# Investigation of faecal volatile organic compounds as biomarkers for the diagnosis of necrotising enterocolitis

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Master of Philosophy by

Arnaud Mayor

September 2015

**Seeking oneself in another place**

**Where the wind blows steady**

**And men stand firm.**

# Abstract

**Investigation of faecal volatile organic compounds as biomarkers for the diagnosis of necrotising enterocolitis**

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Necrotising enterocolitis (NEC) is the most prevalent and harmful illness in the neonatal intensive care unit. It primarily affects premature babies, with higher incidence in low birth-weight patients. NEC usually occurs in the early days of life and may develop rapidly. Its diagnosis is based on medical observation because no early diagnostic tool is currently available.

Over the last two decades, developments in analytical chemistry have allowed scientists to perform untargeted investigation of biological samples. Biomarkers are being sought, for a range of disorders, by investigating volatile organic compounds (VOCs) in bio-fluids. For the analysis of gas samples, solid phase micro-extraction (SPME) is a suitable technique to pre-concentrate volatile compounds prior to analysis using devices such as gas chromatography – mass spectrometry (GC–MS). Recently, much attention has been directed towards the development of sensors and electronic noses to be used as diagnostic tools in hospitals, as they are generally more compact than a GC-MS and do not require specialised personnel.

The work presented here is based on the hypothesis that faeces from patients suffering from necrotising enterocolitis show a specific pattern of volatile organic compounds in the days prior to diagnosis when compared with faeces from healthy patients. The objectives of this work were to develop two methods for the analysis of premature faeces using headspace–SPME–GC–MS and headspace – gas chromatography – sensor (HS–GC–Sensor), to analyse samples from healthy premature infants and premature infants affected by NEC using both instruments and to analyse the data collected.

Methods were developed individually for each analytical technique. Two pipelines were applied for mass spectrometric data analysis while classification models were exclusively built using sensor data.

Results obtained from HS–SPME–GC–MS data showed that the age at sampling had an influence on the number of compounds identified and on their intensities or relative abundance. Heptanal, 2-E-pentenal, hexanal and 2-methylbutanoic acid were identified as relevant compounds. Classifiers were built at days 1 to 6 prior to diagnosis. Accuracy, sensitivity and specificity of up to 74%, 62% and 79%, respectively, were obtained one day prior to diagnosis based on mass spectrometric data, while accuracy, sensitivity and specificity of 100% were obtained based on sensor data at two days prior to diagnosis. Therefore, classification of samples based on headspace analysis of faeces might have potential for the early diagnosis of necrotising enterocolitis.

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# List of Abbreviations

| | |
|---|---|
| ANOVA | Analysis of Variance |
| AMDIS | Automated Mass spectral Deconvolution and Identification System |
| BHH | Birmingham Heartlands Hospital |
| BWH | Birmingham Women's Hospital |
| CS | Caesarean |
| CAR/PDMS | Carboxen®/Polydimethylsiloxane |
| CV | Coefficient of Variation |
| DVB/CAR/PDMS | Divinylbenzene/Carboxen®/Polydimethylsiloxane |
| et al. | et alia (and others in latin) |
| F | Female |
| GC | Gas Chromatography |
| HS | Headspace |
| IQR | Inter Quartile Range |
| k-NN | k-Nearest Neighbour |
| LDA | Linear Discriminant Analysis |
| LWH | Liverpool Women's Hospital |
| M | Male |
| MS | Mass Spectrometry |
| *m/z* | Mass-to-Charge |
| MEBM | Mother Expressed Breast Milk |
| NIST | National Institute of Standard and Technology |
| NEC | Necrotising Enterocolitis |
| NICU | Neonatal Intensive Care Units |
| ppt | Part per trillion |
| PLS | Partial Least Square |
| ppm | Parts per million |
| PCA | Principal Component Analysis |
| RF | Random Forest |
| ROC | Receiver Operating Characteristic |
| RT | Retention Time |
| RSH | Royal Shrewsbury Hospital |
| RWH | Royal Wolverhampton Hospital |
| STH | Sheffield Teaching Hospitals |

| | |
|---|---|
| SPME | Solid Phase Micro-Extraction |
| SD | Standard Deviation |
| SEM | Standard Error of the Mean |
| SVM | Support Vector Machine |
| TIC | Total Ion Count |
| Tukey's HSD test | Tukey's Honest Significance Difference test |
| UHCW | University Hospitals of Coventry and Warwickshire |
| UHL | University Hospitals of Leicester |
| VD | Vaginal Delivery |
| VOCs | Volatile Organic Compounds |

# Chapter 1.

# Introduction

This thesis assesses the differences between faecal volatile organic compounds from preterm human babies with and without necrotising enterocolitis. For this, two analytical platforms have been used: headspace–solid phase micro extraction–gas chromatography–mass spectrometry (HS–SPME–GC–MS) and headspace–gas chromatography–sensor (HS–GC–Sensor).

## 1.1 Necrotising enterocolitis

Necrotising enterocolitis (NEC) is a gastrointestinal disorder characterized by the necrosis of the gut barrier. Prior to necrosis, bloody stools and abdominal distension is often observed and babies usually have difficulty feeding and may vomit. NEC may occur throughout the whole gastrointestinal tract and complications may affect distant organs such as the brain, causing delay in infant development.[1]–[3]

NEC is a common illness in neonatal intensive care units (NICU) worldwide.[3] It may be the final stage of several disorders rather than a define illness. It mostly affects premature neonates, although 5% to 25% of infants diagnosed with NEC are not premature. In most cases the illness is linked to other health conditions, such as congenital heart disease, perinatal asphyxia or maternal drug abuse.[2]–[6] The first recorded observation dates to 1820-30 in Paris[2]; however, the name of the disease first appeared only in the 1950s.[4] NEC cases remained rare until the late 1960s because of the high mortality rate in NICUs worldwide,[2] mainly due to respiratory issues. Further, clinical presentation in 2015 is very different than it was in the past.

Necrotising enterocolitis has an incidence that is inversely proportional to birth weight.[3] It affects about 1 neonate per 1000 live births in US[7] and 0.3 neonate per 1000 live births in the UK and Ireland[5]. A Swedish study covering 22 years (1987-2009) of data collection from new born infants showed an overall incidence rate of 0.34 per 1000 live births.[5] When this dataset was divided into the four time periods of 1987-1992, 1993-1998, 1999-2004 and 2005-2009, the incidence rates were 0.26, 0.22, 0.35 and 0.57 per 1000 live births, respectively. This increase in the incidence of NEC in more recent years may be a result of the higher survival rate in preterm infants associated with improvements in NICU facilities.[5]

When analysing preterm infants with a period of gestation of less than 33 weeks, a Canadian study revealed that, on average, 5.1% of the babies contracted NEC with variable incidence between NICUs ranging from 1.3% to 12.9%.[8] Ahle *et alia (et al.)* have found that when the gestation period was less than 28 weeks NEC occurred in 4.6% of the infants in Sweden. They reported no geographical variation across Sweden, except in Stockholm, where the incidence rate was higher than elsewhere[5].

Globally, the death rate from NEC ranges from 20% to 50%[3], [4]. In the US, in 2000, this percentage was slightly lower at 14%.[7] Meanwhile, in Canada, between 2003 and 2008, the mortality rate was 25%.[8]

### 1.1.1 Pathogenesis

The biological mechanisms, which result in the development of NEC, are incompletely understood. It has been postulated that multiple factors, such as intestinal immaturity and the microbial colonization of the gut[2]–[4], [6], intestinal ischaemia, genetic predisposition or formula feeding[2], [9], may be involved. However, their interactions and time lines remain unknown.[2]–[4] It has also been suggested that NEC might be the final outcome of multiple diseases and not a single specific disease.[2] This observation may impact the volatile organic compounds present in faeces.

A chain reaction leading to necrosis of the gut barrier has been postulated. The immune system associated with the gut mucosa is immature in premature babies, and it cannot accommodate the colonization by every species of bacteria. Some bacteria may damage the intestinal epithelium causing mucosal inflammation and consequent overproduction of inflammatory factors. These inflammatory factors may then damage the epithelial barrier.[6] This hypothesis is supported by two facts, firstly, NEC mainly appears after 8 to 10 days of life[3], a period that coincides with the gut colonization by external bacteria[6], and secondly animal models in a germ free environment do not develop NEC.[3]

Several bacteria have been observed in the preterm gut, however, none is found consistently.[3] *Escherichia, Staphylococcus, Enterococcus, Clostridium* and *Streptococcus* have been described as the most common bacteria associated with NEC.[4], [6] Studies have, however, shown discordant results as the same species have been observed in both unhealthy and healthy patients. Grishin *et al.* suggested that specific strains of bacteria, rather than genera or species, might be the origin of the inflammation.[6]

The first bacteria to reach the gut, or first colonizers, may differ between individuals. Animal models demonstrated that diverse microbiota were observed despite being in a controlled environment.[6] The early microbiota generally shows low complexity (rarely more than 10 species) and low fluidity. The number and variety of bacterial species in the gut consistently increases until 1 year of age, when it finally stabilizes.[6]

### 1.1.2 Diagnosis

Currently, no early diagnostic methods for NEC exist. The diagnosis is clinical and is based on several symptoms such as feed intolerance, abdominal distention and/or bloody stools. However, healthy infants with very low weight may also present some of these symptoms.[3] Other symptoms, including apnoea, lethargy, respiratory failure and circulatory instability, have also been associated with NEC patients.[4]

Radiography of the bowel region shows pneumatosis intestinalis in 70 to 80% of the infected infants.[4] Pneumatosis intestinalis is the formation of hydrogen cysts, which are a cluster of cells forming a sac in the bowel wall.[10] The hydrogen is thought to have a bacterial origin.[4] A second radiographic sign of NEC disease is the accumulation of gas in the portal vein.[3], [11], [12] Observation of radiographic signs are considered to be final diagnostic signs according to Bell's staging criteria.[4] The Bell's staging criteria define NEC severity based on systemic, intestinal and radiological signs. It is rated as follows: I (suspected), II (definite) and III (advanced), and two sub-classes at each stage, A and B, where B is more advanced than A, but not sufficiently to induce a change of stage. Blood is observed in the stool from stage I.A; from stage II.A, pneumatosis intestinalis and abdominal tenderness are observed; from stage III.A, definite peritoneal cavity fluid, distension of abdomen, respiratory and medical acidosis are observed.[13]

### 1.1.3 Treatment

Medical interventions performed in NEC cases are essentially abdominal decompression, bowel rest, use of a broad spectrum intravenous antibiotics and parenteral, or intravenous, nutrition[3], [4]. The use of antibiotics is common practice for the treatment of NEC. Antibiotics administered may vary between NICUs. Enteral antibiotics demonstrated a positive effect on patients to prevent NEC, however their use must be controlled in order to prevent bacterial resistance development[4]. In addition, prolonged use of antibiotics may intensify the disease[3].

It is recognized that a complete restriction of enteral feeding may lead to the development of more severe NEC. Small amounts of breast milk given enterally may reduce the incidence of NEC[3], [9]. Formula feeding is an important risk factor because of the lack of immune-protective factors[2], [6], [9]. The current recommendation is to feed premature infants with small amounts of breast milk, which may prevent NEC and show other beneficial effects[3], [9].

Between 27 and 63% of affected infants need a surgical procedure[4]. Placement of a peritoneal drain without laparotomy has been used, although the literature suggests that mortality rate increases with this practice in comparison to laparotomy. Neu and Walker showed that several cases required laparotomy after a peritoneal drainage[3]. Exploratory laparotomy with resection of diseased bowel can be performed[3].

### 1.1.4 Prevention

Several techniques have been explored to prevent NEC cases, many of them being related to food intake. Breast milk supplemented with L-arginine and egg phospholipid has been suggested. However, the amount of data currently available is insufficient for an accurate assessment of the practice[4], [9]. Trophic feeds do not seem to increase the incidence of NEC, however, as with L-arginine supplementation, more work needs to be performed for a final conclusion. The use of probiotics has been shown to reduce significantly the mortality and incidence of severe NEC. Although this conclusion was drawn in a meta-analysis research comparing several studies including more than 2700 preterm infants, additional studies are still required before probiotics can become part of the standard procedure for NEC cases[14].

Fluid restriction, antenatal steroids and enteral administration of antibiotics have been suggested as potential prevention strategies[4], [9]. The latter is not recommended as it may increase the number of resistant bacteria. Antenatal steroids are recommended for lung disease as they improve lung mechanics and gas exchange by increasing surfactant production and enhancing response to surfactant treatment in neonates. Thus, the incidence of respiratory distress syndrome is reduced. Antenatal steroids also upregulate the gene expression for the epithelial $Na^+$ (sodium) channel present in the colon, therefore improving the salt homeostasis.[15]

## 1.2 Volatile organic compounds

Volatile organic compounds (VOCs) are a group of organic molecules characterized by their low vapour pressure. They are mainly, but not exclusively, carbon based and all functional groups are represented. These compounds can have natural or synthetic origin.[16] They are intermediates of metabolic pathways, thus, their abundances in bio-fluids reflect the current state of the body.[17] This concept is not recent. Hippocrates, in Ancient Greece, suggested smelling a patients' breath in order to diagnose potential disorders.[18] Furthermore, the smell is a known diagnostic tool in traditional Chinese medicine[19] and, more recently, nurses reported differences in the odour of faeces and urine samples from patients with urological and gastrointestinal disorders.[20], [21]

### 1.2.1 Canine detection

The dog's sensitive olfactory apparatus is able to detect compounds down to a few parts per trillion (ppt). Dogs have been used for avalanche victim rescue for decades[22] and are now emerging in medical diagnosis. Several studies have shown that canines are able to differentiate between patients with different diseases. Dogs have successfully identified samples from human patients with prostate cancer and healthy patients (sensitivity and specificity of 91%).[23] However, the use of dogs in medical diagnosis has been criticized. Elliker and co-workers [24] have shown that the failure rate can be high, 70% in their study. In addition, no dog was able to recognize prostate cancer samples during further double-blind testing.[24] In several studies dogs are trained to differentiate cancer patients from healthy patients, instead of identifying samples' from different diseases.[25] Nevertheless, if dogs are able to discriminate between samples based on their scents, it indicates that analytical devices may, potentially, detect the compounds associated with different diseases and be used as diagnostic tools.

### 1.2.2 Applications

Several studies have applied various analytical approaches to search for disease-specific biomarkers from bio-fluids or human tissue. Although many have found significant differences in VOC abundances between disease and control groups [26]–[32], few studies have resulted an application using VOC biomarkers. Examples of application of VOC detection used in clinical practice are the Gastro$^{+}$ Gastrolyzer® and Gastro$CH_4$ECK Gastrolyzer® from Bedfont® Scientific Ltd.[33] Gastro$^{+}$ detects $H_2$ while Gastro$CH_4$ECK detects $H_2$, $CH_4$ and $O_2$ abundances in patients' breath. Both use $H_2$ detection to diagnose carbohydrate malabsorption, lactose intolerance and other gastrointestinal conditions. However, Gastro$CH_4$ECK makes use of methane in addition to $H_2$, which ensures that patients not producing hydrogen can also be diagnosed.[34], [35]

The lack of application of VOC biomarkers is partially due to insufficient method validation and standardisation, high sample variability and compound prevalence across biofluids.[36], [37] For example, results of small cohort studies must agree with larger scale studies and results must be reproducible across laboratories. For this reason, It has been postulated that experts in medical VOC analysis could develop guidelines for standardizing methods and practices.[37] The variation between individuals and the fact that a single compound can be involved in several metabolic pathways makes it difficult to identify disease specific biomarkers. Therefore, it has been suggested that chromatogram patterns may be used for diagnosis instead of single identified compounds.[37]

### 1.2.3 Bio-fluids analysis

Biomarkers may be found in many bio-fluids; breath, urine, blood and faeces being, perhaps, the most commonly analysed. Breath is mainly composed of oxygen, nitrogen and carbon dioxide; however, several VOCs reach the breath from blood via the lungs. VOCs can be found in breath in concentrations of parts per million (ppm) to parts per trillion.[36]

Breath samples are easily available in almost all patients' conditions and matrix effects are minimal. Figure 1 defines the matrix. Breath analysis can be non-invasive, does not need any preparative steps before analysis by techniques such as GC–MS and GC–Sensors, and can be monitored on-line. For these reasons, breath analysis has potential as a diagnostic tool. An example of successful breath analysis is the study performed by Phillips *et al.*, where a model using 30 VOCs allowed classification of patients with lung cancer and healthy patients with a sensitivity and specificity of 84.5% and 81%, respectively.[38]

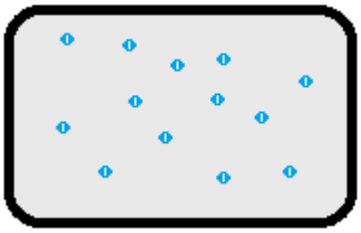

**Figure 1: Illustration of the matrix in a sample.** Considering that the black rectangle is a sample and the blue circles are the molecules of interest, or analytes, the matrix is represented by the grey zone which represents all molecules which are not targeted by the experiment.

Analyses of urine samples have suggested that 2-methyl-3-phenyl-2-propenal, *p*-cymene, anisole, 4-methylphenol and 1,2-dihydro-1,1,6-trimethylnaphtalene could be used as potential biomarker for leukaemia, colorectal cancer and lymphoma.[39] Urine samples are non-invasive, making them easy and comfortable for patients to collect and they can be analysed without additional sample preparation steps. Major disadvantages of urine analysis are its matrix complexity[40] and the non-availability of the samples in some medical conditions.

Blood and serum have been widely used for diagnosis. Blood connects every organ and therefore carries molecules around the body. Wang *et al.*[41] have shown that phenyl methylcarbamate, ethylhexanol and 6-t-butyl-2,2,9,9-tetramethyl-3,5-decadien-7-yne could be used as biomarkers for colorectal cancer. This study involved 16 patients with colorectal cancer and 20 healthy patients. Wilcock *et al.*[42] have analysed samples from 12 preterm neonates, of which 5 developed NEC, and from 8 term neonates using a GC–MS. Differences were identified between samples from preterm and term neonates as well as between samples from patients suffering from NEC and healthy preterm patients. Nevertheless, the discriminating power of the study was not sufficient to consider the proposed compounds as potential biomarkers. A drawback of blood analysis is the invasive nature of the procedure to collect samples, making it less desirable for diagnosis methods, especially for babies and children.

There is an increasing interest in VOCs from faeces because these VOCs are linked to the gut microbiota and faeces collection is non-invasive. VOCs in faeces are, therefore, potential candidates for diagnosing several gastrointestinal disorders.[43] Their analysis for this purpose is recent and few articles have been published. Table 1 presents a summary of the literature review on the topic. Garner *et al.* published two pilot studies[26], [44], in which they aimed to differentiate faecal samples from healthy patients and patients with NEC and with cholera. Su Yin Ng *et al.* analysed samples from 16 patients, 8 were diagnosed to be *Cryptosporidium* positive and 8 negative.[28] Ponnusamy *et al.* studied samples from 11 patients with irritable bowel syndrome and 8 healthy patients.[29] Jenner *et al.* characterised human faecal water from 5 healthy patients.[30] De Lacy Costello *et al.* studied samples from 10 adults and 7 neonates aiming to determine differences between these two groups.[27] Di Cagno *et al.* compared three groups of 7 patients aged between 6 and 12 years old to study the effect of a gluten-free diet on the gut microbiota.[31] Rafter *et al.* studied differences in VOCs in faecal water from 20 patients divided into two groups placed on a high-fat, low-calcium and low-fibre diet versus low-fat, high-calcium and high-fibre diet for 4 days.[32] Most articles reported relevant results, however, there is a clear need for larger scale studies in order to achieve a reliable conclusion. The size of the study needs to be appropriate when considering the sex and age ranges of the subjects studied and variables specific to the research performed. Below further publications with higher sample sizes are presented.

De Meij *et al.* analysed faecal samples from 13 patients suffering from NEC, 31 suffering from sepsis and 14 controls using an e-nose (see 1.4 for sensor description). Preterm neonates with gestational age of less than 30 weeks were recruited from 3 institutions over 7 months. A total of 123 samples were divided in 3 time windows: (1) the day of diagnosis and 1 day before diagnosis; (2) 2 and 3 days before diagnosis; and (3) 4 and 5 days before diagnosis. Sensitivity and specificity when samples from NEC patients were compared to samples from matched control were of 88.9% and 88.9%, respectively, for the time window (1) and of 83.3% and 75.0%, respectively, for the time window (2). When comparing samples from NEC patients and patients suffering from sepsis, sensitivity and specificity were of 88.9% and 56.5%, respectively, for the time window (1) and of 83.3% and 75.0%, respectively, for the time window (2).[45]

Walton *et al.* analysed samples from 87 patients divided into 4 groups: healthy volunteers, Crohn's disease, ulcerative colitis and irritable bowel syndrome patients with 19 (11/8), 26 (4/22), 20 (12/8) and 22 (8/14) patients (males/females), respectively.[46] Diagnosis was made by standard diagnostic criteria. Results showed that butanoic acid, 1-propanol, propanoic acid ethyl ester, butanoic acid methyl ester, butanoic acid ethyl ester, indole, 1-butanol, butanoic acid 3-methyl and phenol compounds were significantly different ($p<0.05$) between the four groups with a Kruskal-Wallis test. A post-hoc test showed that the difference was always, and only, between Crohn's disease patients and the healthy volunteer.

Garner *et al*. analysed samples from 31 patients with *Campylobacter jejuni* infection, 22 with *Clostridium difficile* infection and 18 with ulcerative colitis, recruited from two hospitals, and 30 asymptomatic donors recruited elsewhere.[47] VOCs origins have been hypothesized, based on further analysis with carbon$^{13}$ labelling and 10 asymptomatic donors gave 5 samples over 2 weeks to assess dietary effects on VOCs. The analysis performed using SPME–GC–MS on fresh and frozen samples showed no difference. Results with [1-$^{13}$C] butanoic acid addition showed an increase of $^{13}$C butanol and some esters derived from butanoic acid. These results suggest that, during the incubation time, reduction or oxidation reactions may occur. On 297 VOCs identified in total (median of 101 VOCs per sample), 44 compounds were found in 80% of the asymptomatic donors. The similarity in the number of carbon groups across acids, aldehydes and alcohol suggests that they may be oxidized or reduced from one another. The study was a longitudinal study, using several donors and the abundance of most compounds remained stable, suggesting little day-to-day variation. Finally, using discriminant analysis, the 4 groups of patients have been separated with 100% predictive accuracy and 96% predictive accuracy with a cross-classification and leave-one-out validation method.

De Meij *et al.* recruited 157 patients undergoing endoscopic investigation for colorectal disease, from two institutions in the same city.[48] Patients were divided into three groups: colorectal cancer, advanced adenomas and healthy individuals, with 40, 60 and 57 patients respectively and a median age of 38, 66 and 69 years, respectively.

Samples were analysed using Thermal Desorption–GC–MS (TD–GC–MS). The sensitivity and specificity of the results obtained with an e-noise while comparing colorectal cancer and healthy patients were 85% and 87%, respectively and while comparing advanced adenomas and healthy individuals, they were 62% and 86%, respectively. Results obtained with immunochemical tests were not shown as they differed too much, possibly because of the duration of sample storage.

**Table 1: Summary of the literature review.**

| Reference | Method / Instruments | Number of patients | Topic | Outcome |
|---|---|---|---|---|
| Garner *et al.* [44] | SPME-GC-MS | 13 | NEC | Differences in patterns |
| Garner *et al.* [26] | SPME-GC-MS | 9 | Cholera | 2 compounds identified for discrimination |
| Su Yin Ng *et al.* [28] | Derivatization followed by GC-MS | 16 | *Cryptosporidium* | Differences in patterns |
| Ponnusamy *et al.* [29] | Derivatization followed by GC-MS | 19 | Irritable bowel syndrome | Differences in patterns |
| Jenner *et al.* [30] | Derivatization followed by GC-MS | 5 | Human faecal water | Characterisation of aromatic compounds |
| De Lacy Costello *et al.* [27] | SPME-GC-MS | 17 | Adults compared to neonates samples | Fewer compounds in neonates samples |
| Di Cagno *et al.* [31] | SPME-GC-MS | 21 | Gluten free diet on celiac patients | Differences in patterns |
| Rafter *et al.* [32] | GC-MS | 20 | Diet influence on compounds present in faeces | No differences in fatty acid concentration |
| De Meij *et al.* [45] | E-nose | 58 | NEC and sepsis | Differences using classifier |

| | | | | |
|---|---|---|---|---|
| Walton *et al.* [46] | TD-GC-MS | 87 | Crohn's disease, ulcerative colitis and irritable bowel syndrome | Few compounds identified showed differences |
| Garner *et al*. [47] | SPME-GC-MS | 101 | *Campylobacter jejuni, Clostridium difficile* and ulcerative colitis | Differences using classifier |
| De Meij *et al.* [48] | E-nose | 157 | Colorectal cancer and advanced adenomas | Differences using classifier |

Table presenting a summary of the literature investigating VOCs in faecal samples.

### 1.2.4 Derivatization

Derivatization of samples prior to analysis is performed in some studies involving VOCs. It improves chromatographic separation by reducing the polarity of the compounds and/or enhancing their volatility, which allows the analysis of non-volatile compounds.[49], [50] Therefore, if the compounds of interest cannot be analysed using a gas chromatography they may be derivatized to allow their analysis. However, derivatization is time-consuming, introduces additional variability due to manual procedures and may result in contamination and side products added to the samples.[51] In addition, the complex steps involved in derivatization limit its use by nurses and compromise its application for on-site diagnostic tools.

### 1.2.5 Contaminants

Avoiding contamination in gas analysis is a constant challenge because of the difficulty, often impossibility, of isolating the samples from environmental air. This is a particular issue in breath analysis, where patients breathe constantly and incorporate compounds present in the environment. Usually, these compounds are present in higher concentrations in the air than in the patients' breath.[21] Environmental contaminations are less common when analysing other bio-fluids, but it may nevertheless occur. Therefore, a regular analysis of the environmental air can be applied to detect any potential contaminants introduced in the analysis. Procedures and treatments given to patients may also be source of contamination which could be misinterpreted as disease indicators.

Containers made of plastic can be a source of contamination by carbon based polymers and compounds originating from these polymers .[21], [27], [52], [53] Plastic containers, tubing and other plastic pieces of equipment should be avoided. Solvents, including washing products, used in preparation, or in the environment where the samples are processed, are another source of contamination.[54] The Appendix A summarizes compounds which have been suggested to be contaminants or emanating from the environment. It has been observed that some proposed disease biomarkers were likely to be contaminants.[21]

### 1.2.6 Volatile organic compounds analysis

VOC analysis includes three main steps: extraction, separation and detection. The extraction, with possible pre-concentration of the sample, can be left out with some analytical methods as the matrix effect is not predominant and the instrument has a high sensitivity. In this case, direct headspace analysis is used. It consists of taking a defined volume of air from the sample's headspace and injecting it into the GC–MS, for example. The extraction can be done by solvent-free techniques as dynamic headspace[55], solid phase extraction (SPE)[56], solid phase micro-extraction (SPME)[57], thermal desorption tube (TD)[18] or by stir bar sorptive extraction (SBSE)[58]. Using SPE, SPME, TD and SBSE, compounds are bound to a sorbent material and desorbed afterwards. Compound extraction may also be performed using a solvent, however it results in additional steps and time-spent on sample preparation. Techniques using sorbent, however, may not be representative of VOCs present in the analysed samples, as a result of the competition between molecules for the sorbent.[59] The main advantages of sorbent techniques remain in their chemical neutrality and in the possibility to acquire the samples on-site and to analyse them subsequently.

When applying sorbent techniques, compounds are desorbed and separated; this is usually performed by chromatography, ion mobility techniques or a combination of these. VOCs detection may be achieved using different methods, however we have used GC Mass Spectrometry (MS).[60]

## 1.3 Headspace – solid phase micro-extraction – gas chromatography – mass spectrometry

SPME–GC–MS is well-suited for the analysis of VOCs from biological samples. The complex matrix is avoided while the compounds of interest are extracted from the headspace. GC is a well-known and established method for the analysis of volatile compounds. When coupled to MS instruments, it allows direct identification of analytes. The direct analysis of volatile compounds using SPME–GC–MS reduces potential variability introduced during sample manipulation. However, the affinity of compounds to the phase is not consistent and some compounds may be preferentially selected, which can influence the results.[59]

### 1.3.1 Solid phase micro-extraction

Prior to the chromatographic separation, extraction of compounds may be required, solid phase micro-extraction (SPME) being used most often. It consists of a needle coated with a specific material or phase to which the molecules bind and can be thermally released. Coatings are selective. Water and other major constituents of the matrix are not expected to bind extensively.[61] Analysing each sample with different SPME fibres will give a more complete analysis of the VOCs in the sample. Dixon *et al.* have shown that 100% of the VOCs coverage can only be reached when analysing the sample with all 8 different SPME fibres tested.[62] It is also important to acknowledge that no other techniques have been used to detect VOCs and, therefore, some compounds may have been missed.[61]

SPME can be used to extract compounds from the headspace or the liquid phase of the sample. Headspace is most commonly used because of the advantage of reducing the matrix effect on the coating but, in recent *in vivo* investigation, SPME has been carried by immersion of the fibre in the blood stream or the muscle of fish or mice.[61] Newly created technologies such as stir bar sorptive extraction (SBSE) are based on the same principle as SPME but with a larger surface, which increases its detection limit and accuracy.[63]

### 1.3.2 Gas chromatography

Gas chromatography (GC) is a well-established method in analytical chemistry and has been used for more than 60 years.[64] A GC instrument is composed of an injector, a column placed inside an oven and a detector. Several types of injector are available. GC-ovens can be heated to accelerate the transit of molecules through the column, the carrier gas and its flow rate can be changed and the amount injected can be modified enabling method optimisation. The GC column is the heart of GC instruments, as this is where molecules are separated. Characteristics such as stationary phases, their thickness, column length and diameter can be adjusted specific to the separation to be achieved. The end of the column is connected to a detector, which generates a signal proportional to the number of molecules leaving the column at a given time. Several detectors have been designed, some specific to molecule families and others more universal.[49]

Chromatography is a technique during which molecules are separated while being carried by a mobile phase through a stationary phase. In GC, molecules are separated based on their polarities, shapes and masses resulting in a competitive equilibrium between the mobile and stationary phases. The separation is achieved by the difference in the ratio [molecule in the stationary phase / molecule in the mobile phase] between molecules. Molecules of similar ratio cannot be separated. To achieve that separation parameters, e.g. the mobile and/or stationary phase, can be altered.[65]

The GC instruments provide the retention time (RT) of each compound, which is the time taken by molecules to pass through the entire column. The RT is consistently reproducible. A specific analyte is expected to show the same RT when analysed by GC using the same test conditions.[49] Detectors can add more information about the molecule identity, such as infrared or mass spectra for example. The retention time and the mass spectroscopic information can be combined for a molecule can be directly identified, by matching the obtained spectra to one of several databases.[49], [66]

### 1.3.3 Mass spectrometry

Mass spectrometry started with experiments carried in the late 19$^{th}$ and early 20$^{th}$ centuries by Goldstein, Thompson and Wien. Modern quadrupole MS instruments were first described in 1953 by Paul and Steinwedel.[67] MS has evolved and become very diversified, being applied in several fields and with an increasing quality of data delivered.[67] The analysis of molecule by MS is performed by the creation of gas-phase ions generated from compounds. Molecules are ionized and fragmented during this process, which can be chemically or physically achieved. Ions are then selected and detected. Different types of mass analyser and detector are currently available on the market.[67] Only techniques applied in this study will be described further for brevity.

Electron ionization (EI) source is widely used for analysis of low mass compounds. Molecules enter the ionization chamber in a gaseous form and are collided with electrons emitted by a heated filament. "*Electrons are associated to a define wave of wavelength […]. When this wavelength is close to the bond lengths, the wave is disturbed and becomes complex. If one of the frequencies has an energy corresponding to a transition in the molecule, an energy transfer that leads to various electronic excitations can occur. When there is enough energy, an electron can be expelled.*"[68] On average 1 molecule out of 1000 is ionized. The ions are then extracted from the chamber, focused and accelerated toward the mass analyser.[67] The quadrupole selects ions based on their mass/charge ratio (*m/z*). It consists of four rods perfectly parallel. The polarity of the rods changes from positive to negative at a designated interval. Ions are alternatively attracted and repulsed from the rods and if no collisions with a rod happen, they will, eventually, reach the detector; when a collision occurs they discharge themselves and stop their trajectories. Selection of ion masses is performed by varying the current of the rods.[67] Ions are focused in the middle of the quadrupole and reach the detector. An electron multiplier (EM) is commonly used as a detector. Ions are accelerated at a high velocity and reach a dynode, which will convert the ions into electrons. These electrons are then amplified by a cascade effect producing a usable current.[67]

### 1.3.4 Data analysis

Several computational tools are currently available for the analysis of metabolomics data. Some tools were developed for LC–MS or GC–MS specifically while others can process data from both systems. Perhaps the most common software used for GC/MS data processing is AMDIS (Automated Mass spectral Deconvolution and Identification System).[69], [70] AMDIS can be used in conjunction with NIST (National Institute of Standard and Technology) (http://www.nist.gov/srd/upload/NIST1a11Ver2-0Man.pdf, 04/12/15), a mass spectral database for compound identification. Results obtained from AMDIS have a high false positive rate[71] and the reliability of the identification based on its library matching approach has been critized.[72] Therefore, results from AMDIS must be further processed using external packages. Aggio *et al.*[69] developed the R package 'Metab', which verifies if compounds identified by AMDIS are within their expected retention time window and returns the intensity of the most abundant ions for each compound. Behrends *et al.*[71] developed a Matlab package to visually verify compounds identified by AMDIS and to provide the intensity or abundance, according to users' choice, for every compound in each chromatogram creating spreadsheet without missing data. Other software has been created to analyse GC/MS data with identification, such as 'MetaBox'[73], 'TargetSearch'[74], 'MetaboliteDetector'[75] or 'OpenChrom'[76], [77]. The R package and online platform 'XCMS' performs an analysis of mass spectrometry data, for gas or liquid chromatography.[78], [79] It is based on individual ions intensities at specific retention times, thus, by-passing compound identification.

Figure 2 illustrates a HS– SPME–GC–MS instrumentation.

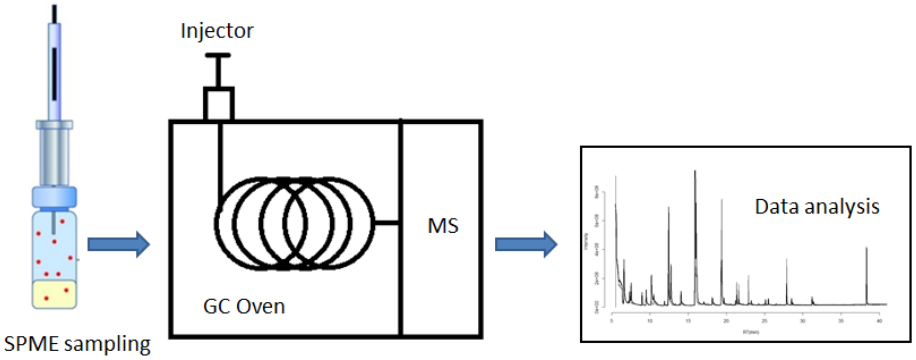


**Figure 2: Illustration of the HS–SPME–GC–MS instrumentation.**

## 1.4 Headspace – gas chromatography – sensor

Oxide semiconductor gas sensors were first reported in early 1960s. Since then, they have been used in many applications. The technology has been investigated to increase its sensitivity and specificity to meet modern criteria in sub-ppm detection.[80] The principle behind the two types of semiconductor as well as main aspects investigated for their improvement will be presented in the next paragraphs.

The semiconductor oxide can be of 2 types, *n* or *p*. The difference between them being that the *n*-types will be covered with a layer of negative charges whereas the *p*-type oxides have holes of positive charges on their surface. *N*-type Semiconductors, eg tin dioxide, indium oxide, tungsten trioxide or zinc oxide, have been used in preference to *p*-type oxide, eg nickel oxide, because their mobility of conduction electrons is higher, which enhances their sensitivity to detect target gas. For the same reason, *n*-type oxides with low mobility of conduction electrons have not been used extensively.[81] This technology principle is based on the interaction between the gas analysed and the solid phase of the sensor, which will induce an electronic variation of the surface and the transduction of this interaction into an electrical resistance. The reaction between the target gas and the semiconductor oxide is done through an oxygen adsorbate layer present on the sensor. The oxygen adsorbate can be $O_2^-$, $O^-$ and $O^{2-}$, with $O^-$ being the most reactive of the three at a temperature between 300-500°C. Tin dioxide has a high stability, both chemically and thermally, therefore it is frequently used to develop sensors.[81]

The surface of the sensor has a key role in its reactivity. Two main aspects have been investigated, the grain size effect and the addition of a noble metal (eg: palladium, silver, platinum). The grain size, expressed as the mean diameter, is increasing along with the temperature of calcination used lastly during the synthesis of the oxide. Metal oxide addition, such as lanthanum, barium or phosphorus oxide, can also control the grain growth. The mean diameter of the oxide has a major influence on the resistance measured with the sensor. It is inversely proportional to the grain size until it reaches a plateau with a grain size of 15nm. Therefore, commercial tin dioxide sensors usually have larger grain size than 15nm to avoid this.[81] The second aspect, the addition of a noble metal, will increase the response given by the sensor at low temperature. The oxidation rate of the target gas is increased as a result of one of two types of interactions which take place.

In the first interaction, the target gas is activated by the noble metal and reacts afterwards with the adsorbed oxygen of the surface. In the second interaction type, the noble metal accepts electrons given by the semiconductor oxide and when the noble metal becomes reduced on contact with the target gas, it then gives the electron back to the semiconductor oxide.[81], [82]

Other additives, metal or oxide, can be used to enhance the ability of a sensor to identify specific compounds. Similarly acidic or basic treatment can improve the response to basic or acidic target compounds, respectively.[81] The physical characteristics, such as the thickness of the metal oxide semiconductor film, play an important role in sensor sensitivity.

In comparison with other sensors, metal oxide semiconductor sensor advantages are their cheapness, short response time, wide range of target compounds coverage and long lifetime, but, their sensitivity and selectivity are low, the environment has an influence on the response and the energy demand is high. Sensor instruments do not deliver mass spectra and therefore, compounds detected cannot be identified.[83]

Figure 3 illustrates the HS–GC–Sensor instrumentation.

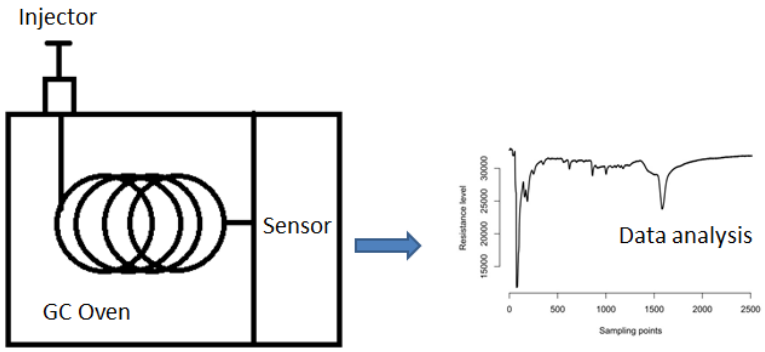


**Figure 3: Illustration of the HS–GC–Sensor instrumentation.**

## 1.5 Hypothesis, aim and objectives

The **D**iagnostic testing **O**f **V**olatile organic compounds (VOCs) in necrotising **E**nterocolitis (the DOVE Study) project will investigate the hypothesis that faeces from patients suffering from necrotising enterocolitis have a specific pattern of VOCs in the days prior to diagnosis which differs from faeces from healthy patients and that this pattern can be characterized using HS–SPME–GC–MS and HS–GC–Sensor.

The aim of the project is to find reliable indicator(s) or a pattern in the signal response to allow the development of early diagnosis tools for NEC in preterm infants.

The objectives are:

1. to develop methods for the analysis of faeces from premature babies using a GC coupled to both a MS and a sensor.
2. to analyse faecal samples from healthy premature infants and from the premature infants with NEC.
3. to perform statistical analysis of the data to determine diagnostic patterns.

# Chapter 2.

# Methods

Content of this chapter is part of: Reade, S., Mayor, A., Aggio, R., Khalid, T., Pritchard, D., Ewer, A., & Probert, C. (2014). Optimisation of Sample Preparation for Direct SPME-GC-MS Analysis of Murine and Human Faecal Volatile Organic Compounds for Metabolomic Studies. *Journal of Analytical & Bioanalytical Techniques*, *5*(2). doi:10.4172/2155-9872.1000184. (Appendix B)

Author contribution to the published work: Arno Mayor designed and implemented the experiments, analysed the data and participated in writing the introduction and the section involving human faecal samples.

This chapter contains a review of the technologies applied in this thesis for detecting, identifying and quantifying volatile organic compounds (VOCs) in biological samples; a description of the statistical methods used for shortlisting potential biomarkers; and a description of sample processing.

# 2.1 Mass spectrometry

In this thesis, headspace–solid phase micro-extraction–gas chromatography–mass spectrometry (HS-SPME–GC–MS) was applied to detect, identify and quantify metabolites present in faeces from premature babies with and without necrotizing enterocolitis (NEC). Many aspects of the HS–SPME–GC–MS analysis of biological samples have been previously optimized by the laboratory team[44], [47], [84], while other aspects were specifically optimized for this study. These optimizations are described below.

## 2.1.1 Gas chromatography – mass spectrometry configuration

A PerkinElmer Clarus 500 GC/MS quadrupole benchtop system (Beaconsfield, UK) was used in combination with a Combi PAL auto-sampler (CTC Analytics, Switzerland) for the analysis of all samples involved with this thesis. The GC column used was a Zebron ZB-624 with inner diameter 0.25 mm, length 60 m, film thickness 1.4 µm (Phenomenex, Macclesfield, UK). The carrier gas used was helium of 99.996% purity (BOC, Sheffield, UK). Vials with magnetic caps of 2 ml (Crawford Scientific, Lanarkshire, UK) and 10 ml (Sigma-Aldrich, Dorset, UK) volume were investigated. Samples were pre-incubated for 30 minutes at 60°C prior to fibre exposure. The SPME fibres used were Carboxen®/Polydimethylsiloxane (CAR/PDMS) 85 µm and Divinylbenzene/Carboxen®/ Polydimethylsiloxane (DVB/CAR/PDMS) 50/30 µm (1cm) (Sigma-Aldrich, Dorset, UK). Both fibres were pre-conditioned before use, in accordance with manufacturer's manual. The fibre was exposed for 20 minutes at 60°C. The fibre was desorbed for 5 minutes at 220°C. The initial temperature of the GC oven was set at 40°C and held for 1 minute before increasing to 220°C at a rate of 5°C/min and held for 4 min with a total run time of 41 min.

