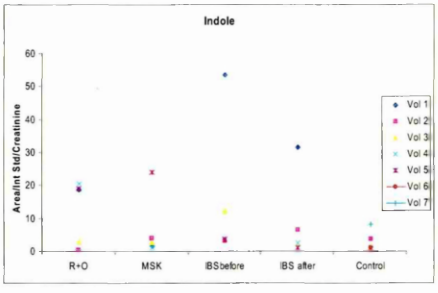

Figure 268. AUC of indole related to the internal standard and creatinine in R+O, MSK, IBS before and after both diets and controls urine analysed using the SPME method.

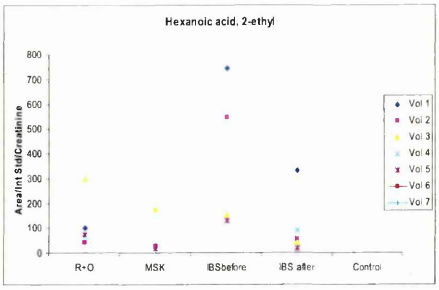

Figure 269. AUC of 2-ethyl hexanoic acid related to the internal standard and creatinine in R+O, MSK, IBS before and after both diets and controls urine analysed using the SPME method.

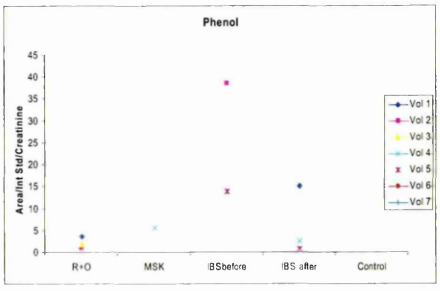

Figure 270. AUC of phenol related to the internal standard and creatinine in R+O, MSK, IBS before and after both diets and controls urine analysed using the SPME method.

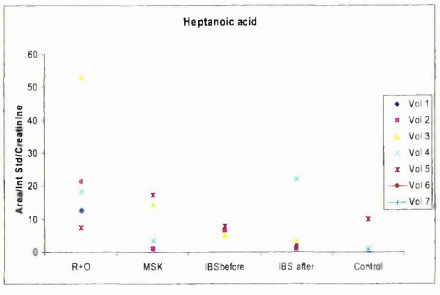

Figure 271. AUC of heptanoic acid related to the internal standard and creatinine in R+O, MSK, IBS before and after both diets and controls urine analysed using the SPME method.

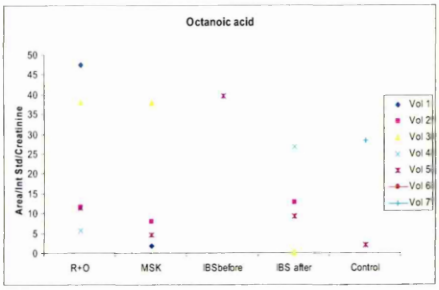

Figure 272. AUC of octanoic acid related to the internal standard and creatinine in R+O, MSK, IBS before and after both diets and controls urine analysed using the SPME method.

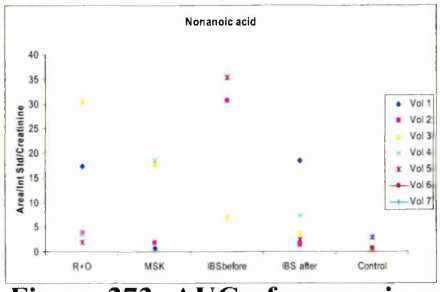

Figure 273. AUC of nonanoic acid related to the internal standard and creatinine in R+O, MSK, IBS before and after both diets and controls urine analysed using the SPME method.

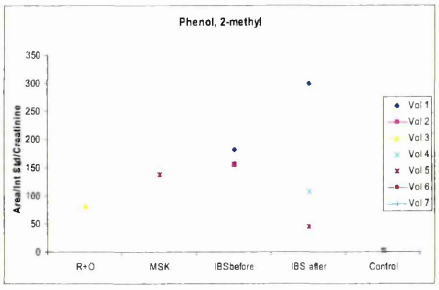

Figure 274. AUC of 2-methyl phenol related to the internal standard and creatinine in R+O, MSK, IBS before and after both diets and controls urine analysed using the SPME method.

Appendix 8.

Autism ethical permission

NHS

North and Mid Hampshire Local Research Ethics Committee
Harness House
Aldermaston Road
Basingstoke
Hampshire
RG24 9NB

Tel: 01256 332288
Fax: 01256 818112
Direct Tel: 01256 312248 and 01256 312245

www.rdsu.soton.ac.uk/rdsu/ethics.htm
General Enquiries: sandra.tapping@nhpct.nhs.uk
maria.keen@nhpct.nhs.uk
Application submissions: lrecsubmissions@nhpct.nhs.uk

Our ref: SKT/mk/L5026

Professor John Hunter
Cranfield University
Barton Road
Silsoe
MK45 4DT

7 July 2004

Dear Professor Hunter

Full title of study: The investigation of the possible role of abnormal colonic fermentation in the pathogenesis of autism
LREC reference number: 04/Q1703/10

The Research Ethics Committee reviewed the above application at the meeting held on 6 July 2004. Please thank Dr Sarah Morgan and Miss Maria Pilar Bilbao-Montoya for attending the meeting to discuss the study.

Documents reviewed

The documents reviewed at the meeting were:

1. Application Form, v. 1, dated 15/6/2004
2. Investigator's CV, dated 15/6/2004
3. Protocol dated 15/6/2004
4. Covering letter dated 15/6/2004
5. Summary/Synopsis dated 15/6/2004
6. Peer Review dated 15/6/2004
7. Copy of questionnaire dated 15/6/2004
8. Letters of invitation to participants dated 15/6/2004
9. Participant Information Sheet, version 1, dated 1/6/2004
10. Participant Consent Form, version 1, dated 14/6/2004
11. Letter from sponsor with supporting documentation

Provisional opinion

The Committee would be content to give a favourable ethical opinion of the research, subject to receiving a complete response to the request for further information below.

Authority to consider your response and to confirm the Committee's final opinion has been delegated to the Chair.

Further information or clarification required:

Letters

1. All the proposed letters should be amended to include the following:

a) Title (i.e. who the letter is addressed to)
b) Version Number
c) Date

2. One letter refers to a cure for autism. This is an ambitious statement which may raise expectations and the letter should be amended. Perhaps instead of starting with "We are trying to find a cure.." you should instead say "We are trying to help children...".

3. In addition the term "volunteers" should not be applied to children with autism since strictly speaking they are not volunteering. It is sufficient to refer to them as "children".

4. The last paragraph in the letters asking participants to contact you by email should be changed to also include a postal address as not everybody has an email address.

Eligibility and Consent

1. Nobody may give consent for an adult over the age of 18. It is therefore suggested that you do not include people with autism who are 18 or over.

2. Volunteers under the age of 16 may be considered competent to give consent in which event you should amend your letters and forms to provide the parent and child the opportunity to jointly consent where appropriate.

3. The consent forms should comply with standard guidelines and be signed by the researcher who has sought consent.

4. Dates do not tie up with those on Information Sheet. Please correct.

Questionnaires

1. It is advised that the questionnaires should be as similar as possible. You should consider the benefits of including questions about the diagnosis since for many it is unlikely that there is a conclusive diagnosis.

2. The subsequent section seeking information about the onset of autism is leading and may raise anxieties and should be removed.

3. The Healthy Volunteers questionnaire does not ask if there is a family history of autism and it is considered this might be useful information.

Patient Information Sheet

1. Please review the P.I.S. and remove jargon and terms that are unlikely to be understood by a lay person. Typing errors also to be corrected.

When submitting a response to the Committee, please send revised documentation where appropriate underlining or otherwise highlighting the changes you have made and giving revised version numbers and dates. Failure to do this will delay consideration of the revisions.

