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URINARY VOLATILE ORGANIC COMPOUND ANALYSIS: A COMPLETE METHODOLOGY FOR GLOBAL PROFILING AND APPLICATION TO PANCREATIC CANCER

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DECLARATION OF ORIGINALITY

I hereby declare that I am the sole author of this thesis and that all the work within it is my own. Any individuals who carried out collaborative work are appropriately credited.

Qing Wen

14 Jan 2022

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ABSTRACT

Cancer continues to pose a major public health and economic burden worldwide. Whilst early diagnosis is frequently associated with improved treatment outcomes, vague initial symptoms means that many cancers are detected at a late, often incurable, state. There remains therefore an important unmet clinical need to develop accurate, acceptable, and affordable methods for cancer early detection.

The objective of this thesis was to develop a high throughput and standardised methodology for the deep profiling of urinary volatile organic compounds (VOCs), and to apply the developed methods for the discovery of urinary VOC biomarkers to non-invasively diagnose gastrointestinal cancers.

Towards this objective the optimal process and conditions of urine sample preparation were determined. Two urinary VOC extraction techniques, solid-phase microextraction (SPME) and HiSorb sorptive extraction, were compared and optimised for urinary VOC analysis. Following method development, HiSorb outperformed SPME for urinary VOC analysis, providing broader VOC detection, reduced batch effects, and high throughput reliable automated sampling. A standardised workflow using HiSorb was therefore developed for urinary VOC analysis to minimise the methodological heterogeneity and maximise the extraction of urinary VOCs. Three gas chromatography mass spectrometry (GC-MS) approaches, including conventional quadrupole-based thermal desorption GC-MS (TD-GC-MS) with a mid-polar column as a benchmark method, TD-GC-time of flight (TOF)-MS (non-polar column), and TD-GC-TOF-MS (polar column), were evaluated with a comprehensive method development being conducted to determine the optimal assay for urinary VOC analysis. Among the eight optimised assays, the best one was able to reliably identify 167 VOCs in urine of healthy subjects. Quality control (QC) measures, including the analysis of pooled QC urine samples, the usage of internal standards, and instrumental quality control, were evaluated and implemented. Removal of non-reproducible, non-linear, and contamination-related VOCs, data normalisation, and urine density correction during data pre-processing were also assessed for quality control purpose. These quality control measures were shown to significantly improve the dataset quality and enable the monitoring and correction of analytical variabilities, ensuring the delivery of reliable results. Subsequently, urine samples from a cohort consisting of pancreatic adenocarcinoma

patients (*n* = 28) and control patients (*n* = 33) were analysed using the optimised methodology. Univariate analysis identified 20 (ChromSpace/Gavin 3 data pre-processing pipeline) and 28 (MSHub/GNPS pipeline) candidate VOC biomarkers for the classification of cancer against control, 8 of which were identified by both pipelines, all with false discovery rate (FDR) corrected p value < 0.25. The majority of the candidate biomarkers identified by the optimal non-polar assay were highly expressed in the cancer group, while the compounds identified by the optimal polar assay were suppressed in cancer. Multivariate partial least squares-discriminant analysis (PLS-DA) showed weak differentiation between groups based on their global VOC profile. Receiver operating characteristic (ROC) curves were plotted using the candidate biomarkers whose adjusted p value < 0.05 (5-(2-thienoyl)butyric acid; butanal, 3-methyl-; furan, 2-methoxy-, and; cyclohexanone, 4R-acetamido-2,3-cis-epoxy-) and < 0.1 (5-(2-thienoyl)butyric acid; butanal, 3-methyl-; furan, 2-methoxy-; cyclohexanone, 4R-acetamido-2,3-cis-epoxy-; 2-butanone, 3-methyl-; 2-vinylfuran; 2,6-di-tert-butyl-4-methyl-phenol; 2-butanol, 2-methyl-; 2-cyclopenten-1-one, 2,3,4-trimethyl-; didodecyl phosphate, and; 2(3h)-furanone, 5-heptyldihydro-), with the obtained area under curves at 0.914 and 0.942, respectively, indicating an excellent diagnostic performance of the models.

This thesis provides standardised high throughput and reliable HiSorb-TD-GC-MS methods for the analysis of urinary VOCs. Preliminary analysis of urine from pancreatic cancer patients supports its application in future large scale clinical trials. Clinical implications and future research directions of these findings are discussed.