A solvent delay was set for the first 6 min and the MS was operated in electron impact ionization EI+ mode, scanning from ion mass fragments 10 to 300 *m/z* with an inter-scan delay of 0.1 sec and a resolution of 1000 at Full Width at Half Maximum (FWHM). The helium gas flow rate was set at 1 ml/min. The sensitivity of the instrument was determined using 2-pentanone. The limit of detection of 3 times the signal/noise ratio of the 2-pentanone with DVB/CAR/PDMS fibre was 16 ppm and with CAR/PDMS fibre was 40 ppm.[57]

Except for the temperature ramp, all parameters were identical for the method development and for the Dove Project. The ramp for the Dove Project was the following: the initial temperature of the GC oven was set to 40°C and held for 1 minute before increasing to 220°C at a rate of 5°C/min and held for 13 min with a total run time of 50 min.

### 2.1.2 Sample mass

Samples of different masses may generate different metabolite profiles when analysed by HS-SPME-GC–MS. This section aims to investigate how homogenised faecal samples of different masses may affect the VOCs identified and to define the optimum sample mass for analysing faecal samples from preterm babies with and without NEC. The initial work (part I) was performed to optimise the mass of human faecal samples. Preterm babies often pass less than 100 mg of faeces. Therefore, mindful of this, the minimum sample mass that would produce the highest number of VOCs with the highest abundances was investigated (part II).

#### 2.1.2.1 Mass optimisation: part I

The number of VOCs identified and their abundances were evaluated according to sample masses ranging from 100 to 700 mg. A single faecal sample was divided into 3 aliquots of 100 mg (100.3 ± 0.6 mg), 3 aliquots of 450 mg (455.1 ± 1.1 mg) and 3 aliquots of 700 mg (700.6 ± 2.8 mg) (mean ± standard deviation or SD) (Figure 4). Only VOCs identified in every aliquot were used for comparing VOC abundances.

Kruskal-Wallis showed no significant differences in the number of identified VOCs between the three masses investigated (Figure 5). Eight compounds were identified in every sample (2,3-butanedione, tetrahydrofurane, ethyl ester propanoic acid, n-propyl acetate, 2-pentenal (E), propyl ester propanoic acid, 2-methylpropanal, 1-propanol) and their intensities were significantly higher in samples of 450 and 700 mg than in samples of 100 mg.

These results indicated that an increase in sample mass did not generate an increase in the number of VOCs detected, however it increased VOC abundance. The lack of difference between samples of 450 and 700 mg suggested that the SPME fibre sorption had reached a plateau. Therefore, 450 mg seemed to be the optimum sample mass.

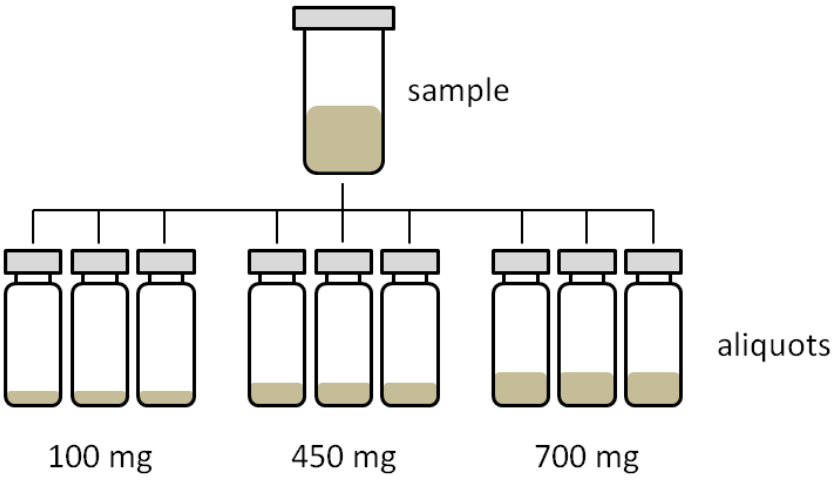


**Figure 4: Design of the part I of the mass optimisation for GC–MS investigation.** Illustration of the aliquots distribution and their respective masses for the part I of the mass optimisation.

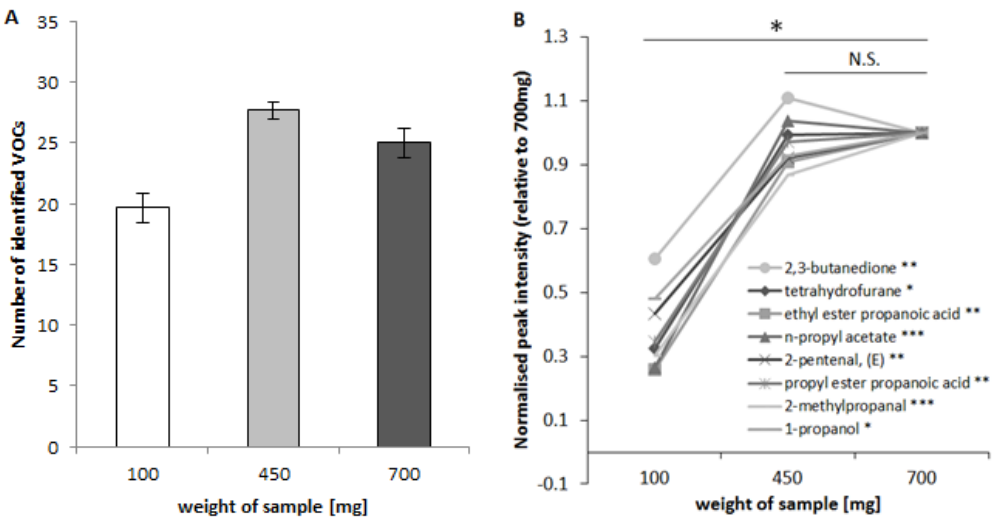


**Figure 5: Part I of the mass optimisation for GC–MS investigation** (A) Bar plot showing the mean of the number of VOCs identified (± standard error of the mean (SEM)) for the three masses tested (n=3/group). (Kruskal-Wallis test; no significant differences) (B) Mean of the intensities of the 8 identified VOCs present in all nine measurements (n=3/group). (*p<0.05, **p<0.01, ***p<0.001; ANOVA with Tukey HSD test, followed by Bonferroni correction)

### 2.1.2.2 Mass optimisation: part II

Aliquots of 50 and 100 mg were compared in order to find the lowest possible sample mass to be used for studying preterm babies. The optimized sample mass must be able to produce a reasonable number of VOCs and reproducible abundances.

Four additional samples were then divided into a set of six aliquots: triplicates of 50 and 100 mg (Figure 6). The number of compounds identified and their abundances were then compared between samples of 50 and 100 mg for each set of aliquots. The majority of the compounds showed less than 5% difference in log-transformed abundances between 50 and 100 mg samples for every set of aliquots (Figure 7). Regarding the number of VOCs identified, there was no difference between samples of 50 and 100 mg (Figure 8).

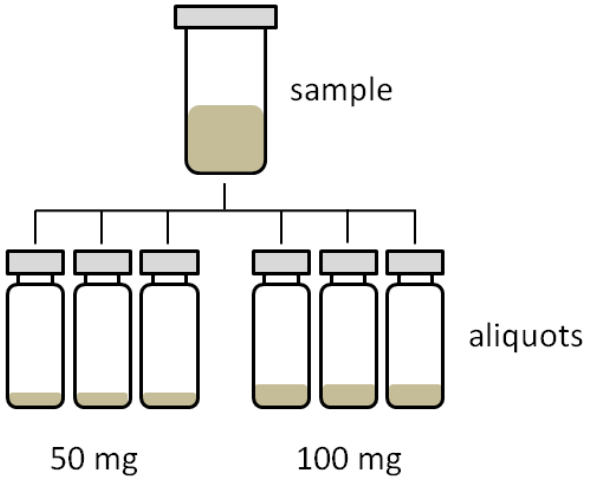


**Figure 6: Design of the part II of the mass optimisation for GC–MS investigation.** Illustration of the aliquots distribution and their respective masses for the part II of the mass optimisation. Four samples were divided this way.

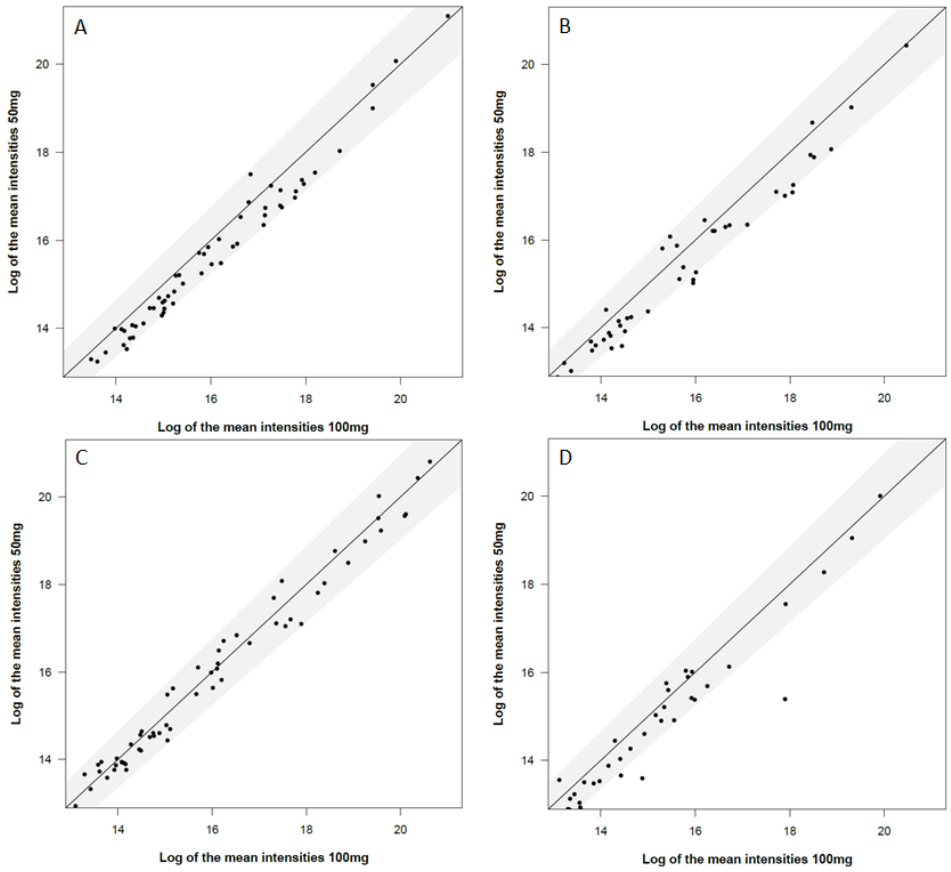


**Figure 7: Abundances of VOCs in replicates of 50 and 100 mg.** Scatterplots comparing the average log values of VOCs abundances from samples of 50 and 100 mg (n=3). The diagonal black segment represents the function x = y, where compounds detected at similar abundances between 50 and 100 mg are located inside of the 5% tolerance region represented by the grey area.

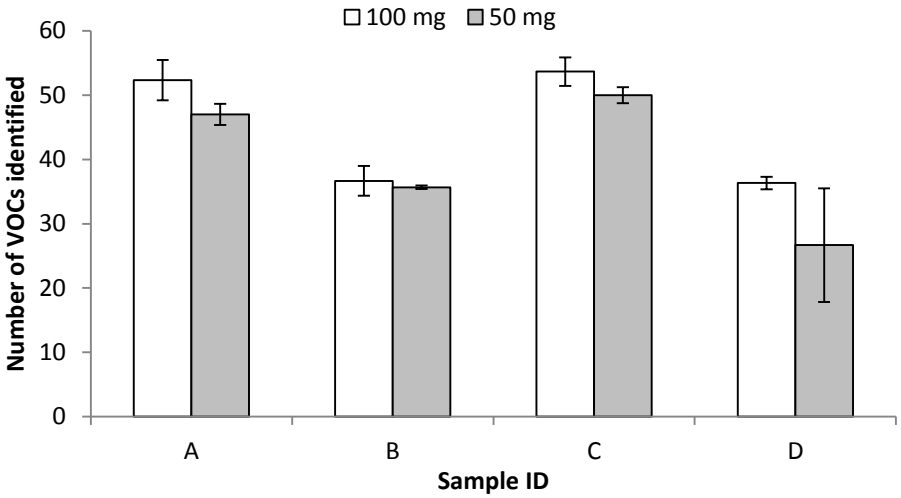


**Figure 8: Number of VOCs identified in replicates of 50 and 100 mg.** Bar plot illustrating the number of VOCs identified (± SEM) for both masses in each samples (A-D). (n=3; no significant differences, Mann-Whitney test)

### 2.1.3 Vial volume

The volume of the vial used to analyse biological samples may determine the metabolite profiles produced by HS–SPME–GC–MS analysis. This section investigates how different vial volumes may affect the VOCs identified and define the optimum vial volume for analysing faecal samples from preterm babies with and without NEC.

Vials of 2 and 10 ml were used to investigate the number of VOCs identified and their abundances. For each vial volume, aliquots of 100 mg were obtained from three different faecal samples (A: n=4; B: n=4; C: n=3) and analysed using the CAR/PDMS fibre. The great majority of the identified compounds showed less than 5% difference in their log transformed abundances between 2 and 10 ml vials (Figure 9). Mann-Whitney tests showed no difference in the numbers of VOCs identified between the vial volumes tested (Figure 10). Although no significant differences were observed, a slightly higher number of VOCs has been identified with vials of 10 ml for samples A and B. Therefore, vials of 10 ml were defined as optimum for the HS–SPME–GC–MS analysis of preterm faecal samples.

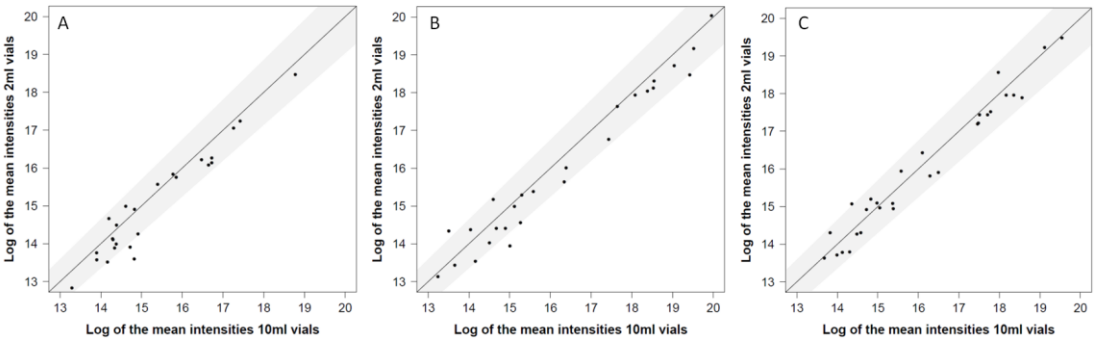


**Figure 9: Abundances of VOCs in replicate in 2 and 10 ml vials.** Scatterplot comparing the average log values of VOCs abundances from samples in 2 and 10 ml vials (A: n=4; B: n=4; C: n=3). The diagonal black segment represents the function x = y, where compounds detected at similar abundances between 2 and 10 ml are located inside of the 5% tolerance region represented by the grey area.

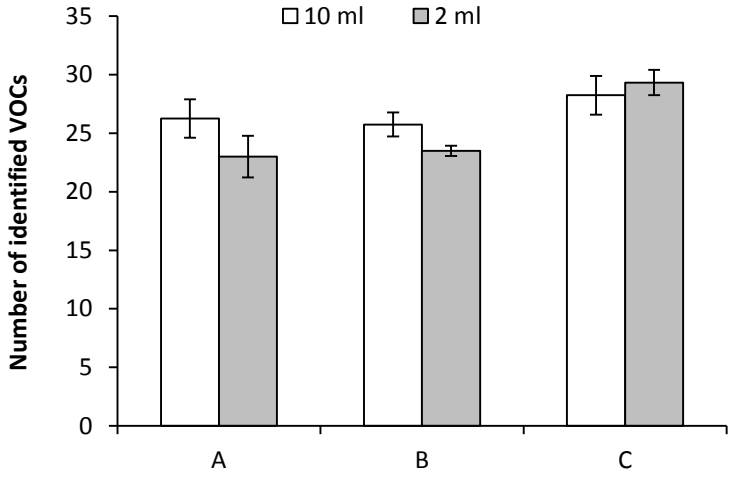


**Figure 10: Number of VOCs identified in replicates in 2 and 10 ml vials.** Bar plot illustrating the mean number of VOCs identified (± SEM) in each sample in 2 and 10 ml vials. (A, B: n=4; C: n=3; no significant differences, Mann-Whitney test**)**

### 2.1.4 Addition of salt, acid and base

Salt can be added to samples prior to headspace analysis with GC instruments. This step is performed to unify the ionic strength within the sample and it also diminishes the retention of the VOCs in the liquid phase, thereby increasing their concentration in the headspace.[49] Acid or base can also be added to improve the metabolite profile of samples analysed by HS–SPME–GC–MS. It produces changes in the pH of samples. Thus, the salt form of acids or bases will diminish with the result that their non-ionic form will enter the headspace. This section aims to investigate how the addition of salt, acid and base solutions may affect the metabolite profile generated by HS–SPME–GC–MS and determine the optimum sample preparation method for the analysis of preterm faecal samples from babies with and without NEC.

To investigate the effect of adding base to faecal samples, one sample was divided into 9 aliquots of 100 mg and stored in 10 ml vials. Three aliquots were analysed with 1 ml of sodium hydroxide 5% in water, three with the addition of 1 ml of sodium hydroxide 10% in water and three aliquots were analysed with no addition of base. To investigate the effect of adding acid to faecal samples, one sample was divided into 9 aliquots of 100 mg and stored in 10 ml vials. Three aliquots were analysed with 1 ml of phosphoric acid 0.85% in water, three with the addition of 1 ml of phosphoric acid 1.7% in water and three with no addition of acid. To investigate the effect of adding salt to faecal samples and because of low sample mass, three samples were divided into 3 aliquots of 100 mg and stored in 10 ml vials. One aliquot from each sample was analysed with either 1 ml, 0.5 ml or no addition of saturated chloride sodium solution. Saturated chloride sodium solution is defined by a concentration higher than 360g/l. All samples were analysed using a CAR/PDMS fibre and the number of VOCs identified were compared using Kruskal-Wallis test. Ms Daisy Noble, a BSc student in the Institute of Translational Medicine, University of Liverpool, under my supervision, conducted these experiments.

Although the addition of salt and base solutions seemed to lower the number of compounds, none of the solutions added led to a significant change in the number of VOCs identified (Kruskal-Wallis test) (Figure 11).

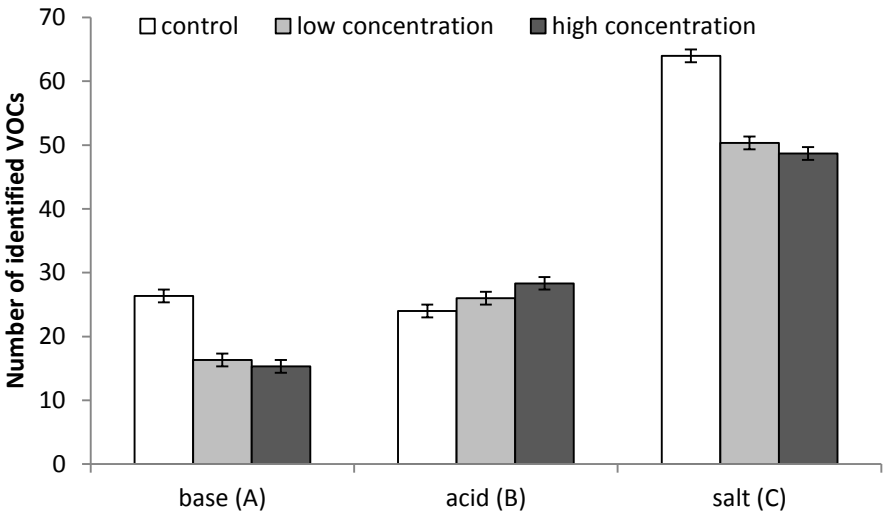


**Figure 11: Number of VOCs identified in replicate after addition of base, acid and salt.** Bar plot illustrating the influence of the addition of 0.5 ml of sodium hydroxide 5% (low) and 10% (high), 0.5 ml of phosphoric acid 0.85% (low) and 1.7% (high), 0.5 (low) and 1 ml (high) of saturated sodium chloride solution on the mean of VOCs (± SEM). The control being samples on their own (n=3/group; no significant differences; Kruskal-Wallis test)

Abundances of compounds identified in each replicate were tested for significant changes when various concentrations of solution were added. Considering the addition of base solution, 5 compounds were common to each replicate whose 4 were significantly different. Further, 3 of these 4 compounds had higher abundance in control samples. Adding acid solution, 8 compounds were identified in each replicate, 5 being significantly different. For 4 out of 5 compounds, the intensities were higher in the control samples. None of the 15 compounds identified in each replicate after addition of salt were significantly different (ANOVA followed by Tukey's HSD test and Bonferroni; based on log of the intensities; p-value>0.05).

Table 2 presents the number of VOCs identified exclusively after addition of a solution at given concentration. For base and salt additions, the highest number of unique VOCs was found when samples were analysed without addition. The addition of 1 ml of phosphoric acid at 1.7% led to the detection of 5 more compounds than no, or lower concentration, addition.

**Table 2: Number of VOCs identified specific to a given concentration after addition of base, acid and salt.**

| Solution added | Concentration | Number of unique compound per concentration |
|---|---|---|
| Base (NaOH) | No addition | 18 |
| | 1 ml of 5% solution | 4 |
| | 1 ml of 10% solution | 1 |
| Acid ($H_3PO_4$) | No addition | 2 |
| | 1 ml of 0.85% solution | 2 |
| | 1 ml of 1.7% solution | 7 |
| Salt (NaCl) | No addition | 15 |
| | 0.5 ml of saturated solution | 3 |
| | 1 ml of saturated solution | 2 |

Table summarizing the number of compounds found in at least 2 out of 3 replicates for a specific concentration after addition of a given solution while the compound was present in no more than 1 replicates for both other concentrations. For example, when evaluating the addition of base solution, 18 compounds were identified only in the control samples, while 4 compounds were only identified in samples that received 1 ml of 5% base solution.

### 2.1.5 Solid phase micro-extraction fibre optimisation

SPME fibres made with different coatings are commercially available. The coating may influence the metabolite profile generated by HS–SPME–GC–MS analysis. The aim of this section is to evaluate how different fibre coatings may affect the VOCs identified and to define the optimal SPME coating.

Two SPME coatings were evaluated: CAR/PDMS and DVB/CAR/PDMS. For this, three samples were divided into 8 aliquots of 100 mg and disposed into 2 ml vials. Four aliquots of each sample were analysed by CAR/PDMS coating and the other 4 aliquots were analysed by DVD/CAR/PDMS coating. In addition 1 sample was divided into 10 aliquots of 100 mg and disposed into 10 ml vials, 5 aliquots were analysed using CAR/PDMS coating and 5 aliquots were analysed with DVB/CAR/PDMS coating.

Figure 12 compares VOCs' abundances using both fibre coatings. When samples were stored in 2 ml vials (samples 1, 2 and 3), intensities were slightly higher using DVB/CAR/PDMS fibre. However, VOCs intensities were similar between fibres with samples stored in 10 ml vials (sample 4). A Mann-Whitney test comparing the number of VOCs identified by each fibre showed no significant differences for all sets of aliquots (Figure 13).

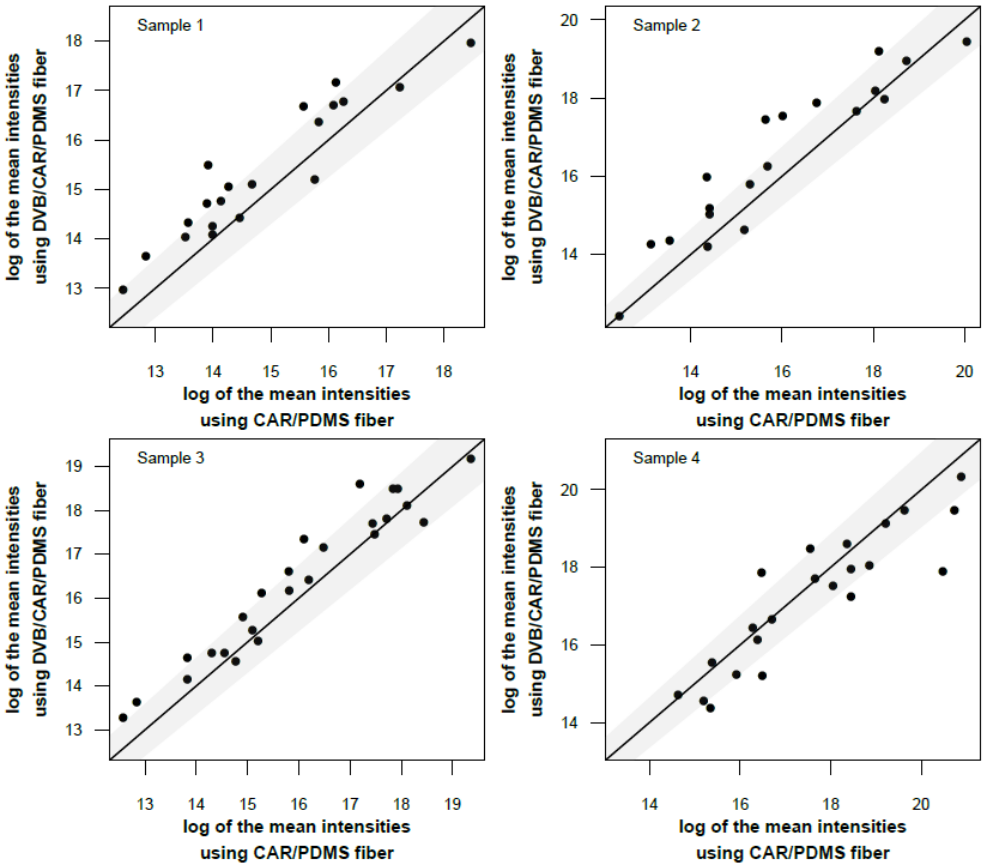


**Figure 12: Abundances in aliquots using CAR/PDMS and DVB/CAR/PDMS SPME fibres.** Scatterplots comparing the average log values of VOC abundances from samples analysed using both type of SPME coatings. The diagonal black segment represents the function x = y, where compounds detected at similar abundances between both types of coatings are located inside of the 5% tolerance region represented by the grey area.

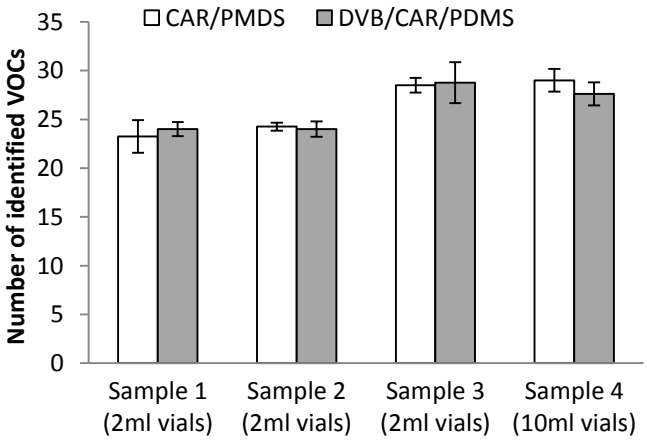


**Figure 13: Number of VOCs identified in replicates using CAR/PDMS and DVB/CAR/PDMS SPME fibres.** Bar plot illustrating the number of compounds (± SEM) found using two types of SPME coating. The samples 1, 2 and 3 had 4 replicates in each condition, measured in 2 ml vials. Sample 4 had 5 replicates in each condition, measured in 10 ml vials. (no significant differences; Mann-Whitney test)

Table 3 presents the number of compounds exclusively identified by each fibre coating. CAR/PDMS coating showed slightly better results with 1 more compound found in two samples. However, samples 3 and 4 did not show any differences.

**Table 3: Summary of the specific compounds identified with each fibre coating.**

| | Number of compounds found only with | |
|---|---|---|
| | CAR/PDMS fibre | DVB/CAR/PDMS fibre |
| Sample 1 | 3 | 2 |
| Sample 2 | 5 | 4 |
| Sample 3 | 4 | 4 |
| Sample 4 | 5 | 5 |

Table presenting the number of compounds identified specifically with both fibre coatings for each sample. Only compounds present in at least half the sample with one coating and completely absent with the other were considered as specific.

In conclusion, the number of compounds identified did not vary significantly between fibres tested and there was no marked trend. The intensities were slightly higher when samples were analysed in 2 ml vials using DVB/CAR/PDMS coating and showed no trend when samples were analysed in 10 ml vials. The numbers of compounds identified with a specific fibre coating were similar between both fibre coatings.

### 2.1.6 Keeping samples at 1°C overnight

The use of an auto-sampler does not allow overnight storage of samples at -20°C, but at 1°C. It might affect the metabolic profiles determined by HS–SPME–GC–MS analysis. This section investigates the influence of storing faecal samples at 1°C for 14 hours on metabolite profiles.

The abundances of identified VOCs were evaluated according to waiting time periods of 0 and 14 hrs at 1°C prior to SPME–GC–MS analysis. One sample was divided into six aliquots and stored in 10 ml glass vials at -20°C. Three aliquots were analysed immediately after being taken from -20°C freezer (0 hrs) and three aliquots were analysed after being left at 1°C on the auto-sampler tray overnight (14 hrs). Each sample was extracted using a CAR/PDMS SPME fibre.

There were no significant differences in the number of VOCs identified (t-test; p-value > 0.05), although there were three compounds detected at slightly lower abundances at 14hrs. These results indicated that faecal samples can be analysed overnight without significant changes on the VOC profiles or abundances (Figure 14).

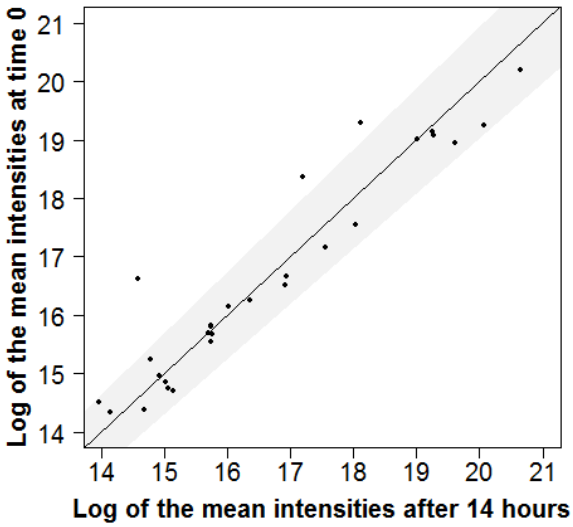


**Figure 14: VOC abundances in samples left for 14 hours at 1°C.** Scatterplot comparing the average log values of VOC abundances from faecal samples analysed straight after leaving the -20°C freezer and after 14 hrs at 1°C (n=3). The diagonal black segment represents the function x=y, where compounds detected at similar abundances between time 0 and time 14 are located inside the 5% tolerance region represented by the grey area.

### 2.1.7 Repeatability and multiple analyses of the same sample

According to the results presented in the previous sections, the following parameters for HS–SPME–GC–MS investigation were defined: faecal samples of masses between 50 to 100 mg, stored in 10 ml vials, without addition of any solution and analysed by CAR/PDMS fibre. The repeatability of the method and the influence of multiple analyses of the same samples were determined to assess the accuracy and limits of the method.

In order to assess the repeatability of the developed method, the VOC profiles of ten samples were used to calculate the variation within samples. Each individual sample was divided in triplicate, stored in 10 ml vials and pre-incubated at 60°C for 30 min before VOC extraction using a CAR/PDMS SPME fibre for 20 min at 60°C prior to desorption into the GC oven. The abundances of the VOCs identified within each sample (n=3 per sample) were used to calculate their coefficient of variation (CV). Three of these samples were reanalysed 3 times in order to determine the effect of multiple analyses of a single sample.

A principal component analysis (PCA) on the VOC profiles of 10 faecal samples, each divided in triplicate, illustrates their repeatability (Figure 15). In addition, the mean, standard deviation and CV were calculated across triplicates for each sample (Table 4). The PCA showed technical replicates present at the same quadrant of the graph, forming clusters, which indicates a high repeatability across samples. On average, 31.3 ± 10.5 VOCs were identified per sample (mean ± SD). The standard deviation for each sample was 2.9 ± 1.3 compounds and 90% of the VOC abundances showed a coefficient of variation smaller than 30%, which is considered adequate for diagnostic sensitivity[85]. The effect of analysing a single sample multiple times was assessed.

The number of VOCs identified and their abundances were compared across 4 GC–MS runs (Figure 16, Table 5). There was a significant decrease in the number of VOCs identified for two sets of aliquots out of 3 considered (Figure 16; Kruskal-Wallis; p-value<0.05). At least 40% of these VOCs were detected at significantly lower or higher abundances after 4 GC–MS runs (ANOVA; p-value < 0.05). These results confirm the hypothesis that the sample's headspace is modified after each HS–SPME–GC–MS analysis. As expected, heating the faecal samples released the majority of VOCs into the headspace air. These VOCs were extracted and removed during the first HS–SPME–GC–MS analysis. During the second, third and fourth analysis, the diversity and abundances of specific VOCs were decreased (Figure 16) suggesting that each sample should, ideally, be analysed only once. The inter-individual variation between samples should also be taken into consideration, explaining the lower number of identified VOCs in sample 3 compared to sample 1 and 2 (Figure 16).

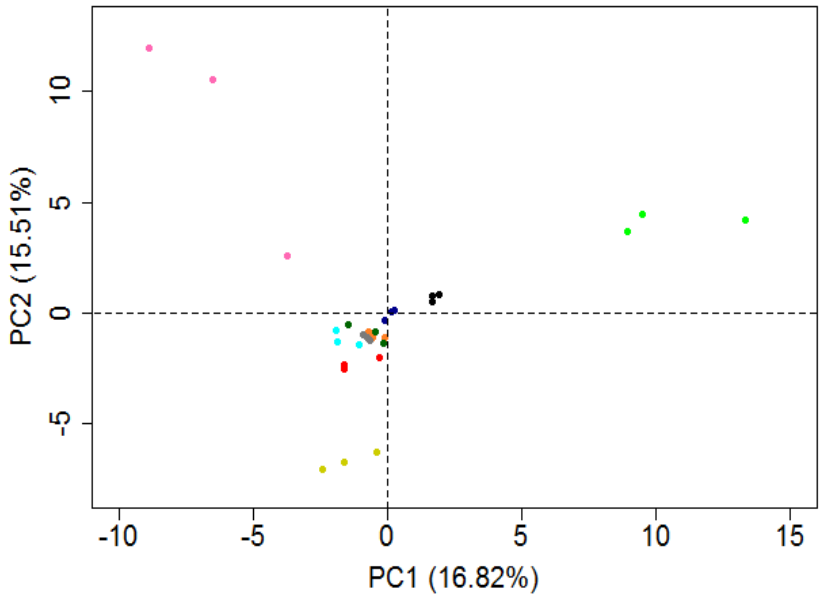


**Figure 15: Principal component analysis (PCA) for method repeatability.** Results of a PCA applied to the VOCs profile of 10 biological replicates, each divided in triplicates and analysed by HS–SPME–GC–MS.

**Table 4: Repeatability of the method for the number of compounds identified and their abundances.**

| | Average number of VOCs ± S.D. (in every triplicate) | VOCs with CV < 30% [%] |
|---|---|---|
| Sample 1 | 24±3 (19) | 100 |
| Sample 2 | 23±1 (18) | 100 |
| Sample 3 | 20±2 (12) | 92 |
| Sample 4 | 48±5 (36) | 89 |
| Sample 5 | 37±4 (26) | 81 |
| Sample 6 | 50±4 (39) | 95 |
| Sample 7 | 32±2 (21) | 90 |
| Sample 8 | 26±4 (18) | 67 |
| Sample 9 | 26±2 (19) | 95 |
| Sample 10 | 27±2 (18) | 94 |

Ten biological replicates of faecal samples were divided in triplicates and analysed by solid phase micro-extraction coupled to gas chromatography-mass spectrometry (SPME–GC–MS). The average number of volatile organic compounds (VOCs) and the standard deviation associated with each biological replicate is presented together with the number of VOCs in every technical replicate and the percentage of compound abundances showing a coefficient of variation (CV) smaller than 30%.

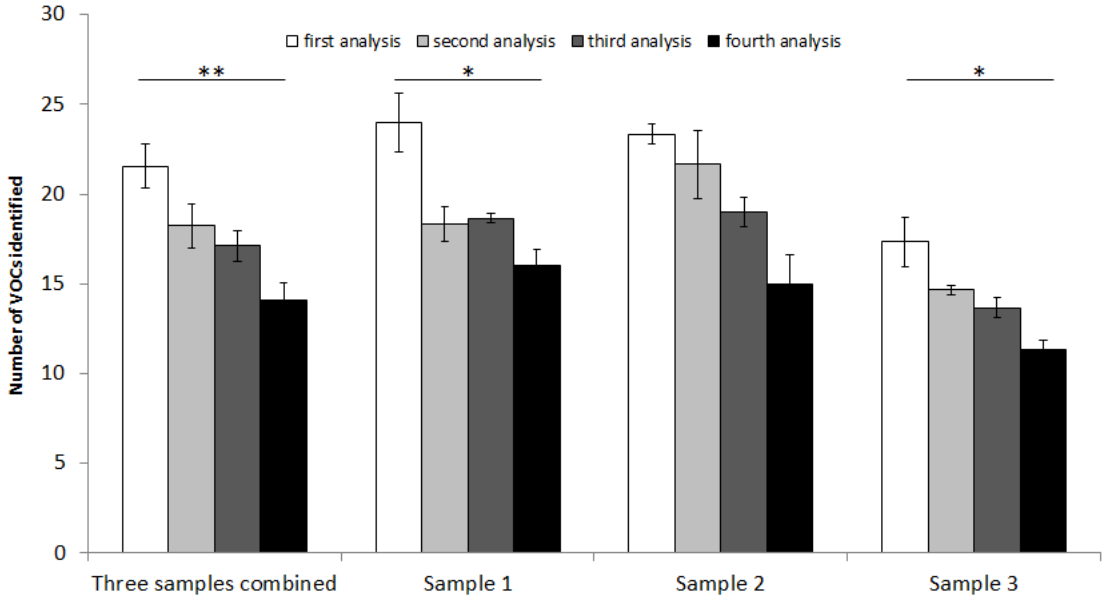


**Figure 16: Effect of multiple analyses of samples on the number of VOCs identified.** Three faecal samples were divided in triplicate and analysed 4 consecutive times by solid phase micro-extraction coupled to gas chromatography-mass spectrometry (SPME–GC–MS). The number of VOCs identified in each analysis is presented for the three samples individually (n=3) and combined (n=9). (mean ± SEM; *p<0.05, **p<0.01 and ***p<0.001; Kruskal-Wallis test)

**Table 5: Effect of multiple analyses of samples on the abundances of compounds.**

| | VOCs present in triplicate | Percentage of VOCs showing significantly different abundances [%] |
|---|---|---|
| Sample 1 | 16 | 56 |
| Sample 2 | 14 | 64 |
| Sample 3 | 10 | 40 |

Three faecal samples were divided in triplicate and analysed 4 consecutive times by solid phase micro-extraction coupled to gas chromatography-mass spectrometry (SPME–GC–MS). The number of volatile organic compounds (VOCs) present in at least 2 consecutive analyses is presented together with the percentage of compounds for which the intensities differed significantly after 4 analyses.

### 2.1.8 Data analysis

The optimisation of the parameters for data analysis using the Automated Mass Spectral Deconvolution System (AMDIS) and Metab[69] is presented in this section, in addition to statistical analyses applied for both the method development and the Dove Project.

#### 2.1.8.1 Parameters study for AMDIS and Metab

AMDIS and Metab have been applied to the analysis of metabolomics data. This section evaluates AMDIS and Metab by comparing their performances against manually curated data. AMDIS and Metab were assessed using 11 chromatograms chosen randomly. Each chromatogram was analysed 3 times and identified compounds were listed. Compounds identified in the first or the second analysis, but not in the third, were added with a special mention.

Files generated by the PerkinElmer GC–MS acquisition software (TurboMass, Perkin Elmer, 2008, version 5.4.2.1617) were in '.raw' format. This type of file needed to be converted to '.CDF' format in order to be analysed in a high-throughput manner using AMDIS, through the batch report option instead of the single file report option. Raw files were converted to CDF files using Databridge (part of TurboMass Suite, version 5.4.2.1617). Results obtained when analysing raw and CDF were compared. Metab results were generated with a time window ranging between 0.1 and 2 minutes in 0.1 minute steps.

Results were reported in terms of sensitivity (sum true positive / sum compounds found manually) and precision (sum true positive / sum compounds found by the package) (see Figure 17 for further explanation). The library used contained 172 compounds. We built it based on preterm faecal samples (Appendix C Table 41). Analysing samples manually using AMDIS software allowed us to save compounds identified in a library. This library could then be used to process data in a high-throughput manner.

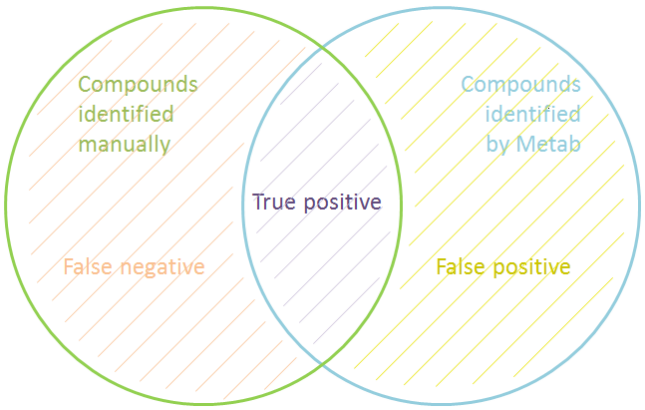


**Figure 17: Illustration of the statistical measure.** Figure illustrating the meaning of the main terms used to describe the results.