The Committee will issue a final ethical opinion on the application within a maximum of 60 days from the date of initial receipt of the application, excluding the time taken by you to respond fully to the above points.

The Committee expects to receive a response from you by no later than 7 November 2004, otherwise we shall consider the application to have been withdrawn.

Membership of the Committee

The members of the Ethics Committee who were present at the meeting are listed on the attached sheet.

Communication with sponsor and host organisations

This communication is confidential to you but you may wish to forward copies to your sponsor and/or host organisation(s) for their information.

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.

REC reference number: 04/Q1703/10 Please quote this number on all correspondence

Yours sincerely,

M·Kee

jpp **Sandra Tapping**
Manager
North and Mid Hampshire Local Research Ethics Committee

Enclosures *List of names and professions of members who were present at the meeting and those who submitted written comments*

Appendix 9.

Compounds found in autistic urine

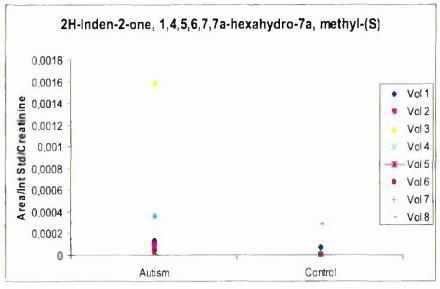

Figure 275. AUC of 2H-Inden-2-one,1,4,5,6,7,7a-hexahydro-7-a, methyl-(S) related to the internal standard and creatinine found in urine analysed using the HAN method of autistic children and healthy children.

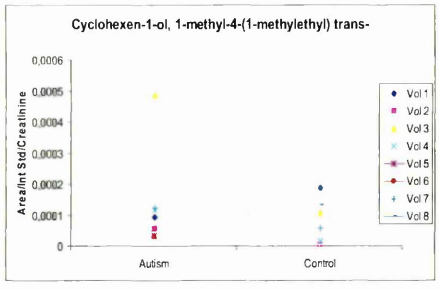

Figure 276. AUC of 1-methyl-4-(1-methylethyl) trans-cyclohexen-1-ol related to the internal standard and creatinine found in urine analysed using the HAN method of autistic children and healthy children.

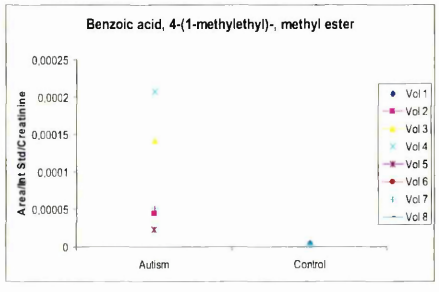

Figure 277. AUC of 4-(1-methylethyl)- benzoic acid, methyl ester related to the internal standard and creatinine found in urine analysed using the HAN method of autistic children and healthy children.

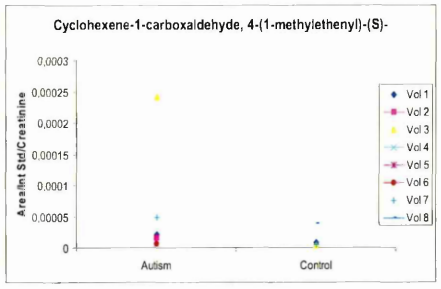

Figure 278. AUC of 1-carboxaldehyde,4-(1-methylethenyl)-(S)-cyclohexene related to the internal standard and creatinine found in urine analysed using the HAN method of autistic children and healthy children.

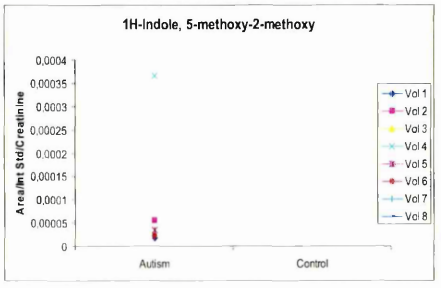

Figure 279. AUC of 1H-INdole, 5-methoxy-2-methoxy related to the internal standard and creatinine found in urine analysed using the HAN method of autistic children and healthy children.

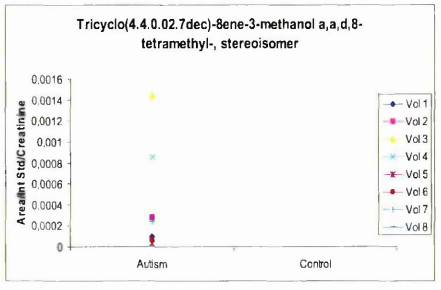

Figure 280. AUC of tricycle(4.4.0.02.7dec)-8ene-3-methanol, α,α,δ-tetramethyl-, estereoisomer related to the internal standard and creatinine found in urine analysed using the HAN method of autistic children and healthy children.

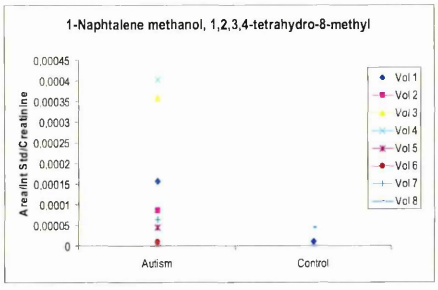

Figure 281. AUC of 1-naphtalene methanol, 1,2,3,4-tetrahydro-8-methyl related to the internal standard and creatinine found in urine analysed using the HAN method of autistic children and healthy children.

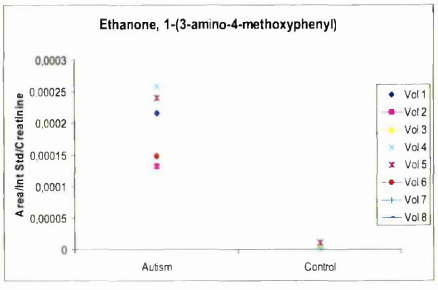

Figure 282. AUC of 1-(3-amino-4-methoxyphenyl) ethanone related to the internal standard and creatinine found in urine analysed using the HAN method of autistic children and healthy children.

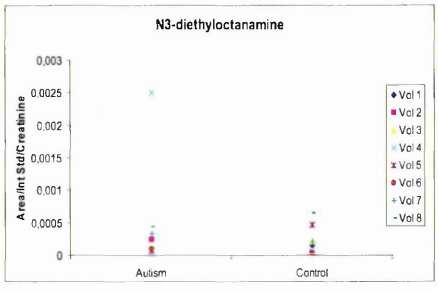

Figure 283. AUC of N3-diethyloctanamine related to the internal standard and creatinine found in urine analysed using the HAN method of autistic children and healthy children.

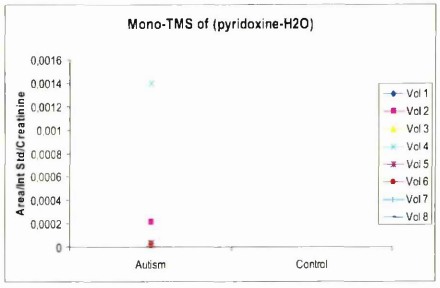

Figure 284. AUC of mono-TMS of (pyridoxine-H20) related to the internal standard and creatinine found in urine analysed using the HAN method of autistic children and healthy children.

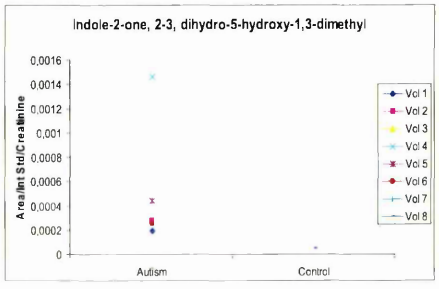

Figure 285. AUC of indole-2-one, 2-3, dihydro-5-hydroxy-1,3-dimethyl related to the internal standard and creatinine found in urine analysed using the HAN method of autistic children and healthy children.