PUBLICATIONS AND PRESENTATIONS

PUBLICATIONS

Wen, Q., Boshier, P., Myridakis, A., Belluomo, I. & Hanna, G. B. Urinary Volatile Organic Compound Analysis for the Diagnosis of Cancer: A Systematic Literature Review and Quality Assessment. *Metabolites* 11, 17 (2020)

PRESENTATIONS

Wen, Q., Chin, S. T. & Hanna, G. B. Novel Headspace Sorptive Extraction on Urinary Volatile Organic Compounds for Oesophago-Gastric Cancer Detection. Mass Spectrometry: Applications to the Clinical Lab 2019 US 11th Annual Conference and Exhibits (MSACL 2019 US), Palm Springs, USA (2019)

Wen, Q., Myridakis, A., Chin, S. T. & Hanna, G. B. Deep Urinary Volatile Organic Compound Profiling with Headspace Sorptive Extraction and GC×GC-MS for Oesophago-Gastric Cancer Detection. Mass Spectrometry: Applications to the Clinical Lab 2019 EU 6th European Congress and Exhibition (MSACL 2019 EU), Salzburg, Austria (2019)

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LIST OF ABBREVIATIONS

ABX	Antibiotics
AD	Pancreatic adenocarcinoma
ATP	Adenosine triphosphate
AUC	Area under the curve
BHT	Butylated hydroxytoluene
BLI	Blue laser imaging
BRCA2	Breast cancer gene 2
BSTFA	N,O-bis(trimethylsilyl)trifluoroacetamide
CA19-9	Cancer antigen 19-9
CAR	Carboxen
CAS	Chemical Abstracts Service
CAWG	Chemical Analysis Working Group
CCD	Central composite design
CDF	Network common data form
CEA	Carcinoembryonic antigen
CI	Chemical ionisation
CP	Chronic pancreatitis
CR	Colorectal cancer
CT	Computed tomography
CV	Cross validation; Coefficient of variation
CWR	Carbon wide range
CYP3A4	Cytochrome P450 3A4
DBC	Dynamically baseline correction
DC	Direct current
DI	Deionised
DM	Diabetes
DNA	Deoxyribonucleic acid
DVB	Divinylbenzene
EI	Electron impact ionisation
ELISA	Enzyme-linked immunosorbent assay
ERCP	Endoscopic retrograde cholangiopancreatography
ESI	Electrospray ionisation
EUS	Endoscopic ultrasonography
EUS-FNA	Endoscopic ultrasonography-guided fine-needle aspiration
FAIMS	Field asymmetric waveform ion mobility spectrometry
FDP	Fibrin degradation products
FDR	False discovery rate
FFPE	Formalin-fixed paraffin-embedded
FIT	Faecal immunochemical test
FOBT	Faecal occult blood test
FOLFIRINOX	Leucovorin calcium, fluorouracil, irinotecan hydrochloride, oxaliplatin
GC	Gas chromatography
GI	Gastrointestinal
GLOBOCAN	Global Cancer Incidence, Mortality and Prevalence
GNPS	Global Natural Products Social Molecular Networking

GPI	Glycosyl phosphatidyl inositol
GWAS	Genome-wide association studies
HMDB	Human Metabolome Database
HPB	Hepato-biliary cancer
H. pylori	*Helicobacter pylori*
HPLC	High-performance liquid chromatography
KEGG	Kyoto Encyclopaedia of Genes and Genomes
LC	Liquid chromatography
LCI	Linked colour imaging
LLOD	Lower limit of detection
LLOQ	Lower limit of quantification
MCCV	Monte-Carlo cross validation
MDCT	Multidetector computed tomography
ME	Magnifying endoscopy
MEDLINE	Medical Literature Analysis and Retrieval System Online
MIPK	Methyl isopropyl ketone
MOOSE	Meta-analysis of Observational Studies in Epidemiology
MRI	Magnetic resonance imaging
MS	Mass spectrometry
MSD	Mass spectrometry detector
MSI	Metabolomics Standard Initiative
MW	Molecular weight
NBI	Narrow band imaging
NET	Pancreatic neuroendocrine tumour
NIST	National Institute of Standards and Technology
NMF	Non-negative matrix factorisation
NMR	Nuclear magnetic resonance spectroscopy
OGD	Oesophago-gastro-duodenoscopy
OPLS-DA	Orthogonal projections to latent structures discriminant analysis
PCa	Prostate cancer
PCA	Principal component analysis
PDAC	Pancreatic ductal adenocarcinoma
PDMS	Polydimethylsiloxane
PET	Positron emission tomography
PFBHA	O-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine
PG	Pepsinogen
PLS-DA	Partial least-squares discriminant analysis
ppm	Parts-per-million
ppb	Parts-per-billion
PRISMA	Preferred Reporting Items for Systematic Reviews and Meta-Analyses
PVT	Pyrrolidinopentiothiophenone
QC	Quality control
QUADAS	Quality Assessment of Diagnostic Accuracy Studies
RF	Radio frequency
RI	Retention indices
RMF	Reverse match factor
RNA	Ribonucleic acid
ROC	Receiver operating characteristic