Table 6 presents the results obtained with Metab for reports generated with '.raw' and '.CDF' files. When using single file report, generated using '.raw' data, the time window should be equal to, or greater than, 0.5 minutes to obtain an optimal sensitivity of 97.3%. The higher precision, 94.7%, was obtained with a time window between 0.2 and 0.4 minute. The 1% drop in precision for a time window of 0.5 minute and the 2.7% drop of sensitivity for a time window of 0.4 minute indicates that a time window of 0.5 minute or greater may generate more accurate results. Results based on AMDIS' reports generated in batch mode showed a maximal sensitivity of 90.9% for a time window of 0.6 minute and an optimal precision of 84.2% with a time window of 0.2 minute. Optimal results were obtained with a time window of 0.3 or 0.4 minute with the precision and the sensitivity dropping by 0.3% and 0.9%, respectively, compared to the maximum obtained using 0.6 and 0.2 minutes, respectively.

**Table 6: Sensitivity and precision obtained with AMDIS in conjunction with Metab at different time window and with two file formats.**

| | Time window [min] | Sensitivity % | Precision % |
|---|---|---|---|
| Single file report with '.raw' files | 0.1 | 89.2 ± 8.3 | 94.6 ± 3.6 |
| | 0.2 | 94.6 ± 7 | 94.7 ± 2.9 |
| | 0.3-0.4 | 94.6 ± 5.3 | 94.7 ± 2.7 |
| | 0.5 | 97.3 ± 4.6 | 93.7 ± 1.7 |
| | 0.6-0.9 | 97.3 ± 4.3 | 93.8 ± 2.4 |
| | 1 | 97.3 ± 5.6 | 93.8 ± 3 |
| | 1.1-1.5 | 97.3 ± 4.3 | 93.8 ± 2.7 |
| | 1.6 | 97.3 ± 4.3 | 93.8 ± 3.2 |
| | 1.7-1.9 | 97.3 ± 4.3 | 93.8 ± 4 |
| | 2 | 96.2 ± 3.7 | 94.3 ± 3 |
| Batch report with '.CDF' files | 0.1 | 84.9 ± 5 | 83.6 ± 5.4 |
| | 0.2 | 88.7 ± 5.1 | 84.2 ± 5.3 |
| | 0.3-0.4 | 90.6 ± 4.2 | 83.3 ± 3.1 |
| | 0.5 | 90.6 ± 4.2 | 81.8 ± 3.6 |
| | 0.6 | 90.9 ± 4.5 | 81.8 ± 3.6 |
| | 0.7 | 90.9 ± 3.5 | 81.8 ± 3.7 |
| | 0.8-0.9 | 90.9 ± 3.5 | 81.8 ± 3 |
| | 1 | 90.9 ± 4.3 | 81.8 ± 3.8 |
| | 1.1 | 90.9 ± 3.5 | 81.8 ± 3 |
| | 1.2-1.4 | 90.9 ± 3.5 | 81.8 ± 3.8 |
| | 1.5-1.6 | 90.9 ± 3.5 | 81.8 ± 3.7 |
| | 1.7-1.9 | 90.9 ± 3.5 | 81.8 ± 3 |
| | 2 | 90.6 ± 4.5 | 81.8 ± 4.8 |

Summary of the statistics based on results obtained with AMDIS followed by Metab with time windows ranking from 0.1 to 2 minutes. Eleven chromatograms in two formats were considered and the library was composed of 172 compounds. Sensitivity was defined as: sum true positive compounds / sum compounds found manually; precision was defined as: sum true positive compounds / sum compounds found by Metab. (median ± interquartile range or IQR)

Table 7 presents compounds reported by Metab and considered to be false negatives when a time window of 0.5 minute was used. Five compounds were not identified using AMDIS and, therefore, they could not be identified using Metab. Four compounds had uncertain identification.

Sensitivity and precision were improved if '.raw' files were analysed suggesting that the software used to convert '.raw' files to '.CDF' files altered the quality of the chromatogram. The batch mode in AMDIS was available only using '.CDF' files and reports using '.raw' files were more tedious to produce as they needed to be generated file-by-file. In addition, Metab fail to identify nineteen compounds and nine could not be identified with certainty.

**Table 7: False negative metabolites from Metab single file analysis using a time window of 0.5 minute.**

| RT [min] | Name | Comment | File |
|---|---|---|---|
| 29.54 | Nonanal | NF | 1 |
| 25.19 | 1-hepten-3-one | – | 2 |
| 26.70 | 2, 4-E,E-heptadienal | UI | 3 |
| 25.41 | 3-octanone | – | |
| 27.02 | 3, 3, 4-trimethyldecane | UI | 4 |
| 26.70 | 2, 4-E,E-heptadienal | – | |
| 26.42 | Hexanoic acid | – | |
| 16.53 | Toluene | NF | |
| 22.31 | Heptanal | NF | 5 |
| 26.06 | Octanal | NF | |
| 25.41 | 3-octanone | – | |
| 10.59 | Ethylacetate | – | |
| 21.48 | 3-methybutanoicacid | – | 6 |
| 26.55 | 2, 6, 11-trimethyldodecane | UI | |
| 31.92 | Ethyl ester dodecanoic acid | – | |
| 10.55 | 2-butanone | NF | |
| 17.41 | 1-pentanol | – | 7 |
| 25.41 | 3-octanone | – | |
| 26.56 | 2, 2, 3, 3-tetramethylpentane | UI | |

Retention times, names, comments and files analysed for each false negative metabolite. When the comment states 'NF', the compound has not been identified using AMDIS; 'UI' means that the identification of the compounds based on NIST library matching was not reliable.

Table 8 presents Metab results obtained with three sets of deconvolution parameters in AMDIS. The results were best with the 'resolution' and 'shape requirements' set as medium and the sensitivity as 'high'. The difference might be explained by the fact that the library was built using this set of parameters.

**Table 8: Sensitivity and precision obtained with AMDIS in conjunction with Metab while varying AMDIS and Metab parameters.**

| Resolution | Sensitivity | Shape requirement | Time window [min] | Sensitivity [%] | Precision [%] |
|---|---|---|---|---|---|
| medium | high | medium | 0.1 | 89.2 ± 8.3 | 94.6 ± 3.6 |
| | | | 0.2 | 94.6 ± 7 | 94.7 ± 2.9 |
| | | | 0.3-0.4 | 94.6 ± 5.3 | 94.7 ± 2.7 |
| | | | 0.5 | 97.3 ± 4.6 | 93.7 ± 1.7 |
| | | | 0.6-0.9 | 97.3 ± 4.3 | 93.8 ± 2.4 |
| | | | 1 | 97.3 ± 5.6 | 93.8 ± 3 |
| medium | medium | medium | 0.1 | 72.4 ± 16.2 | 90.9 ± 1.7 |
| | | | 0.2 | 79.3 ± 7.1 | 91.5 ± 1.1 |
| | | | 0.3-0.5 | 81.1 ± 5 | 91.7 ± 0.9 |
| | | | 0.6-0.9 | 81.1 ± 5.8 | 91.7 ± 0.9 |
| | | | 1 | 81.1 ± 6.8 | 91.7 ± 1.8 |
| high | high | low | 0.1 | 86.2 ± 4.5 | 84.4 ± 7.5 |
| | | | 0.2 | 89.7 ± 4.1 | 84.4 ± 6.5 |
| | | | 0.3 | 90.9 ± 3.6 | 84.6 ± 6.8 |
| | | | 0.4 | 90.9 ± 3.3 | 84.6 ± 6.6 |
| | | | 0.5 | 90.9 ± 3.3 | 83.3 ± 5.6 |
| | | | 0.6-0.9 | 90.9 ± 3.6 | 83.6 ± 5.1 |
| | | | 1 | 90.9 ± 4 | 83.6 ± 6.7 |

Table presenting results from AMDIS followed by Metab with three set of deconvolution parameters in AMDIS. The resolution, sensitivity and shape requirement parameters were varied between low, medium and high. Time window parameter in Metab was ranked between 0.1 and 1 minute (median ± IQR).

To summarize, both sensitivity and precision were higher when analysing '.raw' files. The evaluation of Metab using different time windows showed that 0.5 to 0.9 minutes delivered slightly different results. The time windows of 0.5, 0.6 and 0.9 minutes showed the same sensitivity, 97.3%. However, a time window between 0.6 and 0.9 minute showed a slightly higher precision than 0.5, 93.8% and 93.7%, respectively. The interquartile range was slightly wider with a time window of 0.5 minute for the sensitivity and narrower for the specificity, therefore, a time window of 0.5 minute should be preferentially applied. If the '.CDF' file were analysed using the batch mode available in AMDIS, a time window of 0.3 or 0.4 minutes was optimal with a sensitivity of 90.6 ± 4.2% and a precision of 83.3 ± 3.1%. The sensitivity and specificity were optimal when the resolution, sensitivity and shape requirement parameters in AMDIS were set to medium, high and medium, respectively.

#### 2.1.8.2 Statistical analysis – method development

All HS–SPME–GC–MS data were processed using AMDIS (version 2.71, 2012) in conjunction with the NIST mass spectral library (version 2.0, 2011) and the R package Metab. VOCs were identified using an in-house library of 172 compounds built with AMDIS in combination with the NIST library (Appendix C Table 41). AMDIS was applied using resolution, sensitivity and shape requirements set to medium. Metab was used with time window of 0.1 minute. Compounds present in fewer than 50% of the samples of at least one experimental condition were removed.

All the statistical analysis were performed using R version 3.1.1 & 3.1.2[86], [87] and Microsoft Excel (Microsoft Office Professional Plus 2010).

Shapiro test was applied to test the normality of the distribution. A t-test or a one-way analysis of variance (ANOVA) followed by Tukey's HSD test were applied to test differences between data classes for normally distributed data and Mann-Whitney or Kruskal-Wallis test were applied in case of non-normally distributed data. A principal component analysis (PCA) was used to show similarities within data classes. If required, final p-values were adjusted for multiple comparisons using Bonferroni correction. P-values lower than 0.05 were considered significant.

### 2.1.8.3 Statistical analysis – Dove project

All HS–SPME–GC–MS data were processed using the Automated Mass Spectral Deconvolution System (AMDIS-version 2.71, 2012) in conjunction with the NIST mass spectral library (version 2.0, 2011) and the R package Metab[69]. VOCs were identified using an in-house library of 229 compounds built with AMDIS in combination with the NIST library (Appendix C Table 42). AMDIS was applied using resolution, sensitivity and shape requirements set to medium, high and medium, respectively. Metab was used with a time window of 0.4 minute. In addition, the R package XCMS[78], [88] was used to identify ion mass fragments present at different abundances across conditions.

Differences between the use of AMDIS in conjunction with NIST and Metab and the XCMS package were numerous. Metab identified and quantified compounds based on the library built using AMDIS and NIST, and the report from AMDIS. XCMS package selected features, or *m/z* ratios at specific retention times, and reported their abundances. The absence of compound identification when using XCMS reduced errors, such as false identification (see 2.1.8.1), and saved time as the library building can be tedious and time consuming, requiring the manual analysis of several chromatograms; especially when performing untargeted metabolomics studies. The absence of identification meant that the XCMS package did not allow interpretation of the biological meaning of the results. In order, to compare results with other studies, compounds' names were often needed, therefore, both approaches were applied in order to increase probabilities of finding relevant patterns.

Statistics were performed using R version 3.1.1 & 3.1.2[86], [87], Stata version 9.2 (20 Jul 2007) and Microsoft Excel (Microsoft Office Professional Plus 2010).

For this study, compounds in Metab and features in XCMS were considered for further analysis if they were present in at least 50% of the samples in one or more data class, except for the analysis regarding the number of compounds. Missing values were then replaced using two techniques; (I) imputation by half the lowest intensity of that compound across categories, (II) imputation using k-Nearest Neighbours algorithm. These methods were selected after consideration of the literature[89], [90] and discussion with Mrs Rosemary Greenwood, University Hospital Bristol NHS Foundation Trust. For XCMS, intensities equal to 0 were considered as missing data. Table 9 presents the occurrence of missing values in final results.

**Table 9: Occurrence of missing data in final results.**

| | | Missing data [%] | Number of features (XCMS) or compounds (Metab) |
|---|---|---|---|
| XCMS | Both statuses | 2.07% | 356 |
| Metab | Control only | 24.52% | 46 |
| | Both statuses | 27.43% | 50 |

Table presenting the percentage of missing data in the final spreadsheets obtained with XCMS and Metab. The total number of ions, respectively identified compounds, is also presented.

Using the XCMS package, only 2.1% of the data were equal to 0, where 27.4% of the data were 'missing' using Metab package (Table 9), therefore, missing values substitution had more impact on results from Metab package. Of the two different techniques applied to implement missing data, the substitution of missing data with half the lowest value of each compound changed the mean and median. In contrast, k-NN applied an algorithm which gave a value based on the sample's neighbours in a mathematical space, thus reducing disparity between substituted and measured values.[89]

Shapiro test was applied to test the normality of the distribution. T-test or one-way analysis of variance (ANOVA) followed by Tukey's HSD test were applied to test differences between data classes for normally distributed data and Mann-Whitney or Kruskal-Wallis were applied in case of non-normally distributed data. If required, final p-values were adjusted for multiple comparisons using Bonferroni correction. Principal component analysis (PCA) was used to show similarities within data classes and differences between data classes. Parametric two-way ANOVA was applied for comparing number of compounds across different experimental conditions. It was performed by fitting an analysis of variance model through the R function lm.

In order to build a statistical model that allows the classification of unknown samples, features that best described the differences between data classes were selected. Three different algorithms for feature selection were applied: a step-wise linear discriminant analysis and two different random forest classification algorithms, rfe and Boruta89. These algorithms were implemented in R package caret [91]. As 3 feature selection algorithms were applied, a single feature may be selected more than once. Then, the number of occasions on which each feature was selected on each day, was summed.

Features selected more than once were used for modelling at each day prior to diagnosis for Metab and XCMS results with missing values replaced using both k-NN algorithm and half the lowest compound intensity. Thus, four datasets were considered, 2 sets of data for Metab (1 set of data for each data imputation strategy) and 2 sets of data for XCMS (1 set of data for each data imputation strategy).

For each of the four data sets, random forest and k-Nearest Neighbour algorithms were applied for sample classification. Accuracy and kappa statistics reported by each statistical model technique were calculated for each day using only features selected more than once for each specific day. These results were validated using repeated 10-fold cross validation. Based on these results, one missing data imputation algorithm was selected for Metab results and one for XCMS results, which resulted in two sets of data to be further analysed.

For these two sets of data, random forest (RF), linear discriminant analysis (LDA), k-Nearest Neighbour (k-NN), partial least square (PLS), support vector machine (SVM) radial, linear and polynomial algorithm were applied for classification at each day prior to diagnosis. Features selected more than twice across six days prior to diagnosis were considered, therefore the same features were used for each time point. These results were validated using repeated double cross-validation (inner loop of 3-fold cross-validation repeated 5 times; and outer loop of 3-fold repeated 30 times). Accuracy, sensitivity and specificity obtained by each statistical modelling techniques and each validation scheme were reported. Accuracy was defined as the sum of correctly classified samples divided by the total number of samples; sensitivity was defined as correctly positively classified samples divided by the sum of positive samples; specificity was defined as correctly negatively classified samples divided by the sum of negative samples.

Functions from the following R packages have been used for the data analysis and data mining: Metab[69], MASS[92], impute[93], xcms[78], [88], [94], FactoMineR[95], beeswarm[96], gplots[97], metabolomics[98], multtest[99], random[100], baseline[101], caret[91], pROC[102], reshape2[103], Boruta[104], e1071[105], klaR[106], ipred[107], kknn[108], pls[109] and kernlab[110].

### 2.1.9 Optimised method

Several parameters were investigated and optimised for the analysis of faecal samples from preterm babies. Samples of 50 to 100 mg, vials of 10 ml, CAR/PDMS fibre, VOCs extraction within 14 hours and samples analysed once were the resultant optimised settings.

Optimal sensitivity and specificity were obtained with the resolution, sensitivity and shape requirement in AMDIS set as medium, high and medium, respectively. In addition, the use of a time window of 0.4 minute in Metab was optimal with a sensitivity of 90.6 ± 4.2% and a precision of 83.3 ± 3.1%.

## 2.2 Sensor technology

In this thesis, headspace–gas chromatography–sensor (HS–GC–Sensor) was applied to detect patterns of VOCs present in faeces from premature babies with and without necrotizing enterocolitis (NEC). Many aspects of the HS–SPME–GC–MS analysis of biological samples have been previously optimized[111] and other aspects were optimized for this study as described below.

### 2.2.1 HS–GC–Sensor configuration

The HS–GC–Sensor is an in-house developed instrument[112]. It is composed of a gas chromatography column coupled to a metal oxide gas sensor and an electronic circuit monitored by a computer software. The gas chromatography column separates VOCs in a mixture while the metal oxide gas sensor detects VOCs, which result in changes in the resistance level of the electronic circuit. The computer software then registers, at every 0.5 seconds, these changes in resistance level during the analysis of a given biological sample. Specific metabolic states are expected to produce changes in the metabolites present in biological samples such as urine or faeces. The HS–GC–Sensor was developed to detect these changes and, coupled to computer algorithms, classify or diagnose unknown samples.

The GC column used in this thesis was a SPB-1 sulphur, length 30 m, internal diameter 0.32 mm, film thickness 4 µm (Supelco, Sigma Aldrich). The carrier gas used was synthetic air composed of oxygen at 21.21% and nitrogen at 78.79% (BOC, Guildford, UK). Vials with phenolic cap and PTFE/silicone septum of 10 ml (Sigma-Aldrich, Dorset, UK) volume were used. Samples were pre-incubated for 10 minutes at 50°C prior to sampling of 2 $cm^3$ of headspace. The injector temperature was set at 180°C. The initial temperature of the GC oven was set at 40°C and held for 3.4 min before increasing to 100°C at a rate of 2, 2.5 and 5°C/min and held for 10 min, for a total run time of 43.4 min, 37.4 min and 25.4 min, respectively. The carrier gas pressure at the entrance of the column was set at 32 psi and the synthetic air flow rate on the sensor was set at 100 ml.

## 2.2.2 Sample mass

The mass of a sample analysed by HS–GC–Sensor may affect the resistance levels. This section describes the sample mass optimisation applied to HS–GC–Sensor. One faecal sample was divided into aliquots of 100, 300 and 500 mg in triplicate (104.4 ± 1.7 mg, 308.6 ± 1.1 mg and 506.5 ± 1.7 mg) and analysed by HS–GC–Sensor using a ramp of 5°C/min. Figure 18 presents the mean resistance profiles of each tested sample mass. ANOVA was performed on each data point when no intensities were equal to 0. No significant difference was observed, between each stool mass, at any data point (ANOVA followed by Bonferroni correction for multiple measurements). These results indicated that, up to the data point 1400, the mean resistance level was proportional to the sample mass used. A larger mass also resulted in improved resolution; however, this was not marked for the first 1100 data points. From data point 1200 onwards, sample mass of 100 mg seemed to have a higher resistance variation than larger masses. A sample mass of 500 mg would appear to be optimal, but as no significant differences were detected across samples masses, 100 mg could also be used.

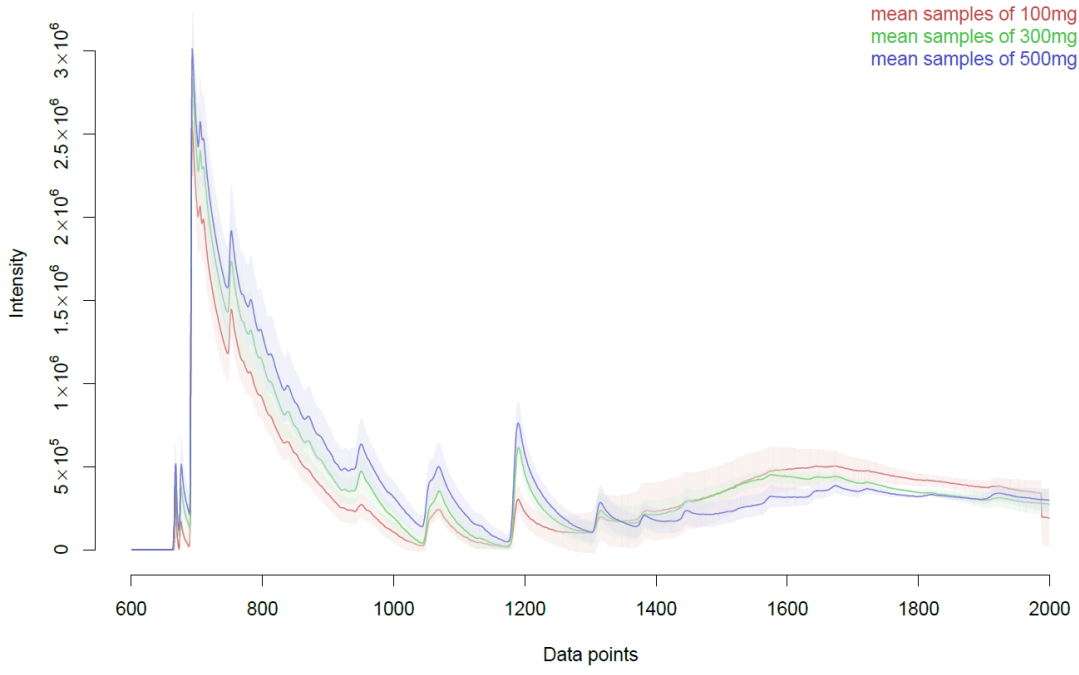


**Figure 18: Mass optimisation GC–sensor investigation.** One human sample was divided in triplicate aliquots of 100, 300 and 500 mg and analysed by headspace gas chromatography sensor (HS–GC–Sensor). Mean profiles of all three masses investigated (continuous line) and their standard deviation (light shade) were illustrated.

## 2.2.3 Ramp optimisation

Different configurations of temperature ramping were tested in order to determine its effect on the resolution of the separation. This section investigates 3 configurations of temperature ramp and determines the optimal one for analysing preterm faecal samples from babies with and without NEC.

Figure 19 presents the mean of the profiles for the ramp optimisation. One faecal sample was divided into 9 aliquots of 100 mg and analysed in triplicate using ramps of temperature of 2, 2.5 and 5°C/min (97.6 ± 2.8 mg, 99.0 ± 7.1 mg and 97.0 ± 4.7 mg, respectively). The resolution was slightly improved with a ramp of 2 or 2.5°C/min compared to a 5°C/min ramp. As the compounds affinity with the stationary phase was increased, compounds co-elution might be reduced. However, peak intensities at the end of the run might be reduced as the width of the peaks was increased.

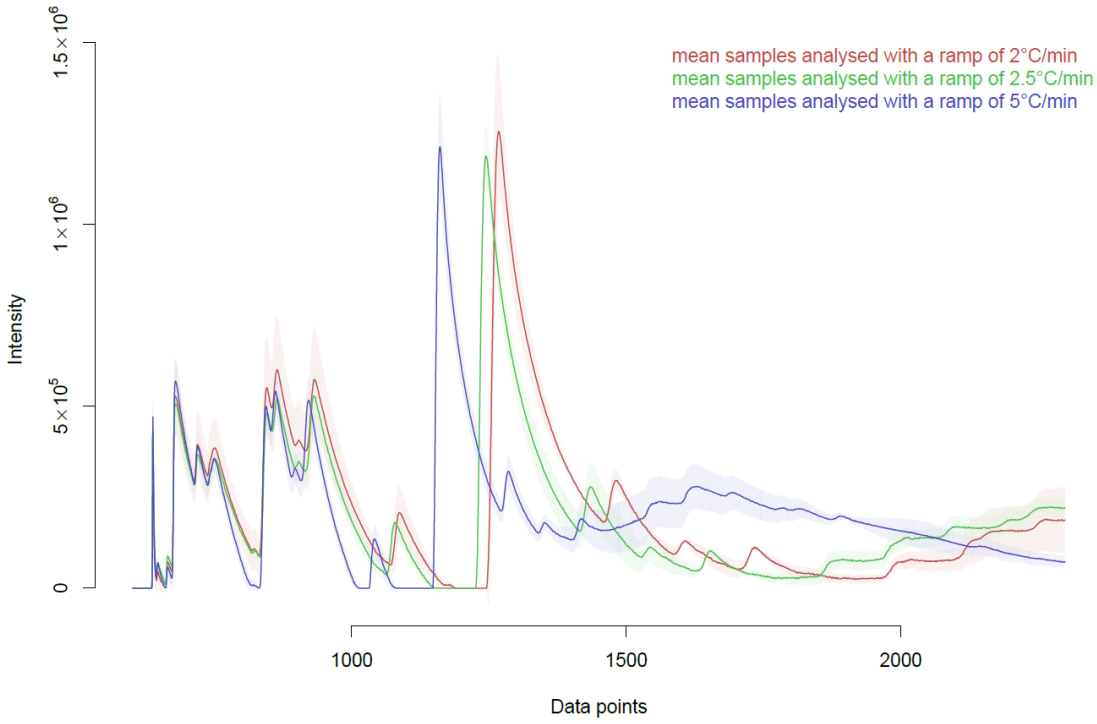


**Figure 19: Ramp comparison GC–sensor investigation.** One sample was divided in triplicates aliquots of 100 mg and analysed by headspace gas chromatography sensor (HS–GC–Sensor). Mean profiles of all three ramps investigated (continuous line) and their standard deviation (light shade) are illustrated.

### 2.2.4 Repeatability

The repeatability of the method was assessed by using VOC profiles of three aliquots of 100 mg to calculate the variation within samples. Three aliquots of 99.0 ± 7.1 mg (mean ± SD) from one sample were analysed over two consecutive days using a 2.5°C/min ramp. More than 75% of the data point showed a variation of less than 30% and a median coefficient of variation of 15.1%, which indicated acceptable repeatability. Data points equal to 0 were discarded.

### 2.2.5 Reference gas

A reference gas was analysed every day in order to assess the repeatability of the response between days of measurements. This section disscusses how the reference gas was used and the results obtained with that practice.

A reference gas composed of 50 ppm ethanol and 50 ppm methanol in 20.9% oxygen and 79.09% nitrogen (Air Products PLC, Crewe, UK) was injected 2 to 3 times at the beginning and, ideally, the end of each day of measurement. The injection of 2 ml of the reference gas was performed manually and the column remained at 40°C. Peak intensities were visually checked to assess values correctness prior to statistical analysis.

All samples were run over 94 days. For eleven days a single set of reference gas injections was applied therefore, within days variability could not be tested for those days. On the 83 days left with multiple set of measurements, differences within each day were tested using t-test or ANOVA. Methanol was statistically different for 4 days and ethanol for 6 other days, at a level of significance of 5% and with Bonferroni correction applied. Three days showed statistically different abundances of methanol and ethanol.

### 2.2.6 Data analysis

In this section, the statistical analyses applied are presented for both the method development and the Dove Project.

#### 2.2.6.1 Statistical analysis – method development

Chromatograms were aligned using an in-house developed algorithm developed by Aggio *et al.*[113]. Samples were aligned in relation to a single reference chromatogram. To determine the reference chromatogram, each chromatogram was compared to every other chromatogram when shifting by 31 measurement points (15 points to the left and 15 to the right). Pearson's correlation was computed at each shift and the chromatogram with the highest mean correlation coefficient was selected as reference chromatogram.

Statistics were performed using R version 3.1.1 & 3.1.2[86], [87].

One-way analysis of variance (ANOVA) was applied to test differences between sensor signals. P-values were adjusted for multiple comparisons using Bonferroni correction. P-values $< 0.05$ were considered to be significant.

#### 2.2.6.2 Statistical analysis – Dove study

The analysis of the dataset generated by the GC-Sensor was performed by Dr Aggio.

The analysis was similar to that described by Aggio *et al.* [113], except for the validation process and the length of the chromatograms. There was a small number of samples available at each day prior to diagnosis and so leave-one-out cross validation was applied and only the first 900 sampling points (or 450 seconds) were considered.

The seven stages of the pipeline applied to the data could be briefly described as follows. Sensor data were loaded into R software; the baseline of each chromatogram was corrected; chromatograms were normalized and aligned; data were transformed using wavelet coefficients; features were selected using two algorithms based on random forest; classifiers were built using LDA, PLS, RF, k-NN, SVM radial, linear and polynomial; validation was applied on classifiers. Principal component analysis (PCA) was used to show similarities between statuses.

### 2.2.7 Optimised method

A sample mass of 100 mg was chosen for practical reasons. A temperature ramp of 2.5°C/min was applied for the investigation, as it may reduce the co-elution of compounds. The repeatability of the measurement was found to be acceptable with more than 75% of the data points varying less than 30%.

## 2.3 Dove study

The Dove project has been funded to analyse volatile organic compounds emitted from the faeces of preterm infants as a predictor of preclinical necrotising enterocolitis. The ethics committee approval is 11/WM/0078 (Appendix D), with parents giving consent following project presentation (Appendix E).

The Dove project was performed in parallel with the Dovetail project, which aimed to sequence faecal samples and characterize bacteria species present in the gut of preterm infants. A total of 1,326 patients were recruited over more than 2 years: from these NEC and controls were defined according to their clinical status: NEC was defined according to clinical features and the Bell's stage; controls were neonates without NEC. The controls were babies with a range on non-NEC disorders associated with prematurity: in this thesis they have been designed healthy controls. Table 10 shows the distribution of patients. The recruitment criterion was a gestation period shorter than 34 weeks. Patients were excluded if they were unlikely to survive or had significant gastrointestinal anomalies. Eight hospitals took part in the study: Birmingham Heartlands Hospital (BHH), Birmingham Women's Hospital (BWH), Liverpool Women's Hospital (LWH), Royal Shrewsbury Hospital (RSH), Royal Wolverhampton Hospital (RWH), Sheffield Teaching Hospitals (STH), University Hospitals of Coventry and Warwickshire (UHCW) and University Hospitals of Leicester (UHL).

**Table 10: Table presenting the number of patients per status included in the study.**

| Status | Number of patients |
|---|---|
| Confirmed NEC | 64 |
| Suspected | 192 |
| Healthy Control | 1047 |
| Other disorders | 23 |

'Other disorders' refers to patients without NEC but disorders which presented similar symptoms.

Faecal samples were collected daily, if possible, according to a clear protocol (see Appendix F), for the entire hospitalisation of the patients. Samples were stored in glass pots with plastic cap and kept at -20°C. Samples were shipped to the laboratory based in Liverpool on dry-ice. In Liverpool, samples were kept at -20°C and organised in a MySQL database.

Patient details were collected by the nurses on-site. These details were stored in a database constructed by Ph.D. student Nicholas Ellaby, Institute of Integrative Biology, University of Liverpool.

### 2.3.1 Healthy control assignment to confirmed NEC

Two heathy control patients were assigned to each confirmed NEC patient according to a number of factors: date of birth; date of discharge or death; the mode of delivery; the birth weight; the gestation duration; the gender; the type of pregnancy; the enteral feeding; intravenous antibiotics administrated; and the status. For confirmed NEC patients, the first date of diagnosis was also considered. These factors were selected based on discussions with statisticians and clinicians involved in the study. The final assignment was performed automatically by a computer script developed in Visual Basic and implemented in an excel folder.

Match factors for each control were calculated based on the criteria presented in Table 11. The global match factor was calculated summing match factors of each criterion and had a maximum of 47. The lowest global match factor reported was -27.9, the 1$^{st}$ quartile 22.7, the median 27.7, the 2$^{nd}$ quartile 30.0 and the highest global match factor was 36.1. Further statistical values regarding the assignment are presented in Table 12.

**Table 11: Calculation details regarding the assignment of healthy controls to confirmed NEC patients.**

| Criteria | Calculation | Criterion match factors calculation |
|---|---|---|
| Gestation duration | Absolute value of confirmed NEC gestation duration minus healthy control gestation duration | 10 – i * 10/6 with a minimal factor equal to 0 |
| Delivery | Classified as vaginal or caesarean | 10 if identical and 0 if not |
| Enteral feeding type | Classified as mother expressed breast milk only, formula milk only, mix of both or no feed given | 6 if identical and 0 if not |
| Hospital | BHH, BWH, LWH, RSH, RWH, STH, UHCW and UHL | 6 if identical and 0 if not |
| Birth Weight | Absolute value of confirmed NEC birth weight minus healthy control birth weight | 5 if $0 \leq i \leq 20$<br>3 if $21 \leq i \leq 50$<br>1 if $51 \leq i \leq 75$<br>0 if $i > 75$ |
| Intravenous antibiotics (iAB) | Shared iAB divided by the sum of healthy control's iAB plus sum of confirmed NEC's iAB minus the sum of shared iAB | i * 5 |
| Gender | Classified as female or male | 5 if identical and 0 if not |

Table presenting the criteria taken into consideration for patients assignment. The calculation column described either the classification applied for categorical data or the formula applied on numerical data. A 'i' in the third column symbolised the numerical answer calculated when data are numerical. E.g. if the confirmed patient had a gestation duration of 200 days and the healthy control had a gestation duration of 197 days, the absolute value of the difference was 3 days and the criterion match factor was 5 (10 – 3 * 6 / 10).

**Table 12: Percentage of matched healthy controls according to various factors.**

| Criteria | | Number of controls | Percentage of control [%] |
|---|---|---|---|
| Same unit | | 41 | 59.4 |
| Same feeding group | | 51 | 73.9 |
| Same delivery | | 35 | 50.7 |
| Same sex | | 44 | 63.8 |
| Difference in length of gestation [day] | 0 | 3 | 4.3 |
| | 1 | 6 | 8.7 |
| | 2 | 8 | 11.6 |
| | 3 | 6 | 8.7 |
| | 4 | 2 | 2.9 |
| | 5 | 1 | 1.5 |
| | 6 | 3 | 4.3 |
| | > 6 | 40 | 58.0 |
| iAB match factor > 0.75 | | 3 | 4.4 |
| 0.75 >= iAB match factor > 0.5 | | 11 | 15.9 |
| 0.5 >= iAB match factor > 0.25 | | 44 | 63.8 |
| 0.25 > iAB match factor > 0 | | 11 | 15.9 |
| Birth weight difference | of 0 to 20 g | 8 | 11.6 |
| | of 21 to 50 g | 6 | 8.7 |
| | of 51 to 75 g | 3 | 4.3 |
| | higher than 75 g | 52 | 75.4 |

Table presenting the number of healthy controls and their respective relative percentages in accordance with the criteria. (iAB = intravenous antibiotics) E.g.: 41 healthy controls (59.4%) were from the same unit as their matched confirmed NEC. A total of 69 healthy controls were matched to confirmed NEC cases.

### 2.3.2 Samples analysed

A total of 251 samples were analysed in the study, 166 samples were from 70 healthy control patients and 85 samples from 34 confirmed NEC patients. Table 13 presents the sample daily distribution and the number of patients included in the GC–MS investigation and Table 14 presents the sample daily distribution and the number of patients included in the GC–Sensor investigation. Only six days prior to diagnosis were considered as NEC usually develops quickly.[114]

The sample distribution for both statuses using both instruments and for each characteristic's level is presented in detail in Table 15. To apply classifiers on GC–MS data, only one sample per patients per day was considered to avoid bias, therefore, 4 samples were removed at random from the data. Table 16 presents this sample distribution. Table 17 presents the various levels of each characteristic used to classify samples during data analysis.

**Table 13: Samples distribution along time line for GC–MS analysis.**

| Status | N of Patient | Day-1 | Day-2 | Day-3 | Day-4 | Day-5 | Day-6 | Total sample |
|---|---|---|---|---|---|---|---|---|
| Healthy control | 70 | 34 | 22 | 42 | 18 | 31 | 19 | 166 |
| Confirmed NEC | 34 | 17 | 9 | 20 | 15 | 16 | 8 | 85 |

Table presenting the number of samples per day prior to diagnosis and the number of patients enrolled in the study, with respect to the GC–MS data.

**Table 14: Samples distribution along time line for GC–Sensor analysis.**

| Status | N of Patient | Day-1 | Day-2 | Day-3 | Day-4 | Day-5 | Day-6 | Total sample |
|---|---|---|---|---|---|---|---|---|
| Healthy control | 63 | 33 | 18 | 37 | 15 | 25 | 16 | 144 |
| Confirmed NEC | 27 | 10 | 9 | 15 | 8 | 14 | 6 | 62 |

Table presenting the number of samples analysed per day prior to diagnosis and the number of patients enrolled in the study, with respect to the GC–Sensor data.

**Table 15: Sample distribution and patients characteristics.**

| Characteristics | Levels / Values | HS–SPME–GC–MS | | HS–GC–Sensor | |
|---|---|---|---|---|---|
| | | Confirmed NEC | Healthy Control | Confirmed NEC | Healthy Control |
| Delivery | Vaginal delivery | 17 | 28 | 15 | 25 |
| | Caesarean delivery | 17 | 42 | 12 | 38 |
| Gestation duration [days] | Minimum | 165 | 163 | 166 | 164 |
| | Median | 184.5 | 199 | 184 | 201 |
| | Maximum | 229 | 231 | 229 | 231 |
| Birth weight [g] | Minimum | 500 | 485 | 500 | 485 |
| | Median | 795 | 1045 | 835 | 1070 |
| | Maximum | 2116 | 2098 | 2116 | 2098 |
| Sex | Male | 20 | 27 | 12 | 38 |
| | Female | 14 | 43 | 15 | 25 |
| Food | Mix | 25 | 53 | 20 | 46 |
| | Formula only | 1 | 5 | 1 | 5 |
| | MEBM only | 8 | 12 | 6 | 12 |
| Bell's grading | Stage II A or B | 2 | N/A | 2 | N/A |
| | Stage III A or B | 32 | N/A | 25 | N/A |
| Unit | UHL | 6 | 14 | 4 | 14 |
| | UHCW | 3 | 13 | 3 | 12 |
| | STH | 7 | 10 | 6 | 9 |
| | BHH | 4 | 5 | 2 | 5 |
| | BWH | 13 | 19 | 11 | 16 |
| | RSH | 1 | 6 | 1 | 5 |
| | RWH | 0 | 3 | N/A | 2 |

Table presenting the sample distribution for the GC–MS and GC–Sensor investigation according to patients' characteristics for both statuses. One confirmed NEC case had donor breast milk only added to mother expressed breast milk and was therefore classified as mix. (N/A: not applicable)

**Table 16: Samples distribution along time line for modelling data of GC–MS investigation.**

| status | N of Patient | Day-1 | Day-2 | Day-3 | Day-4 | Day-5 | Day-6 | Total sample |
|---|---|---|---|---|---|---|---|---|
| Confirmed NEC | 34 | 17 | 9 | 19 | 15 | 16 | 8 | 84 |
| Healthy control | 70 | 34 | 21 | 41 | 17 | 31 | 19 | 163 |

Table presenting the number of samples per day prior to diagnosis used for classification. Four samples were removed to avoid bias. The number of patients and the total number of samples is also presented.

**Table 17: List of demographic and environmental factors tested, their classes and their definition.**

| Factor | Classes | Comment |
|---|---|---|
| Age at sampling grouped by 10 days | 1-160 | Grouped by 10 days, labels being the mean of each range slice |
| Age at sampling grouped by 5 days | 1-160 | Grouped by 5 days, labels being the mean of each range slice |
| Birth weight | 400-2200 | Grouped by 100 grams, labels being the mean of the range slice |
| Delivery type | CS | Caesarean |
| | VD | Vaginal delivery |
| Feeding type | Formula only | Feed only with formula milk |
| | MEBM only | Feed only with mother expressed breast milk (MEBM) |
| | Mix | Feed with a mix of formula and MEBM |
| Gender | F | Female |
| | M | Male |
| Gestation duration | 24-34 | Rounded down weekly |
| Hospital | BHH | Birmingham Heartlands Hospital |
| | BWH | Birmingham Women's Hospital |
| | LWH | Liverpool Women's Hospital |
| | RSH | Royal Shrewsbury Hospital |
| | RWH | Royal Wolverhampton Hospital |
| | STH | Sheffield Teaching Hospitals |
| | UHCW | University Hospitals of Coventry and Warwickshire |
| | UHL | University Hospitals of Leicester |

Table presenting each factor used to divide samples based on their levels. The description of the levels is presented in the comment column.

# Chapter 3.

# Results

This chapter presents the results obtained from the HS–SPME–GC–MS and the HS–GC–Sensor analyses. The GC–sensor data were analysed using the modelling pipeline described in chapter 2.2.6.

# 3.1 Mass spectrometry

Several analyses were performed using results generated by the mass spectrometer. Uni-variate statistical analyses were applied to compare the number of compounds identified across data classes. Compounds reported by Metab and features reported by XCMS were analysed quantitatively using uni-variate, multi-variate and modelling tools.

## 3.1.1 Results based on Metab output

A pipeline allowing the identification of the compounds was applied to the data. A library was built using AMDIS software and NIST database. This library, AMDIS and Metab were used to generate a report containing compounds identified and their abundances per sample. This section presents the results of the analyses performed on these data.

### 3.1.1.1 Univariate analysis of the number of compounds identified in healthy control samples

Analysis of the number of compounds identified per samples was performed on 329 healthy control samples. This section compares the number of compounds identified within healthy control samples and defines factors associated with the potential differences.

Figure 20 shows the number of compounds found in 329 samples from healthy patients presented according to the different factors analysed (Table 17).