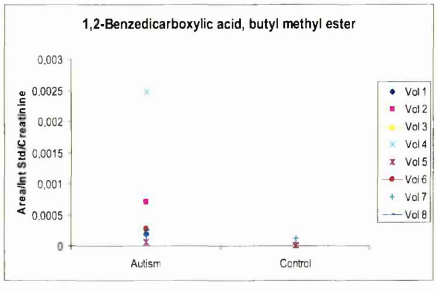

Figure 286. AUC of 1,2-benzedicarboxylic acid, butyl methyl ester related to the internal standard and creatinine found in urine analysed using the HAN method of autistic children and healthy children.

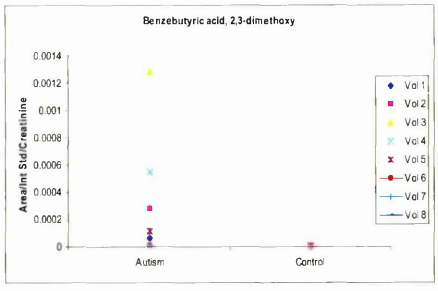

Figure 287. AUC of 2,3-dimethoxy benzebutyric acid related to the internal standard and creatinine found in urine analysed using the HAN method of autistic children and healthy children.

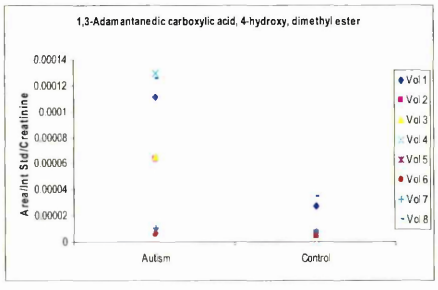

Figure 288. AUC of 1,3-Adamantane dic carboxylic acid, 4-hydroxy, dimethyl ester related to the internal standard and creatinine found in urine analysed using the HAN method of autistic children and healthy children.

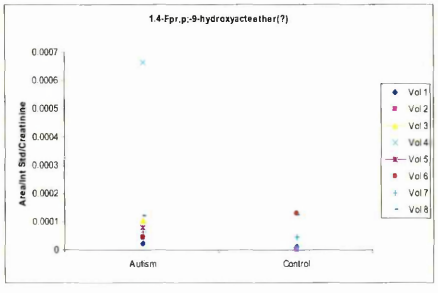

Figure 289. AUC of 1,4-Fpr,p;8-hydroxyacetate ether related to the internal standard and creatinine found in urine analysed using the HAN method of autistic children and healthy children.

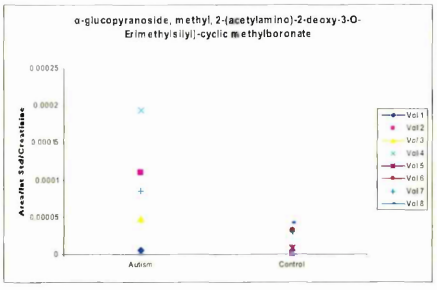

Figure 290. AUC of α-glucopyranoside, methyl,2-(acetylamino)-2-deoxy-3-O-brimethylsilyl)-cyclic methylboronate related to the internal standard and creatinine found in urine analysed using the HAN method of autistic children and healthy children.

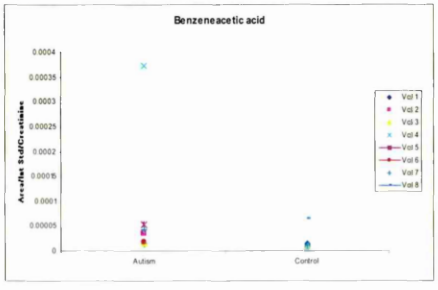

Figure 291. AUC of benzeneacetic acid related to the internal standard and creatinine found in urine analysed using the HAN method of autistic children and healthy children.

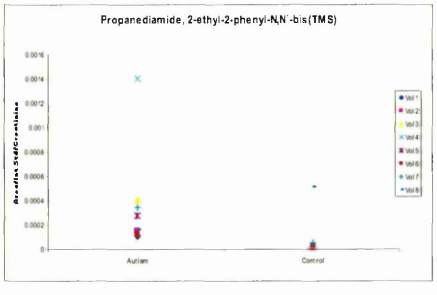

Figure 292. AUC of 2-ethyl-2-phenyl-N,N′-bis (TMS) related to the internal standard and creatinine found in urine analysed using the HAN method of autistic children and healthy children.

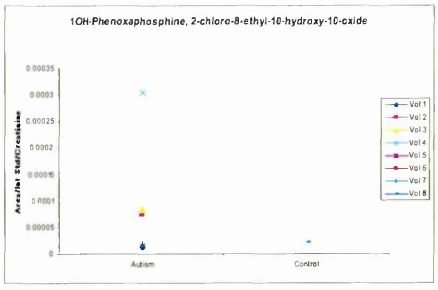

Figure 293. AUC of 10H-phenoxaphosphine, 2-chloro-8-ethyl-10-hydroxy-10-oxide related to the internal standard and creatinine found in urine analysed using the HAN method of autistic children and healthy children.

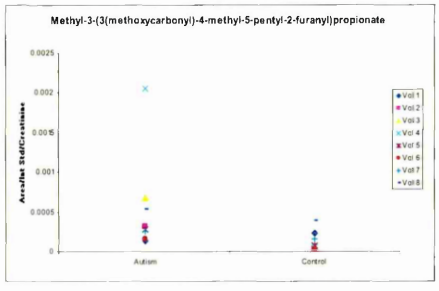

Figure 294. AUC of methyl-3-(3(methoxycarbonyl)-4-methyl-5-pentyl-2-furanyl) propionate related to the internal standard and creatinine found in urine analysed using the HAN method of autistic children and healthy children.

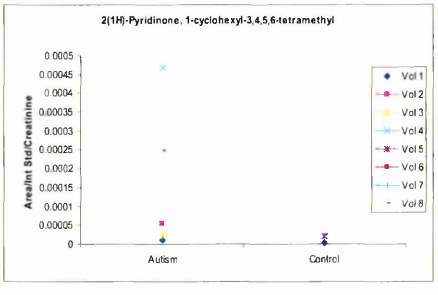

Figure 295. AUC of 2(1H)-pyridinone,1-cyclohexyl-3,4,5,6-tetramethyl related to the internal standard and creatinine found in urine analysed using the HAN method of autistic children and healthy children.

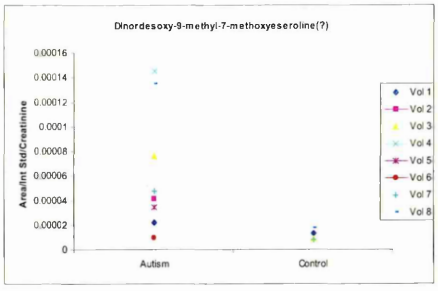

Figure 296. AUC of dinordesoxy-3-methyl-7-methoxyeseroline related to the internal standard and creatinine found in urine analysed using the HAN method of autistic children and healthy children.

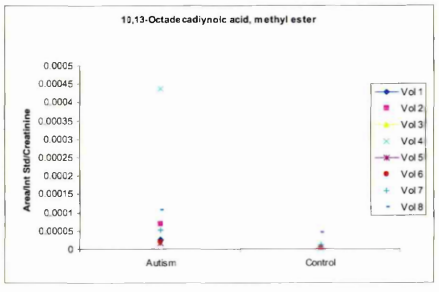

Figure 297. AUC of 10,13-octadecadiynoic acid methyl ester related to the internal standard and creatinine found in urine analysed using the HAN method of autistic children and healthy children.