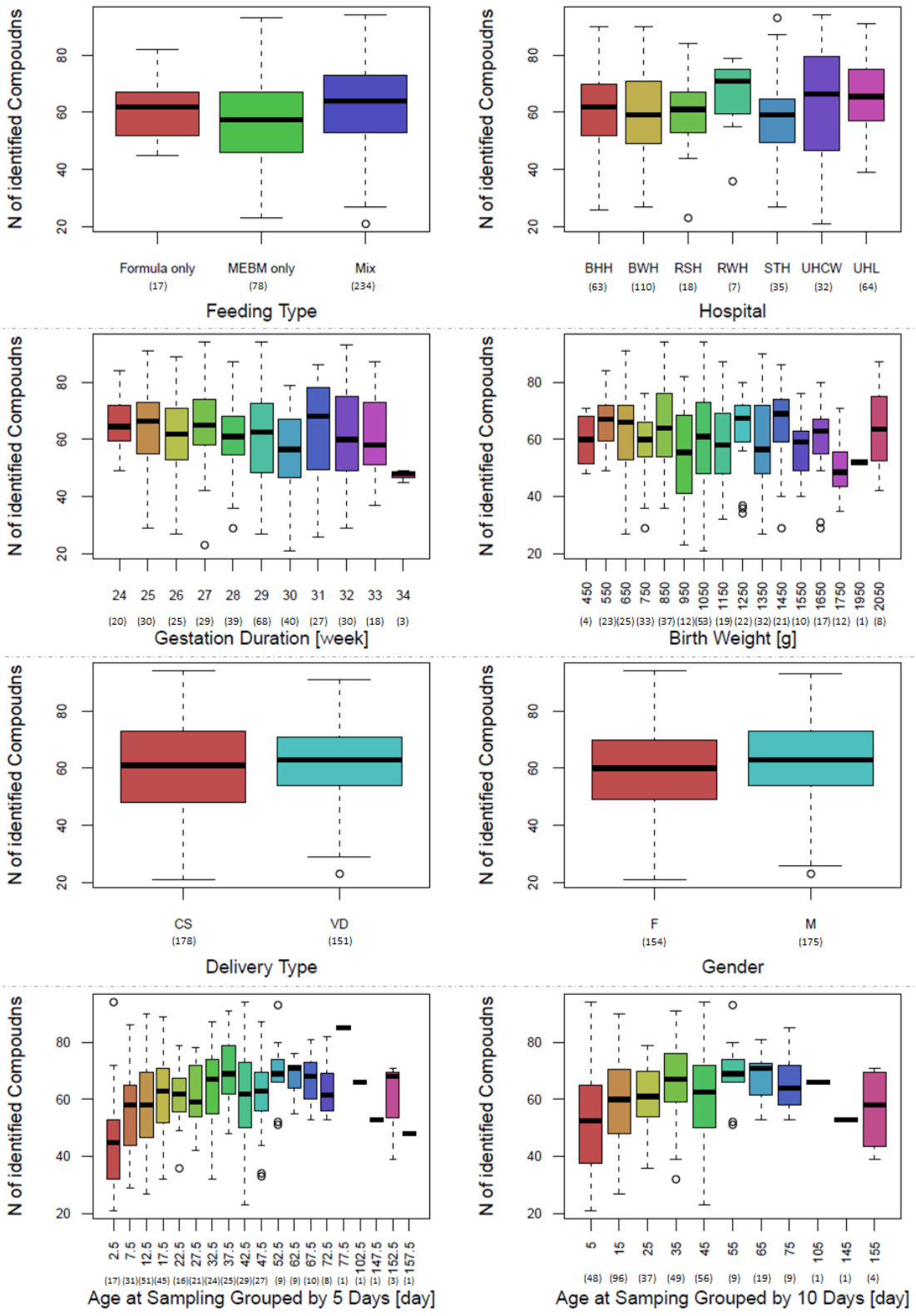


**Figure 20: Number of compounds identified from healthy patients samples grouped according to various factors.** Boxplot presenting the number of compounds identified in 329 samples based on factors (see Table 17 for details). The number of sample per sub-group is shown in brackets.

Overall, 62 ± 20 compounds were identified in 329 samples (median ± IQR or interquartile range). A Mann-Whitney test was applied to compare delivery types and gender whereas Kruskal-Wallis tests were used for the other factors. The Spearman's correlation coefficient was calculated for the gestation duration, the birth weight and the age at sampling grouped by 5 and 10 days; they were then tested for significance. Results are presented in Table 18.

Three factors showed a significant difference between sub-groups, these were the feeding type and the age at sampling grouped by 5 and 10 days. The age at sampling showed a greater difference if the samples from babies older than 100 days were removed before a Kruskal-Wallis test. In general, more compounds were observed as the age at sampling increased. However, no correlation was found with the Spearman's correlation coefficient.

**Table 18: Comparison of the number of compounds identified in healthy patients samples clustered according to various factors.**

| | p-value similarity | p-value correlation |
|---|---|---|
| Feeding type | 0.018 | N/A |
| Hospital | 0.774 | N/A |
| Gestation duration | 1 | >0.05 |
| Birth weight | 0.652 | >0.05 |
| Delivery type | 1 | N/A |
| Gender | 0.489 | N/A |
| Age at sampling grouped by 5 days | 0.032 | >0.05 |
| Age at sampling grouped by 10 days | 0.007 | >0.05 |
| Age at sampling lower than 100 days grouped by 5 days | 0.002 | >0.05 |
| Age at sampling lower than 100 days grouped by 10 days | <0.001 | >0.05 |

Table presenting results of the test for the difference within each factor considered and correlation between factors and the number of compounds (Mann-Whitney test for 2 sub-groups and Kruskal-Wallis test if more than 2 sub-groups present; Spearman *rho* statistics used for the correlation; Bonferroni correction applied with both tests).

#### 3.1.1.2 Univariate analysis of the number of compounds identified in matched healthy control and confirmed NEC samples

Analysis of the number of compounds identified per sample was performed on samples from each condition and classified using different factors. In total, 166 samples from healthy controls were compared to 85 samples from confirmed NEC, approximately 2 samples from healthy control patients for each confirmed NEC case. This section compares the number of compounds identified between statuses (confirmed NEC and healthy control) and defines factors associated with potential differences.

Figure 21 and Figure 22 present the number of compounds found in 85 confirmed NEC and 166 healthy control samples when grouped according to the factors described in Table 17. A median of 62 ± 22 compounds per sample were identified in 166 healthy control samples and 63 ± 25 compounds per sample were identified in confirmed NEC samples (median ± IQR). Mann-Whitney tests were applied to compare delivery types and gender, while Kruskal-Wallis tests were used for the other factors. The Spearman's correlation coefficient was calculated for the gestation duration, the birth weight and age at sampling. Gender was the only factor showing a significant difference for matched healthy control samples. A slight trend could be observed when samples were grouped by age at sampling; however, it was neither significantly different nor correlated.

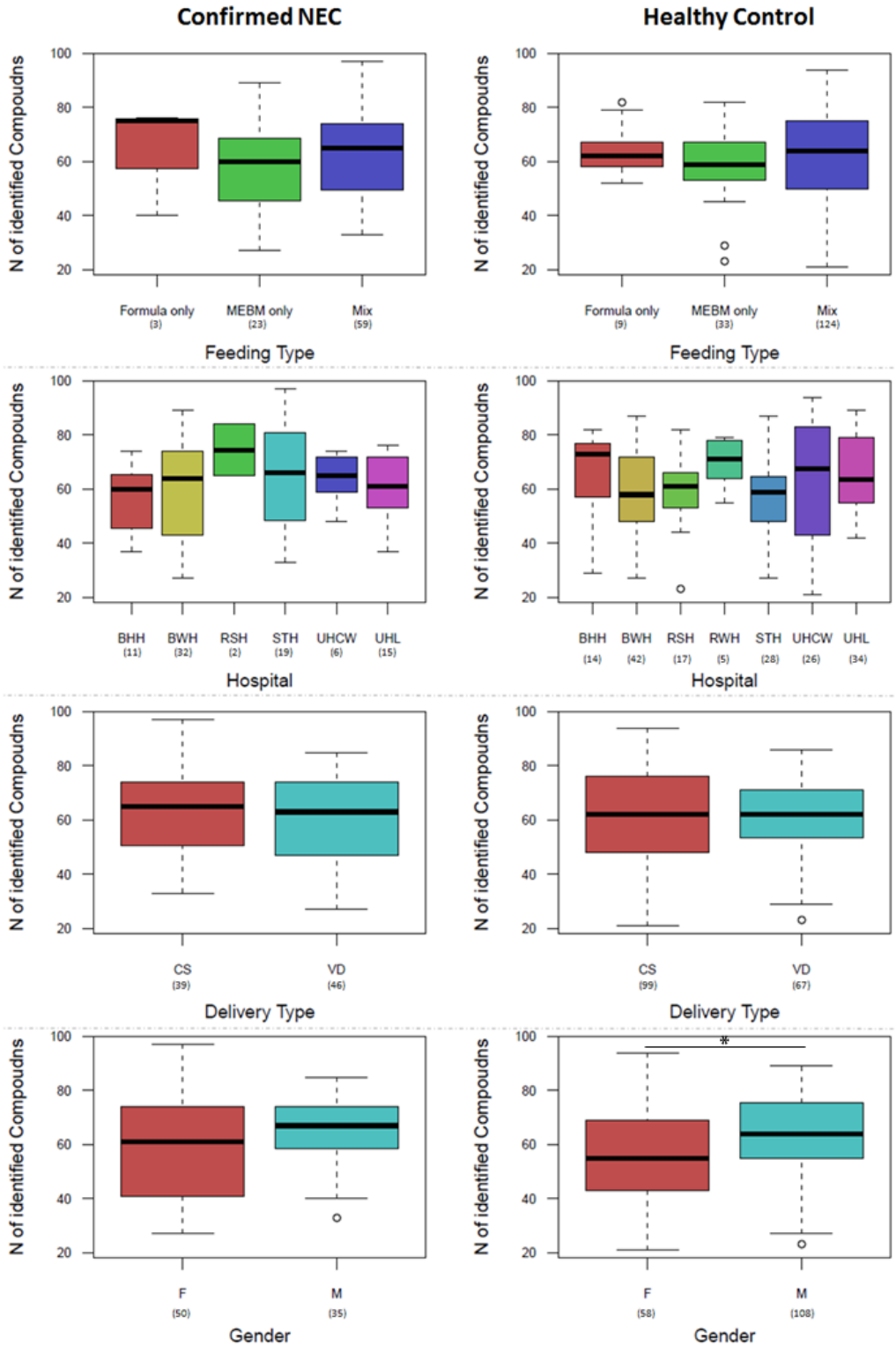


**Figure 21: Number of compounds identified in samples from confirmed NEC cases and healthy controls grouped according to various factors.** Boxplot presenting the number of compounds identified in 85 samples from NEC patients and 166 healthy controls divided according to the feeding type, the hospital, the delivery type and the gender (see Table 17 for details). The number of sample per sub-group is shown in brackets. (* $p < 0.05$; Mann-Whitney tests were applied to compare delivery types and gender, while Kruskal-Wallis tests were used for the feeding type and the hospital)

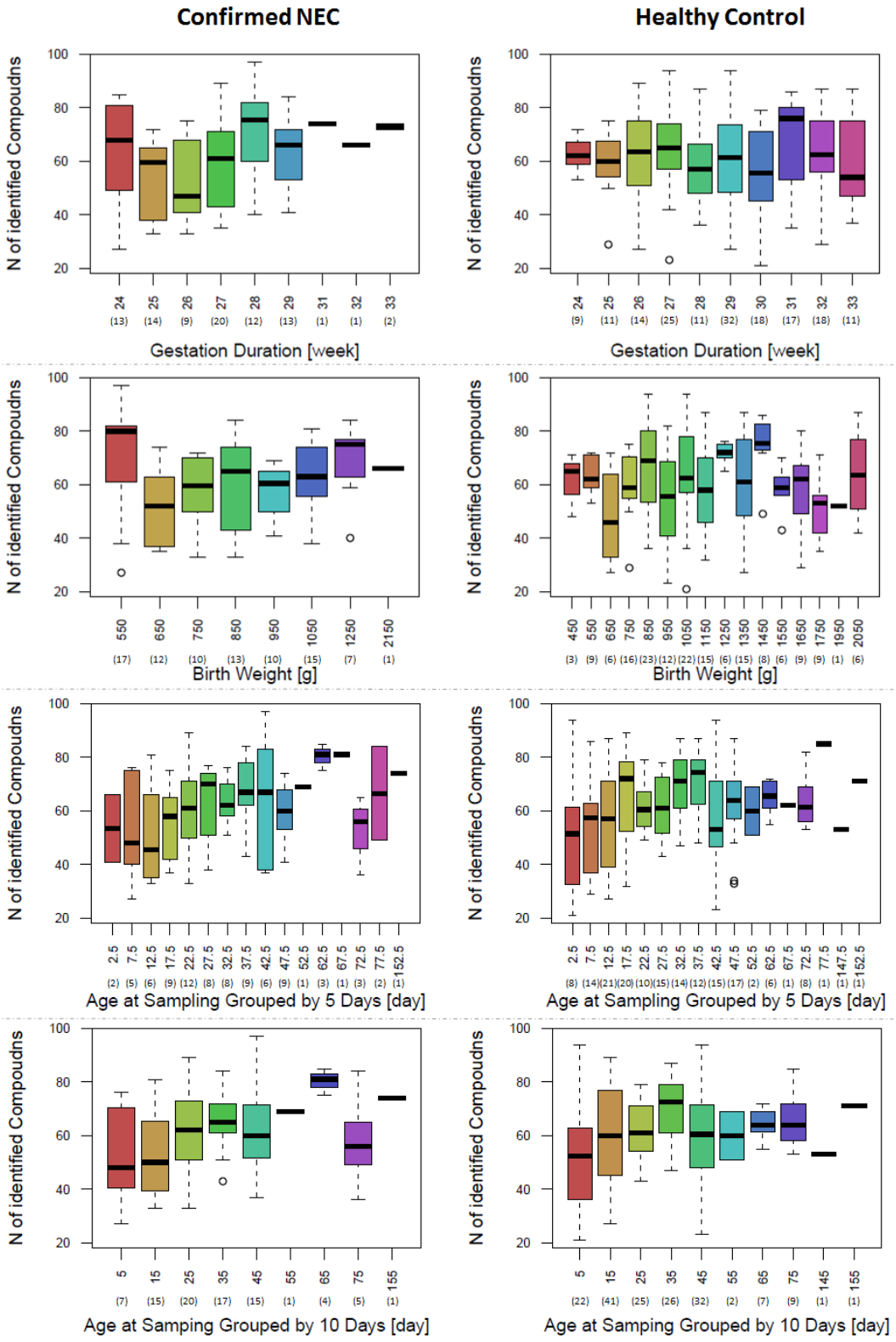


**Figure 22: Number of compounds identified in samples from confirmed NEC cases and healthy controls grouped according to various factors.** Boxplot presenting the number of compounds identified in 85 samples from NEC patients and 166 healthy controls divided according to the gestation duration, the birth weight and the age at sampling grouped by 5 and 10 days (see Table 17 for details). The number of sample per sub-group is shown in brackets. ($p > 0.05$; Kruskal-Wallis test)

In addition, to compare various factors while considering health statuses, two-way ANOVA were applied. This test was chosen as Kruskal-Wallis test could not be applied to consider interaction between factors. Table 19 presents the results of two-way ANOVA for the number of compounds identified when comparing the status and the various factors presented above. It showed no significant difference between confirmed NEC and matched healthy controls, which are also illustrated in Figure 21 and Figure 22. The gender was the only factor associated with a significantly different number of identified compounds (Figure 21). This difference may have happened by chance despite the utilisation of Bonferroni correction. There was no significant interaction between statuses and factors.

**Table 19 Comparison of each independent factor, status and interactions.**

| Factor | p-value | | |
|---|---|---|---|
| | Between confirmed NEC and healthy control | Difference within factor | Interaction |
| Feeding type | 1 | 1 | 1 |
| Hospital | 1 | 1 | 1 |
| Delivery type | 1 | 1 | 1 |
| Gender | 1 | 0.010 | 1 |
| Gestation duration | 1 | 1 | 1 |
| Birth weight | 1 | 0.091 | 1 |
| Age at sampling grouped by 5 days | 1 | 0.469 | 1 |
| Age at sampling grouped by 10 days | 1 | 0.116 | 1 |

Table presenting the results of two-way ANOVA to test influence of factors and statuses on the number of compounds and their interactions. (Bonferroni correction was applied)

Figure 22 showed a potential trend for the age at sampling grouped by 5 and 10 days for confirmed NEC and healthy control where the number of VOCs identified seemed to increase according to the age at sampling. However, neither the difference in the number of compounds identified across ages at sampling, nor correlation between the number of compounds identified and the age at sampling were significant (Table 19).

Figure 23 presents the boxplots with the number of compounds found in samples from confirmed NEC and healthy controls for days 1 to 6 prior to diagnosis. No significant differences were observed while comparing confirmed NEC and healthy controls (Mann-Whitney test; p-values > 0.05), moreover, the number of compounds identified at each day of sampling prior to diagnosis was compared within health status. Neither differences nor correlations were significant (Kruskal-Wallis test used for differences; Spearman *rho* statistics used for correlation; p-values > 0.05).

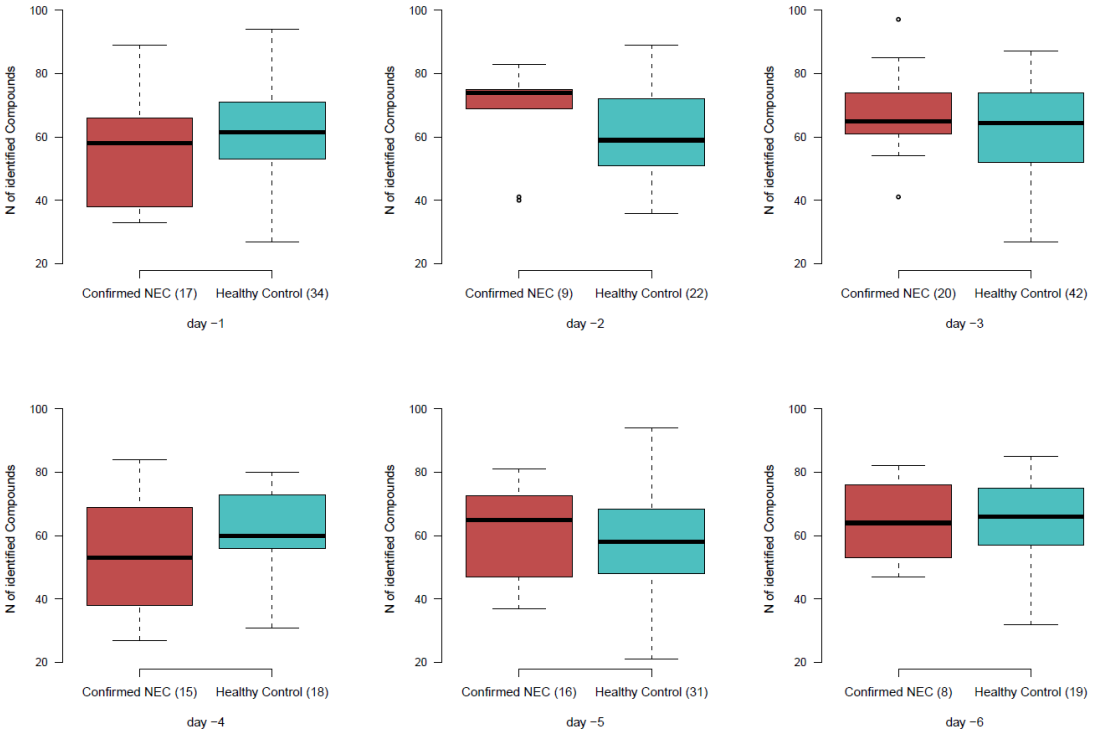


**Figure 23: Number of identified compounds in confirmed NEC and healthy control samples at each day prior to diagnosis.** Boxplot presenting the number of compounds identified in samples from both statuses at days -1 to -6 prior to diagnosis. The number of samples per sub-group is shown in brackets. (p > 0.05; Mann-Whitney tests)

### 3.1.1.3 Compounds chemical family

This section presents the different chemical families detected in samples from confirmed NEC and healthy controls at each day prior to diagnosis.

Figure 24 presents the profile of chemical families detected in samples from confirmed NEC and healthy controls; Figure 25 presents the profile of chemical families detected according to the number of days prior to diagnosis. A total of 166 samples from healthy control and 85 samples from confirmed NEC patients were considered.

Mann-Whitney was applied on each compound's family to test for significance between confirmed NEC and healthy control samples. P-values were corrected using Bonferroni correction and none of them was significantly different at 5%. Kruskal-Wallis was applied on each compound's family to test for significance between days prior to diagnosis within confirmed NEC and healthy control samples. Again, p-values were corrected using Bonferroni correction and none of them was significantly different at 5%.

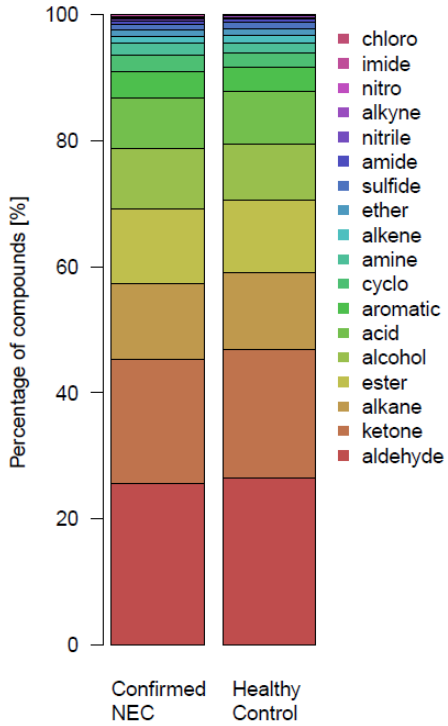


**Figure 24: Chemical families detected in samples from confirmed NEC and healthy control.** Stacked bar plot illustrating the distribution of the chemical families associated with compounds found in samples from confirmed NEC and healthy control patients. ($p > 0.05$; Mann-Whitney followed by Bonferroni correction)

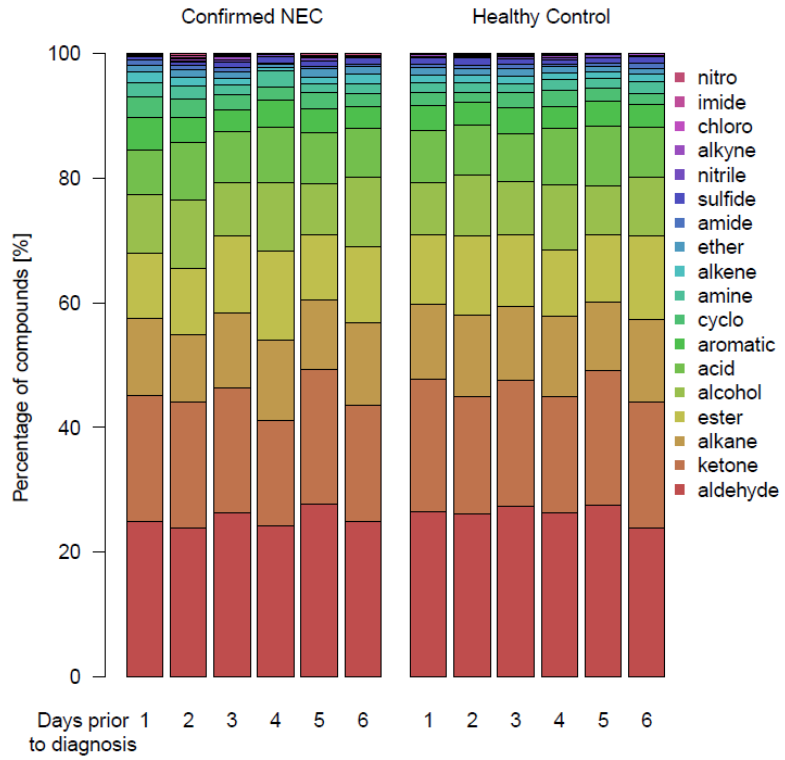


**Figure 25: Chemical families detected at each day prior to diagnosis for confirmed NEC and healthy control.** Stacked bar plot illustrating the distribution of the chemical families detected at days 1 to 6 prior diagnosis. Families of compounds were tested for differences within statuses ($p > 0.05$; Kruskal-Wallis followed by Bonferroni correction)

### 3.1.1.4 Compounds prevalence in different group of samples

In total, the AMDIS library built during this work contained 229 compounds. However, few compounds were expected to be highly prevalent among analysed samples. The aim of this section is to investigate which compounds were highly prevalent in the different groups of samples investigated: confirmed NEC cases plus matched healthy controls (251 samples); confirmed NEC cases (85); matched healthy controls (166); and healthy controls (329). Compounds present in 80% of at least one of these 4 groups of samples were presented in Table 20. Few compounds were prevalent in more than 80% of the samples. In total, 21 compounds had a high frequency (>80%) in healthy control samples, 17 in confirmed NEC plus control samples, 16 in confirmed NEC samples and 19 in matched healthy control samples.

**Table 20: Most prevalent compound identified in different groups of sample.**

| RT [min] | Compound names | Confirmed NEC and healthy control (n=251) | Confirmed NEC (n=85) | Healthy control (n=166) | Healthy control (n=329) |
|---|---|---|---|---|---|
| 6.6 | Ethanol | 99% | 99% | 99% | 98% |
| 22.3 | Heptanal | 98% | 96% | 98% | 98% |
| 24.3 | 2, 2, 4, 6, 6-pentamethylheptane | 97% | 99% | 96% | 96% |
| 12.7 | Acetic acid | 96% | 98% | 96% | 97% |
| 18.4 | Hexanal | 96% | 98% | 96% | 97% |
| 29.5 | Nonanal | 95% | 95% | 95% | 95% |
| 10.3 | 2, 3-butanedione | 95% | 94% | 95% | 93% |
| 7.4 | Acetone | 94% | 92% | 95% | 97% |
| 9.0 | 2-methylpropanal | 92% | 89% | 93% | 94% |
| 16.2 | Propanoic acid | 90% | 91% | 90% | 93% |
| 25.8 | 2, 2, 4, 4-tetramethyloctane | 90% | 94% | 87% | 85% |
| 20.7 | Xylene | 86% | 84% | 87% | 88% |
| 9.6 | 1-propanol | 86% | 84% | 87% | 86% |
| 7.2 | Propanal | 86% | 80% | 89% | 91% |
| 25.7 | Benzaldehyde | 82% | 82% | 83% | 85% |
| 24.8 | 2-pentylfuran | 82% | 84% | 81% | 84% |
| 19.5 | Butanoic acid | 80% | 76% | 81% | 80% |
| 13.9 | 1-penten-3-one | 79% | 73% | 83% | 81% |
| 21.1 | 2-E-hexenal | 78% | 72% | 82% | 84% |
| 14.2 | Pentanal | 75% | 73% | 76% | 81% |
| 26.1 | Octanal | 75% | 68% | 79% | 81% |

Table listing the most prevalent compounds in the 4 sets of samples, their prevalence is quoted as a percentage of the sample.

### 3.1.1.5 Univariate analysis of the intensities of compounds found in healthy control samples to investigate the influence of multiple factors

Univariate analysis was performed to investigate the influence of multiple factors on VOCs intensities emitted from 329 healthy control samples.

A total of 46 compounds were identified in the 329 healthy controls samples analysed. Their abundances were then compared within levels of factors described in Table 17. Table 21 shows the number of compound associated with p-values lower than 0.05 when missing values were substituted using either half the lowest value across status for each compound or k-NN algorithm (t-test for 2 sub-groups and ANOVA if more than 2 sub-groups were present; with Bonferroni correction). No Spearman's correlation coefficient was significant between the intensities of the compounds and the factors considered.

**Table 21: Number of compounds with significantly different intensities while healthy control samples were analysed according to various factors.**

| | Number of compounds | |
|---|---|---|
| | Half lowest value | k-NN |
| Age at sampling grouped by 10 days | 14 | 8 |
| Age at sampling grouped by 5 days | 12 | 6 |
| Birth weight | 14 | 9 |
| Delivery type | 0 | 0 |
| Feeding type | 2 | 0 |
| Gender | 1 | 0 |
| Gestation duration | 9 | 4 |
| Hospital | 7 | 3 |
| Total | 59 | 30 |

Table presenting the number of compounds which intensities showed a p-value lower than 0.05 when tested for differences between levels based on 329 samples clustered according to the factors presented in Table 17. Missing values were replaced either by half the lowest value across status for each compound or by k-NN averaging (T-test for 2 sub-groups and ANOVA test if more than 2 sub-groups present; with Bonferroni correction).

Some factors seemed to have a stronger influence on the intensities of the VOCs identified. Age at sampling and birth weight showed a high number of VOCs present at significantly different intensities across ages and birth weights, respectively. Gestation duration and hospital also showed some VOCs at significantly different intensities. In contrast, the delivery type, feed type and gender seemed to have very low or no influence in the abundances of VOCs found, with none or very few compounds present at significantly different intensities. This trend was observed with both imputation methods, although the number of significantly different compounds was higher when half the lowest value was used instead of k-NN algorithm.

#### 3.1.1.6 Univariate analysis of the intensities of compounds found in confirmed NEC and matched healthy control samples to investigate the influence of factors

In addition to the investigation on healthy control samples described above (see 3.1.1.5), VOCs found in NEC samples and matched controls were further analysed in order to determine which factors might affect their intensities or relative abundances. This section presents the factors that influence the intensity of VOCs found in these samples.

The abundances of all 50 compounds identified in the 166 healthy control samples and 85 confirmed NEC samples were compared between the levels of each factors described in Table 17. Table 22 presents the number of compounds showing p-values lower than 0.05 when missing values were substituted by either half the lowest value for that compound (across samples from both statuses) or using k-Nearest Neighbour (t-test for 2 sub-groups and ANOVA test if more than 2 sub-groups present; with Bonferroni correction).

There was a higher number of VOCs present at significantly different intensities within levels of most factors tested when analysing healthy control samples than confirmed NEC cases. The imputation method used on the missing data produced small differences in the results. Age at sampling seemed to impact VOC intensities for both groups, while the birth weight and the hospital influenced VOC intensities on healthy control samples.

**Table 22: Number of compounds which showed significantly different intensities while confirmed NEC and healthy control samples were analysed according to various factors.**

| | Number of compounds in confirmed NEC samples | | Number of compounds in healthy control samples | |
|---|---|---|---|---|
| | Half lowest value | k-NN | Half lowest value | k-NN |
| Age at sampling grouped by 10 days | 2 | 3 | 7 | 8 |
| Age at sampling grouped by 5 days | 0 | 0 | 5 | 6 |
| Birth weight | 0 | 1 | 5 | 2 |
| Delivery type | 0 | 0 | 0 | 0 |
| Feeding type | 1 | 0 | 0 | 0 |
| Gender | 0 | 0 | 2 | 0 |
| Gestation duration | 0 | 0 | 0 | 2 |
| Hospital | 1 | 0 | 5 | 5 |
| Total | 4 | 4 | 24 | 23 |

Table presenting the number of compounds with intensities having a p-value less than 0.05 when tested for differences between levels based on 85 confirmed NEC and 166 healthy control samples grouped according to the factors (see Table 17 for details). Missing values were replaced by either by half the lowest value for each compound or by k-NN averaging (T-test for 2 sub-groups and ANOVA test if more than 2 sub-groups present; with Bonferroni correction).

Spearman's correlation coefficient was calculated for the intensities of VOCs found in confirmed NEC cases and healthy control, with missing values substituted by half the lowest value and k-NN. It showed a significant correlation for N-propylacetate and propanol. Missing values were substituted by half the lowest value in both cases.

N-propylacetate (RT: 14.4 min) was correlated with the age at sampling when grouped by 5 days for the confirmed NEC samples ($p < 0.05$; Spearman *rho* statistics), however it was not significantly different (ANOVA p-value greater than 0.05). Propanol (RT: 9.6 min) was correlated with the age at sampling when grouped by 5 days for the healthy control samples ($p < 0.05$; Spearman *rho* statistics) and was significantly different (ANOVA p-value $< 0.001$) (Figure 26).

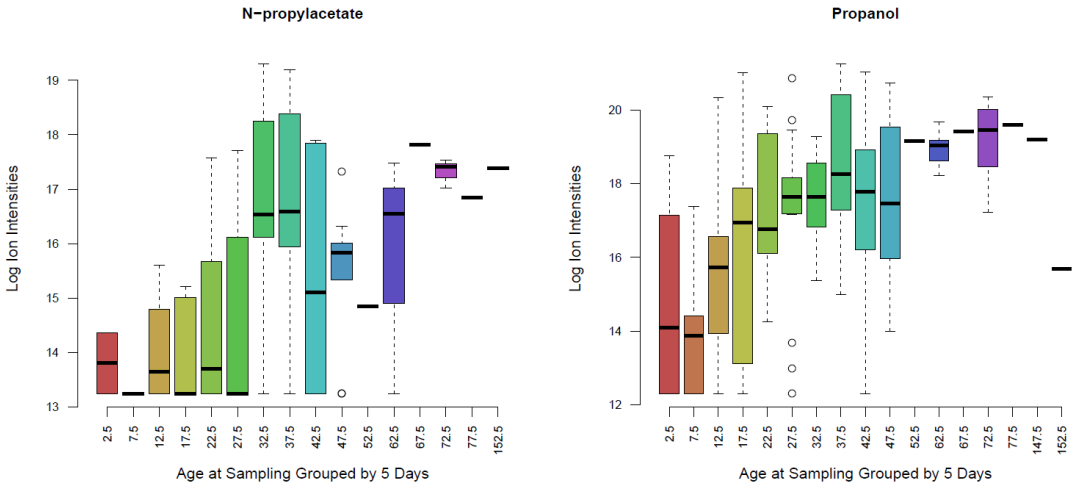


**Figure 26: Compounds with intensities correlated to the age at sampling.** Boxplot showing VOCs with significant correlation between their abundances and the age at sampling grouped by 5 days. N-propylacetate (RT: 14.4 min) was correlated in confirmed NEC samples while propanol (RT: 9.6 min) was correlated in healthy control samples ($p < 0.05$; Spearman *rho* statistics).

### 3.1.1.7 Univariate analysis on the intensities of compounds comparing confirmed NEC and matched healthy control samples

VOC profiles were analysed using univariate statistical tools in order to identify differences between confirmed NEC and healthy controls. Qualitative and quantitative analyses were applied. This section presents VOCs that showed significant differences between confirmed NEC and healthy control patients at individual days prior to diagnosis.

The log transformed intensities of each compound found in confirmed NEC and healthy control samples were compared at each day prior to diagnosis. Missing values were replaced by half the lowest value and using k-NN. In addition, samples of each data class, confirmed NEC and healthy control, were grouped by 2 and 3 days and, subsequently, all days were combined.

Table 23 presents the identities (ID) of compounds showing frequencies or abundances in confirmed NEC samples significantly different than in healthy control samples (p-value < 0.01; Chi-square with continuity correction was applied for frequencies comparison and t-test for intensities comparison). Table 24 presents their respective names.

**Table 23: Comparison of frequencies and intensities of compounds found in confirmed NEC and healthy control samples.**

| Day(s) investigated | Frequencies comparison | k-NN | Half lowest value |
|---|---|---|---|
| Day -1 | 15 | - | 15 |
| Day -2 | 14 | 4 | - |
| Day -3 | - | - | **18** |
| Day -4 | 5 | 12, 10, 8, 13, 3 | 6, 5 |
| Day -5 | - | - | - |
| Day -6 | - | 2 | 11, 17 |
| Day -1 & -2 | - | - | **7** |
| Day -3 & -4 | - | - | - |
| Day -5 & -6 | 16 | - | - |
| Day -1, -2 & -3 | 19 | - | **18** |
| Day -4, -5 & -6 | - | - | 9 |
| All days | 1, 7 | - | 1, **7** |

Summary of identification number (in relation to Table 24) of significantly different compounds at different time points or group of time points. Missing values were substituted with half the lowest value of the compounds intensity across groups and k-NN algorithm ($p < 0.01$; Chi-square with continuity correction for frequencies comparison and t-test for intensities comparison). For compounds more abundant in confirmed NEC samples, the IDs are bold.

**Table 24: Names and identification number of compounds identified in relation to Table 23.**

| ID | Name | RT [min] | ID | Name | RT [min] |
|---|---|---|---|---|---|
| 1 | 2-butanone | 10.6 | 11 | Propyl ester 2-methylbutanoic acid | 23.3 |
| 2 | 2-methylbutanal | 12.8 | 12 | 2, 6, 10-trimethyldodecane | 26.0 |
| 3 | N-propyl acetate | 14.4 | 13 | 2, 7, 10-trimethyldodecane | 26.4 |
| 4 | Toluene | 16.5 | 14 | Hexanoic acid | 26.4 |
| 5 | 2-E-pentenal | 17.1 | 15 | 2,2,6-trimethylcyclohexanone | 27.7 |
| 6 | 2-E-hexenal | 21.1 | 16 | 3,4-dimethylcyclohexanol | 30.7 |
| **7** | **4-heptanone** | **21.2** | 17 | 6-methyl-2-heptanone | 30.9 |
| 8 | 3-methylbutanoic acid | 21.5 | **18** | **Ethyl ester decanoic acid** | **37.6** |
| 9 | 2-methylbutanoic acid | 21.8 | 19 | Propanal | 7.2 |
| 10 | Heptanal | 22.3 | | | |

List of the compound names, retention time (RT) and identification number (ID) related to Table 23. For compounds more abundant in confirmed NEC samples, the IDs are bold.

When Bonferroni correction was applied, only 3 compounds showed significantly different abundances between confirmed NEC and healthy control samples. All three compounds had missing values replaced by half the lowest value. 2,2,6-trimethylcyclohexanone was present at significantly higher abundance in healthy controls samples the day equivalent to one day prior to diagnosis. The compounds 2-E-pentenal and 2-E-hexenal were present at higher abundances in healthy control samples the day equivalent to four days prior to diagnosis (Figure 27).

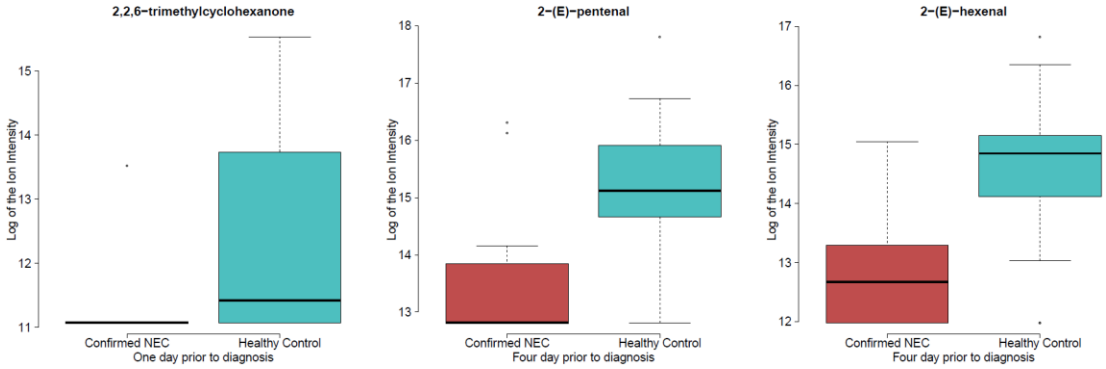


**Figure 27: Compounds found at significantly different abundances between confirmed NEC and healthy controls.** Log of the compounds intensities at different days prior to diagnosis for both statuses illustrated with a boxplot. Missing data were replaced using half the lowest value of each compound. ($p < 0.05$, t-test followed by Bonferroni correction).

### 3.1.1.8 Comparisons of the intensities of compounds within confirmed NEC or matched healthy control samples

This section evaluates the variability of VOC abundances within status.

ANOVA followed by Tukey HSD test was applied on confirmed NEC data to compare days prior to diagnosis. Five compounds were different at 1% significance; they are presented in Figure 28 with Tukey HSD test results. None of the abundances was significantly correlated with the days prior to diagnosis ($p > 0.05$; Pearson's correlation coefficients).

The same tests, followed by Bonferroni correction, were applied on matched healthy control data to compare days prior to diagnosis. Again five compounds were significantly different (Figure 29). In addition, Pearson's correlation coefficients were calculated for each compound and only propanol showed a significant decrease in abundance as the days prior to diagnosis decrease (p-value < 0.05).

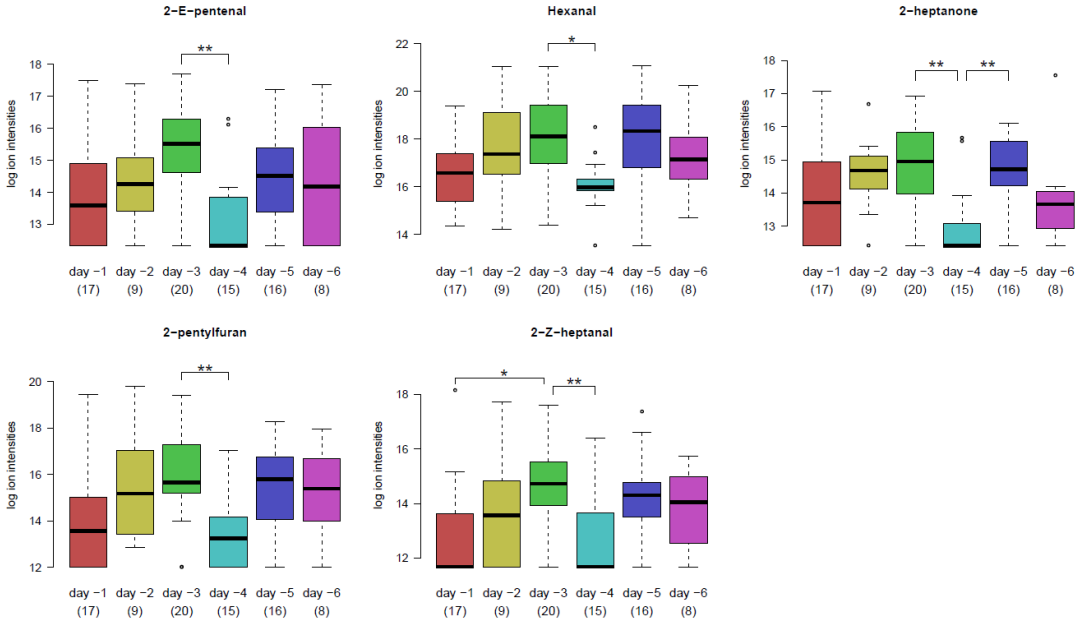


**Figure 28: Intensities of significant compounds within confirmed NEC patients.** This panel shows the compounds present at significantly different abundances, at a level of 1%, between days prior to diagnosis in confirmed NEC samples. Missing values were implemented using half the lowest value (* $p < 0.05$ and ** $p < 0.01$ for Tukey-HSD test; ANOVA and Tukey-HSD test). No abundances were significantly correlated with the days prior to diagnosis ($p > 0.05$; Pearson's correlation coefficients).

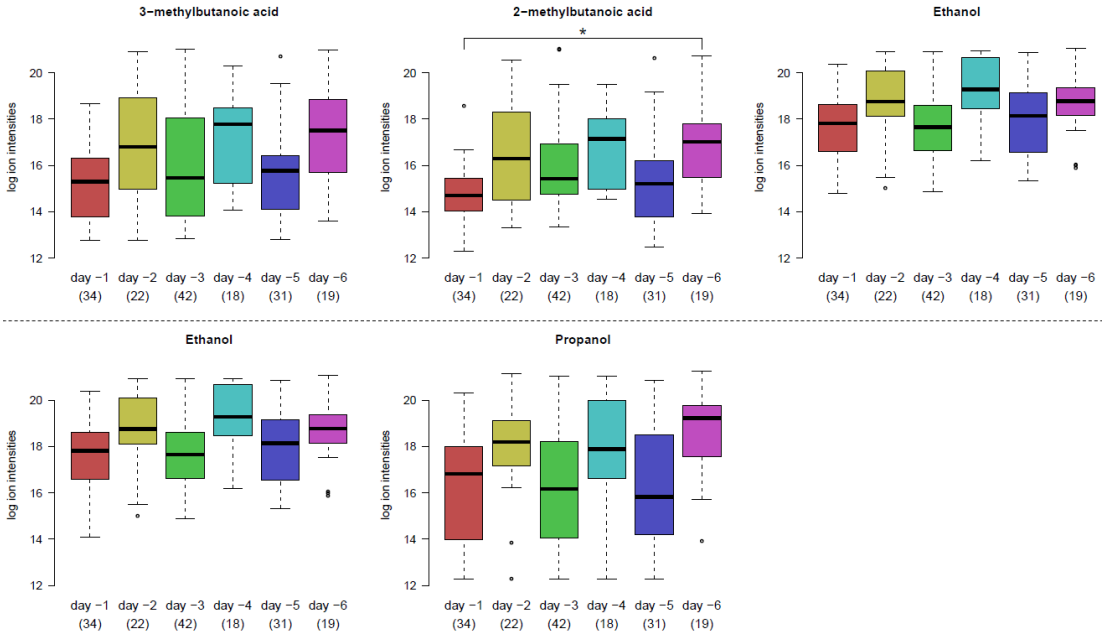


**Figure 29: Intensities of compounds of interest within healthy controls.** This panel shows the compounds present at significantly different abundances between days prior to diagnosis in healthy control samples. 2-methylbutanoic acid was different at a level of significance of 1% while the others were different at a level of significance of 5%. The top row shows VOC abundances when k-NN was used for imputing missing values, while the bottom row shows VOC abundances when half the lowest value was used for missing values substitution (* $p < 0.05$ for Tukey-HSD test; ANOVA and Tukey-HSD test, followed by Bonferroni correction). Propanol was the only significantly correlated compound ($p < 0.05$; Pearson's correlation coefficients).

#### 3.1.1.9 Modelling VOC profile for sample classification

Classification of samples based on VOC abundances was performed using different statistical techniques. This section presents in detail every step applied for this process and the results obtained.

Statistical models were built based on the VOC profile reported by Metab. The first step was to select the compounds or features that best described the differences between confirmed NEC and healthy controls. For this, 50 compounds identified in 50% or more of the samples of at least one condition, confirmed NEC or healthy control, along with the age at sampling in days, food type, hospital, gestation duration in days, birth weight in grams, delivery type and sex were submitted to a step-wise linear discriminant analysis[91] and two random forest[91], [104] algorithms for feature selection. The analysis was performed at each day prior to diagnosis and with missing values replaced using either half the lowest value of each compound across status or k-NN algorithm (Table 25). The gestation duration and the birth weight were the most selected features.

At each day prior to diagnosis, RF and k-NN algorithms were applied to classify samples based on every feature that was selected by at least one of the feature selection algorithm (Table 25). Table 26 presents the accuracies and kappa values obtained from sample classification after repeated 10-fold cross validation.

The results of sample classification (Table 26) were improved when missing data were replaced using k-NN algorithm, therefore, further analyses were performed only on this data. Features selected more than twice across the 6 days prior to diagnosis, as presented in Table 25, were used for each day prior to diagnosis. In this case, RF, LDA, k-NN, PLS, SVM using radial, linear and polynomial kernel algorithms were applied. The results were validated repeated double cross-validation (Table 27 and Table 28). The sensitivity reported using RF was lower than reported by SVM linear; however the specificity reported by RF was higher than reported by SVM linear. The best result was recorded at 4 day prior to diagnosis, when the number of samples considered for each class was similar. It indicates that the unbalanced nature of the dataset used for some days may affect the results of sample classification.

**Table 25: Features selection for modelling and sample classification based on Metab results.**

| Selected features | RT [min] | Half the lowest value (number of algorithms selecting the feature) | | | | | | | k-NN (number of algorithms selecting the feature) | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Days prior to diagnosis | | | | | | | Days prior to diagnosis | | | | | | |
| | | 1 | 2 | 3 | 4 | 5 | 6 | sum | 1 | 2 | 3 | 4 | 5 | 6 | sum |
| Birth weight | | 1 | 1 | 2 | 2 | 0 | 2 | 8 | 1 | 0 | 3 | 3 | 0 | 3 | 10 |
| Gestation | | 3 | 0 | 3 | 0 | 2 | 0 | 8 | 3 | 0 | 2 | 0 | 2 | 0 | 7 |
| Hexanal | 18.4 | 0 | 0 | 0 | 2 | 1 | 0 | 3 | 1 | 0 | 0 | 1 | 2 | 0 | 4 |
| Acetone | 7.4 | 0 | 2 | 2 | 0 | 0 | 0 | 4 | 0 | 2 | 1 | 0 | 0 | 0 | 3 |
| 2-Z-heptanal | 25.0 | 0 | 0 | 1 | 0 | 2 | 0 | 3 | 0 | 3 | 0 | 0 | 0 | 0 | 3 |
| 2-methylbutanoic acid | 21.8 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 2 | 3 |
| 2-pentylfuran | 24.8 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 3 | 0 | 0 | 3 |
| Pentanal | 14.2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 1 | 0 | 3 |
| 2-butanone | 10.6 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 3 | 3 |
| 1-penten-3-one | 13.9 | 2 | 0 | 0 | 1 | 0 | 0 | 3 | 0 | 0 | 0 | 2 | 0 | 0 | 2 |
| 2, 3-butanedione | 9.0 | 0 | 0 | 0 | 0 | 0 | 3 | 3 | 0 | 0 | 0 | 0 | 0 | 2 | 2 |
| 2-methylpropanal | 22.3 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 2 |
| Heptanal | 10.3 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 2 | 0 | 0 | 2 |
| 2-E-octenal | 28.6 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 2 |
| 2-cyclopenten-1-one, 4-acetyl-2, 3, 4, 5, 5-pentamethyl | 34.3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 0 | 2 |
| 2-methylbutanal | 12.8 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 2 |
| 3,4-dimethyl cyclohexanol | 20.3 | 0 | 1 | 0 | 0 | 1 | 2 | 4 | 0 | 1 | 0 | 0 | 0 | 0 | 1 |
| Ethylbenzene | 21.2 | 1 | 0 | 0 | 0 | 1 | 0 | 2 | 0 | 0 | 0 | 1 | 0 | 0 | 1 |
| 4-heptanone | 30.7 | 0 | 1 | 0 | 0 | 1 | 0 | 2 | 0 | 0 | 0 | 0 | 1 | 0 | 1 |
| 2-E-pentenal | 17.1 | 0 | 0 | 0 | 2 | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 0 | 1 | 1 |

| | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 3-methylbutanoic acid | 21.5 | 0 | 0 | 0 | 2 | 0 | 0 | 2 | 0 | 0 | 0 | 1 | 0 | 0 | 1 |
| Carbon disulfide | 7.8 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 1 |
| Propanoic acid | 16.2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 |
| Ethyl acetate | 10.6 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 1 |
| 2-ethylfuran | 13.5 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 1 |
| Sex | | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 1 |
| 3-methylbutanal | 12.5 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 1 |
| 3, 3, 4-trimethyldecane | 27.0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 |
| 2-E-hexenal | 7.2 | 0 | 0 | 0 | 3 | 0 | 0 | 3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Propanal | 21.0 | 1 | 0 | 1 | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Acetic acid | 27.8 | 0 | 0 | 0 | 0 | 0 | 2 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 1-decen-3-one | 28.7 | 0 | 0 | 0 | 0 | 0 | 2 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 5-Ethylcyclopent-1-enecarboxaldehyde | 12.7 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Benzeneacetaldehyde | 25.2 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

Table presenting the variables evaluated for modelling and the number of feature selection algorithms that positively selected each of these features. This analysis was performed on results reported by Metab when missing values were replaced using either half the lowest value or k-NN algorithm. Three feature selection algorithms were applied, a step-wise linear discriminant analysis and two random forest (RF) algorithms. The 'sum' columns present the number of times each feature was selected across all days using each NA's substitution techniques.

**Table 26: Results of sample classification, based on all and selected features in Metab results, using two classifiers.**

| Arguments implemented in the model | Missing data substitution techniques | Model tested | Lowest accuracy | Mean accuracies [%] (kappa values) | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | % | Day: 1 | 2 | 3 | 4 | 5 | 6 |
| Selected features | Half the lowest value | k-NN | 64.2 | 71.4 (0.28) | 64.2 (0.01) | 77.8 (0.44) | 80.7 (0.62) | 71.7 (0.34) | 78.8 (0.31) |
| | | RF | 51.5 | 73.4 (0.34) | 51.5 (-0.13) | 74.0 (0.34) | 86.2 (0.71) | 71.9 (0.34) | 83.9 (0.46) |
| | k-NN | k-NN | 66.4 | 66.4 (0.20) | 67.8 (0.15) | 71.2 (0.31) | 82.4 (0.62) | 70.8 (0.37) | 85.6 (0.55) |
| | | RF | 60.5 | 60.5 (0.09) | 77.1 (0.34) | 63.5 (0.13) | 73.8 (0.46) | 72.9 (0.38) | 77.6 (0.34) |

K-Nearest Neighbour (k-NN) and random forest (RF) algorithm were applied on Metab results with missing data replaced using k-NN and half the lowest value. Selected features, specific for each day, were used (see Table 25). Results were validated using repeated 10-fold cross validation. Accuracy was defined as the sum of correctly classified and rejected samples divided by the total number of samples.

**Table 27: Sample classification at each day prior to diagnosis based on Metab data.**

| Day prior to diagnosis | 1 | | | 2 | | | 3 | | | 4 | | | 5 | | | 6 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Acc % | Sens % | Spec % | Acc % | Sens % | Spec % | Acc % | Sens % | Spec % | Acc % | Sens % | Spec % | Acc % | Sens % | Spec % | Acc % | Sens % | Spec % |
| RF | 69.1 | 38.6 | 84.4 | 67.1 | 26.4 | 84.8 | 63.2 | 28.3 | 79.3 | 76.0 | 72.7 | 79.1 | 66.7 | 34.6 | 83.4 | 72.0 | 24.6 | 91.6 |
| LDA | 74.2 | 62.9 | 79.8 | 52.5 | 35.1 | 60.2 | 63.4 | 46.5 | 71.1 | 62.5 | 59.3 | 65.7 | 60.3 | 50.2 | 65.6 | 58.3 | 47.5 | 62.9 |
| k-NN | 64.9 | 30.4 | 82.2 | 62.2 | 28.7 | 76.6 | 61.8 | 54.3 | 65.2 | 78.5 | 76.5 | 80.5 | 65.6 | 55.6 | 70.7 | 66.3 | 23.8 | 84.2 |
| PLS | 69.6 | 48.8 | 80.0 | 61.0 | 21.9 | 77.1 | 65.0 | 27.2 | 82.6 | 74.9 | 77.1 | 73.2 | 63.1 | 32.7 | 78.8 | 65.1 | 28.8 | 79.9 |
| SVM Radial | 65.5 | 7.3 | 93.7 | 66.4 | 0 | 90.4 | 65.2 | 9.3 | 91.2 | 75.7 | 69.2 | 81.3 | 62.2 | 4.4 | 92.0 | 67.4 | 0.4 | 90.1 |
| SVM Linear | 75.7 | 58.9 | 84.1 | 63.0 | 33.2 | 76.3 | 64.1 | 46.1 | 72.6 | 69.0 | 65.4 | 72.6 | 60.8 | 43.3 | 69.8 | 70.8 | 45.0 | 81.7 |
| SVM Polynomial | 74.3 | 39.6 | 91.3 | 67.6 | 4.3 | 90.7 | 62.7 | 16.0 | 84.4 | 77.5 | 72.2 | 82.2 | 62.0 | 16.7 | 85.6 | 69.1 | 15.4 | 89.1 |

Random forest (RF), linear discriminant analysis (LDA), k-Nearest Neighbour (k-NN), partial least square (PLS), support vector machine (SVM) radial, linear and polynomial algorithm were applied for classification of Metab generated data and missing values replaced using k-NN algorithm. Results were validated using repeated double cross-validation. Results were expressed using percentage of accuracy (acc), sensitivity (sens) and specificity (spec). Accuracy was defined as the sum of correctly classified samples divided by the total number of samples; sensitivity was defined as correctly positively classified samples divided by the sum of positive samples; specificity was defined as correctly negatively classified samples divided by the sum of negative samples.

**Table 28: Median values of accuracy, sensitivity and specificity across 6 days prior to diagnosis based on Metab data.**

| | Median accuracy % | Median sensitivity % | Median specificity % |
|---|---|---|---|
| RF | 68.1 | 31.4 | 83.9 |
| LDA | 61.4 | 48.9 | 65.6 |
| k-NN | 65.2 | 42.4 | 78.6 |
| PLS | 65.1 | 30.7 | 79.4 |
| SVM Radial | 66.0 | 5.8 | 90.8 |
| SVM Linear | 66.6 | 45.6 | 74.4 |
| SVM Polynomial | 68.3 | 16.3 | 87.4 |

Summary of the modelling results expressed using percentage of median values of accuracy, sensitivity and specificity for each model applied (Random forest (RF), linear discriminant analysis (LDA), k-Nearest Neighbour (k-NN), partial least square (PLS), support vector machine (SVM) radial, linear and polynomial algorithm) for the 6 days prior to diagnosis. Results were validated using repeated double cross-validation. Accuracy was defined as the sum of correctly classified samples divided by the total number of samples; sensitivity was defined as correctly positively classified samples divided by the sum of positive samples; specificity was defined as correctly negatively classified samples divided by the sum of negative samples.

### 3.1.2 Results based on XCMS output

XCMS was an algorithm used to profile the ion mass fragments associated with specific conditions, such as confirmed NEC and healthy controls, and also to identify which of these ion mass fragments were present at significantly different abundances across conditions. This section presents results of the analysis based on XCMS-generated data.

#### 3.1.2.1 Univariate analysis of the intensities of features found in confirmed NEC and healthy control samples to investigate the influence of factors

The abundances of ion mass fragments reported by XCMS were investigated using univariate tools to determine the influence of different factors, in a manner similar to the Metab-based analysis. The *m/z*, or mass to charge ratio, of the ion mass fragments reported by XCMS and their associated retention times could be used to identify the name of the associated compound using the library built in AMDIS.

Abundances for each 356 features identified in 166 healthy controls' and 85 confirmed NEC's samples were compared between the levels for each factors described in Table 17. Table 29 presents the number of compounds with p-values lower than 0.05 when missing values were substituted by either half the lowest value for each feature (across samples from both statuses) or using k-NN (t-test for 2 sub-groups and ANOVA test if more than 2 sub-groups present; with Bonferroni correction applied).

**Table 29: Number of features which showed significant different intensities while confirmed NEC and matched healthy control samples were analysed according to various factors.**

| | Number of features in confirmed NEC samples | | Number of features in healthy control samples | |
|---|---|---|---|---|
| | Half lowest value | k-NN | Half lowest value | k-NN |
| Age at sampling grouped by 10 days | 6 | 6 | 37 | 33 |
| Age at sampling grouped by 5 days | 1 | 1 | 35 | 34 |
| Birth weight | 5 | 3 | 32 | 25 |
| Delivery type | 0 | 0 | 6 | 6 |
| Feeding type | 0 | 0 | 0 | 0 |
| Gender | 0 | 0 | 2 | 2 |
| Gestation duration | 3 | 2 | 0 | 0 |
| Hospital | 0 | 0 | 23 | 22 |
| Total | 15 | 12 | 135 | 122 |

The number of features found by XCMS with intensities having a p-value lower than 0.05 when tested for differences between levels based on 85 confirmed NEC and 166 healthy control samples grouped according to factors. Missing values were replaced by either half the lowest value for each compound or k-NN algorithm (t-test for 2 sub-groups and ANOVA test if more than 2 sub-groups present; with Bonferroni correction applied).

A higher variation within healthy control samples was observed compared to confirmed NEC samples. The age at sampling and the birth weight seemed to be major factors influencing feature intensities for both statuses and the hospital might also have an impact on the compounds intensities in healthy control samples.

Spearman's correlation coefficient showed a significant correlation for two ions at specific retention time. The results were identical for both substitution methods applied on the missing values. A *m/z* of 66 at a retention time of 28.05 minutes was positively correlated with the age at sampling when grouped by 5 days for confirmed NEC samples; and a *m/z* of 85 at a retention time of 16.52 minutes was correlated with the age at sampling when grouped by 5 days for both the healthy controls and the confirmed NEC (Figure 30).

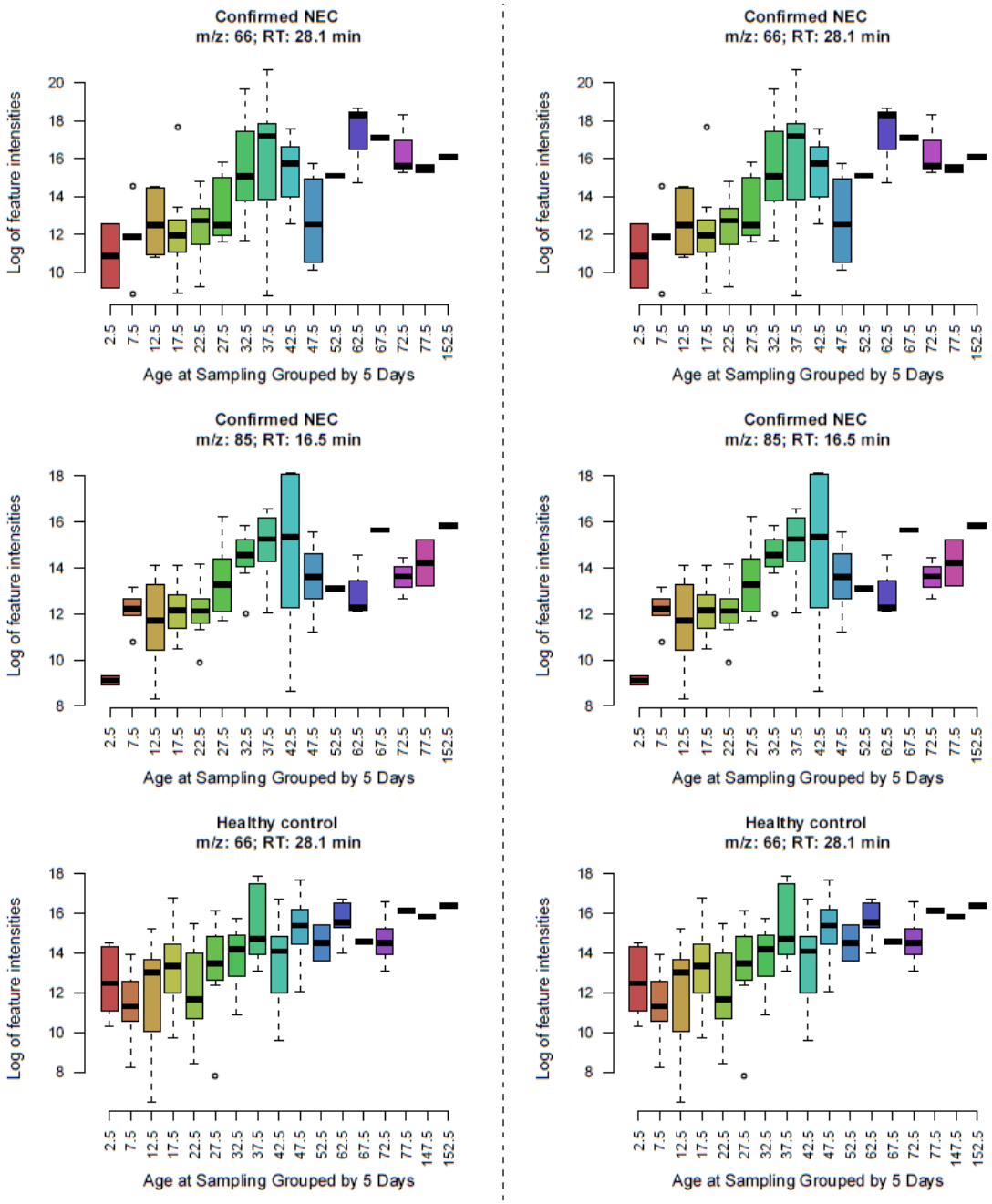


**Figure 30: Features with intensities correlated to the age at sampling.** Boxplot showing features with significant correlation between their abundances and the age at sampling grouped by 5 days. On the left side, missing values were substituted by half the lowest value and on the right side using k-NN. ($p < 0.05$; Spearman *rho* statistics).

#### 3.1.2.2 Univariate analysis of the intensities of features comparing confirmed NEC and matched healthy control samples

In order to compare healthy controls and confirmed NEC samples, the data were divided according to days prior to diagnosis. Each day was investigated separately, grouped by 2 and 3 days and, subsequently, all days were considered as a single dataset.

Table 30 and Table 31 present the day(s) investigated, the potential compound name and the significantly different ions between confirmed NEC and healthy controls. T-test was applied for intensities comparison without Bonferroni correction. Table 30 presents results when missing data were replaced using k-NN algorithm and Table 31 presents results when missing data were replaced using half the lowest value.

The identification of compound name was achieved by matching ions and their retention times to compounds present in the AMDIS library. Only retention times with two ions or more were considered for compound identification. The results were similar for both missing data substitution techniques. Indole and 2-pentenal were significantly different 1 day prior to diagnosis when missing data were replaced with half the lowest value.

In Table 30 and Table 31, the same ion may occur more than once for the same compound if ions were significantly different at multiple retention times.

**Table 30: Comparison of feature intensities found in confirmed NEC and healthy control samples with missing values implemented using k-NN algorithm.**

| Day(s) investigated | Compound (RT [min]) | ions | Number of ions | Greater intensity |
|---|---|---|---|---|
| Day 3 | Ethanol (6.6-6.7) | 13, 15, 15, 19, 24, 26, 27, 31, 33, 41, 42, 43, 44, 45, 45, 46, 47, 47 | 18 | NEC |
| Day 3 | Nonanal (29.4-29.5) | 55, 68, 98, 114 | 4 | NEC |
| Day 4 | Hexanal (18.2-18.3) | 55, 56, 57, 67, 72, 82 | 6 | Control |
| Day 4 | Xylene (20.5-20.6) | 50, 51, 62, 63, 65, 77, 78, 89, 91, 92, 102, 106, 106, 107 | 14 | Control |
| Day 4 | Xylene (21.6-21.7) | 50, 51, 63, 65, 77, 91, 102, 103, 107 | 9 | Control |
| Day 4 | Heptanal (22.2) | 70, 72 | 2 | Control |
| Day 4 | D-limonene (26.0-26.1) | 67, 68 | 2 | Control |
| Day 4 | Nonanal (29.5) | 55, 56 | 2 | Control |
| Day 5 | Ethanol (6.7) | 43, 44 | 2 | NEC |
| Day 5 | Hexanal (18.3) | 41, 55 | 2 | NEC |
| Day 5 | Nonanal (29.4-29.5) | 55, 56, 67, 68, 70, 82, 98 | 7 | NEC |
| Day 6 | 2-methylbutanoic acid (21.5-21.7) | 50, 74 | 2 | Control |
| Day 5 & 6 | Nonanal (29.4-29.5) | 56, 67, 70 | 3 | NEC |
| Day 4, 5 & 6 | Styrene (21.5-21.7) | 39, 74 | 2 | Control |
| All days | Ethanol (6.7) | 27, 30, 30 | 3 | NEC |
| All days | Indole (38.4) | 62, 63, 89, 116 | 4 | NEC |

Summary of significantly different ions at different days prior to diagnosis investigated and their potential identification, with comparison to AMDIS library. Missing values were substituted using k-NN algorithm ($p < 0.05$; t-test).

**Table 31: Comparison of feature intensities found in confirmed NEC and healthy control samples with missing values substituted by half the lowest value.**

| Day(s) investigated | Compound (RT [min]) | Ions [*m/z*] | Number of ions | Greater intensity |
|---|---|---|---|---|
| Day 1 | 2-pentenal (17.0-17.1) | 81, 82 | 2 | Control |
| Day 1 | Indole (38.4) | 62, 116 | 2 | NEC |
| Day 3 | Ethanol (6.6-6.7) | 13, 15, 15, 19, 24, 26, 27, 31, 33, 41, 42, 44, 45, 45, 46, 47, 47 | 17 | NEC |
| Day 3 | Nonanal (29.4-29.5) | 55, 68, 98, 114 | 4 | NEC |
| Day 4 | Hexanal (18.2-18.3) | 55, 56, 57, 67, 72, 82 | 6 | Control |
| Day 4 | Xylene (20.5-20.6) | 50, 51, 62, 63, 65, 77, 78, 89, 91, 92, 102, 106, 106, 107 | 14 | Control |
| Day 4 | Xylene (21.6-21.7) | 50, 51, 63, 65, 77, 91, 102, 103, 107 | 9 | Control |
| Day 4 | Heptanal (22.2) | 70, 72 | 2 | Control |
| Day 4 | D-limonene (26.0-26.1) | 67, 68 | 2 | Control |
| Day 4 | Nonanal (29.5) | 55, 56 | 2 | Control |
| Day 5 | Ethanol (6.7) | 42, 43, 44 | 3 | NEC |
| Day 5 | Hexanal (18.3) | 41, 55, 56 | 3 | NEC |
| Day 5 | Nonanal (29.4-29.5) | 55, 56, 67, 68, 70, 82, 98 | 7 | NEC |
| Day 6 | 2-methylbutanoic acid (21.6-21.7) | 50, 74 | 2 | Control |
| Day 5 & 6 | Nonanal (29.4-29.5) | 56, 67, 70 | 3 | NEC |
| Day 1, 2 & 3 | Ethanol (6.6-6.7) | 19, 47 | 2 | NEC |
| All days | Ethanol (6.7) | 27, 30, 30 | 3 | NEC |
| All days | Indole (38.4) | 58, 62, 63, 89, 116 | 5 | NEC |

Summary of significantly different ions at different days prior to diagnosis investigated and their potential identification, with comparison to AMDIS library. Missing values were substituted using half the lowest value ($p < 0.05$; t-test).

### 3.1.2.3 Comparison of the intensities of features within confirmed NEC and matched healthy control samples

This section aims to evaluate the variability of VOC abundance within statuses.

ANOVA was applied on confirmed NEC data to compare days prior to diagnosis. Six ions allowed the identification of nonanal. Their mass to charge ratios were of 55, 56, 82, 95, 98, 114; at retention times of 29.4-29.5 minutes.

ANOVA was also applied on healthy control data to compare days prior to diagnosis (Table 32). The compound name, deduced by matching ions and their retention times to compounds present in the AMDIS library, is presented as well as the significant ions and the number of significant ions. Only retention times with two ions, or more, were considered for compound identification. The same ion may occur more than once for the same compound if ions were significantly different at multiple retention times.

Missing data were replaced using half the lowest value and applying k-NN algorithm. ANOVA were applied to test differences.

**Table 32: Comparison of feature intensities, found in healthy control samples, during the 6 days prior to diagnosis.**

| | half the lowest value | | k-NN | |
|---|---|---|---|---|
| Compound name (RT [min]) | Ions [*m/z*] | Number of ions | Ions [*m/z*] | Number of ions |
| Ethanol (6.6-6.7) | 13, 14, 15, 15, 19, 24, 25, 25, 26, 26, 27, 29, 29, 30, 30, 31, 33, 41, 42, 43, 44, 45, 45, 47 | 24 | 13, 14, 15, 15, 19, 24, 25, 25, 26, 26, 27, 29, 29, 30, 30, 31, 33, 41, 42, 43, 44, 45, 45, 47, 47 | 25 |
| Isopropanol (7.4-7.7) | 15, 37, 37, 38, 39, 39, 41, 42, 42, 45, 45, 59, 59 | 13 | 15, 37, 37, 38, 39, 39, 41, 42, 42, 45, 45, 59, 59 | 13 |
| Dichloromethane (8.2-8.3) | 49, 71, 84, 84, 88 | 5 | 49, 71, 84, 84, 88 | 5 |
| Cyclopentane (8.3-8.4) | 42, 70 | 2 | 42, 70 | 2 |
| 3-methylbutanal (12.3-12.7) | 15, 29, 41, 57 | 4 | 15, 29, 41, 57 | 4 |

| | | | | |
|---|---|---|---|---|
| 3-methylbutylmethanoate (16.0-16.2) | 38, 39, 48, 49, 50, 51, 52, 52, 53, 75, 76, 78, 80, 89 | 14 | 26, 39, 48, 49, 50, 51, 78, 80, 89 | 9 |
| Propanoic acid (16.1-16.2) | 26, 74, 74 | 3 | 38, 52, 52, 53, 74, 74, 75, 76 | 8 |
| Pyridine (16.2-16.4) | 38, 78, 79, 80 | 4 | 38, 78, 79, 80 | 4 |
| Toluene (16.5-16.6) | 37, 62, 63, 64, 64, 65, 65, 79, 90, 91, 91, 92, 92, 93 | 14 | 37, 62, 63, 64, 64, 65, 65, 79, 90, 91, 91, 92, 92, 93 | 14 |
| Xylene (20.5-20.6) | 39, 50, 53, 62, 63, 64, 76, 78, 89, 102, 107 | 11 | 39, 50, 53, 62, 63, 64, 76, 78, 89, 102, 107 | 11 |
| Xylene (21.6-21.7) | 50, 63, 65, 76, 77, 78, 91, 102, 103, 106, 107 | 11 | 50, 63, 65, 76, 77, 91, 102, 103, 106, 107 | 10 |
| Styrene (21.5-21.7) | 51, 104 | 2 | 39, 51, 74, 78, 104 | 5 |
| Heptanal (22.2-22.3) | 70, 72, 82, 94 | 4 | 70, 72, 82, 94 | 4 |
| 3-methylenecycloheptene (22.6) | 80, 92, 93, 93 | 4 | 80, 92, 93, 93 | 4 |
| 1-butylheptylbenzene (34.9) | 189, 232 | 2 | – | – |
| Indole (38.4-38.5) | 63, 64, 89, 90, 116, 117, 118, 62, 63, 64, 89, 90, 116 | 13 | 58, 58, 62, 63, 63, 64, 64, 89, 89, 90, 90, 116, 116, 117, 118 | 15 |
| Unknown 1 (11.14-11.23) | 47, 82, 83, 83, 85, 87 | 6 | 47, 82, 83, 83, 85, 87 | 6 |
| Unknown 2 (12.44-12.55) | 12, 62, 62, 64, 78 | 5 | 12, 62, 62, 64, 78 | 5 |
| Unknown 3 (25.43-25.54) | 105, 106, 120 | 3 | 105, 106, 120 | 3 |

Potential identifications suggested based on ions which were significantly different. ANOVA test was applied to compare ion intensities between days prior to diagnosis within healthy control samples. Results using both missing data substitution techniques are presented. ($p < 0.05$; ANOVA)

### 3.1.2.4 Modelling VOC profile for sample classification

Classification of samples based on features abundances was performed using different statistical modelling techniques. This section presents in detail every step applied for this process and the results achieved. The approach was similar to classifications performed on Metab data (see 3.1.1.9). However, statistical models were built based on the feature profile reported by XCMS.

Features that best described the differences between confirmed NEC and healthy controls were selected from the 356 features identified in 50% or more of the samples of at least one condition (confirmed NEC or healthy control), in addition to the age at sampling in days, food type, unit, gestation duration in days, birth weight in grams, delivery type and sex. The analysis applied a step-wise linear discriminant analysis and two random forest classification algorithms. This analysis was performed separately for each individual day prior to diagnosis and with missing values replaced using either half the lowest value of each compound across status or k-NN algorithm (Table 33). Features selected varied considerably between days and according to the technique of imputation of missing data. Gestation duration and birth weight were among the most important features to differentiate confirmed NEC and healthy control samples (Table 33).

K-NN and random forest algorithms were applied on the selected features at each day prior to diagnosis. Results were validated using repeated 10-fold cross-validation. Accuracies and Kappa values were calculated for both models based on data with missing values substituted using k-NN algorithm and half the lowest value (Table 34). Results were improved when missing data were replaced with half the lowest value for each compound (Table 34). Therefore, further analyses were performed using this method.

Seven classifiers were applied on the data: RF, LDA, k-NN, PLS, SVM using radial, linear and polynomial kernel algorithms. Accuracies, sensitivities and specificities were calculated at each day prior to diagnosis using classification models with repeated double cross-validation (Table 35). To summarize, the median accuracy, sensitivity and specificity across the 6 days prior to diagnosis, for each model applied on the data were calculated and were presented in the Table 36. LDA, RF and SVM linear algorithm performed fairly similarly with median within 5% of variation.

**Table 33: Features selection for modelling and sample classification based on XCMS data.**

| *m/z* or feature | RT [min] | Half the lowest value | | | | | | | k-NN | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Day:1 | 2 | 3 | 4 | 5 | 6 | sum | 1 | 2 | 3 | 4 | 5 | 6 | sum |
| Gestation duration | | 3 | 0 | 0 | 0 | 2 | 0 | 5 | 3 | 0 | 0 | 0 | 2 | 0 | 5 |
| 176 | 17.0 | 0 | 3 | 0 | 0 | 0 | 0 | 3 | 0 | 2 | 0 | 0 | 0 | 0 | 2 |
| Birth weight | | 0 | 0 | 1 | 2 | 0 | 0 | 3 | 0 | 0 | 1 | 2 | 0 | 0 | 3 |
| 55 | 18.3 | 0 | 0 | 0 | 3 | 0 | 0 | 3 | 0 | 0 | 0 | 3 | 0 | 0 | 3 |
| 44 | 6.7 | 0 | 0 | 0 | 0 | 3 | 0 | 3 | 0 | 0 | 0 | 0 | 3 | 0 | 3 |
| 50 | 16.2 | 2 | 0 | 0 | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 18 | 6.2 | 0 | 0 | 2 | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 24 | 6.7 | 0 | 0 | 2 | 0 | 0 | 0 | 2 | 0 | 0 | 2 | 0 | 0 | 0 | 2 |
| 101 | 6.2 | 0 | 0 | 2 | 0 | 0 | 0 | 2 | 0 | 0 | 2 | 0 | 0 | 0 | 2 |
| 136 | 25.2 | 0 | 0 | 0 | 2 | 0 | 0 | 2 | 0 | 0 | 0 | 2 | 0 | 0 | 2 |
| 39 | 21.5 | 0 | 0 | 0 | 2 | 0 | 0 | 2 | 0 | 0 | 0 | 2 | 0 | 0 | 2 |
| 70 | 22.2 | 0 | 0 | 0 | 2 | 0 | 0 | 2 | 0 | 0 | 0 | 2 | 0 | 0 | 2 |
| 43 | 6.7 | 0 | 0 | 0 | 0 | 2 | 0 | 2 | 0 | 0 | 0 | 0 | 2 | 0 | 2 |
| 192 | 17.2 | 0 | 0 | 0 | 0 | 0 | 2 | 2 | 0 | 0 | 0 | 0 | 0 | 2 | 2 |
| 249 | 23.4 | 0 | 0 | 0 | 0 | 0 | 2 | 2 | 0 | 0 | 0 | 0 | 0 | 3 | 3 |
| 51 | 16.2 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 84 | 8.2 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 281 | 23.4 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 103 | 21.7 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 19 | 7.6 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 20 | 6.2 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 101 | 6.3 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 18 | 6.2 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

| | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 15 | 6.7 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 14 | 6.7 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 81 | 17.0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 235 | 23.3 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 95 | 20.8 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 1 |
| 165 | 17.0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 1 |
| 97 | 17.1 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 1 |
| 16 | 6.2 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 1 |
| 98 | 29.4 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 1 |
| 15 | 6.7 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 1 |
| 82 | 17.1 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 1 |
| 51 | 21.6 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 1 |
| 93 | 25.4 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 1 |
| 102 | 21.7 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 1 |
| 232 | 34.0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 1 |
| 56 | 18.3 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 1 |
| 75 | 23.4 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 1 |
| 63 | 21.6 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 1 |
| 265 | 23.4 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 1 |
| 119 | 17.1 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 1 |
| 116 | 38.4 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 2 | 0 | 0 | 0 | 0 | 0 | 2 |
| 41 | 18.3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 1 |
| 33 | 6.7 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 1 |
| 50 | 21.6 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 |
| 56 | 29.5 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 1 |
| 175 | 33.7 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 1 |
| 84 | 8.2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 1 |

| | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 153 | 7.3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 1 |
| 85 | 16.5 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 1 |
| 66 | 28.1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 1 |
| 250 | 23.2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 1 |
| 19 | 6.7 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 1 |
| 283 | 23.3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 |
| 126 | 23.4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 |
| 221 | 23.3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 1 |
| 43 | 10.4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 2 |
| 13 | 6.7 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 2 |

Table presenting the number of times each feature has been selected at each day prior to diagnosis based on XCMS results when missing data were replaced using either half the lowest value for each features or k-NN algorithm. The mass to charge ratio (*m/z*) and retention time in minutes are presented for features identified. Three feature selection algorithms were applied on the data, a step-wise linear discriminant analysis and two random forest classification algorithm. The 'sum' columns present how many times each feature were selected across all days using each NA's substitution techniques.

**Table 34: Results of samples classification, based on selected features in XCMS data, using two classifiers.**

| Arguments implemented in the model | Missing data substitution techniques | Model tested | lowest accuracy % | mean accuracies [%] (kappa values) Day: 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|---|---|---|
| Selected features | Half the lowest value | k-NN | 69.2 | 69.3 (0.297) | 74.3 (0.364) | 72.6 (0.194) | 82.8 (0.645) | 76.6 (0.471) | 69.2 (0.172) |
| | | RF | 68.8 | 72.8 (0.373) | 80.4 (0.548) | 68.8 (0.188) | 81.8 (0.631) | 72.3 (0.345) | 69.3 (0.179) |
| | k-NN | k-NN | 68.5 | 69.2 (0.269) | 72.8 (0.318) | 70.2 (0.151) | 80.6 (0.581) | 75.8 (0.493) | 68.5 (0.153) |
| | | RF | 66.5 | 74.9 (0.423) | 80.0 (0.53) | 66.5 (0.164) | 81.2 (0.61) | 72.6 (0.36) | 69.6 (0.188) |

K-Nearest Neighbour (k-NN) and random forest (RF) algorithm were applied on XC-MS results with missing data replaced using k-NN and half the lowest value. Selected features, specific for each day, were used (see Table 33). Results were validated using repeated 10-fold cross validation. Accuracy was defined as the sum of correctly classified and rejected samples divided by the total number of samples.

**Table 35: Sample classification at each day prior to diagnosis based on XCMS data.**

| Day prior to diagnosis | 1 | | | 2 | | | 3 | | | 4 | | | 5 | | | 6 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Acc % | Sens % | Spec % | Acc % | Sens % | Spec % | Acc % | Sens % | Spec % | Acc % | Sens % | Spec % | Acc % | Sens % | Spec % | Acc % | Sens % | Spec % |
| RF | 68.7 | 42.9 | 81.6 | 64.6 | 25.6 | 81.6 | 72.1 | 41.7 | 86.2 | 77.5 | 80.4 | 75.0 | 71.5 | 49.2 | 83.1 | 60.4 | 18.3 | 77.7 |
| LDA | 68.8 | 48.1 | 79.1 | 65.5 | 46.8 | 73.7 | 73.7 | 47.5 | 85.9 | 74.4 | 79.4 | 70.0 | 69.8 | 54.6 | 77.7 | 60.0 | 26.3 | 74.3 |
| k-NN | 65.9 | 43.0 | 77.4 | 68.1 | 28.3 | 85.2 | 70.4 | 36.4 | 86.3 | 80.4 | 90.3 | 71.6 | 75.0 | 59.0 | 83.3 | 58.7 | 22.9 | 73.6 |
| PLS | 69.1 | 45.0 | 81.2 | 63.0 | 21.4 | 80.9 | 69.9 | 36.9 | 85.3 | 71.9 | 74.0 | 70.2 | 64.3 | 34.2 | 79.8 | 67.1 | 30.8 | 80.8 |
| SVM Radial | 65.9 | 10.9 | 92.9 | 63.7 | 1.3 | 89.9 | 69.9 | 10.7 | 96.0 | 79.1 | 78.4 | 79.7 | 68.7 | 19.4 | 92.4 | 65.3 | 0.0 | 89.6 |
| SVM Linear | 69.4 | 43.8 | 82.2 | 66.4 | 40.2 | 77.8 | 74.5 | 42.8 | 89.3 | 77.0 | 75.9 | 77.8 | 71.9 | 56.0 | 80.2 | 59.1 | 15.8 | 76.5 |
| SVM Polynomial | 67.5 | 22.4 | 89.8 | 63.1 | 10.0 | 85.7 | 71.8 | 25.7 | 93.0 | 77.3 | 80.3 | 74.7 | 70.8 | 29.6 | 91.3 | 64.9 | 1.3 | 88.5 |

Random forest (RF), linear discriminant analysis (LDA), k-Nearest Neighbour (k-NN), partial least square (PLS), support vector machine (SVM) radial, linear and polynomial algorithm were applied for classification on XCMS data, with missing values replaced using half the lowest value. Results were validated using repeated double cross-validation. Results were expressed using percentage of accuracy (acc), sensitivity (sens) and specificity (spec). Accuracy was defined as the sum of correctly classified samples divided by the total number of samples; sensitivity was defined as correctly positively classified samples divided by the sum of positive samples; specificity was defined as correctly negatively classified samples divided by the sum of negative samples.