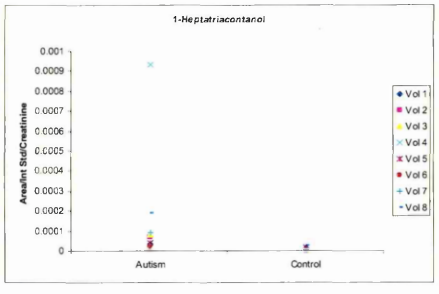

Figure 298. AUC of 1-heptatricontanol related to the internal standard and creatinine found in urine analysed using the HAN method of autistic children and healthy children.

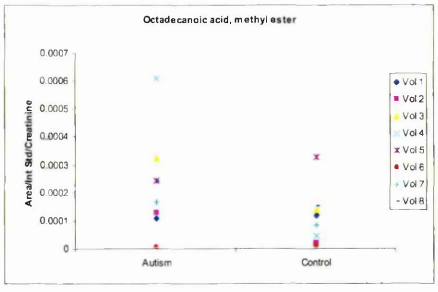

Figure 299. AUC of octadecanoic acid methyl ester related to the internal standard and creatinine found in urine analysed using the HAN method of autistic children and healthy children.

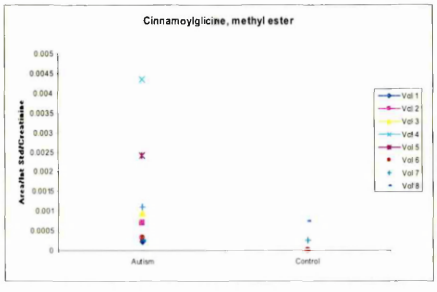

Figure 300. AUC of cinnamoylglicine methyl related to the internal standard and creatinine found in urine analysed using the HAN method of autistic children and healthy children.

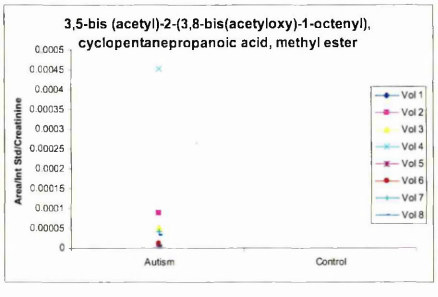

Figure 301. AUC of 3,5-bis-(acetyl)-2-(3,8-bis(acetyloxy)-octenyl), cyclopentanepropanoic acid methyl ester related to the internal standard and creatinine found in urine analysed using the HAN method of autistic children and healthy children.

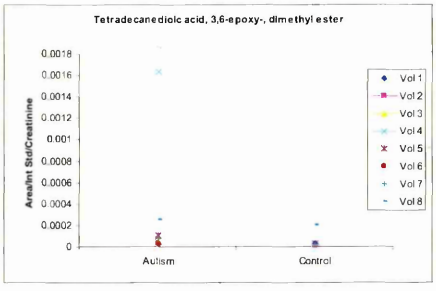

Figure 302. AUC of 3,6-epoxy, tetradecanedioic acid dimethyl ester related to the internal standard and creatinine found in urine analysed using the HAN method of autistic children and healthy children.

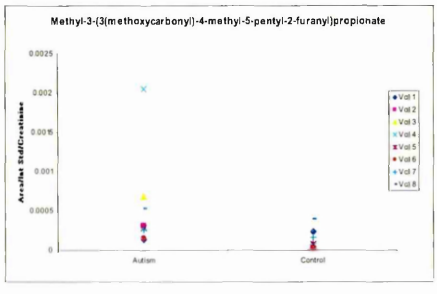

Figure 303. AUC of 3,methyl-3-(3(methoxycarbonyl)-4-methyl-5-pentyl-2-furanyl) propionate related to the internal standard and creatinine found in urine analysed using the HAN method of autistic children and healthy children.

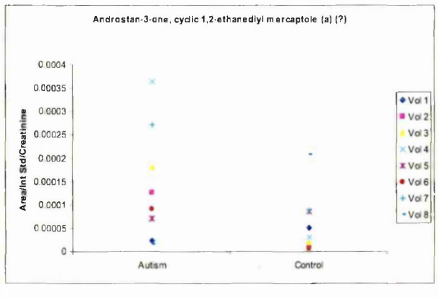

Figure 304. AUC of androstan-3-one, cyclic 1,2-ethanediyl mercaptole(a) related to the internal standard and creatinine found in urine analysed using the HAN method of autistic children and healthy children.

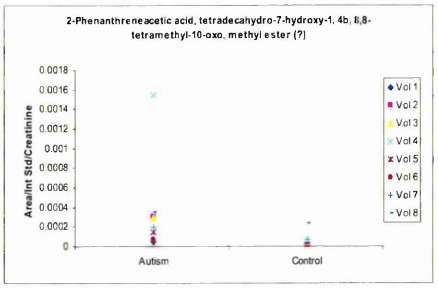

Figure 305. AUC of 2-phenanthreneacetic acid, tetradecahydro-7-hydroxy-1, 4b, 8,8-tetramethyl-10-oxo, methyl ester related to the internal standard and creatinine found in urine analysed using the HAN method of autistic children and healthy children.

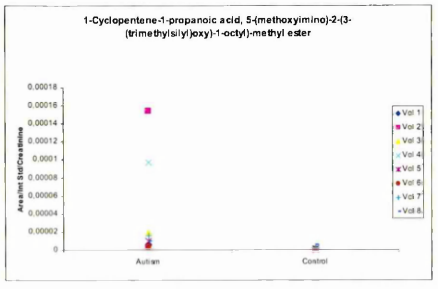

Figure 306. AUC of 1-cyclopentene-1-propanoic acid, 5-(methoxyimino)-2-(3-(trimethylsilyl)oxy)1-octyl)-methyl ester related to the internal standard and creatinine found in urine analysed using the HAN method of autistic children and healthy children.

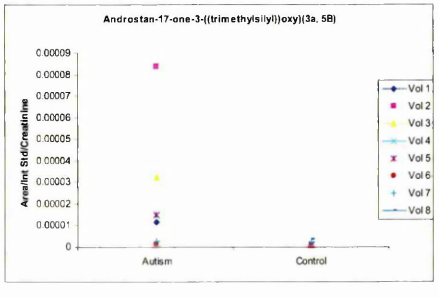

Figure 307. AUC of androstan-17-one-3-((trimethylsilyl)oxy)(3a,5B) related to the internal standard and creatinine found in urine analysed using the HAN method of autistic children and healthy children.

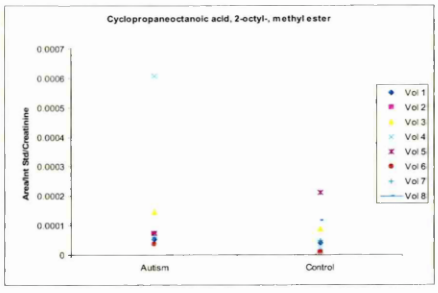

Figure 308. AUC of 2-octyl cyclopropaneoctanoic acid methyl ester related to the internal standard and creatinine found in urine analysed using the HAN method of autistic children and healthy children.

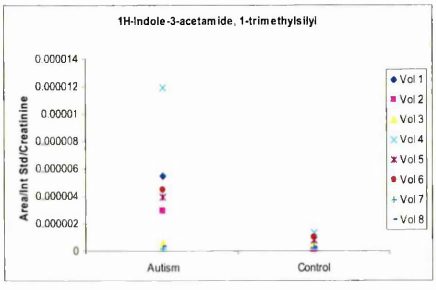

Figure 309. AUC of 1H-Indole-3-acetamide, 1-trimethylsilyl related to the internal standard and creatinine found in urine analysed using the UB method of autistic children and healthy children.

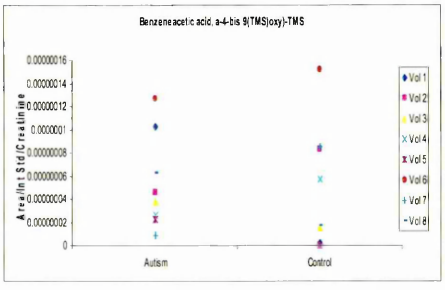

Figure 310. AUC of α-4-bis 3(TMS)oxy)- benzeneacetic acid-TMS related to the internal standard and creatinine found in urine analysed using the UB method of autistic children and healthy children.