**Table 36: Median values of accuracy, sensitivity and specificity across 6 days prior to diagnosis based on XCMS data.**

| | Median accuracy % | Median sensitivity % | Median specificity % |
|---|---|---|---|
| RF | 70.1 | 42.3 | 81.6 |
| LDA | 69.3 | 47.8 | 76.0 |
| k-NN | 69.2 | 39.7 | 80.3 |
| PLS | 68.1 | 35.5 | 80.9 |
| SVM Radial | 67.3 | 10.8 | 91.2 |
| SVM Linear | 70.6 | 43.3 | 79.0 |
| SVM Polynomial | 69.1 | 24.0 | 89.2 |

Summary of the modelling results expressed using median values of percentage of accuracy, sensitivity and specificity for each model applied (Random forest (RF), linear discriminant analysis (LDA), k-Nearest Neighbour (k-NN), partial least square (PLS), support vector machine (SVM) radial, linear and polynomial algorithm) for the 6 days prior to diagnosis. Results were validated using repeated 10-fold cross-validation. Accuracy was defined as the sum of correctly classified samples divided by the total number of samples; sensitivity was defined as correctly positively classified samples divided by the sum of positive samples; specificity was defined as correctly negatively classified samples divided by the sum of negative samples.

### 3.1.3 Summary of the compounds of interest

This section highlights significant compounds identified using Metab and XCMS approaches. Using Metab results, qualitative and quantitative comparison of confirmed NEC and healthy control patients (Table 23) and compounds selected twice or more during sample classification (Table 25) were considered. Using XCMS results, compounds considered were identified based on ions with significantly different intensities between confirmed NEC and healthy control patients (Table 30 and Table 31).

In total four compounds were significantly different while treating data using both pipelines: 2-E-pentenal (RT: 17.1 min), hexanal (RT: 18.2 min), 2-methylbutanoic acid (RT: 21.8 min) and heptanal (RT: 22.3 min).

## 3.2 Sensor technology

The sensor instrument did not allow identification of compounds. Instead, features were reported that indicated a change in the headspace gas/sensor interaction at a specific time point. Therefore, results were expressed exclusively using classification; features were selected and implemented into seven algorithms. This section presents results obtained at each day prior to diagnosis.

Random forest (RF), linear discriminant analysis (LDA), k-Nearest Neighbour (k-NN), partial least square (PLS), support vector machine (SVM) using radial linear and polynomial Kernel algorithms, were applied for classification at each day prior to diagnosis. Table 37 presents results after leave-one-out validation applied. The number of samples available at each day for confirmed NEC and healthy control was presented in Table 14.

SVM polynomial Kernel and SVM radial Kernel algorithm had a minimum of 80% of accuracy, sensitivity and specificity at each day prior to diagnosis (Table 37). In order to summarize Table 37, medians of accuracy, sensitivity and specificity across the 6 days prior to diagnosis were calculated for each algorithm (Table 38). Medians of accuracy, sensitivity and specificity were higher than 89% for Support vector machine polynomial kernel algorithm.

Figure 31 illustrates these results using PCAs based on features selected at each day prior to diagnosis. The discrimination between healthy controls and confirmed NEC samples was relatively high with few overlapping samples for days 1, 3 and 5 prior to diagnosis. It reflected the high accuracy, sensitivity and specificity reported by the classification algorithms presented in Table 37.

**Table 37: Sample classification at each day prior to diagnosis based on Sensor data.**

| Day prior to diagnosis | 1 | | | 2 | | | 3 | | | 4 | | | 5 | | | 6 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Acc % | Sens % | Spec % | Acc % | Sens % | Spec % | Acc % | Sens % | Spec % | Acc % | Sens % | Spec % | Acc % | Sens % | Spec % | Acc % | Sens % | Spec % |
| k-NN | 88.6 | 70.0 | 94.1 | 96.3 | 88.9 | 100.0 | 86.5 | 73.3 | 91.9 | 100.0 | 100.0 | 100.0 | 92.3 | 92.9 | 92.0 | 95.5 | 83.3 | 100.0 |
| PLS | 93.2 | 70.0 | 100.0 | 88.9 | 77.8 | 94.4 | 86.5 | 60.0 | 97.3 | 100.0 | 100.0 | 100.0 | 89.7 | 85.7 | 92.0 | 100.0 | 100.0 | 100.0 |
| RF | 86.4 | 40.0 | 100.0 | 92.6 | 77.8 | 100.0 | 86.5 | 53.3 | 100.0 | 91.3 | 75.0 | 100.0 | 89.7 | 78.6 | 96.0 | 86.4 | 50.0 | 100.0 |
| LDA | 90.9 | 80.0 | 94.1 | 92.6 | 88.9 | 94.4 | 84.6 | 73.3 | 89.2 | 65.2 | 50.0 | 73.3 | 87.2 | 92.9 | 84.0 | 54.5 | 50.0 | 56.3 |
| SVM Radial | 95.5 | 90.0 | 97.1 | 100.0 | 100.0 | 100.0 | 86.5 | 86.7 | 86.5 | 95.7 | 100.0 | 93.3 | 84.6 | 92.9 | 80.0 | 95.5 | 100.0 | 93.8 |
| SVM Linear | 88.6 | 80.0 | 91.2 | 100.0 | 100.0 | 100.0 | 88.5 | 66.7 | 97.3 | 91.3 | 87.5 | 93.3 | 84.6 | 85.7 | 84.0 | 95.5 | 83.3 | 100.0 |
| SVM Polynomial | 95.5 | 90.0 | 97.1 | 96.3 | 100.0 | 94.4 | 86.5 | 80.0 | 89.2 | 91.3 | 100.0 | 86.7 | 89.7 | 92.9 | 88.0 | 95.5 | 100.0 | 93.8 |

Random forest (RF), linear discriminant analysis (LDA), k-Nearest Neighbour (k-NN), partial least square (PLS), support vector machine (SVM) radial, linear and polynomial algorithm were applied for classification of Sensor data. Results were validated using leave-on-out validation. Results were expressed using percentage of accuracy (acc), sensitivity (sens) and specificity (spec). Accuracy was defined as the sum of correctly classified samples divided by the total number of samples; sensitivity was defined as correctly positively classified samples divided by the sum of positive samples; specificity was defined as correctly negatively classified samples divided by the sum of negative samples.

**Table 38: Median values of accuracy, sensitivity and specificity across 6 days prior to diagnosis based on Sensor data.**

| | Median accuracy % | Median sensitivity % | Median specificity % |
|---|---|---|---|
| k-NN | 93.9 | 86.1 | 97.1 |
| PLS | 91.5 | 81.7 | 98.6 |
| RF | 88.1 | 64.2 | 100.0 |
| LDA | 85.9 | 76.7 | 86.6 |
| SVM Radial | 95.5 | 96.4 | 93.5 |
| SVM Linear | 90.0 | 84.5 | 95.3 |
| SVM Polynomial | 93.4 | 96.4 | 91.5 |

Summary of the modelling results expressed using median values of percentage of accuracy, sensitivity and specificity for the 6 days prior to diagnosis and for each model applied (random forest (RF), linear discriminant analysis (LDA), k-Nearest Neighbour (k-NN), partial least square (PLS), support vector machine (SVM) radial, linear and polynomial algorithm). Results were validated using leave-on-out validation. Accuracy was defined as the sum of correctly classified samples divided by the total number of samples; sensitivity was defined as correctly positively classified samples divided by the sum of positive samples; specificity was defined as correctly negatively classified samples divided by the sum of negative samples.

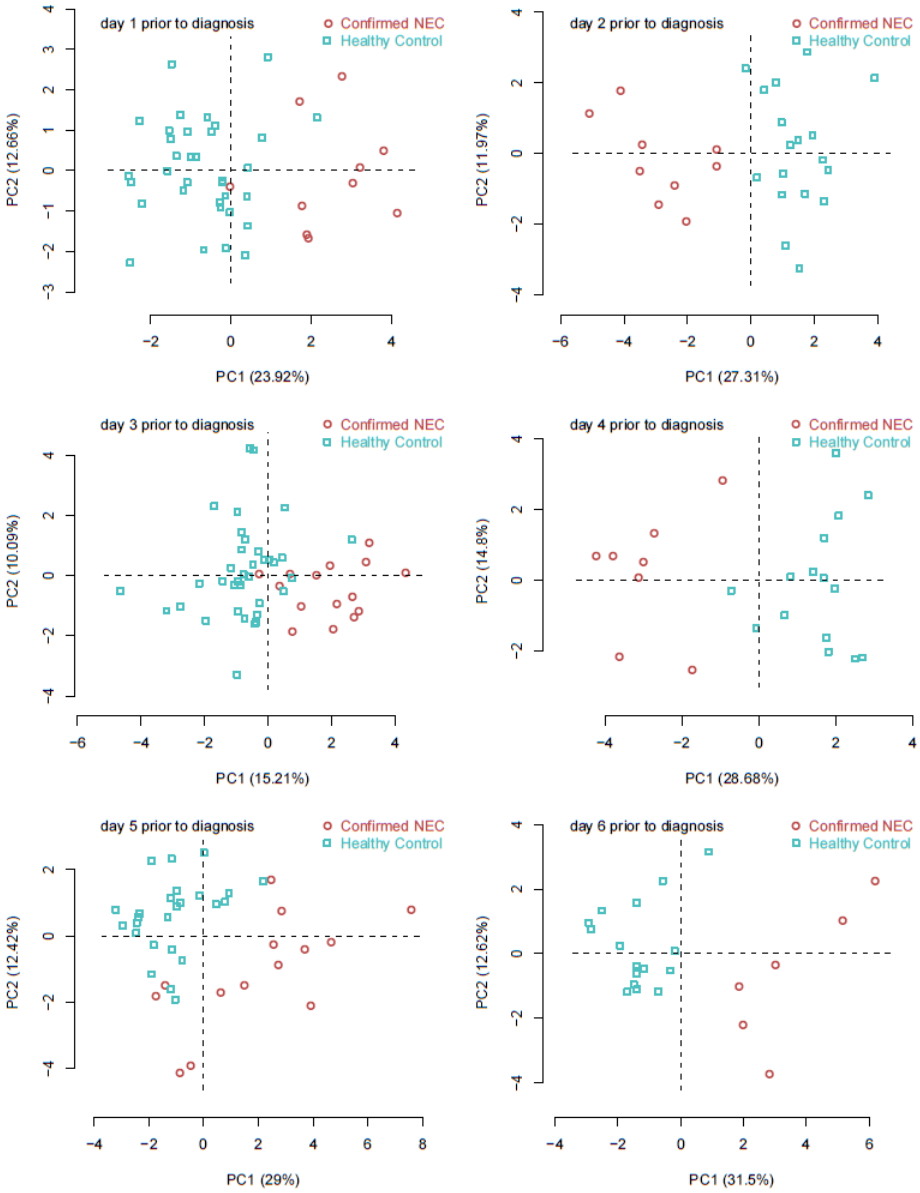


**Figure 31: Principal component analysis (PCA) of selected features from sensor data.** This figure shows the PCAs applied to the features selected at each day prior to diagnosis from sensor data used to build classifier.

# Chapter 4.

# Discussion

Over 14,000 samples were collected from 1326 premature babies over 27 months with the expectation that some of the babies will have developed NEC. Sixty-four babies developed confirmed NEC. The aspiration was to build diagnostic models based on faecal headspace gases. Premature babies do not defaecate daily and when they do the volume of stool is extremely small. A total of 30 on 64 confirmed NEC patients had to be discarded due to lack of sample in six days prior to diagnosis: this arose because babies were treated elsewhere before being sent to a study hospital with the diagnosis of NEC. From over 14,000 samples, only 251 samples could be used to study NEC. In order to search for potential trends related specifically to healthy neonates, another 163 samples from healthy neonates were added to 166 samples from matched healthy controls samples to investigated trends in healthy neonates.

## 4.1 Mass spectrometry

Two pipelines were applied to analyse the data. The first involved the use of the AMDIS software in conjunction with the NIST library and the Metab package[69] and the second applied XCMS package[78], [88]. Their simultaneous application might allow a better coverage of the data.

Faeces from preterm infants in early days of life were expected to show fewer volatile organic compounds (VOCs) than adult samples. A median of 101 compounds was previously reported from adult faecal samples.[47] When considering 329 samples from healthy preterm patients, a median of 62 VOCs per sample was identified (3.1.1.1). This lower number of identified VOCs might be due to the relatively simple diet of neonates compared to adults[27] and a less complex gut microbiota.[6] The gut microbiota of adults is diverse and contains at least one hundred sixty bacteria species[115] up to several hundred bacteria species,[116] where neonate microbiota is limited, with as few as 1 to 8 major bacterial species.[6] A median of 62 compounds and 63 compounds were identified in 166 matched healthy control samples and 85 confirmed NEC samples, respectively (3.1.1.2). At each day prior to diagnosis the numbers of VOCs identified was similar (Figure 23). This demonstrated the relative constancy of compounds emitted from confirmed NEC and matched healthy preterm faecal samples. However, the interquartile ranges were of 22 and 25 compounds for matched healthy control samples and confirmed NEC samples, respectively. These values suggest some variability in the number of compounds per sample.

During the factors investigation, if the number of samples considered from healthy control patients varied the results were varied as well. The effect of feeding type and the age at sampling influenced the number of VOCs identified in 329 healthy control samples (Table 18). However, when only 166 samples were considered, the gender was reported as a significant factor (Figure 21 and Figure 22). This discordance, when reducing the number of samples, illustrates the variability of this dataset. It might be a result of the multiple factors involved in the study (eg: delivery type, different hospitals).

Compounds were grouped by families, based on their functional groups, and the prevalence of these families was compared (3.1.1.3). Compounds families were found to be similar between confirmed NEC and matched healthy control samples. This observation did not confirm that previously observed by Garner *et al.* who found dissimilarities in the number of esters between NEC and control samples.[44] Differences in findings between studies probably arose as the number of patients and samples considered differed greatly. Several classes of compounds were determined; however the vast majority of identified compounds belonged to 6 classes: aldehydes, ketones, alkanes, esters, alcohols and carboxylic acids. Except for carboxylic acids, results were in agreement with a previous study of neonate faeces.[27]

The prevalence of compounds was also investigated. Sixteen compounds occurred in at least 80% of the 85 samples from confirmed NEC patients (Table 20). A few compounds were potential contaminants; ethanol and 1-propanol were measured in disinfectant, acetone in lab air and 2,2,4,6,6-pentamethylheptane in plastic tubing (see Appendix A). Table 39 presents the 16 common compounds and reports their occurrence in other body-fluids.[117] Only 2,2,4,6,6-pentamethylheptane has not been previously observed in body-fluids which suggests that it was likely to be a contaminant.

**Table 39: Summary of the most prevalent compounds identified in confirmed NEC samples and correlation with literature.**

| RT [min] | Compound names | Observed in human body | RT [min] | Compound names | Observed in human body |
|---|---|---|---|---|---|
| 6.6 | Ethanol | Yes | 18.4 | Hexanal | F |
| 7.2 | Propanal | F | 20.7 | Xylene | F |
| 7.4 | Acetone | F | 22.3 | Heptanal | F |
| 9.0 | 2-methylpropanal | F | 24.3 | 2,2,4,6,6-pentamethylheptane | No |
| 9.6 | 1-propanol | F | 24.8 | 2-pentylfuran | F |
| 10.3 | 2,3-butanedione | F | 25.7 | Benzaldehyde | F |
| 12.7 | Acetic acid | F | 25.8 | 2,2,4,4-tetramethyloctane | Yes |
| 16.2 | Propanoic acid | F | 29.5 | Nonanal | F |

Presentation of the 16 most prevalent compounds identified in confirmed NEC samples along with an annotation if they have already been observed in human body-fluids (Yes / No) and, further, in faeces (F). Molecules were matched against the list built by De Lacy Costello *et al.*[117]

Seventeen compounds were shared by 80% or more of the 251 samples from confirmed NEC and matched healthy controls, which is considerably less than the 44 VOCs shared by 80% of 151 samples in adult cohort.[47] It might be explained by the origin of VOCs. Faecal VOCs may come mainly from the gut microbiota and it has been suggested that early microbiota has a high inter-individual diversity.[6], [43], [118] Further, this inconsistency of compounds between samples supports a concept suggested by Van de Kant *et al.* to investigate pattern, and not single compound, differences.[37]

The influence of demographic and environmental factors (see Table 17 for details) was investigated on the number of compounds identified and on intensity of compounds and features. This influence was of interest to clarify the data in case the factors have to be implemented in the data analysis. When the number of compounds identified in 329 samples from healthy controls patients was considered, there was a significant difference with the donor's age at sampling and their feeding type (Table 18). When 166 samples from matched controls were considered, only gender had a significant effect on the number of compounds identified (Figure 21 and Figure 22). However, age at sampling, birth weight, neonatal unit and gestation duration had a significant effect on the intensities of compounds identified in 329 healthy control samples (Table 21). Excepting gestation duration, these factors significantly influenced compound intensities in matched healthy control samples, while only age at sampling had an influence on compound intensities identified in confirmed NEC samples (Table 22). Similar results were obtained when considering the influence of factors on features intensities identified using XCMS (Table 29). Age at sampling, birth weight and neonatal unit were the main factors influencing feature intensities in matched healthy control samples, while age at sampling and birth weight had an influence on feature intensities identified in confirmed NEC samples. In the light of these observations, age at sampling had an influence on the number of compounds identified in healthy controls and on the intensity of compounds and features identified in samples from both statuses. Meanwhile, birth weight and neonatal unit had a moderate impact on compound and feature intensities.

Considering the factor influencing the results, the criteria presented in the Table 11 could be optimized if another project was to take place. The birth weight and the unit may be scored as 10 where the delivery type may be scored as 6. The birth weight calculation could be: 10 if $0 \leq i \leq 75$; 6 if $76 \leq i \leq 150$; 3 if $151 \leq i \leq 225$; 0 if $i > 226$.

When comparing compounds intensities to factors, the total number of compounds showing significantly different intensities in confirmed NEC and matched healthy control samples was different (Table 22). Four compounds had different intensities in confirmed NEC samples and more than 20 compounds had different intensities with respect to matched healthy control samples. The same observation was seen with features identified using XCMS, where in total up to 10 times more features were significantly different in matched healthy control samples compared to confirmed NEC samples (Table 29).

Days prior to diagnosis were compared using XCMS features identified in samples from confirmed NEC patients and only nonanal was significantly different. Moreover, while doing the same comparison using samples from matched healthy controls, 19 compounds identified were significantly different (3.1.2.3). These observations suggested a greater variability within healthy control samples than within confirmed NEC samples. It might be a result of a less diverse microbiota in ill babies.

While investigating days individually or grouped together, several compounds, identified using Metab package, had different concentration at a level of significance of 1% (Table 23). Moreover, 2-E-hexenal, 2-E-pentenal and 2,2,6-trimethylcyclohexanone had different concentration at a level of significance of 5% after application of Bonferroni correction for multiple comparisons (Figure 27). 2-E-hexenal and 2-E-pentenal had already been detected in faeces[27], [47], while 2,2,6-trimethylcyclohexanone had not. The latter was mostly absent from confirmed NEC samples explaining the boxplot being a single line at a low abundance (half the lowest concentration of that compound across samples). It is noteworthy that no compounds intensities were seen to be significantly different between classes, after application of Bonferroni correction, when missing data were replaced using k-NN algorithm. This is likely to be due to the bigger noise in healthy control samples, the high variability due to multiple factors and the nature of the imputation algorithm.

Considering results from XCMS package, several features had different intensities between confirmed NEC and healthy control samples at a level of significance of 5% when days were considered individually or grouped together (Table 30 and Table 31). Moreover, when more than two ions had similar retention times, they were matched to the library built in AMDIS to allow potential identification. Ethanol, hexanal, heptanal, nonanal, two isomers of xylene, D-limonene, 2-methylbutanoic acid and indole were observed using both missing data imputation techniques. Except for the two isomers of xylene, each compound has previously been identified in faeces and listed in the Human Metabolome Database.[119]–[121]

Compounds of interest identified using Metab and XCMS packages were 2-E-pentenal, 2-methylbutanoic acid, hexanal and heptanal. Furthermore, these four compounds were not listed as potential contaminants in the Appendix A and have already been observed in the human body.[121] 2-methylbutanoic acid, also called 2-methylbutyric acid, is a short-chain fatty acid. It has already been identified in adult faeces[47], [122], [123] and faecal samples[124]. It has been postulated that its origin is derived from branch-chain amino acid

metabolic pathways.[124] Hexanal, heptanal and 2-E-pentenal are aldehydes. Hexanal has been observed in adult[123] and child[125] faeces. It was postulated that its origin might be dietary, as it was found in carrots and potato tubers, however, the source in preterm infants must be different. Heptanal has been observed in adult faeces[47], [62], [125] and neonate faeces[27] as well. It has been positively associated with inflammatory bowel disease when compared with irritable bowel syndrome. It has also been identified in high abundance in Crohn's disease patients samples[84],and iIts prevalence significantly decreased in samples from patients diagnosed with non-alcoholic fatty liver disease.[123] 2-E-pentenal was observed in neonate faeces by De Lacy Costello *et al.*[27]

Modelling algorithms were applied on results obtained with Metab and XCMS packages. Following a feature selection step (see Table 25 and Table 33) and preliminary results (see Table 26 and Table 34), seven classifiers were applied on two sets of data. When using Metab, missing values were replaced using k-NN algorithm. When using XCMS, missing values replaced using half the lowest value of each compounds. Samples from four days prior to diagnosis delivered the greatest accuracy, sensitivity and specificity values with both sets of data (Table 27 and Table 35). This is likely to be due to the number of samples from confirmed NEC and matched healthy controls being very similar (Table 16). Nevertheless, accuracy, sensitivity and specificity as great as 74.2%, 62.9% and 79.8% were obtained one day prior to diagnosis with LDA based on Metab data. Furthermore, median accuracy, sensitivity and specificity across 6 days were of 66.6%, 45.6% and 74.5%, respectively, using SVM linear kernel algorithm based on the Metab data and of 69.3%, 47.8% and 76%, respectively, using LDA based on XCMS data (Table 28 and Table 36).

An external statistician performed a backwards stepwise logistic regression on categorical data from Metab (see Appendix G). With this type of algorithm, the same model will be applied at each day prior to diagnosis. This might be an advantage for patient classification in comparison to algorithm such as PLS. ROC curve values were given for each day prior to diagnosis and when all days were considered together. No-cross validation was performed. Areas under the ROC curve were greater than 75%, demonstrating accuracy higher than 80% when all days were considered together and between 79.2% and 89.5% for each day. Accuracies obtained were greater than with other method that used seven classifiers. However, no cross-validation was performed and this might have reduced these accuracies. Nevertheless, this external analysis tended to confirm the findings presented in this thesis.

Healthy control samples had a greater variation than confirmed NEC samples when intensities of compounds and features were considered. However, the number of VOCs identified and families of compounds were similar between confirmed NEC and healthy control samples. Moreover, few compound intensities showed strong differences between classes, as only 3 compounds were different when Bonferroni correction was applied. These observations suggested that confirmed NEC samples have a lower variability than healthy control samples and that the range of intensities in confirmed NEC samples might be contained in the range of healthy control samples. Although the number of healthy control samples, being almost twice the number of confirmed NEC samples (166 and 85, respectively), might be a reason for this behaviour, the nature of the samples might also influence the VOCs emitted.

No other studies using GC–MS technology and investigating VOCs were found in the literature considering early diagnosis of NEC. Current research for NEC biomarkers obtained sensitivity and specificity as high as 93% and 90%, respectively, using urinary protein.[126] Furthermore, faecal calprotectin delivered sensitivity and specificity of 86% and 93%, respectively. However, faecal calprotectin levels did not change before abdominal signs and was therefore not applicable for early diagnosis.[126] Breath hydrogen has been tested for the differentiation between healthy controls and NEC patients. Sensitivity and specificity were relevant but the test generated too many false positive responses to be of interest.[127]

Despite a relatively high variability within samples considering the number of compounds and their intensities, promising results were obtained with classification of samples with median accuracy, sensitivity and specificity as great as 69.3%, 47.8% and 76%, when considering the six days prior to diagnosis.

## 4.2 Sensor technology

Results considering the sensor technology were straightforward as compounds identification could not be performed using this analytical platform. Following a feature selection step, seven classifiers were applied on the data (Table 37). Leave-on-out cross validation applied on sensor data was less robust than the validation applied on mass spectrometric data. The number of samples available for the GC–Sensor investigation explained this modification in data analysis compared to HS–SPME–GC–MS data investigation. As few as 6 confirmed NEC samples were available six days prior to diagnosis (Table 14). Two reasons explained this fact. First, the project placed a higher priority on mass spectrometric results, as more information was obtained from these results; secondly, the amount of sample available was small, see chapter 2.2.2.

SVM radial kernel algorithm delivered the best results with medians accuracy, sensitivity and specificity across 6 days prior to diagnosis of 95.5%, 96.4% and 93.5%, respectively (Table 38). PCA plots demonstrated the separation between confirmed NEC and healthy control samples (Figure 31).

A recently published study of faecal analysis using e-nose allowed sensitivity and specificity of 88.9% when samples collected a day before the diagnosis and the day of diagnosis were analysed.[45] Moreover, when samples of the same time window were compared with samples from patients suffering from sepsis, sensitivity and specificity were of 88.9% and 56.5%, respectively.[45] This study had a similar goal as this thesis. Pattern analysis was applied on data collected, as presented in 1.2.3. Their results were similar to what we obtained; this confirmed the potential usefulness of VOCs for an early detection of NEC using faecal samples. Further, similarities in the methodology of both studies allowed us to consider their study as an external validation.

# Chapter 5.

# Conclusion

In this thesis, VOCs emitted from faeces of neonates were investigated as potential biomarkers for NEC. We hypothesised that VOC patterns in healthy control samples might differ from patterns found in patients with NEC. Mass spectrometric results did not allow clear discrimination of the samples with both the sensitivity and accuracy remaining relatively low for clinical testing. However, results obtained using the sensor technology platform were similar to biomarkers found in the literature. Despite a lower number of patients being investigated using the sensor technology, the VOC patterns found were able to clearly discriminate confirmed NEC from healthy control samples. More importantly, this technology showed potential to diagnose the disease from 2 to 5 days prior to diagnosis, which may allow great improvement in the treatment of this disease. Our results agree with results reported previously[45]. One of the novelties of this project was the simultaneous application of two analytical platforms, which considerably enriched the information obtained from faecal samples. In order to develop an effective diagnostic tool, it will need to be, ideally, applicable worldwide. The next step will be the validation of our finding in a multinational study. Similarities in the results obtained from different devices will need to be certified and it might prove useful to reduce the instrument size and to simplify its utilization for medical staff.

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# Appendix A.

# Potential contaminants list

**Table 40: Potential contaminants identified in other studies.**

| Name | CAS number | Origin | References |
|---|---|---|---|
| Ethanol | 64-17-5 | From disinfectant | [54] |
| 1-propanol | 71-23-8 | From disinfectant | [54] |
| Propan-2-ol | 67-63-0 | Skin disinfectants | [53], [54] |
| 3-hydroxy-2-butanone | 513-86-0 | Found in lab air | [54] |
| 3-pentanone | 96-22-0 | Found in lab air | [54] |
| Acetone | 67-64-1 | Found in lab air | [54] |
| 2-ethyl-1-hexanol | 104-76-7 | Likely from plastic | [27] |
| Bis (2-ethylhexyl) phthalate | 117-81-7 | Likely from plastic | [27] |
| 2,2,4,6,6-pentamethylheptane | 13475-82-6 | Likely from plastic tube; EVA, PP, PVC | [52], [53] |
| 1,2-diphenlycyclobutane | 3018-21-1 | Plastic (ABS, PS) | [52] |
| 4-phenylcyclohexene | 4994-16-5 | Plastic (ABS) | [52] |
| Cis-1,2-diphenlycyclobutane | 7694-30-6 | Plastic (ABS, ABS/PVC alloy) | [52] |
| 1-dodecanol | 112-53-8 | PVC | [52] |
| 1-hexadecanol | 36653-82-4 | PVC | [52] |
| 1-nonanol | 143-08-8 | PVC | [52] |
| 1-tetradecanol | 112-72-1 | PVC, SEBS | [52] |
| 2-ethylhexanol | 104-76-7 | ABS, EVA, PGA, PVC, PVC/NBR | [52] |
| 4-nonylphenol | 104-40-5 | ABS, PIPMA, PVC, SBR | [52] |
| 2,6-di-tert-butyl-p-cresol | 128-37-0 | ABS, NR, EVA, PE, PPEAA, PP, PS, PUR, PVC, SBR | [52] |
| 9,12-octadecadienoic acid | 60-33-3 | NR, PE | [52] |
| (9E) 9-octadecenoic acid | 112-79-8 | PVC | [52] |
| (9Z) 9-octadecenoic acid | 112-80-1 | ABS, NR, PVC | [52] |
| Dodecanoic acid | 143-07-7 | ABS, NR, PVC, PE | [52] |
| Hexadecanoic acid | 57-10-3 | ABS, ABS/PVC alloy, NR, PE, PS, PVC | [52] |
| Hexadecanoic acid butyl ester | 111-06-8 | PVC | [52] |
| Methyl 7-octadecenoate | 28010-28-8 | ABS | [52] |
| Octadecanoic acid | 57-11-4 | ABS, NR, PE | [52] |
| Octadecanoic acid butyl ester | 123-95-5 | PVC | [52] |
| 1-heptadecene | 6765-39-5 | PVC | [52] |
| 1-nonadecene | 18435-45-5 | ABS, PE, PVC | [52] |
| 1-octadecene | 112-88-9 | ABS, NR, PE, PVC | [52] |
| 2,6,10,14-tetramethylhexadecane | 638-36-8 | PE, PP, PVC | [52] |
| 2,6,11-trimethyldodecane | 31295-56-4 | PP, SEBS | [52] |
| 3,7-dimethyldecane | 17312-54-8 | PP, SEBS | [52] |
| E-5-eicosene | 74685-30-6 | NR, PE, PVC | [52] |
| Cyclododecane | 294-62-2 | ABS, PIPMA, PVC | [52] |
| Cyclohexadecane | 295-65-8 | ABS, ABS/PVC alloy, PE, PVC | [52] |
| Cyclotetradecane | 295-17-0 | PPEAA, PUR, PVC | [52] |
| N-docosane | 629-97-0 | ABS, PE, PP, PS, PVC, SBR, SEBS | [52] |
| Octane | 17252-77-6 | | [21] |
| Decane | 124-18-5 | | [21] |

| | | | |
|---|---|---|---|
| Undecane | 1120-21-4 | | [21] |
| N-dodecane | 112-40-3 | ABS, PVC, PP, SEBS | [52] |
| N-eicosane | 112-95-8 | ABS, PE, PP, PS, PVC | [52] |
| N-heneicosane | 629-94-7 | PE, PP, PVC, SEBS | [52] |
| N-heptacosane | 593-49-7 | PP | [52] |
| N-heptadecane | 629-79-7 | ABS, NR, EVA, PE, PP, PS, PUR, PVC | [52] |
| N-hexadecane | 544-76-3 | NR, EVA, PE, PP, PUR, PVC | [52] |
| N-nonadecane | 629-92-5 | EVA, PE, PP, PVC | [52] |
| N-octadecane | 593-45-3 | EVA, PE, PP, PS, PVC, SEBS | [52] |
| N-pentadecane | 629-62-9 | NR, EVA, PE, PIPMA, PP, PS, PUR, PVC, SEBS | [52] |
| N-tetracosane | 646-31-1 | PE, PP, PS, PVC | [52] |
| N-tetradecane | 629-59-4 | NR, PE, PIPMA, PP, PS, PUR, PVC, SBR, SEBS | [52] |
| Squalene | 7683-64-9 | EVA, PE, PS, SBR | [52] |
| 2-ethylhexanoic acid | 149-57-5 | PIPMA, PGA, PVC | [52] |
| Adipic acid | 124-04-9 | PVC | [52] |
| Benzoic acid | 65-85-0 | PVC | [52] |
| Phenol | 108-95-2 | ABS, ABS/PVC alloy, PVC | [52] |
| P-tert-butyl benzoic acid | 98-73-7 | PVC | [52] |
| 2,2,4-trimethyl-3-carboxyisopropyl pentanoic acid isobutyl ester | - | PIPMA, PVC | [52] |
| 2-ethyl hexanoic acid butyl ester | 68443-63-0 | PIPMA, PVC | [52] |
| 2-ethyl hexanoic acid dodecyl ester | - | PVC | [52] |
| Butyl benzoate | 136-60-7 | PIPMA, PVC | [52] |
| Diethyl phtalate | 84-66-2 | NR, PUR | [52] |
| Diisooctyl adipate | 1330-86-5 | ABS, PVC | [52] |
| 1,3-diphenyl-1,3-propanedione | 120-46-7 | PVC | [52] |
| Benzophenone | 119-61-9 | NR, PE, PIPMA, PVC, SBR | [52] |
| 2-phenyl-2-butna | 617-94-7 | ABS, ABS/PVC alloy, SEBS | [52] |
| Acetophenone | 98-86-2 | ABS | [52] |
| Dibenzylamine | 103-49-1 | NR | [52] |
| Di(2-ethylhexyl) ether | 10143-60-9 | PVC | [52] |
| E-15-heptadecenal | - | ABS, PVC | [52] |
| 2-ethylhexanoic acid (2-EHA) | 149-57-5 | PIPMA, PGA, PVC | [52] |
| Adipic acid | 124-04-9 | PVC | [52] |
| Benzoic acid | 65-85-0 | PVC | [52] |
| Phenol | 108-95-2 | ABS, ABS/PVC alloy, PVC | [52] |
| P-tert-butyl benzoic acid | 98-73-7 | PVC | [52] |

Presentation of the potential contaminants names, CAS number, suggested origin and references. Acronyms: acrylonitrile butadiene styrene (ABS), ethylene vinylacetate (EVA), natural rubber (NR), polyethylene (PE), polyglycolide (PGA), polyisopropyl methylacrylate (PIPMA), polypropylene (PP), poly(propylene - ethylene - acrylic acid) (PPEAA), polystyrene (PS), polyurethane (PUR), polyvinylchloride (PVC), nitrile butadiene rubber (NBR), styrene - butadiene rubber (SBR), styrene - ethylene - butadiene - styrene co-polymer (SEBS) We constructed the table.

# Appendix B.

# Publication of the method development

Analytical & Bioanalytical Techniques

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Research Article Open Access

# Optimisation of Sample Preparation for Direct SPME-GC-MS Analysis of Murine and Human Faecal Volatile Organic Compounds for Metabolomic Studies

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## Abstract

The analysis of volatile organic compounds (VOCs) emitted from biological fluids such as blood, urine, breath and faeces, has been receiving more attention from researchers and clinicians due to their contribution to the metabolome and potential use as diagnostic tools in clinical settings. The faecal metabolome represents the final product of a complex interaction involving the gut microbiota and cell metabolism and an accurate measurement of the faecal volatile organic metabolome enables a better understanding of disease-related metabolic pathways and, eventually, identifying potential biomarkers. However, there is a lack of published evidence evaluating the sample preparation steps for faecal metabolome analysis and no well-defined protocol has been established. Consequently, different research groups employ diverse methodologies, which, ultimately, prevent comparison of results between laboratories.

We evaluated different aspects of sample preparation when processing murine and human faecal samples through a pipeline involving solid phase micro-extraction (SPME) coupled to gas chromatography-mass spectrometry (GC-MS). We identified the sample volume, the SPME fibre coating, the extraction conditions and the vial volume that produce the most accurate and reproducible results. Finally, we propose an optimized method for the direct SPME-GC-MS analysis of VOCs in murine and human faecal samples.

To the best of our knowledge, this is the first work evaluating different aspects of sample preparation for direct SPME-GC-MS analysis of VOCs and the first method proposed for the analysis of murine and human faecal samples. In addition, our proposed method can be coupled to the Automated Mass Spectral Deconvolution and Identification System (AMDIS) and the R software package, Metab, in order to produce results in a reliable and high-throughput manner.

**Keywords:** Volatile organic compounds (VOCs); Gas chromatography-mass spectrometry (GC-MS); Solid phase micro-extraction (SPME); Faeces

## Introduction

Metabolomics is considered one of the newest *omics* technologies and has grown rapidly in the last 10 years. It involves screening a large number of low molecular mass metabolites (<1.5Kd) present in biological samples [1,2]. Metabolomic techniques have been largely and successfully applied in the identification of biomarkers and the dissection of metabolic pathways associated with specific diseases [3]. More recently, a number of studies have emphasized the role of volatile organic compounds (VOCs) in identifying disease-specific metabolome patterns [4-8].

VOCs are a large and diverse group of carbon-based molecules whose accurate identification has the potential to revolutionize patient care. Most vapours emitted from biological samples (e.g. breath, sweat, blood, urine and faeces) contain VOCs which may have a potential link to specific diseases [9]. For example, 3-methylhexane, decane, caryophyllene naphthalene have been detected at significantly lower levels in the breath of breast cancer patients, while lauric acid and palmitic acid have been detected at high levels in biopsied tissue from melanoma patients [10,11]. In addition, VOCs analysis of urine, breath or faeces is non-invasive and can be performed at a low cost, which makes it suitable for use as a screening tool in clinical diagnosis and in monitoring the efficacy of therapies [12].

The faecal metabolome is a result of the complex interaction between the intestinal microbiome and the cell metabolism [13]. Therefore, the analysis of VOCs present in faecal samples is of particular relevance when dealing with gastrointestinal (GI) diseases. Healthcare professionals commonly observe unusual smells from the faeces of patients suffering from GI diseases such as celiac disease, Crohn's disease, chronic pancreatitis or intestinal infections [14]. Therefore, there is an increasing use of faecal metabolomics to investigate both the pathophysiology of GI diseases and the role of the intestinal microbiota in the metabolism in states of health and disease.

The analysis of VOCs relies on an accurate and reliable extraction of metabolites from the biological sample [15]. A solid phase micro-extraction (SPME) fibre is a solvent-free extraction technique ideally suited to metabolite extraction, as it minimises contact with possible

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infectious agents from blood, stool and urine samples [16]. SPME can be coupled to high-performance liquid chromatography (HPLC), inductively coupled plasma (ICP) and gas chromatography-mass spectrometry (GC-MS), however, SPME-GC-MS is considered one of the most popular methods for the analysis of VOCs emitted from faecal samples [16,17]. The different possible SPME configurations and the distinct sample preparation steps directly affect the results produced by SPME-GC-MS analyses [18]. Parameters such as the SPME fibre coating, the extraction conditions (temperature and time) and the sample volume used ultimately determine the number of VOCs identified their abundances and the repeatability across replicates [18]. Therefore, an evaluation of each of these parameters is essential in order to obtain reliable and reproducible results.

Although SPME-GC-MS has been used extensively for the analysis of VOCs from faecal samples [4,19-21], there is a lack of published evidence evaluating sample preparation steps and potential SPME extraction configurations prior to GC-MS analysis. Using human faecal samples, Dixon and co-workers [22] investigated the effect of different extraction times on VOCs when using 4 different SPME fibres. In addition, they compared the number of VOCs identified when using 8 different SPME fibres and an extraction time of 20 min. Couch et al. [23] studied 2 SPME extraction times (20 min and 18 hrs) using 3 different fibres. On the other hand, a thorough literature review resulted in no published protocols or methods regarding sample preparation for the direct SPME-GC-MS analysis of murine faecal samples. Therefore, different research groups have to evaluate the same method-related parameters prior to analysis of experimental samples. In addition, there is a lack of standardization between laboratories performing metabolomics. To the best of our knowledge, there is no single gold standard method for the direct SPME-GC-MS analysis of VOCs extracted from human and/or murine faecal samples, which results in different findings when analysing the same subject [20-22] and prevents comparing results between labs. Thus, there is a current need for well-established method for the analysis of VOCs in murine and human faecal samples.

Here, we have evaluated the effect of different parameters for sample preparation and different SPME configurations on the analysis of VOCs from murine and human faecal samples. The number of VOCs identified and their abundances have been assessed when using different sample masses, distinct vial volumes, salting out of samples, and the effect of leaving samples at 1°C whilst loaded onto auto-sampler tray for 14 hours (overnight), applying different SPME extraction times and temperatures and using distinct SPME fibre coatings. Finally, we propose an optimized method for a reliable and high-throughput analysis of VOCs present in faecal samples.

## Materials and Methods

### Mouse faecal samples

Four inbred wild-type C57BL/6 mice were purchased from Charles River Laboratories (Margate, UK) at 9 weeks old and acclimatised under standard specific pathogen free (SPF) animal house conditions at the University of Liverpool for a minimum of 1 week prior to faecal sample collection. Animals were housed in a single cage under conventional conditions with food and water *ad libitum* and kept on a 12:12 hrs light-dark cycle. Samples of 3, 5, 10 and 20 pellets of mice faeces were collected during 3 consecutive weeks, placed in 10 ml glass vials and stored at -20°C. An additional four male inbred wild-type C57BL/6 mice were purchased at 5 weeks old and housed individually in the same conditions. Samples of 10 pellets were collected from each of these four additional mice in a single week and used to assess the effect of vial volume, extraction time and temperature and SPME fibre coating, on the number and abundances of VOCs identified, as described below

### Human faecal samples

Eleven faecal samples of variable masses were collected fresh from 5 premature babies hospitalized across the UK. The 5 donors were chosen at random from a larger cohort study. In accordance to a clear protocol, samples were collected by nurses and stored at -20°C. The sampling took place after written parental consent, with research ethics committee approval (11/WM/0078).

### GC/MS conditions

A Perkin Elmer Clarus 500 GC/MS quadruple bench top system (Beacons field, UK) was used in combination with a Combi PAL auto-sampler (CTC Analytics, Switzerland) for the analysis of all samples. The GC column used was a Zebron ZB-624 with inner diameter 0.25 mm, length 60 m, film thickness 1.4 µm (Phenomenex, Maccles field, UK). The carrier gas used was helium of 99.996% purity (BOC, Sheffield, UK). The SPME fibres used were CAR-PDMS 85 µm and DVB-CAR-PDMS 50/30 µm (1 cm) (Sigma-Aldrich, Dorset, UK). Both fibres were pre-conditioned before use, in accordance with the manufacturer manual. Vials with magnetic caps of 2 ml (Crawford Scientific, Lanarkshire, UK) and 10 ml (Sigma-Aldrich, Dorset, UK) volume were used. The fibre desorption conditions were 5 minutes at 220°C. The initial temperature of the GC oven was set at 40°C and held for 1 minute before increasing to 220°C at a rate of 5°C/min and held for 4 min with a total run time of 41 min. A solvent delay was set for the first 6 min and the MS was operated in electron impact ionization EI+ mode, scanning from ion mass fragments 10 to 300 *m/z* with an inter-scan delay of 0.1 sec and a resolution of 1000 at FWHM (Full Width at Half Maximum). The helium gas flow rate was set at 1 ml/min. The sensitivity of the instrument was determined with 2-pentanone only and will vary for other compounds. The limit of detection, as being 3 times the signal/noise ratio, of the method for 2-pentanone with DVB-CAR-PDMS is 16 ppm and with CAR-PDMS is 40 ppm.