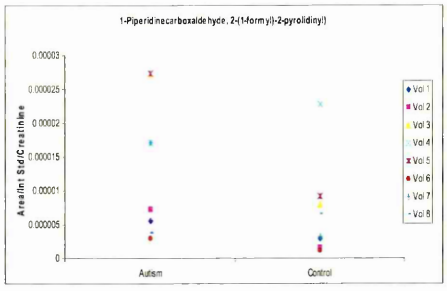

Figure 311. AUC of 1-piperidinecarboxaldehyde, 2-(1-formyl)-2-pyrolidinyl) related to the internal standard and creatinine found in urine analysed using the UB method of autistic children and healthy children.

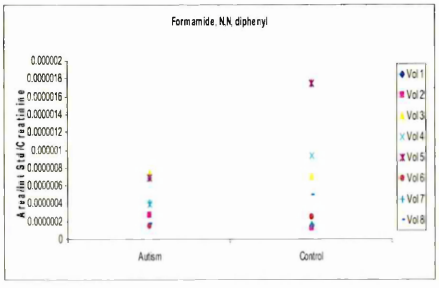

Figure 312. AUC of N,N, diphenyl formamide related to the internal standard and creatinine found in urine analysed using the UB method of autistic children and healthy children.

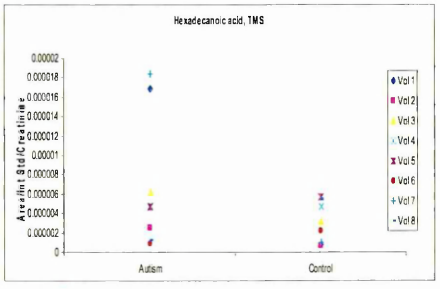

Figure 313. AUC of hexadecanoic acid, TMS related to the internal standard and creatinine found in urine analysed using the UB method of autistic children and healthy children.

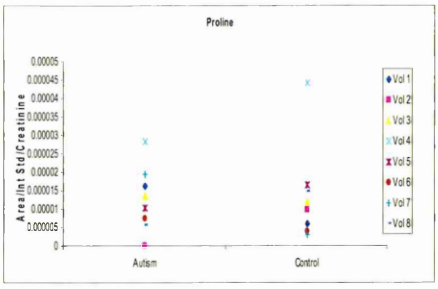

Figure 314. AUC of proline related to the internal standard and creatinine found in urine analysed using the UB method of autistic children and healthy children.

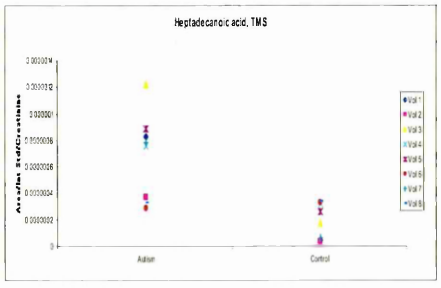

Figure 315. AUC of heptadecanoic acid, TMS related to the internal standard and creatinine found in urine analysed using the UB method of autistic children and healthy children.

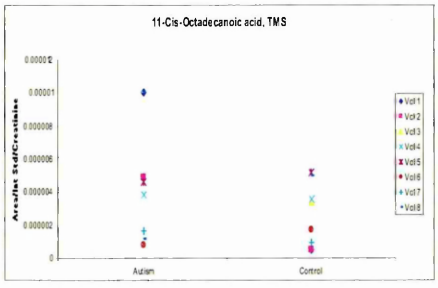

Figure 316. AUC of 11-cis octadecanoic acid, TMS related to the internal standard and creatinine found in urine analysed using the UB method of autistic children and healthy children.

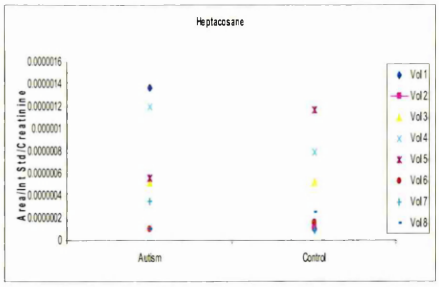

Figure 317. AUC of heptacosane related to the internal standard and creatinine found in urine analysed using the UB method of autistic children and healthy children.

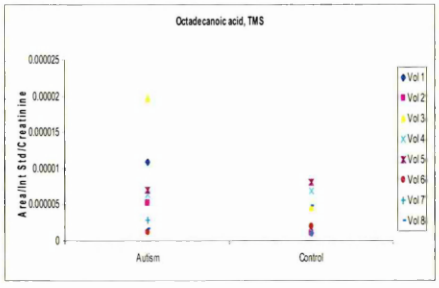

Figure 318. AUC of octadecanoic acid, TMS related to the internal standard and creatinine found in urine analysed using the UB method of autistic children and healthy children.

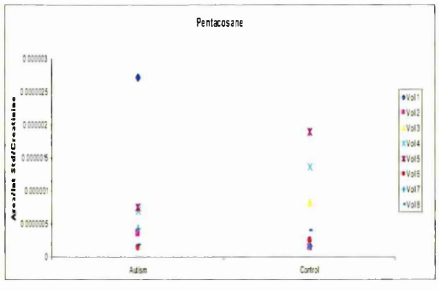

Figure 319. AUC of pentacosane related to the internal standard and creatinine found in urine analysed using the UB method of autistic children and healthy children.

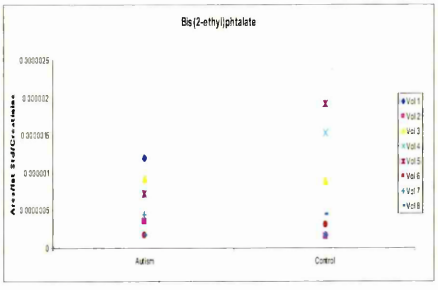

Figure 320. AUC of bis(2-ethyl)phtalate related to the internal standard and creatinine found in urine analysed using the UB method of autistic children and healthy children.

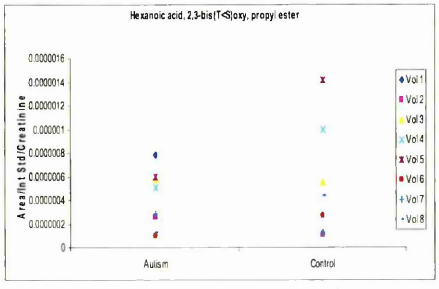

Figure 321. AUC of 2,3-bis(TMS)oxy, hexanoic acid, propyl ester related to the internal standard and creatinine found in urine analysed using the UB method of autistic children and healthy children.

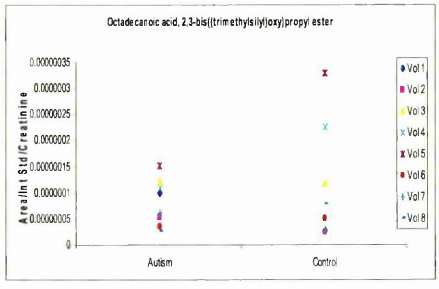

Figure 322. AUC of 2,3-bis((TMS) oxy) octadecanoic acid methyl ester related to the internal standard and creatinine found in urine analysed using the UB method of autistic children and healthy children.

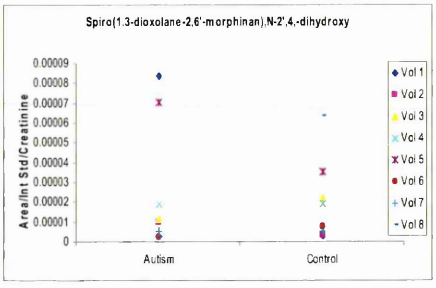

Figure 323. AUC of spiro(1,3-dioxolane-2.6′-morphinan), N-2′, 4-dihydroxy related to the internal standard and creatinine found in urine analysed using the HB method of autistic children and healthy children.

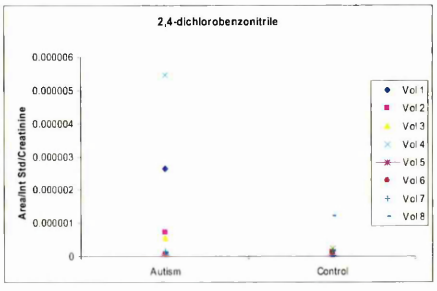

Figure 324. AUC of 2,4-dichlorobenzonitrile related to the internal standard and creatinine found in urine analysed using the HB method of autistic children and healthy children.

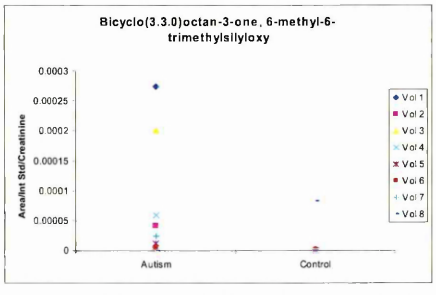

Figure 325. AUC of bicycle(3.3.0) octan-3-one, 6-methyl-6-trimethylsilyoxy related to the internal standard and creatinine found in urine analysed using the HB method of autistic children and healthy children.

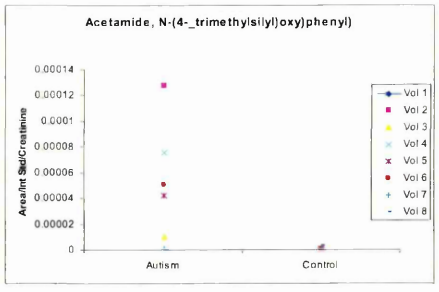

Figure 326. AUC of N-(4-trimethylsilily)oxy)phenyl) acetamide related to the internal standard and creatinine found in urine analysed using the HB method of autistic children and healthy children.

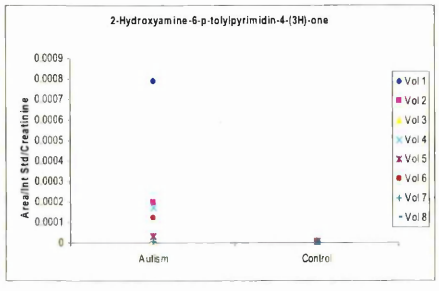

Figure 327. AUC of 2-hydroxyamine-6-p-tolylpyrimidin-4-(3H)-one related to the internal standard and creatinine found in urine analysed using the HB method of autistic children and healthy children.

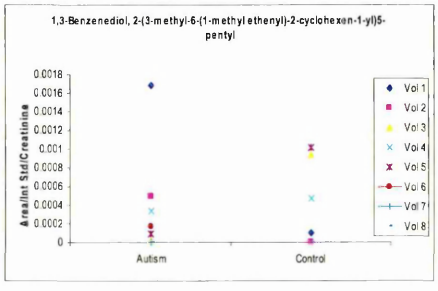

Figure 328. AUC of 1,3-benzediol,2-(3-methyl-6-(1-methyl ethenyl)-2-cyclohexen-1-yl)-5-pentyl related to the internal standard and creatinine found in urine analysed using the HB method of autistic children and healthy children.

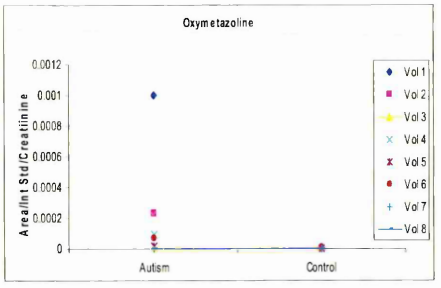

Figure 329. AUC of oxymetaxoline related to the internal standard and creatinine found in urine analysed using the HB method of autistic children and healthy

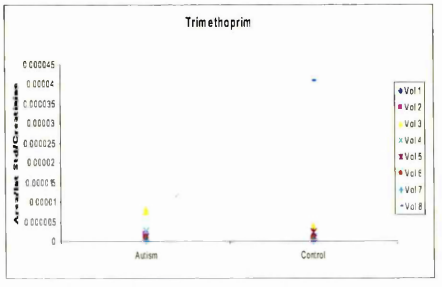

Figure 330. AUC of trimethoprim related to the internal standard and creatinine found in urine analysed using the HB method of autistic children and healthy children.

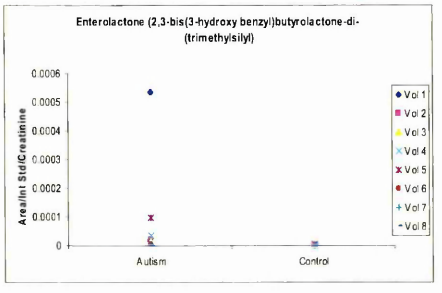

Figure 331. AUC 2,3-bis(3-hydroxybenzyl)butyrolactone-di- enterolactone, TMS related to the internal standard and creatinine found in urine analysed using the HB method of autistic children and healthy children.

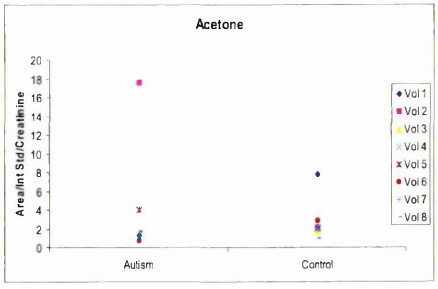

Figure 332. AUC of acetone related to the internal standard and creatinine found in urine analysed using the SPME method of autistic children and healthy children.

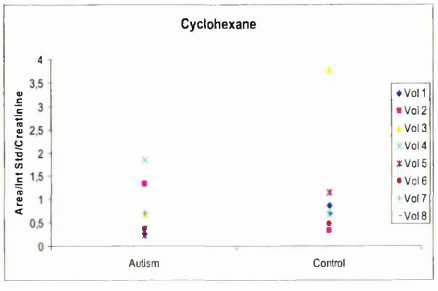

Figure 333. AUC of cyclohexane related to the internal standard and creatinine found in urine analysed using the SPME method of autistic children and healthy children.

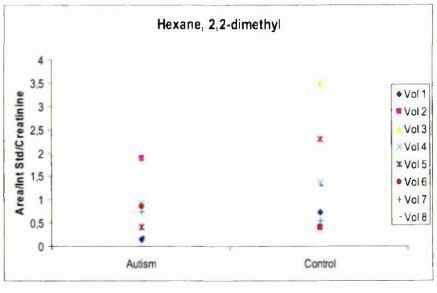

Figure 334. AUC of 2,2-dimethyl hexane related to the internal standard and creatinine found in urine analysed using the SPME method of autistic children and healthy children.

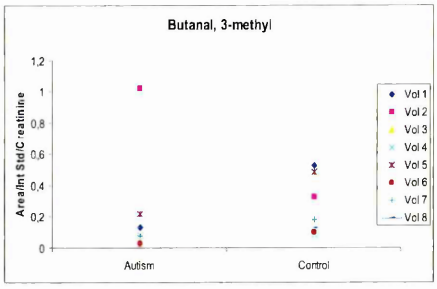

Figure 335. AUC of 3-methyl butanal related to the internal standard and creatinine found in urine analysed using the SPME method of autistic children and healthy children.