### Sample mass optimisation

The number of VOCs identified and their abundances were evaluated according to different sample masses. Murine samples of 3, 5, 10 and 20 pellets, in triplicate, were pre-incubated at 60°C for 30 min before VOC extraction using a CAR-PDMS SPME fibre at 60°C for 20 min prior to desorption into the GC oven. Only VOCs identified in every sample were then used for comparing the VOC abundances according to the sample mass used. The same procedure was applied to human faecal samples, where one single sample was initially divided into triplicates of 100, 450 and 700 mg. Then 4 additional samples were divided into triplicates of 50 and 100 mg. A pilot study (data not published) using human faecal samples between 130 and 1320 mg showed a plateau in the number of VOCs identified initiating at 700 mg. Therefore, sample masses greater than 700 mg were not investigated here.

### Vial volume optimisation

The number of VOCs identified and their abundances were evaluated according to different vial volumes used for SPME-GC-MS analysis. For each vial volume of 2 and 10 ml, murine samples of 10 pellets (n=4) and aliquots of 100 mg from one human samples (n=4) were analysed. Each sample was pre-incubated at 60°C for 30 min

before VOC extraction using a CAR-PDMS SPME fibre at 60°C for 20 min prior to desorption into the GC oven.

### Salting–out optimisation

The number of VOCs identified was evaluated according to the addition of 0, 0.5 or 1 ml of saturated sodium chloride solution. Three human faecal samples were divided into 9 aliquots of 100 mg, stored in 10 ml vials and analysed with either 0 (n=3), 0.5 (n=3) or 1 ml (n=3) of sodium chloride solution added. Each sample was pre-incubated at 60°C for 30 min before VOC extraction using a CAR-PDMS SPME fibre for 20 min at 60°C prior to desorption into the GC oven.

### Leaving samples at 1°C overnight (14 hrs)

The abundances of identified VOCs were evaluated according to waiting time periods of 0 and 14 hrs at 1°C prior to SPME-GC-MS analysis. One human sample was divided into six aliquots and stored in 10 ml glass vials at -20°C. Three aliquots were analysed immediately after being taken from -20°C freezer (0 hrs), while three aliquots were analysed after being left at 1°C on the auto-sampler tray overnight (14 hrs). Each sample was pre-incubated at 60°C for 30 min before VOC extraction onto a CAR-PDMS SPME fibre at 60°C for 20 min prior to desorption into the GC oven.

### Extraction time optimisation

The number of VOCs identified was evaluated according to different SPME extraction times. Murine samples of 10 pellets were pre-incubated at 60°C for 30 min before VOC extraction using a CAR-PDMS SPME fibre at 60°C for 10 (n=3), 20 (n=3) or 30 min (n=3) prior to desorption into the GC oven.

### Extraction temperature optimisation

The number of VOCs identified was evaluated according to different SPME extraction temperatures. Murine samples of 10 pellets were pre-incubated at 60°C for 30 min before VOC extraction using a CAR-PDMS SPME fibre at 50 (n=3), 60 (n=3) or 70°C (n=3) for 20 min prior to desorption into the GC oven.

### SPME fibre optimisation

The number of VOCs identified was evaluated according to two SPME fibres. Murine samples of 10 pellets were pre-incubated at 60°C for 30 min before VOCs extraction using CAR-PDMS (n=5) or DVB-CAR-PDMS (n=5) SPME fibres at 60°C for 20 min prior to desorption into the GC oven. The same procedure was applied to a single human faecal sample divided into 10 aliquots of 100 mg.

### Repeatability and multiple analyses

In order to assess the repeatability of the final method proposed in this paper, the VOC profiles of ten human samples were further used to calculate the variation within samples. Each individual sample was divided in triplicate, stored in 10 ml vials and pre-incubated at 60°C for 30 min before VOCs extraction using a CAR-PDMS SPME fibre for 20 min at 60°C prior to desorption into the GC oven. The abundances of the VOCs identified within each sample (n=3 per sample) were used to calculate their coefficient of variation. Finally, 3 of these human samples were reanalysed 3 additional times in order to determine the effect of multiple analyses of a single sample.

### Data processing

All GC-MS data were processed using the Automated Mass Spectral Deconvolution System (AMDIS-version 2.71, 2012) in conjunction with the NIST mass spectral library (version 2.0, 2011) and the R package Metab [24]. VOCs were identified using an in-house library built with AMDIS in combination to the NIST library. All statistics were performed using R version 3.0.2 [25,26]. A t-test or a one-way analysis of variance (ANOVA) followed by Tukey's HSD test were applied to test differences between data classes. A principle component analysis (PCA) was used to show similarities within data classes. Final p-values were adjusted for multiple comparisons using Bonferroni correction. P-values<0.05 were considered as significant.

## Results

### Sample mass optimization

In order to optimize the sample mass for direct SPME-GC-MS analysis, we compared the number and abundances of VOCs identified in murine faecal samples (mean ± SEM) of 3 (40.0 ± 14.1 mg), 5 (76.7 ± 25 mg), 10 (133.3 ± 7 mg) and 20 (233.0 ± 25 mg) pellets; and in human faecal samples of 100 mg (100.3 ± 0.6 mg), 450 mg (455.1 ± 1.1 mg) and 700 mg (700.6 ± 2.8 mg) (Figure 1) and then 50 mg (52.4 ± 0.4 mg) and 100 mg (102.5 ± 0.2 mg) (Figure 2). Murine samples of 3 pellets showed a significantly lower number of VOCs than 20 pellets (p=0.05), with no significant difference in the number of VOCs between 10 and 20 pellets (p=0.87). Six compounds (pentanal, pentane, propanal, hexanal, 2,3-butandione and benzaldehyde) were consistently present in every murine sample with the compounds pentane and pentanal detected at significantly lower abundances in 3 and 5 pellets compared to 10 and 20 pellets. The compound propanal was present at significantly different levels between 3 and 20 pellets. In human samples, 450 and 700 mg showed a significantly higher number of VOCs than samples of 100 mg. There was no difference in the number of VOCs between 450 and 700 mg. Eight compounds were identified in every human sample (2, 3-butanedione, tetrahydrofurane, ethyl ester propanoic acid, n-propyl acetate, 2-pentenal (E), propyl ester propanoic acid, 2-methylpropanal, 1-propanol) and their intensities were significantly higher in samples of 450 and 700 mg than samples of 100 mg. The additional comparison between 50 and 100 mg showed no difference in both the number of VOCs identified (data not shown) and their abundances. All the statistical analyses for sample mass optimisation were performed by ANOVA followed by Tukey's HSD test and Bonferroni.

### Vial volume and salting–out optimisation

Vials of 2 ml showed a higher number of VOCs than vials of 10 ml when analysing murine samples (Figure 3A). However, there were no significant differences in VOCs abundances between vials (T-test; p-value>0.05). On the other hand, human samples showed no significant differences in both the number and the abundances of VOCs detected (T-test; p-value>0.05) (Figure 3B and 3C). The addition of sodium chloride showed no significant differences in the number and abundances of VOCs (ANOVA followed by Tukey's HSD test and Bonferroni; p-value>0.05) (Figure 3D).

### Leaving samples at 1°C overnight (14 hrs)

Although there were no significant differences in the number of VOCs identified (T-test; p-value > 0.05) there were three compounds detected at slightly lower abundances at 14hrs (Figure 4D).

### Extraction time and temperature optimization

Although there was no difference in the number of VOCs reported by the exposure times tested, 20 minutes produced the highest number

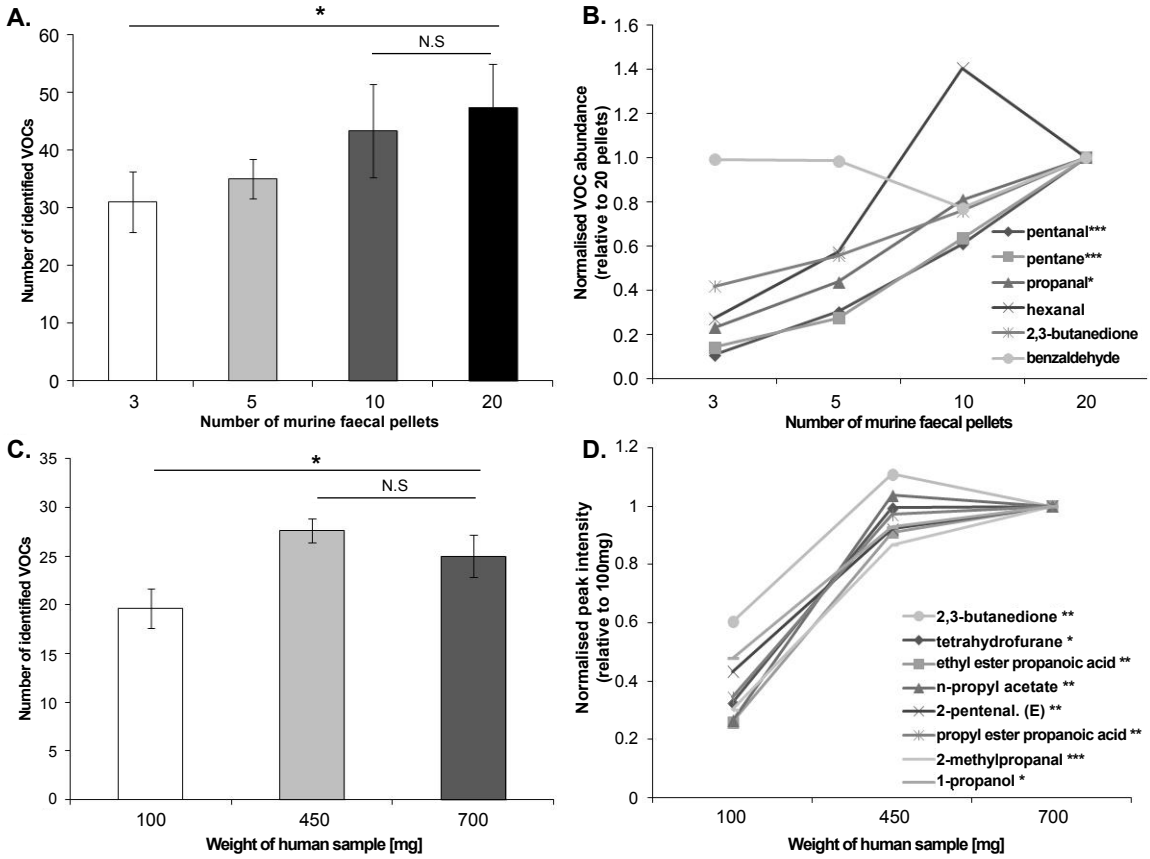


**Figure 1: Sample mass optimisation.(A-B)** The number and abundances of volatile organic compounds (VOCs) identified in murine faecal samples of 3, 5, 10 and 20 pellets, with VOC abundances normalized to their abundances in samples of 20 pellets. **(C-D)** Human faecal samples of 50, 100, 450 and 700 mg (n=3/group), with VOC abundances normalised to their abundances in 700 mg samples; ANOVA with Tukeys HSD followed by Bonferroni; *p<0.05, **p<0.01 and ***p<0.001.

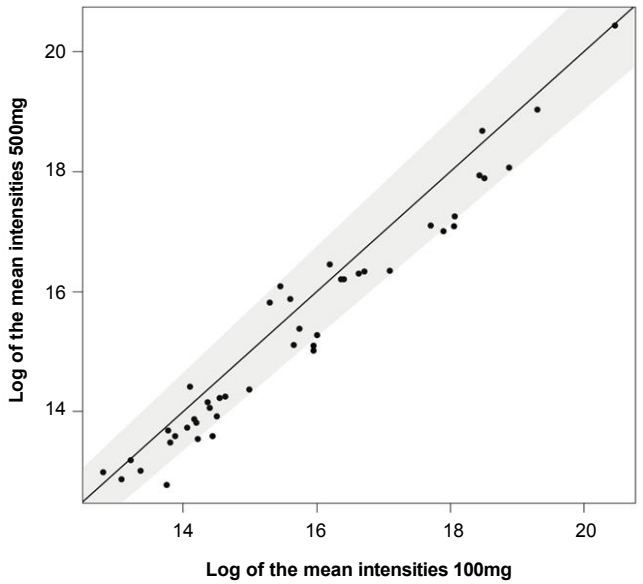


**Figure 2: Abundances of volatile organic compounds (VOCs) in samples of 50 and 100 mg.** Scatterplot comparing the average log values of VOCs abundances from human faecal samples of 50 and 100 mg (n=4). The diagonal black segment represents the function x=y, where compounds detected at similar levels between 50 and 100 mg are located inside of the 5% tolerance region represented by the grey area.

of VOCs (p=0.5) (Figure 4A). The exposure temperatures of 60 and 70°C showed a significantly higher number of VOCs than 50°C (p=0.03). Although there was no significant difference between 60 and 70°C (p=0.33), the exposure temperature of 60°C produced the highest number of VOCs (Figure 4B) (ANOVA followed by Tukey's HSD test and Bonferroni).

## SPME fibre optimization

DVB-CAR-PDMS showed a significantly higher number of VOCs when analysing murine samples (T-test; p=0.04). Seven compounds were exclusively detected by DVB-CAR-PDMS, while 2 compounds were exclusively detected by CAR-PDMS. Human samples showed no significant difference between SPME coatings, although five compounds were exclusively reported by CAR-PDMS and another five compounds were exclusively reported by DVB-CAR-PDMS (Figure 4C and Table 1).

## Optimized method

Based on the results presented above, we propose a SPME-GC-MS method for analysing murine and human faecal samples (Figure 5). We suggest murine samples are analysed using 10 to 20 pellets, stored in 2 ml glass vials, incubated for 30 min at 60°C and extracted by DVB-CAR-PDMS for 20 min. Alternatively, human samples of 450 mg should be stored in 2 or 10 ml glass vials, incubated for 30 min at 60°C and extracted by CAR-PDMS or DVB-CAR-PDMS for 20 min.

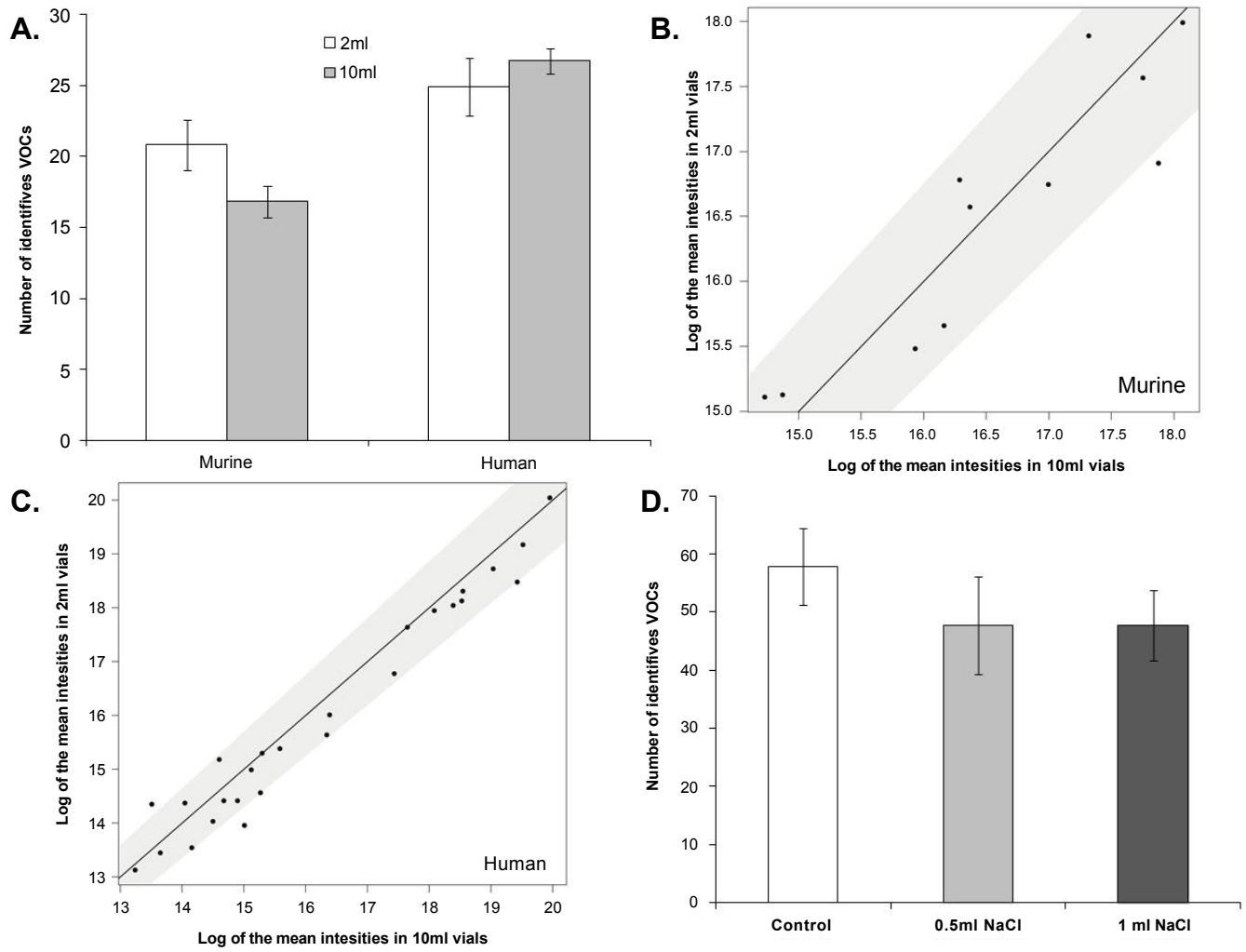


**Figure 3: Vial volume optimisation and salting-out. (A)** Faecal samples collected from murine (n=4/volume, N=3) and human (n=4/volume; 10 ml=102.8 ± 1.7 mg, 2 ml=101.9 ± 1.2 mg, N=3) using two different vial volumes, 2 ml and 10 ml (mean ± SEM; t-test). **(B-C)** Comparison of the two vial volumes studied, showing the mean of the abundance for each identified compounds (murine=11, humans=26) per size of vial (n=4/time) for both murine **(B)** and human samples **(C)**, plotted against the other. The grey area indicates the 5% tolerance around the optimal function x=y. **(D)** Faecal samples collected from an individual human treated with 0.5 ml NaCl and 1 ml NaCl and compared to a control sample (n=3/treatment; control=97.9 ± 2 mg, 0.5 ml NaCl=100.4 ± 3.6 mg, 1 ml NaCl=99.5 ± 3.2 mg) (mean ± SEM); ANOVA with Tukeys HSD followed by Bonferroni; *p<0.05, **p<0.01 and ***p<0.001.

| Murine | | Human | |
|---|---|---|---|
| CAR-PDMS | DVB-CAR-PDMS | CAR-PDMS | DVB-CAR-PDMS |
| 2,2-dimethylpropanoic acid | 2-methyl-1-propanal | propanoic acid | 2-methyl-2-butenal |
| 2-methylpentanal | 2-pentanone | 2-methylpropanoic acid | butanoic acid, propyl ester |
| | 2-methylfuran | 2,2-dimethylpropanoic acid | cyclopentane |
| | 2-nonenal | N,N-dimethylacetamide | 2-pentenal, (E) |
| | 3-methylbutanal | 1-propanol | 2-ethyl-1-hexanol |
| | 1-penten-3-ol | | |
| | 2-heptanal | | |

**Table 1: SPME fibre optimisation.** Murine and human faecal samples were analysed using a CAR-PDMS (n=5) or DVB-CAR-PDMS (n=5) SPME fibre. There were 2 and 5 specific compounds found exclusively by CAR-PDMS in murine and human samples respectively, and 7 and 5 found exclusively by DVB-CAR-PDMS in murine and human samples, respectively.

## Repeatability and multiple analyses

In order to assess the repeatability of our proposed method, we performed a principal component analysis (PCA) on the VOC profiles of 10 human faecal samples, each divided in triplicate (Figure 6). In addition, the mean, standard deviation and coefficient of variation (CV) were calculated across triplicates for each sample (Table 2). The PCA yielded results showing that each sample was clustered together. In average, 31.3 ± 10.5 VOCs were identified per human sample (mean ± SD). The standard deviation for each sample was 2.9 ± 1.3 compounds and, in average, 90% of the VOC abundances showed a coefficient of variation smaller than 30%, which is considered adequate for diagnostic

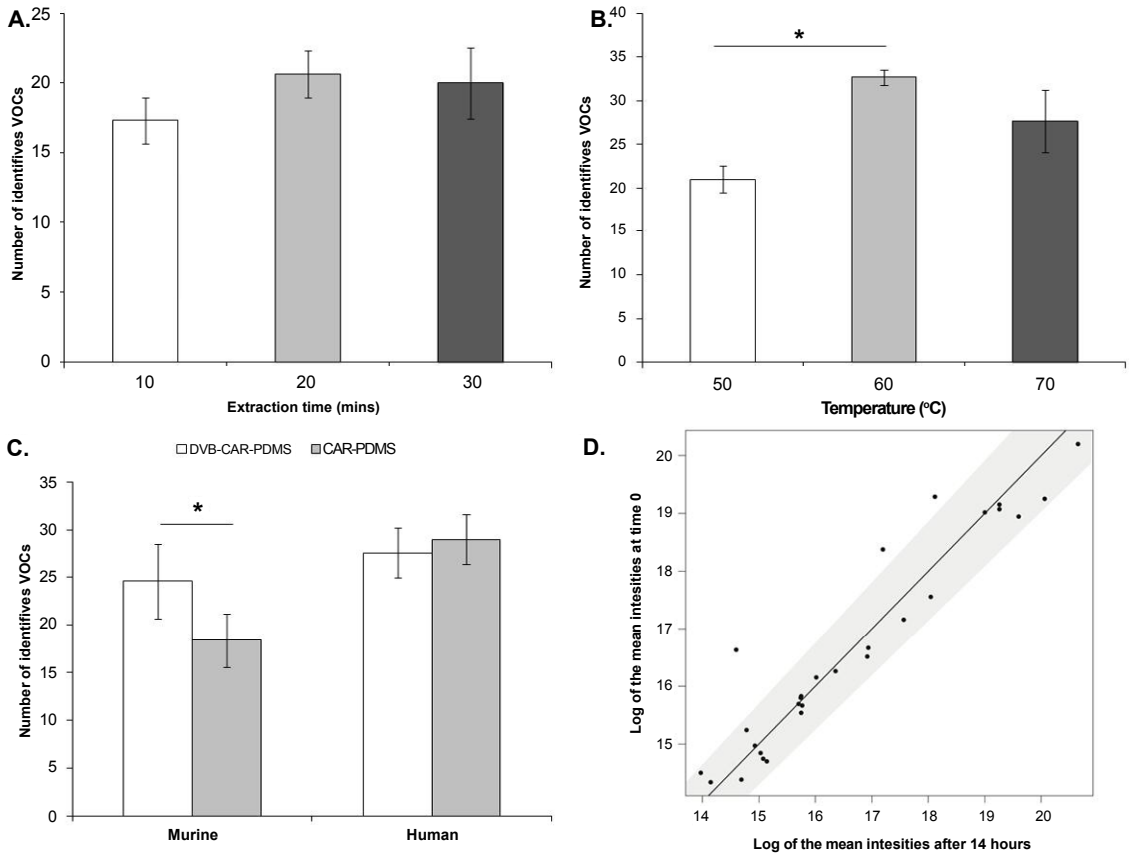


**Figure 4: Solid phase micro-extraction (SPME) optimisation and volatile organic compound (VOC) abundances in samples left for 14 hours at** 1°C. The number of VOCs identified in murine faecal samples according to the **(A)** extraction time and the **(B)** temperature applied to SPME coupled to gas chromatography- mass spectrometry (GC-MS) analysis (n=3); ANOVA with Tukeys HSD followed by Bonferroni. **(C)** The number of VOCs identified in murine and human faecal samples according to the SPME fibre used (n=3); T-test. **(D)** Scatterplot comparing the average log values of VOC abundances from human faecal samples analysed straight after leaving the -20°C freezer and after 14 hrs at 1°C(n=3). The diagonal black segment represents the function x=y, where compounds detected at similar levels between time 0 and time 14 are located inside of the 5% tolerance region represented by the grey area.*p<0.05, **p<0.01 and ***p<0.001.

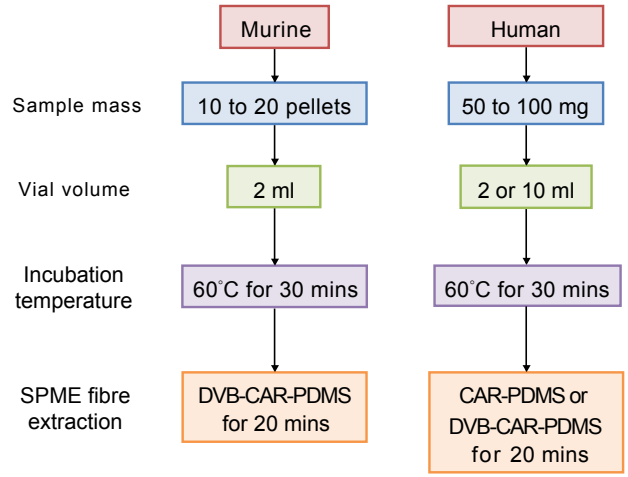


**Figure 5: Proposed optimised methods.** A diagram summarising our proposed optimised methods for solid phase micro-extraction coupled to gas chromatography-mass spectrometry (SPME-GC-MS) analysis of volatile organic compounds (VOCs) in murine and human faecal samples. Ideally, multiple SPME fibre coatings should be applied in order to extract a higher diversity of VOCs.

sensitivity [27]. Furthermore, the effect of analysing a single sample multiple times was assessed. The number of VOCs identified and their abundances were compared across 4 GC-MS runs (Table 3). There was a significant decrease in the number of VOCs identified (Figure 7) and 40% of these VOCs were detected at significantly lower or higher abundances after 4 GC-MS runs (ANOVA; p-value<0.05).

## Discussion

In this section, we discuss the results obtained when evaluating different aspects of sample preparation and SPME configurations for direct SPME-GC-MS analysis. Murine and human faecal samples have shown similar behaviour regarding the mass selection; however, they differed considering SPME coatings affinity and the vials volume optimisation.

### Sample mass optimization

The sample mass optimization showed the same pattern for murine and human samples (Figure 1). An increase in sample mass led to an increase in both the number of VOCs detected and their abundances, therefore reaching a limit of VOCs identified; a result of fibre overload. However, as the amount of mass per sample may be limited according to the study being conducted, we searched for the minimum sample

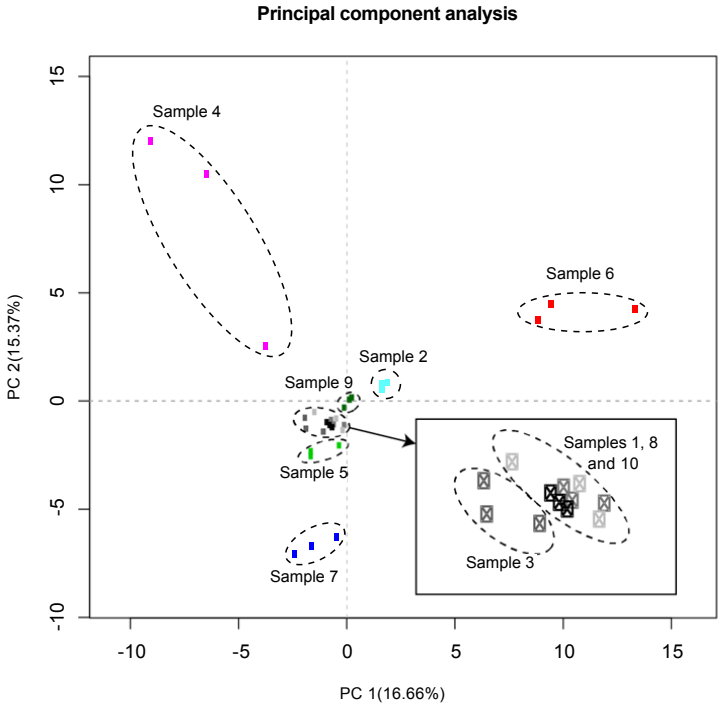


**Figure 6: Principal component analysis (PCA) for method repeatability.** The results of a PCA applied to the volatile organic compounds (VOCs) profile of 10 biological replicates, each divided in triplicates and analysed by solid phase micro-extraction coupled to gas chromatography-mass spectrometry (SPME-GC-MS).

| Samples | Number of VOCs ± S.D. (VOCs in every technical replicate) | VOCs with CV <30% (%) |
|---|---|---|
| Sample 1 | 24 ± 3 (19) | 100 |
| Sample 2 | 23 ± 1 (18) | 100 |
| Sample 3 | 20 ± 2 (12) | 92 |
| Sample 4 | 48 ± 5 (36) | 89 |
| Sample 5 | 37 ± 4 (26) | 81 |
| Sample 6 | 50 ± 4 (39) | 95 |
| Sample 7 | 32 ± 2 (21) | 90 |
| Sample 8 | 26 ± 4 (18) | 67 |
| Sample 9 | 26± 2 (19) | 95 |
| Sample 10 | 27± 2 (18) | 94 |

**Table 2: Repeatability of method.** Ten biological replicates of human faecal samples were divided in triplicates and analysed by solid phase micro-extraction coupled to gas chromatography-mass spectrometry (SPME-GC-MS). The average number of volatile organic compounds (VOCs) and the standard deviation associated to each biological replicate is presented together with the number of VOCs in every technical replicate and the percentage of compound abundances showing a coefficient of variation (CV) smaller than 30%.

mass that would produce the highest number of VOCs with the highest abundances. Murine samples of >20 faecal pellets were not included in this present study as no significant difference was found between samples of 10 and 20 pellets and it was found experimentally difficult to collect >20 pellets from individual mice. Therefore, we would predict that any number of murine faecal pellets higher than 20 would also produce a non-significant result. Figure 1C showed an increase in the number of VOCs identified between samples of 100 and 450 mg and between samples of 100 and 700 mg. The lack of difference between samples of 450 and 700 mg indicates that the SPME fibre reached its limit of absorbance at 450 mg. Therefore, samples of 10 pellets and samples of 450 mg showed the best results for murine and human samples respectively. Although human samples of 450 mg showed higher number of VOCs identified and higher abundances, samples of this mass are not always available. For example, studies with premature babies generally involve samples of 100 mg or less [28-30]. Therefore, we compared the results between samples of 50 and 100 mg in order to find the lowest possible sample mass able to produce a reasonable number of VOCs and reproducible abundances. Results showed no significant differences, which suggests that samples from 50 to 100 mg are appropriate in these cases (Figure 2).

## Vial volume and salting–out optimisation

Although no significant differences were observed between the vials tested when analysing both murine and human samples (Figure 3), 2 ml vials showed a much higher number of VOCs identified than 10 ml vials when analysing murine samples. However, when analysing human samples, 10 ml vials showed a slightly higher number of VOCs identified than 2 ml vials (2 ml=24, 10 ml=26 VOCs). Therefore, 2 ml vials are recommended for the analysis of murine samples and 2 ml or 10 ml vials when analysing human samples. This difference of behaviour might be explained by sample concentration. Certain compounds are more concentrated and at higher abundances in human samples compared to murine samples. Therefore, if the volume of headspace decreases then the levels of compounds in human samples will remain mostly unchanged whereas in murine samples the concentration increases leading to better coverage of the VOCs profiles in the samples. In addition, no significant differences were observed when salt was added to the samples. This could reflect the solid or semi-solid nature of the sample, which is only partially dissolved in the sodium chloride solution.

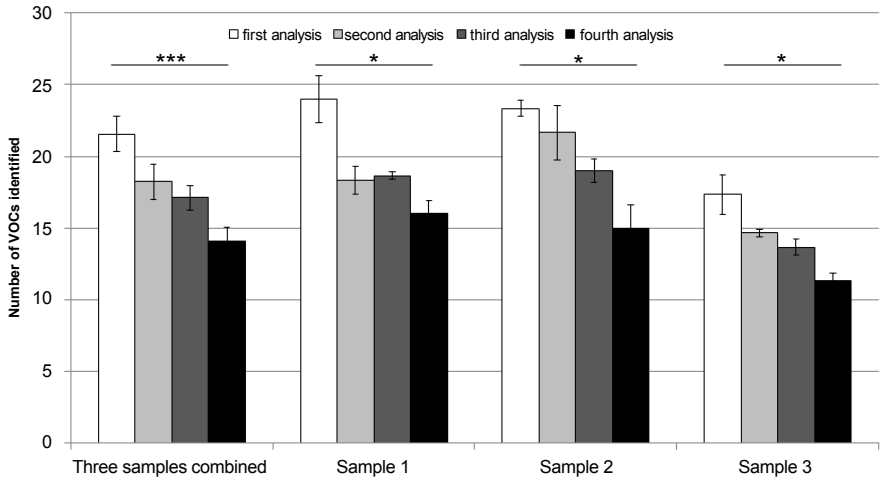


**Figure 7: Effect of multiple analyses of samples on the number of VOCs identified.** Three human faecal samples were divided in triplicate and analysed 4 consecutive times by solid phase micro-extraction coupled to gas chromatography-mass spectrometry (SPME-GC-MS). The number of VOCs identified in each analysis is presented for the three samples individually (n=3) and combined (n=9) (mean ± SEM); ANOVA with Tukeys HSD followed by Bonferroni; *p<0.05, **p<0.01 and ***p<0.001.

| SPME-GC-MS analyses | VOCs present in triplicate | Percentage of significantly different abundances(%) |
|---|---|---|
| Sample 1 | 16 | 56 |
| Sample 2 | 14 | 64 |
| Sample 3 | 10 | 40 |

**Table 3:** Effect of multiple analyses of samples on the abundances. Three human faecal samples were divided in triplicate and analysed 4 consecutive times by solid phase micro-extraction coupled to gas chromatography-mass spectrometry (SPME-GC-MS). The number of volatile organic compounds (VOCs) present in at least 2 consecutive analyses is presented together with the percentage of compounds for which the intensities differed significantly after 4 analyses.

## Leaving samples at 1°C overnight (14 hrs)

There were no significant changes in VOC abundances when samples were left for 14h at 1°C prior to SPME-GC-MS analysis (Figure 4D), which indicates that samples can be analysed overnight without significantly changing the VOC profiles or abundances. This result is particularly important for those experiments using GC-MS combined to auto-samplers that have a cooling tray with a minimum temperature of 1°C, where samples are generally sitting for up to 14 hours prior to injection.

## Extraction time and temperature optimization

Typically, the SPME extraction process is considered complete when the analyte concentration has reached distribution equilibrium between the sample matrix and the fibre coating. Until the distribution equilibrium is not reached, the extraction process is considered in pre-equilibrium state. Our results showed that an increase in extraction time and temperature resulted in higher numbers of VOCs identified (Figure 4A and 4B). However, extraction times higher than 20 minutes and extraction temperatures higher than 60°C showed no improvement. These results indicate that in the pre-equilibrium state, a small change in extraction time and temperature result in a large change in the amount of analyte being absorbed. However, once the distribution gets close to the equilibrium, there are either no changes or only small changes in the amount of analyte absorbed. Therefore, the extraction time and temperature are not critical once equilibrium is nearly reached. These results are in agreement with previous work from our group and others (Figure 4A and 4B) [4,22,31].

## SPME fibre optimization

Although there were no differences between the SPME fibres tested when analysing human samples, five compounds were exclusively detected by each specific fibre. On the other hand, the DVB/CAR/PDMS fibre was able to identify a higher number of compounds than the CAR/PDMS fibre when analysing murine samples (Figure 4C), with seven compounds exclusively identified by DVB/CAR/PDMS. Therefore, when possible, we suggest the use of different SPME fibres in order to extract a greater diversity of compounds, which is in agreement with previous work from Dixon and collaborators [22]. However, if a single SPME fibre must be used, we suggest DVB/CAR/PDMS for murine studies whereas both types of SPME fibre can be equally applied for human samples.

## Optimized method

Our optimized method (Figure 5) shows two main differences when applied to murine or human samples. When a single fibre is available, murine samples should be analysed by DVB/CAR/PDMS fibre and using 2 ml vials, while human samples can be analysed by DVB/CAR/PDMS or CAR/PDMS and using 2 or 10 ml vials. Our proposed method involves almost no sample preparation steps and results in reproducible and reliable VOC identification and quantification. Furthermore, our method can be easily combined to AMDIS software and Metab package in order to frame a powerful pipeline able to analyse VOCs in faecal samples in a reliable and high-throughput manner.

## Repeatability and multiple analyses

The PCA results (Figure 6) showed technical replicates clustering together, which indicates low variability across samples and high reproducibility. The PCA results are consistent with the low standard deviation across technical replicates (Table 2) and with the fact that the great majority of VOC abundances (90%) showed a CV lower than 30%. Finally, multiple SPME-GC-MS analyses of a single sample showed significant influence on the results, with a significant decrease in the number of identified VOCs (Figure 7) and difference in the

intensity of at least 40% of the VOCs (Table 3) after 4 SPME-GC-MS re-runs of each sample. These differences confirmed the hypothesis that the sample's headspace is affected by its analysis. Heating the faecal samples releases the majority of VOCs into the headspace air which are extracted and removed during the first SPME-GC-MS analysis. During the second, third and fourth analysis, the diversity and levels of specific VOCs are decreased, as confirmed in Figure 7. Therefore, each sample should be analysed once. The inter-individual variation between samples should also be taken into consideration, explaining the lower number of identified VOCs in sample 3 compared to sample 1 and 2 (Figure 7).

This proposed optimised method for the analysis of human and murine faecal samples using SPME-GC-MS is specific for the GC-MS conditions described in the 'Materials and Methods' section. Therefore, any change introduced by altering these configurations may result in different outcomes compared to those described in this study.

## Conclusion

We have evaluated several aspects involving the direct VOC analysis of murine and faecal samples via SPME-GC-MS. As a result, we proposed a new optimised method and demonstrated its high repeatability across technical replicates. To the best of our knowledge, this is the first study evaluating different aspects of SPME-GC-MS analysis of murine and human faecal samples and the first study suggesting an optimised method. Furthermore, our proposed method can be combined to the data analysis tools AMDIS and Metab in order to produce reliable results in a high-throughput way.

### Acknowledgment

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# Appendix C.

# Libraries

**Table 41: Library used for the data analysis during the method development.**

| Name | RT [min] | 1st ion | 2nd ion | 3rd ion | 4th ion |
|---|---|---|---|---|---|
| Ethanol | 6.639 | 31 | 45 | 46 | 29 |
| 2-propenal | 7.097 | 56 | 55 | 27 | 26 |
| Propanal | 7.219 | 58 | 29 | 28 | 27 |
| Acetone | 7.373 | 43 | 58 | 42 | 15 |
| Isopropanol | 7.579 | 45 | 43 | 41 | 39 |
| Carbon disulfide | 7.799 | 76 | 44 | 78 | 38 |
| Acetonitrile | 7.903 | 41 | 40 | 39 | 38 |
| Dicholormethane | 8.193 | 84 | 49 | 86 | 51 |
| Cyclopentane | 8.33 | 42 | 70 | 55 | 41 |
| Propanal, 2-methyl | 9.015 | 43 | 41 | 72 | 39 |
| 1-propanol | 9.619 | 31 | 59 | 42 | 60 |
| Butanal | 10.223 | 44 | 72 | 41 | 29 |
| 2,3-butanedione | 10.281 | 43 | 86 | 42 | 15 |
| 2-butanone | 10.55 | 43 | 72 | 29 | 57 |
| Ethyl acetate | 10.593 | 43 | 45 | 70 | 61 |
| Tetrahydrofurane | 11.16 | 42 | 41 | 72 | 71 |
| Formic acid | 11.606 | 46 | 45 | 29 | 44 |
| Column bleed | 11.829 | 147 | 148 | 73 | 66 |
| 1-propanol, 2-methyl | 11.995 | 43 | 41 | 42 | 33 |
| Butanal, 3-methyl | 12.54 | 44 | 41 | 43 | 58 |
| Acetic acid | 12.742 | 43 | 45 | 60 | 42 |
| Butanal, 2-methyl | 12.831 | 57 | 41 | 58 | 29 |
| 2-butenal, (E) | 12.9 | 70 | 41 | 39 | 69 |
| 3-buten-2-one, 3-methyl | 13.281 | 41 | 43 | 84 | 69 |
| Furan, 2-ethyl | 13.497 | 81 | 96 | 53 | 39 |
| 1-penten-3-one | 13.858 | 55 | 84 | 27 | 29 |
| 2-pentanone | 13.936 | 43 | 86 | 41 | 71 |
| 2-Penten-1-ol, (Z) | 13.991 | 57 | 29 | 67 | 43 |
| 2,3-pentanedione | 14.163 | 43 | 57 | 29 | 100 |
| Propanoic acid, ethyl ester | 14.181 | 57 | 29 | 43 | 102 |
| Pentanal | 14.203 | 44 | 41 | 58 | 29 |
| N-propyl acetate | 14.36 | 43 | 61 | 73 | 42 |
| Propanoic acid, 2-methyl-, ethyl ester | 15.986 | 43 | 71 | 41 | 29 |
| Acetoin | 16.114 | 45 | 43 | 88 | 42 |
| 1-butanol, 3-methyl-, formate | 16.19 | 55 | 43 | 70 | 42 |
| Propanoic acid | 16.215 | 74 | 45 | 73 | 28 |
| 3-penten-2-one | 16.343 | 69 | 41 | 43 | 39 |
| 2-butenal, 2-methyl | 16.422 | 84 | 55 | 41 | 69 |
| Pyridine | 16.424 | 79 | 52 | 51 | 50 |
| 1-pentanol | 16.444 | 41 | 42 | 55 | 70 |
| Toluene | 16.533 | 91 | 92 | 65 | 39 |
| 2-pentenal, (E) | 17.101 | 55 | 84 | 83 | 41 |
| Column bleed | 17.124 | 207 | 28 | 209 | 133 |

| | | | | | |
|---|---|---|---|---|---|
| Column bleed | 17.152 | 207 | 191 | 209 | 208 |
| 1-pentanol | 17.411 | 42 | 55 | 41 | 70 |
| Butanoic acid, ethyl ester | 17.688 | 71 | 43 | 88 | 41 |
| Propanoic acid, propyl ester | 18.043 | 57 | 75 | 43 | 29 |
| Propanoic acid, 2-methyl | 18.291 | 43 | 41 | 73 | 39 |
| Hexanal | 18.369 | 44 | 41 | 56 | 43 |
| Furan, 2,3-dihydro-4-methyl | 18.487 | 84 | 55 | 83 | 39 |
| 5-isoxazolecarboxylic acid, 4,5-dihydro-5-methyl-, methyl ester, (R) | 18.503 | 84 | 83 | 49 | NA |
| 2-oxabicyclo[2.2.2]octan-6-ol, 1,3,3-trimethyl | 19.095 | 71 | 43 | 126 | 56 |
| Column bleed | 19.316 | 221 | 222 | 73 | 14 |
| Butanoic acid, 2-methyl-, ethyl ester | 19.611 | 57 | 102 | 41 | 85 |
| Acetyl valeryl | 19.628 | 57 | 43 | 41 | 85 |
| Butanoic acid | 19.64 | 60 | 73 | 42 | 41 |
| Butanoic acid, 3-methyl-, ethyl ester | 19.748 | 88 | 85 | 57 | 60 |
| Propanoic acid, 2,2-dimethyl | 19.811 | 57 | 41 | 29 | 39 |
| Propane, 1-chloro-2,2-dimethyl | 20.244 | 57 | 91 | NA | 1 |
| Propanoic acid, 2-methylpropyl ester | 20.306 | 57 | 56 | 29 | 87 |
| Ethylbenzene | 20.344 | 91 | 106 | 77 | 51 |
| Xylene | 20.696 | 91 | 106 | 105 | 77 |
| 1-butanol, 3-methyl-, acetate | 20.732 | 43 | 70 | 55 | 41 |
| 3-furaldehyde | 20.822 | 96 | 95 | 39 | 38 |
| Butanoic acid, 2-methyl-3-oxo-, methyl ester | 20.829 | 43 | 88 | 55 | 87 |
| Acetic acid, diethyl | 20.854 | 43 | 32 | 88 | 87 |
| 2-hexenal, (E) | 21.055 | 41 | 55 | 69 | 83 |
| 4-heptanone | 21.194 | 71 | 43 | 114 | 41 |
| 1-hexanol | 21.335 | 56 | 55 | 41 | 43 |
| Butanoic acid, propyl ester | 21.453 | 71 | 43 | 89 | 41 |
| Butanoic acid, 3-methyl | 21.484 | 60 | 43 | 41 | 87 |
| Styrene | 21.798 | 104 | 103 | 78 | 77 |
| Xylene | 21.802 | 91 | 106 | 105 | 77 |
| Butanoic acid, 2-methyl | 21.805 | 74 | 57 | 41 | 87 |
| 2-heptanone | 22 | 43 | 58 | 71 | 59 |
| Heptanal | 22.313 | 70 | 44 | 43 | 41 |
| 3-methylenecycloheptene | 22.568 | 32 | 108 | 93 | 77 |
| 2-acetoxy-3-butanone | 22.663 | 43 | 87 | 130 | 45 |
| Pyrazine, 2,5-dimethyl | 22.674 | 108 | 42 | 39 | 40 |
| N,N-dimethylacetamide | 22.914 | 44 | 87 | 43 | 72 |
| Cyclohexanone | 23.11 | 55 | 42 | 98 | 69 |
| Butanoic acid, 2-methyl-, propyl ester | 23.293 | 103 | 57 | 85 | 41 |
| Column bleed | 23.392 | 18 | 281 | 283 | 249 |
| Column bleed 2 | 23.395 | 281 | 282 | 265 | 207 |
| Butanoic acid, 3-methyl-, propyl ester | 23.414 | 85 | 103 | 43 | 57 |
| Methional | 23.57 | 48 | 104 | 76 | 47 |
| 1-ethyl-5-methylcyclopentene | 23.97 | 81 | 110 | 67 | 79 |

| | | | | | |
|---|---|---|---|---|---|
| 3-heptyne, 5-methyl | 23.988 | 81 | 110 | 67 | 79 |
| 3-hepten-2-one | 24.105 | 32 | 55 | 97 | 43 |
| 1-butanol, 3-methyl-, propanoate | 24.114 | 57 | 70 | 55 | 75 |
| Hexanal, 2-ethyl | 24.218 | 72 | 57 | 41 | 43 |
| Decane | 24.245 | 57 | 43 | 71 | 41 |
| Heptane, 2,2,4,6,6-pentamethyl | 24.325 | 57 | 56 | 41 | 71 |
| Ethanone, 1-cyclopropyl-2-(4-pyridinyl) | 24.477 | 32 | 93 | 41 | 69 |
| Bicyclo[3.1.1]heptane, 6,6-dimethyl-2-methylene-, (1S) | 24.601 | 93 | 69 | 41 | 91 |
| Benzene, (1-butylhexyl) | 24.654 | 91 | 105 | 147 | 17 |
| Furan, 2-pentyl | 24.781 | 81 | 82 | 138 | 53 |
| 2-heptanal, (Z) | 25.001 | 41 | 57 | 43 | 83 |
| Hexanoic acid, ethyl ester | 25.034 | 88 | 43 | 99 | 29 |
| 1-hepten-3-one | 25.185 | 32 | 17 | 70 | 55 |
| 1-decen-3-one | 25.206 | 70 | 55 | 43 | 97 |
| 2,3-octanedione | 25.227 | 43 | 71 | 99 | 41 |
| 1-octen-3-ol | 25.269 | 57 | 72 | 67 | 54 |
| Dimethyl trisulfide | 25.369 | 126 | 79 | 57 | 45 |
| 3-heptanone, 6-methyl | 25.414 | 43 | 57 | 72 | 99 |
| 3-octanone | 25.414 | 57 | 43 | 72 | 99 |
| 5-hepten-2-one, 6-methyl | 25.6 | 43 | 108 | 41 | 69 |
| Benzaldehyde | 25.711 | 106 | 105 | 77 | 51 |
| 2,2,4,4-tetramethyloctane | 25.824 | 57 | 99 | 41 | 43 |
| Pyrazine, trimethyl | 25.942 | 122 | 42 | 81 | 39 |
| Octanal | 26.062 | 43 | 84 | 41 | 44 |
| D-limonene | 26.241 | 68 | 93 | 67 | 79 |
| Isoamyl nitrite | 26.274 | 43 | 41 | 60 | 57 |
| Alkane(decane, 2,4,6-trimethyl-) | 26.392 | 43 | 71 | 57 | 85 |
| Benzene, (1-propylheptyl) | 26.398 | 91 | 133 | 218 | 175 |
| Pantolactone | 26.409 | 71 | 68 | 57 | 43 |
| Hexanoic acid | 26.468 | 60 | 73 | 41 | 43 |
| 2-propanol, 1,1,1-trichloro-2-methyl- | 26.515 | 59 | 43 | 16 | 127 |
| Decane, 2,6,6-trimethyl | 26.526 | 57 | 71 | 43 | 70 |
| Dodecane, 2,6,11-trimethyl | 26.552 | 57 | 71 | 43 | 70 |
| Pentane, 2,2,3,3-tetramethyl | 26.559 | 57 | 71 | 70 | 43 |
| 2H-pyran-2-one, tetrahydro | 26.685 | 41 | 56 | 100 | 28 |
| 2,4-heptadienal, (E,E) | 26.703 | 81 | 110 | 53 | 67 |
| Eucalyptol | 26.758 | 43 | 81 | 71 | 84 |
| Cyclopentanone, 2,4,4-trimethyl | 26.922 | 83 | 55 | 126 | 56 |
| Decane, 3,3,4-trimethyl | 27.017 | 71 | 70 | 57 | 43 |
| 1-hexanol, 2-ethyl | 27.136 | 57 | 41 | 43 | 70 |
| Cyclohexanone, 2,2,6-trimethyl | 27.686 | 82 | 69 | 140 | 56 |
| Octane, 2,3,6,7-tetramethyl | 27.787 | 18 | 71 | 43 | 57 |
| 5-Ethylcyclopent-1-enecarboxaldehyde | 27.789 | 67 | 95 | 124 | 41 |
| 3-Cyclohexene-1-carboxaldehyde, 1- | 27.819 | 67 | 95 | 124 | 55 |

| | | | | | |
|---|---|---|---|---|---|
| methyl | | | | | |
| Dodecane | 27.893 | 43 | 71 | 57 | 85 |
| Phenol | 28.046 | 94 | 66 | 65 | 39 |
| Furan, 2-hexyl | 28.278 | 32 | 81 | 60 | 82 |
| Pyrazine, 3-ethyl-2,5-dimethyl | 28.445 | 135 | 136 | 42 | 39 |
| 2-octenal, (E) | 28.609 | 41 | 70 | 55 | 82 |
| Benzeneacetaldehyde | 28.668 | 91 | 32 | 120 | 92 |
| column | 28.834 | 73 | 267 | 268 | 74 |
| 3,5-octadien-2-one | 29.02 | 95 | 81 | 43 | 124 |
| 3-octanol, 3,7-dimethyl | 29.152 | 73 | 55 | 69 | 70 |
| 3-carene | 29.386 | 93 | 41 | 91 | 71 |
| Nonanal | 29.544 | 57 | 41 | 43 | 56 |
| Furan, 2-butyltetrahydro | 29.859 | 18 | 71 | 41 | 55 |
| Acetic acid, 2-ethylhexyl ester | 30.417 | 70 | 43 | 57 | 55 |
| 2-heptanone, 6-methyl | 30.886 | 71 | 95 | 43 | 110 |
| Dodecane | 30.969 | 57 | 43 | 71 | 85 |
| 2,6-nonadienal, (E,E) | 31.716 | 41 | 69 | 70 | 85 |
| Dodecanoic acid, ethyl ester | 31.917 | 88 | 41 | 55 | 43 |
| 2-nonenal, (E) | 31.971 | 55 | 43 | 70 | 41 |
| (+)-2-bornanone | 32.187 | 95 | 81 | 108 | 152 |
| 2-dodecylcyclohexanone | 32.28 | 69 | 98 | 55 | 43 |
| Formic acid, 2-ethylhexyl ester | 33.187 | 57 | 70 | 112 | 55 |
| 2-chloro-6-fluorobenzonitrile | 33.697 | 155 | 157 | 120 | 100 |
| Benzene, 1,3-bis(1,1-dimethylethyl) | 33.731 | 175 | 57 | 190 | 176 |
| 3-carene | 33.823 | 93 | 91 | 121 | 41 |
| 2,4-nonadienal, (E,E) | 33.958 | 81 | 41 | 67 | 138 |
| Column bleed | 34.072 | 73 | 281 | 221 | 32 |
| Column bleed | 34.21 | 73 | 207 | 147 | 32 |
| 2-cyclopenten-1-one, 4-acetyl-2,3,4,5,5-pentamethyl | 34.287 | 152 | 137 | 109 | 123 |
| N-butyric acid 2-ethylhexyl ester | 34.368 | 71 | 70 | 43 | 112 |
| Benzene, (1-pentylhexyl) | 34.602 | 91 | 18 | 161 | 105 |
| Benzene, (1-butylheptyl) | 34.901 | 91 | 147 | 175 | 105 |
| 2-decenal, (E) | 35.05 | 70 | 55 | 41 | 83 |
| Benzene, (1-propyloctyl) | 35.602 | 91 | 133 | 189 | 232 |
| Cyclohexene, 1-methyl-3-(1-methylethenyl)-, (±) | 35.847 | 121 | 93 | 95 | 136 |
| Pentanoic acid, 2-ethylhexyl ester | 36.994 | 85 | 70 | 57 | 112 |
| y-terpinene | 37.148 | 93 | 121 | 136 | 68 |
| Decanoic acid, ethyl ester | 37.575 | 88 | 101 | 155 | 43 |
| Indole | 38.538 | 117 | 90 | 89 | 63 |
| Column bleed | 39.168 | 281 | 147 | 73 | 207 |
| Column bleed | 39.209 | 207 | 28 | 281 | 17 |
| Benzene, (1-pentylheptyl) | 40.695 | 91 | 161 | 246 | 281 |

Presentation of compound names, retention times and the first 4 ions used by Metab for each compound present in the library used during the data analysis of the method development. Library built using AMDIS and NIST database.

**Table 42: Library applied during the data analysis of the study.**

| Name | RT [min] | 1st ion | 2nd ion | 3rd ion | 4th ion |
|---|---|---|---|---|---|
| Ethanol | 6.639 | 31 | 45 | 46 | 29 |
| 2-propenal | 7.097 | 56 | 55 | 27 | 26 |
| Propanal | 7.219 | 58 | 29 | 28 | 27 |
| Acetone | 7.373 | 43 | 58 | 42 | 15 |
| Isopropanol | 7.579 | 45 | 43 | 41 | 39 |
| Carbon disulfide | 7.799 | 76 | 44 | 78 | 38 |
| Acetonitrile | 7.903 | 41 | 40 | 39 | 38 |
| Dicholormethane | 8.193 | 84 | 49 | 86 | 51 |
| Cyclopentane | 8.33 | 42 | 70 | 55 | 41 |
| Propanal, 2-methyl | 9.015 | 43 | 41 | 72 | 39 |
| 1-propanol | 9.619 | 31 | 59 | 42 | 60 |
| Butanal | 10.223 | 44 | 72 | 41 | 29 |
| 2,3-butanedione | 10.281 | 43 | 86 | 42 | 15 |
| 2-butanone | 10.55 | 43 | 72 | 29 | 57 |
| Ethyl acetate | 10.593 | 43 | 45 | 70 | 61 |
| Tetrahydrofurane | 11.16 | 42 | 41 | 72 | 71 |
| Formic acid | 11.606 | 46 | 45 | 29 | 44 |
| Column bleed | 11.829 | 147 | 148 | 73 | 66 |
| 1-propanol, 2-methyl | 11.995 | 43 | 41 | 42 | 33 |
| Butanal, 3-methyl | 12.54 | 44 | 41 | 43 | 58 |
| Acetic acid | 12.742 | 43 | 45 | 60 | 42 |
| Butanal, 2-methyl | 12.831 | 57 | 41 | 58 | 29 |
| 2-butenal, (E) | 12.9 | 70 | 41 | 39 | 69 |
| 3-buten-2-one, 3-methyl | 13.281 | 41 | 43 | 84 | 69 |
| 1-butanol | 13.294 | 56 | 41 | 43 | 31 |
| Furan, 2-ethyl | 13.497 | 81 | 96 | 53 | 39 |
| 1-penten-3-one | 13.858 | 55 | 84 | 27 | 29 |
| 2-pentanone | 13.936 | 43 | 86 | 41 | 71 |
| 2-penten-1-ol, (Z) | 13.991 | 57 | 29 | 67 | 43 |
| 2,3-pentanedione | 14.163 | 43 | 57 | 29 | 100 |
| Propanoic acid, ethyl ester | 14.181 | 57 | 29 | 43 | 102 |
| Pentanal | 14.203 | 44 | 41 | 58 | 29 |
| N-propyl acetate | 14.36 | 43 | 61 | 73 | 42 |
| 2-propanone, 1-hydroxy | 14.411 | 43 | 31 | 74 | 29 |
| Propanoic acid, 2-methyl-, ethyl ester | 15.986 | 43 | 71 | 41 | 29 |
| Acetoin | 16.114 | 45 | 43 | 88 | 42 |
| 1-butanol, 3-methyl-, formate | 16.19 | 55 | 43 | 70 | 42 |
| Propanoic acid | 16.215 | 74 | 45 | 73 | 28 |
| 3-penten-2-one | 16.343 | 69 | 41 | 43 | 39 |
| 2-butenal, 2-methyl | 16.422 | 84 | 55 | 41 | 69 |
| Pyridine | 16.424 | 79 | 52 | 51 | 50 |
| 1-pentanol | 16.444 | 41 | 42 | 55 | 70 |
| Toluene | 16.533 | 91 | 92 | 65 | 39 |

| | | | | | |
|---|---|---|---|---|---|
| 2-pentenal, (E) | 17.101 | 55 | 84 | 83 | 41 |
| Column bleed | 17.124 | 207 | 28 | 209 | 133 |
| Column bleed | 17.152 | 207 | 191 | 209 | 208 |
| 1-pentanol | 17.411 | 42 | 55 | 41 | 70 |
| Butanoic acid, ethyl ester | 17.688 | 71 | 43 | 88 | 41 |
| Propanoic acid, propyl ester | 18.043 | 57 | 75 | 43 | 29 |
| Propanoic acid, 2-methyl | 18.291 | 43 | 41 | 73 | 39 |
| Hexanal | 18.369 | 44 | 41 | 56 | 43 |
| Furan, 2,3-dihydro-4-methyl | 18.487 | 84 | 55 | 83 | 39 |
| 5-isoxazolecarboxylic acid, 4,5-dihydro-5-methyl-, methyl ester, (R) | 18.503 | 84 | 83 | 49 | NA |
| Cyclobutanone, 2,2,3-trimethyl | 18.855 | 55 | 70 | 42 | 41 |
| Butanoic acid, 1-methylethyl ester | 19.064 | 43 | 71 | 89 | 41 |
| 2-oxabicyclo[2.2.2]octan-6-ol, 1,3,3-trimethyl | 19.095 | 71 | 43 | 126 | 56 |
| Column bleed | 19.198 | 221 | 73 | 222 | 103 |
| Column bleed | 19.316 | 221 | 222 | 73 | 14 |
| 2,3-butanediol | 19.594 | 45 | 43 | 57 | 29 |
| Butanoic acid, 2-methyl-, ethyl ester | 19.611 | 57 | 102 | 41 | 85 |
| Acetyl valeryl | 19.628 | 57 | 43 | 41 | 85 |
| Butanoic acid | 19.64 | 60 | 73 | 42 | 41 |
| Butanoic acid, 3-methyl-, ethyl ester | 19.748 | 88 | 85 | 57 | 60 |
| Propanoic acid, 2,2-dimethyl | 19.811 | 57 | 41 | 29 | 39 |
| Propane, 1-chloro-2,2-dimethyl | 20.244 | 57 | 91 | NA | 1 |
| Propanoic acid, 2-methylpropyl ester | 20.306 | 57 | 56 | 29 | 87 |
| Ethylbenzene | 20.344 | 91 | 106 | 77 | 51 |
| Xylene | 20.696 | 91 | 106 | 105 | 77 |
| 1-butanol, 3-methyl-, acetate | 20.732 | 43 | 70 | 55 | 41 |
| 3-furaldehyde | 20.822 | 96 | 95 | 39 | 38 |
| Butanoic acid, 2-methyl-3-oxo-, methyl ester | 20.829 | 43 | 88 | 55 | 87 |
| Acetic acid, diethyl | 20.854 | 43 | 32 | 88 | 87 |
| 2-hexenal, (E) | 21.055 | 41 | 55 | 69 | 83 |
| 2-pentanone, 4-hydroxy-4-methyl | 21.159 | 43 | 59 | 32 | 58 |
| 4-heptanone | 21.194 | 71 | 43 | 114 | 41 |
| Isocrotonic acid | 21.228 | 86 | 39 | 41 | 68 |
| 1-hexanol | 21.335 | 56 | 55 | 41 | 43 |
| Butanoic acid, propyl ester | 21.453 | 71 | 43 | 89 | 41 |
| Butanoic acid, 3-methyl | 21.484 | 60 | 43 | 41 | 87 |
| Styrene | 21.798 | 104 | 103 | 78 | 77 |
| Xylene | 21.802 | 91 | 106 | 105 | 77 |
| Butanoic acid, 2-methyl | 21.805 | 74 | 57 | 41 | 87 |
| 2-heptanone | 22 | 43 | 58 | 71 | 59 |
| Cyclohexanol | 22.267 | 57 | 82 | 54 | NA |
| Heptanal | 22.313 | 70 | 44 | 43 | 41 |
| 3-methylenecycloheptene | 22.568 | 32 | 108 | 93 | 77 |
| 2-acetoxy-3-butanone | 22.663 | 43 | 87 | 130 | 45 |

| | | | | | |
|---|---|---|---|---|---|
| Pyrazine, 2,5-dimethyl | 22.674 | 108 | 42 | 39 | 40 |
| Pentanoic acid | 22.891 | 60 | 73 | 41 | 45 |
| N,N-dimethylacetamide | 22.914 | 44 | 87 | 43 | 72 |
| Cyclohexanone | 23.11 | 55 | 42 | 98 | 69 |
| Decane, 2,6,7-trimethyl | 23.142 | 43 | 71 | 70 | 57 |
| Column bleed | 23.242 | 281 | 282 | 283 | 133 |
| Butanoic acid, 2-methyl-, propyl ester | 23.293 | 103 | 57 | 85 | 41 |
| Column bleed | 23.392 | 18 | 281 | 283 | 249 |
| Column bleed 2 | 23.395 | 281 | 282 | 265 | 207 |
| Butanoic acid, 3-methyl-, propyl ester | 23.414 | 85 | 103 | 43 | 57 |
| Butanoic acid, 2-methylpropyl ester | 23.498 | 71 | 56 | 43 | 57 |
| 2-heptenal, (E) | 23.512 | 55 | 83 | 57 | 56 |
| Methional | 23.57 | 48 | 104 | 76 | 47 |
| 1-ethyl-5-methylcyclopentene | 23.97 | 81 | 110 | 67 | 79 |
| 3-heptyne, 5-methyl | 23.988 | 81 | 110 | 67 | 79 |
| 3-hepten-2-one | 24.105 | 32 | 55 | 97 | 43 |
| 1-butanol, 3-methyl-, propanoate | 24.114 | 57 | 70 | 55 | 75 |
| Hexanal, 2-ethyl | 24.218 | 72 | 57 | 41 | 43 |
| Decane | 24.245 | 57 | 43 | 71 | 41 |
| Heptane, 2,2,4,6,6-pentamethyl | 24.325 | 57 | 56 | 41 | 71 |
| Ethanone, 1-cyclopropyl-2-(4-pyridinyl) | 24.477 | 32 | 93 | 41 | 69 |
| Nonane, 2,6-dimethyl | 24.544 | 71 | 43 | 57 | 70 |
| Bicyclo[3.1.1]heptane, 6,6-dimethyl-2-methylene-, (1S) | 24.601 | 93 | 69 | 41 | 91 |
| Benzene, (1-butylhexyl) | 24.654 | 91 | 105 | 147 | 17 |
| Furan, 2-pentyl | 24.781 | 81 | 82 | 138 | 53 |
| Heptane, 2,2,4,6,6-pentamethyl | 24.927 | 57 | 56 | 41 | 85 |
| 2-heptanal, (Z) | 25.001 | 41 | 57 | 43 | 83 |
| Hexanoic acid, ethyl ester | 25.034 | 88 | 43 | 99 | 29 |
| 1-hepten-3-one | 25.185 | 32 | 17 | 70 | 55 |
| 1-decen-3-one | 25.206 | 70 | 55 | 43 | 97 |
| 2,3-octanedione | 25.227 | 43 | 71 | 99 | 41 |
| 1-octen-3-ol | 25.269 | 57 | 72 | 67 | 54 |
| Pentanoic acid, 4-methyl | 25.313 | 57 | 73 | 74 | 55 |
| Dimethyl trisulfide | 25.369 | 126 | 79 | 57 | 45 |
| 3-heptanone, 6-methyl | 25.414 | 43 | 57 | 72 | 99 |
| 3-octanone | 25.414 | 57 | 43 | 72 | 99 |
| Butyrolactone | 25.481 | 42 | 28 | 41 | 56 |
| 5-hepten-2-one, 6-methyl | 25.6 | 43 | 108 | 41 | 69 |
| Benzaldehyde | 25.711 | 106 | 105 | 77 | 51 |
| Octane, 6-ethyl-2-methyl | 25.82 | 57 | 71 | 43 | 85 |
| 2,2,4,4-tetramethyloctane | 25.824 | 57 | 99 | 41 | 43 |
| Decane, 3-methyl | 25.84 | 57 | 71 | 85 | 43 |
| Pyrazine, trimethyl | 25.942 | 122 | 42 | 81 | 39 |
| Dodecane, 2,6,10-trimethyl | 25.949 | 57 | 71 | 43 | 70 |
| Octanal | 26.062 | 43 | 84 | 41 | 44 |

| | | | | | |
|---|---|---|---|---|---|
| 2-propenoic acid, 4-methylpentyl ester | 26.2 | 32 | 55 | 69 | 18 |
| D-limonene | 26.241 | 68 | 93 | 67 | 79 |
| Isoamyl nitrite | 26.274 | 43 | 41 | 60 | 57 |
| Alkane(decane, 2,4,6-trimethyl-) | 26.392 | 43 | 71 | 57 | 85 |
| Pentane, 2,2,3,3-tetramethyl | 26.392 | 57 | 71 | 70 | 43 |
| Benzene, (1-propylheptyl) | 26.398 | 91 | 133 | 218 | 175 |
| Dodecane, 2,7,10-trimethyl | 26.399 | 57 | 71 | 43 | 41 |
| Decane, 2,6,6-trimethyl | 26.408 | 57 | 71 | 70 | 56 |
| Pantolactone | 26.409 | 71 | 68 | 57 | 43 |
| Hexanoic acid | 26.468 | 60 | 73 | 41 | 43 |
| 2-propanol, 1,1,1-trichloro-2-methyl- | 26.515 | 59 | 43 | 16 | 127 |
| Decane, 2,6,6-trimethyl | 26.526 | 57 | 71 | 43 | 70 |
| Pentane, 3-ethyl-2,2-dimethyl | 26.537 | 57 | 56 | 43 | 41 |
| Decane, 2,2-dimethyl | 26.539 | 57 | 56 | 71 | 43 |
| Dodecane, 2,6,11-trimethyl | 26.552 | 57 | 71 | 43 | 70 |
| Pentane, 2,2,3,3-tetramethyl | 26.559 | 57 | 71 | 70 | 43 |
| 1,4-hexadiene, 3-ethyl | 26.561 | 81 | 71 | 53 | 110 |
| Undecane, 6,6-dimethyl | 26.614 | 57 | 56 | 32 | 71 |
| 2H-pyran-2-one, tetrahydro | 26.685 | 41 | 56 | 100 | 28 |
| 2,4-heptadienal, (E,E) | 26.703 | 81 | 110 | 53 | 67 |
| Eucalyptol | 26.758 | 43 | 81 | 71 | 84 |
| Cyclopentanone, 2,4,4-trimethyl | 26.922 | 83 | 55 | 126 | 56 |
| Decane, 3,3,4-trimethyl | 27.017 | 71 | 70 | 57 | 43 |
| Butanoic acid, 3-methylbutyl ester | 27.067 | 70 | 71 | 43 | 55 |
| 1-hexanol, 2-ethyl | 27.136 | 57 | 41 | 43 | 70 |
| 2,2,4,4-tetramethyloctane | 27.415 | 57 | 55 | 41 | 99 |
| 2,2,4,4-tetramethyloctane | 27.426 | 57 | 55 | 41 | 43 |
| Cyclohexanone, 2,2,6-trimethyl | 27.686 | 82 | 69 | 140 | 56 |
| Nonane, 4,5-dimethyl | 27.753 | 71 | 57 | 43 | 85 |
| Octane, 2,3,6,7-tetramethyl | 27.787 | 18 | 71 | 43 | 57 |
| 5-ethylcyclopent-1-enecarboxaldehyde | 27.789 | 67 | 95 | 124 | 41 |
| 3-cyclohexene-1-carboxaldehyde, 1-methyl | 27.819 | 67 | 95 | 124 | 55 |
| Dodecane | 27.893 | 43 | 71 | 57 | 85 |
| Phenol | 28.046 | 94 | 66 | 65 | 39 |
| Furan, 2-hexyl | 28.143 | 81 | 82 | 53 | 152 |
| Decane, 3,3,8-trimethyl | 28.44 | 57 | 71 | 70 | 41 |
| Pyrazine, 3-ethyl-2,5-dimethyl | 28.445 | 135 | 136 | 42 | 39 |
| 2-octenal, (E) | 28.609 | 41 | 70 | 55 | 82 |
| Benzeneacetaldehyde | 28.668 | 91 | 32 | 120 | 92 |
| Column bleed | 28.834 | 73 | 267 | 268 | 74 |
| Formic acid, 2-methylbutyl ester | 29.008 | 57 | 70 | 55 | 41 |
| 3,5-octadien-2-one | 29.02 | 95 | 81 | 43 | 124 |
| 3-octanol, 3,7-dimethyl | 29.152 | 73 | 55 | 69 | 70 |
| 3-carene | 29.386 | 93 | 41 | 91 | 71 |
| Nonanal | 29.544 | 57 | 41 | 43 | 56 |

| | | | | | |
|---|---|---|---|---|---|
| (E)-4-oxohex-2-enal | 29.695 | 83 | 55 | 27 | 84 |
| Furan, 2-butyltetrahydro | 29.859 | 18 | 71 | 41 | 55 |
| Undecane, 3-methyl | 29.963 | 57 | 85 | 71 | 56 |
| 1-nonen-4-ol | 30.291 | 55 | 43 | 83 | 41 |
| Acetic acid, 2-ethylhexyl ester | 30.417 | 70 | 43 | 57 | 55 |
| 4-octene, 2,3,7-trimethyl-, [S-(E)] | 30.466 | 69 | 83 | 55 | 111 |
| 3,4-dimethylcyclohexanol | 30.734 | 71 | 43 | 95 | 41 |
| 2-heptanone, 6-methyl | 30.886 | 71 | 95 | 43 | 110 |
| Dodecane | 30.969 | 57 | 43 | 71 | 85 |
| 2,5-pyrrolidinedione, 1-methyl | 31.328 | 28 | 113 | 56 | 27 |
| 1-octanol, 3,7-dimethyl | 31.51 | 70 | 41 | 69 | 71 |
| Undecane, 2-methyl | 31.702 | 71 | 57 | 85 | 43 |
| 2,6-nonadienal, (E,E) | 31.716 | 41 | 69 | 70 | 85 |
| 1-nonene, 4,6,8-trimethyl | 31.865 | 69 | 71 | 85 | 83 |
| Dodecanoic acid, ethyl ester | 31.917 | 88 | 41 | 55 | 43 |
| 2-nonenal, (E) | 31.971 | 55 | 43 | 70 | 41 |
| (+)-2-bornanone | 32.187 | 95 | 81 | 108 | 152 |
| 2-dodecylcyclohexanone | 32.28 | 69 | 98 | 55 | 43 |
| Heptane, 4-propyl | 32.602 | 32 | 57 | 71 | 41 |
| Decanal | 32.608 | 55 | 57 | 43 | 41 |
| Furan, 2-butyltetrahydro | 32.929 | 71 | 55 | 43 | 41 |
| 11-tricosene | 33.034 | 55 | 57 | 69 | 97 |
| Formic acid, 2-ethylhexyl ester | 33.187 | 57 | 70 | 112 | 55 |
| 2-chloro-6-fluorobenzonitrile | 33.697 | 155 | 157 | 120 | 100 |
| Benzene, 1,3-bis(1,1-dimethylethyl) | 33.731 | 175 | 57 | 190 | 176 |
| 3-carene | 33.823 | 93 | 91 | 121 | 41 |
| 2,4-nonadienal, (E,E) | 33.958 | 81 | 41 | 67 | 138 |
| Column bleed | 34.072 | 73 | 281 | 221 | 32 |
| Column bleed | 34.21 | 73 | 207 | 147 | 32 |
| 2-cyclopenten-1-one, 4-acetyl-2,3,4,5,5-pentamethyl | 34.287 | 152 | 137 | 109 | 123 |
| N-butyric acid 2-ethylhexyl ester | 34.368 | 71 | 70 | 43 | 112 |
| Benzene, (1-pentylhexyl) | 34.602 | 91 | 18 | 161 | 105 |
| Benzene, (1-butylheptyl) | 34.901 | 91 | 147 | 175 | 105 |
| 2-decenal, (E) | 35.05 | 70 | 55 | 41 | 83 |
| Cyclopentane, 1,3-dimethyl | 35.075 | 70 | 41 | 55 | 43 |
| 2-undecanone | 35.279 | 58 | 43 | 71 | 59 |
| Benzene, (1-propyloctyl) | 35.602 | 91 | 133 | 189 | 232 |
| Cyclohexene, 1-methyl-3-(1-methylethenyl)-, (±) | 35.847 | 121 | 93 | 95 | 136 |
| 1H-pyrrole-2,5-dione, 3-ethenyl-4-methyl | 36.682 | 66 | 137 | 65 | 39 |
| 2,4-decadienal | 36.823 | 81 | 18 | 41 | 67 |
| Pentanoic acid, 2-ethylhexyl ester | 36.994 | 85 | 70 | 57 | 112 |
| y-terpinene | 37.148 | 93 | 121 | 136 | 68 |
| 3-nonen-5-yne, 4-ethyl | 37.293 | 121 | 77 | 91 | 94 |
| Decanoic acid, ethyl ester | 37.575 | 88 | 101 | 155 | 43 |

| | | | | | |
|---|---|---|---|---|---|
| 2-undecenal | 37.829 | 55 | 41 | 57 | 70 |
| Indole | 38.538 | 117 | 90 | 89 | 63 |
| Column bleed | 39.168 | 281 | 147 | 73 | 207 |
| Column bleed | 39.209 | 207 | 28 | 281 | 17 |
| 2-cyclohexen-1-ol, 2,6,6-trimethyl | 39.235 | 84 | 55 | 43 | 207 |
| Benzene, (1-pentylheptyl) | 40.695 | 91 | 161 | 246 | 281 |
| Dodecanoic acid, ethyl ester | 44.156 | 88 | 101 | 43 | 41 |
| Dodecanoic acid, propyl ester | 48.712 | 18 | 61 | 43 | 60 |

Presentation of compound names, retention times and the first 4 ions used by Metab for each compound present in the library used during the data analysis of the Dove study results. Library built using AMDIS and NIST database.

# Appendix D.

# Ethics committee approval

**R&D approval**

All investigators and research collaborators in the NHS should notify the R&D office for the relevant NHS care organisation of this amendment and check whether it affects R&D approval of the research.

**Statement of compliance**

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

We are pleased to welcome researchers and R & D staff at our NRES committee members' training days – see details at http://www.hra.nhs.uk/hra-training/

| 11/WM/0078: | Please quote this number on all correspondence |
|---|---|

Yours sincerely,

pp

**Dr Ronald Jubb**
**Vice Chair**

E-mail: NRESCommittee.WestMidlands-Edgbaston@nhs.net

*Enclosures:* *List of names and professions of members who took part in the review*

*Copy to:* *Mrs Kelly Hard, Birmingham Women's Hospital*
*Mr Brendan Laverty*

**NRES Committee West Midlands - Edgbaston**

**Attendance at Sub-Committee of the REC meeting on 17 December 2012**

| *Name* | *Profession* | *Capacity* |
|---|---|---|
| Dr Ronald Jubb | Retired Consultant Rheumatologist | Expert |
| Professor John Marriott | Pharmaceutical Chemist/Academic Pharmacist | Expert |

# Appendix E.

# Patient information sheet


Birmingham Women's NHS
NHS Foundation Trust

## Parent information Sheet

**Diagnostic testing of Volatile Organic Compounds (VOCs) in Necrotising Enterocolitis. The DOVE Study.**

Your baby is being invited to take part in a research study. Before you decide, it is important that you understand why the research is being done and what it will involve. Please ask us if there is anything that you do not understand or would like more information.

**What is the purpose of the Study?**
Necrotising enterocolitis (NEC) is an illness that affects premature or unwell babies causing inflammation of their gut. It is sometimes difficult to diagnose until later on in the disease. It would be very helpful to be able to identify children with the disease earlier as this would allow us to treat children with suspected NEC more effectively. We plan to look at the concentration organic chemicals called VOCs in your babies stool. In order to do this we will take small samples from their nappy.. This will be stored and the test will be done in batches later. The result of this test will not alter your baby's treatment. The analysis may lead to a rapid diagnosis bed-side test and may help us to choose more appropriate treatment for NEC in the future.

**Why has my baby been chosen?**
We are collecting stool samples from as many premature babies as possible in order to establish the level of VOCs in babies who go on to develop NEC and those who do not. Your baby is being invited to take part because as they are premature and we know that all premature babies are potentially at risk of developing NEC. By comparing the results of this test in babies showing features of NEC with those who do not we hope to find out if this test will be useful for identifying this disease earlier.

**Do I have to participate?**
No, you are free to refuse to take part in this study on behalf of your child. If you do not decide to take part it will not alter your baby's treatment in any way. If you decide to take part we will ask you to sign a consent form on behalf of your baby and give you a copy of this information sheet and the consent form for you to keep. If you decide to take part you are still free to withdraw at any time.
If you decide to take part in the study you baby's care will also remain the same.

**What will happen if I agree to participate on behalf of my baby?**
Your baby's treatment will continue as normal. If you agree to take part, a small sample of stool will be taken from your baby's nappy every day up to the time the baby is discharged. The samples will be stored in a freezer prior to analysis. If you have any concerns or complaints during the study please let the team know as soon as possible. You may wish to contact the PALS team also, you have the right to use the Hospital complaints procedure and they will be able to help you with this.


Birmingham Women's NHS
NHS Foundation Trust

**What are the possible side effects of taking part?**

There are no side effects of this study. We do not expect there to be any benefits to individual babies taking part. However the information we hope to gain from this research may help the treatment of babies in the future.

**Will the results of this study be kept confidential?**
The researcher will need to examine your baby's medical notes to find out about what happened to your baby following the stool sample being taken. All information will be anonymous and your baby will not be identifiable in any publication. The baby's details will not be discussed outside the hospital.

**What will happen to the results of the study?**
The results of the study will not be known until the data is analysed. The results will be reported in professional publications or meetings but babies will not be individually identified. We will make our results widely available so that doctors and nurses can use the information to help babies in the future.

**Who is funding and organising the research?**
The study is funded by Action Medical Research Charity. The study is being organised by University of Birmingham.

**Who has reviewed the study?**
Action Medical Research Charity and West Midland Research Ethics Committee have reviewed and approved the study.

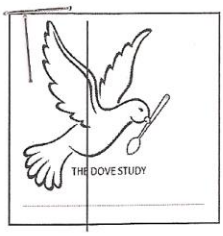
**What do I do now?**
Thank you for considering taking part in this research. If you have any further questions about the study please do not hesitate to contact me.

**Contact Details:**
Dr Andy Ewer, Consultant Neonatologist, Birmingham Women's Hospital. 0121 472 1377 ext. 4403.

# Appendix F.

# Sampling SOP for hospital staff

22-1-13




# STANDARD OPERATING PROCEDURE.

## SOP NUMBER:1:COLLECTION OF STOOL SAMPLES.

| AUTHOR: | | | |
|---|---|---|---|
| NAME: | ROLE: | SIGNATURE: | DATE: |
| NAME: | ROLE: | SIGNATURE: | DATE: |
| REVIEWED BY: | | | |
| NAME: | ROLE: | SIGNATURE: | DATE: |
| APPROVED BY: | | | |
| NAME: | ROLE: | SIGNATURE: | DATE: |
| DOCUMENT CONTROL: | | | |
| VERSION NUMBER: | IMPLEMENTATION DATE: | REVIEW DATE: | SIGNATURE: |

## BACKGROUND:

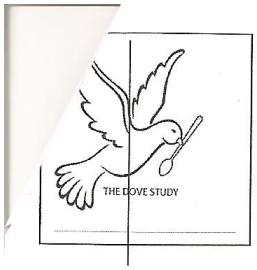
The background to this SOP is to give guidance on the collection of stool samples for the DOVE study.

This is to be viewed in conjunction with the normal policies of the hospital at which the baby is being cared for.

## SCOPE:

This SOP is written for all staff members involved in the collection of stool samples for the DOVE study.

As this study is being carried out over 5 hospitals, the SOP will address issues relating to the collection, labelling and storage of the stool samples. Cross infection control measures should be employed in accordance with standard procedures at the individual hospital.




# STANDARD OPERATING PROCEDURE.

## PROCEDURE:

. Once the sample has been obtained and put into the freezer as described, the data collection form needs to be completed, with the date and time of sample and the intials of the person who has collected the samples.

. If an infant does not have their bowels opened in 24hours, this needs to be documented on the Data Collection form, and the next sample collected when the infant next opens it's bowel's.

. If the sample collection is forgotten for any reason please ensure that the next available sample is collected.

## RELATED SOP'S:

The other related SOP is SOP number 2: Care of the DOVE freezer.

## REFERENCES:

The Control of Substances Hazardous to Health regulations 2002 as amended.

The Health and Safety at Work Act 1974.

Personal Protective Equipment at work 2002.

The Policy for Effective and Appropiate Hand Hygiene. Birmingham Women's Hospital 2nd March 2010.

The Policy for the use of gloves in the Clinical area. Birmingham Women's Hospital, December 2008.

# Appendix G.

# Statistical modelling performed by an external statistician

An external statistician developed and applied a script to perform a backwards stepwise logistic regression. This appendix presents the method applied and results obtained.

**Method**

Using Metab data, with missing values replaced by 1, Mrs Greenwood developed a Stata script to perform a backwards stepwise logistic regression. Features were selected among the 50 VOCs and 7 factors (i.e. the age at sampling, food type, hospital, gestation duration, birth weight, delivery type and sex), and the same model was applied at each day prior to diagnosis and on all days grouped together.

**Results**

VOCs used by the model were 2-butanone (10.55 min), 2-heptanone (22.00 min), 2-Z-heptanal (25.00 min), 1-decen-3-one (25.21 min), octanal (26.06 min) and carbon disulphide (7.8 min). Moreover, the gestation duration and the gender were also implemented.

The area under the receiver operating characteristic (ROC) curve was calculated at each day prior to diagnosis and when all days were considered (**Table 43**).

**Table 43: Results of the logistic regression model.**

| Day prior to diagnosis | All | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|---|
| N of observation | 251 | 51 | 31 | 62 | 33 | 47 | 27 |
| Area under ROC curve | 0.832 | 0.858 | 0.793 | 0.792 | 0.826 | 0.849 | 0.895 |

Table presenting the number of samples available at each day prior to diagnosis and with all days grouped. Furthermore, the area under the ROC curve obtained using a backwards stepwise logistic regression applied on the data at each time point was presented.