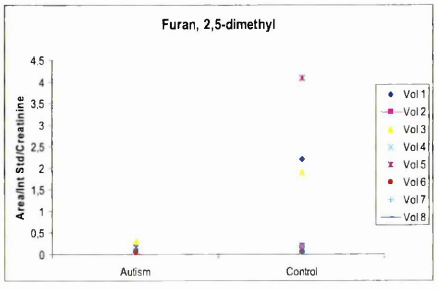

Figure 336. AUC of 2,5-dimethyl furan related to the internal standard and creatinine found in urine analysed using the SPME method of autistic children and healthy children.

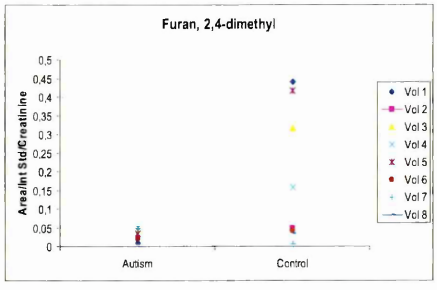

Figure 337. AUC of 2,4-dimethyl furan related to the internal standard and creatinine found in urine analysed using the SPME method of autistic children and healthy children.

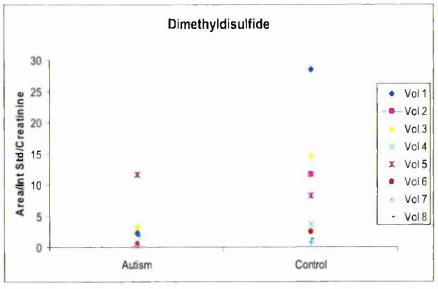

Figure 338. AUC of dimethyldisulfide related to the internal standard and creatinine found in urine analysed using the SPME method of autistic children and healthy children.

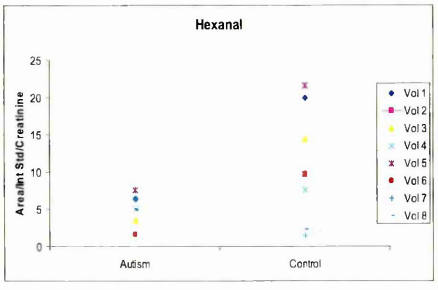

Figure 339. AUC of hexanal related to the internal standard and creatinine found in urine analysed using the SPME method of autistic children and healthy children.

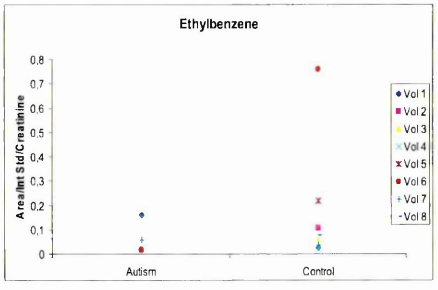

Figure 340. AUC of ethylbenzene related to the internal standard and creatinine found in urine analysed using the SPME method of autistic children and healthy children.

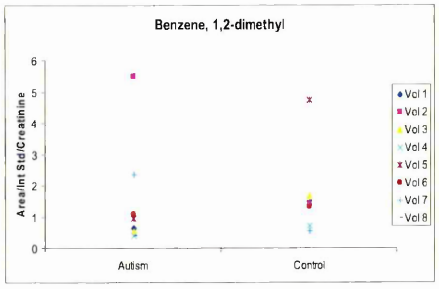

Figure 341. AUC of 1,2-dimethyl benzene related to the internal standard and creatinine found in urine analysed using the SPME method of autistic children and healthy children.

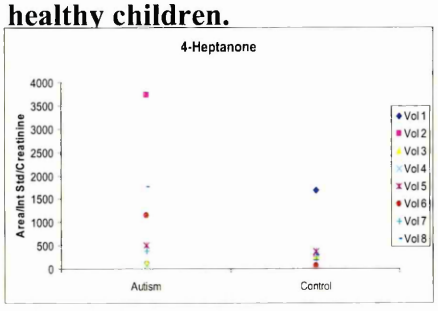

Figure 342. AUC of 4-heptanone related to the internal standard and creatinine found in urine analysed using the SPME method of autistic children and healthy children.

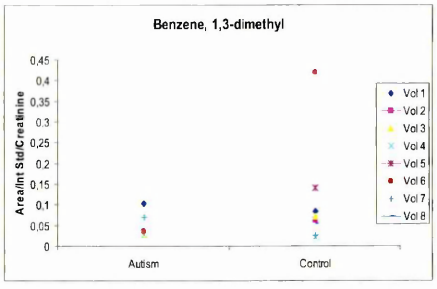

Figure 343. AUC of 1,3-dimethyl benzen related to the internal standard and creatinine found in urine analysed using the SPME method of autistic children and

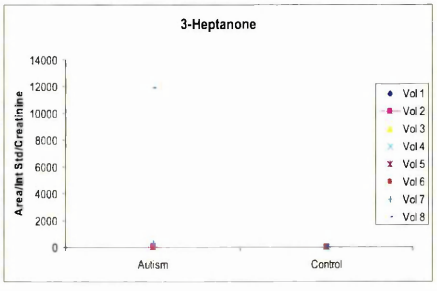

Figure 344. AUC of 3-heptanone related to the internal standard and creatinine found in urine analysed using the SPME method of autistic children and healthy children.

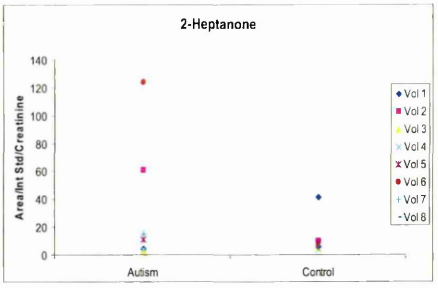

Figure 345. AUC of 2-heptanone related to the internal standard and creatinine found in urine analysed using the SPME method of autistic children and healthy children.

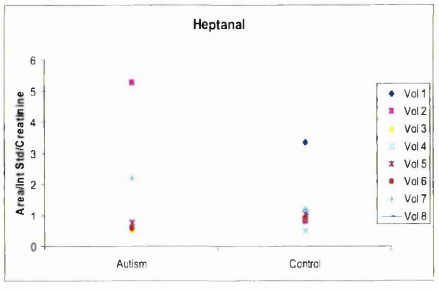

Figure 346. AUC of heptanal related to the internal standard and creatinine found in urine analysed using the SPME method of autistic children and healthy children.

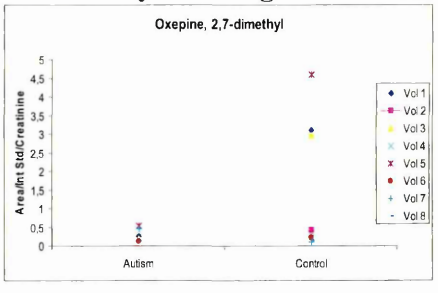

Figure 347. AUC of 2,7-dimethyl oxepine related to the internal standard and creatinine found in urine analysed using the SPME method of autistic children and healthy children.

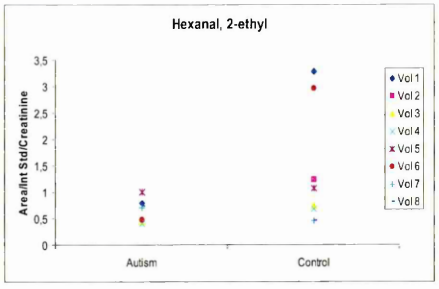

Figure 348. AUC of 2-ethyl hexanal related to the internal standard and creatinine found in urine analysed using the SPME method of autistic children and healthy children.

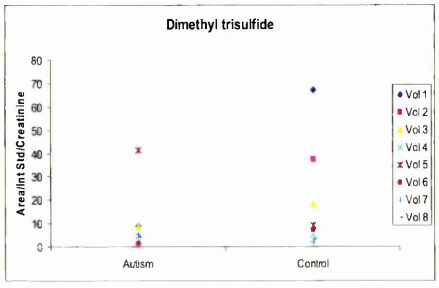

Figure 349. AUC of dimethyl trisulfide related to the internal standard and creatinine found in urine analysed using the SPME method of autistic children and healthy children.

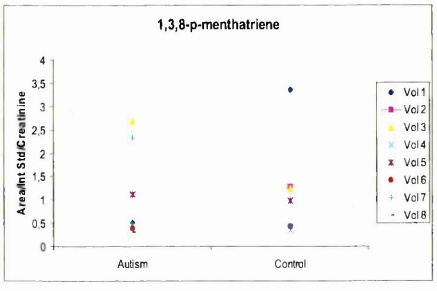

Figure 350. AUC of 1,3,8-p-menthatriene related to the internal standard and creatinine found in urine analysed using the SPME method of autistic children and healthy children.

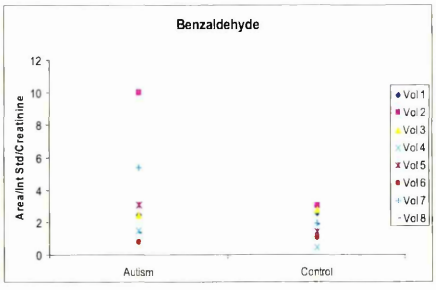

Figure 351. AUC of benzaldehyde related to the internal standard and creatinine found in urine analysed using the SPME method of autistic children and healthy children.

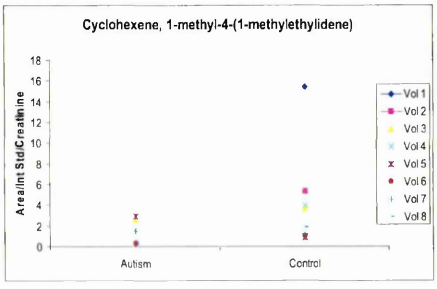

Figure 352. AUC of 1-methyl-4-(1-methylethylidene) cyclohexene related to the internal standard and creatinine found in urine analysed using the SPME method of autistic children and healthy children.

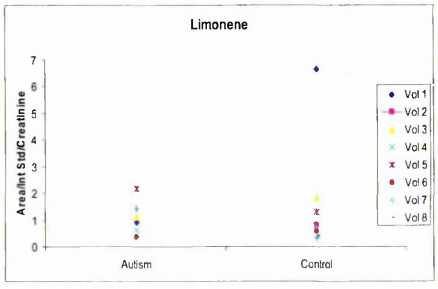

Figure 353. AUC of limonene related to the internal standard and creatinine found in urine analysed using the SPME method of autistic children and healthy children.

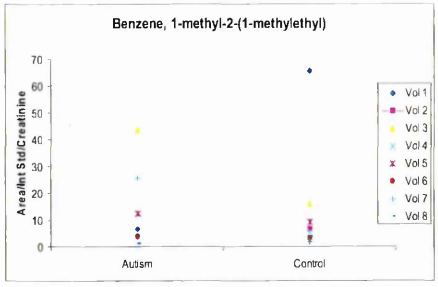

Figure 354. AUC of 1-methyl-2-(1-methylethyl) benzene related to the internal standard and creatinine found in urine analysed using the SPME method of autistic children and healthy children.

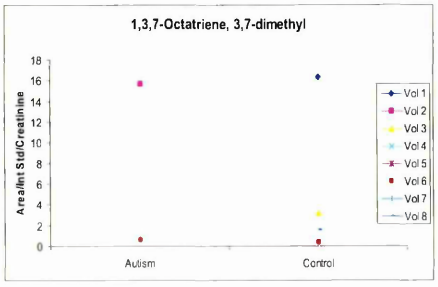

Figure 355. AUC of 3,7-dimethyl 1,3,7-octatriene, related to the internal standard and creatinine found in urine analysed using the SPME method of autistic children and healthy children.

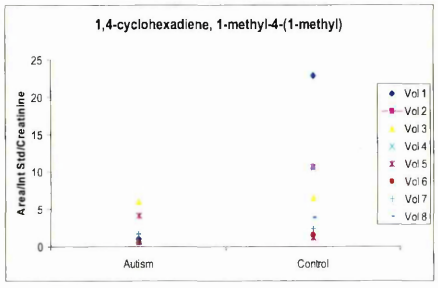

Figure 356. AUC of 1-methyl-4-(1-methyhl) 1,4-cyclohexadiene related to the internal standard and creatinine found in urine analysed using the SPME method of autistic children and healthy children.

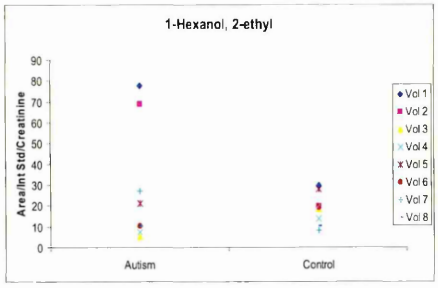

Figure 357. AUC of 2-ethyl 1-hexanol related to the internal standard and creatinine found in urine analysed using the SPME method of autistic children and healthy children.

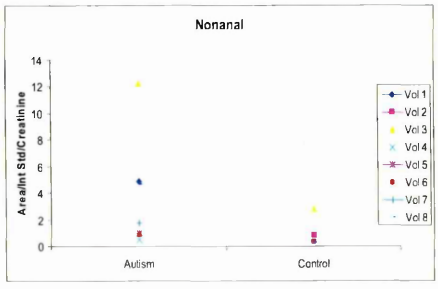

Figure 358. AUC of nonanal related to the internal standard and creatinine found in urine analysed using the SPME method of autistic children and healthy children.

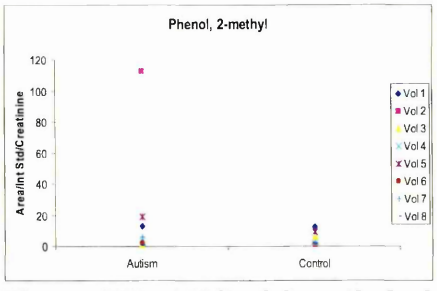

Figure 359. AUC of 2-methyl phenol related to the internal standard and creatinine found in urine analysed using the SPME method of autistic children and healthy children.

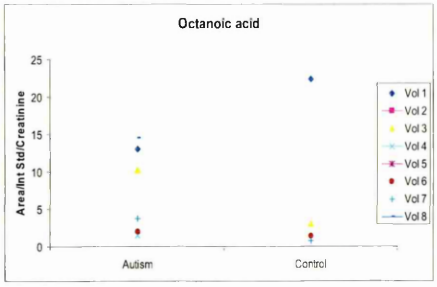

Figure 360. AUC of octanoic acid related to the internal standard and creatinine found in urine analysed using the SPME method of autistic children and healthy children.

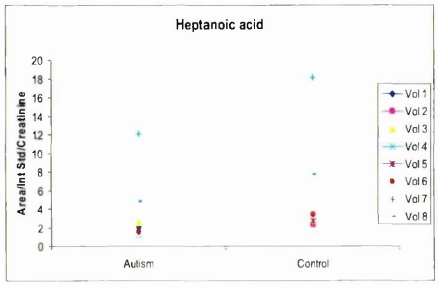

Figure 361. AUC of heptanoic acid related to the internal standard and creatinine found in urine analysed using the SPME method of autistic children and healthy children.

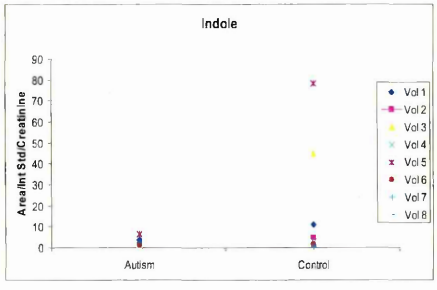

Figure 362. AUC of indole related to the internal standard and creatinine found in urine analysed using the SPME method of autistic children and healthy children.