RT	Retention time
SD	Serial dilution, standard deviation
SIFT	Selected ion flow tube mass spectrometry
SIM	Selected ion monitoring
SNP	Single-nucleotide polymorphism
SPME	Solid-phase microextraction
STARD	Standards for Reporting of Diagnostic Accuracy Studies
SVM	Support vector machines
TAA	tert-Amyl alcohol
TBE	Tribromoethanol
TCA	Tricarboxylic acid
TD	Thermal desorption
TF	Transfer line
TFF	Trefoil Factor
TIC	Total ion chromatograms
TOF	Time of flight
TPME	Three phase micro extraction
UGI	Upper gastrointestinal series
USG	Urine specific gravity
UV	Unit-variance
VIP	Variable importance for the projection
VOC	Volatile organic compounds
WES	Whole-exome sequencing

SECTION 1

INTRODUCTION

1 INTRODUCTION

1.1 Cancer

Cancer has become a major public health and economic burden worldwide. During industrialisation, urbanization and economic development, the overall incidence and prevalence of cancer is increasing due to aging and growing of global population, as well as the increase in risk factors such as tobacco/alcohol use, obesity, infection, poor dietary patterns, and advancement of diagnostics.[1,2] In 2020, a total of 19.3 million new cancer cases were recorded. Between 2012 and 2020, the number of new cancer diagnosis worldwide has increased by 36%.[3,4] It is projected that in the next two decades, there will be a 56% rise in terms of annual new cancer diagnosis, to 30.2 million in 2040.[5] In 2020, approximately 9.96 million cancer-related deaths occurred worldwide. It is predicted that by 2040 there will be 16.3 million deaths annually from cancer.[3,5] Based on GLOBOCAN estimates, cancer is the first or second leading cause of death before the age of 70 years in 112 of 183 countries.[6]

Breast cancer has overtaken lung cancer to become the most common cancer in the world, with nearly 2.3 million cases (11.7% of total cases) diagnosed in 2020. Other common cancers include lung (11.4%), colorectal (10.0%), prostate (7.3%), skin (non-melanoma) (6.2%), gastric (5.6%), liver (4.7%), cervix and uterine (3.1%), and oesophageal (3.1%) cancers.[3,6] Lung cancer (1.8 million cases, 18% of total deaths) remains the leading cause of cancer death. Other leading causes of cancer death are colorectal (9.4%), liver (8.3%), gastric (7.7%), breast (6.9%), oesophageal (5.5%), pancreatic (4.7%), and prostate (3.8%) cancers.[3,6] New cases and deaths for the ten most common cancers in 2020 are demonstrated in Figure 1.

Cancer statistics also show that the burden pattern varies in different places of the world, as a result of exposure to different risk factors which are highly associated with the development level of the local society. For example, in the UK, breast (15%), prostate (13%), lung (13%) and bowel (11%) cancers are the most common cancers, accounting for more than half (53%) of all new cases in 2017.[7] Lung (21%), bowel (10%), prostate (7%) and breast (7%) cancers together caused nearly half (45%) of all cancer deaths in 2018.[8] In less developed areas, cancer occurrence can be associated largely with risk factors including infection, poor hygiene, unhealthy diets, chronic

inflammation, etc. For instance, in sub-Saharan Africa, one in three cancers is associated with a chronic infection.[9] 66% of the worldwide burden of lip and oral cavity cancer occurs in less developed areas, with Indian subcontinent contributed 33% of all cases.[10]

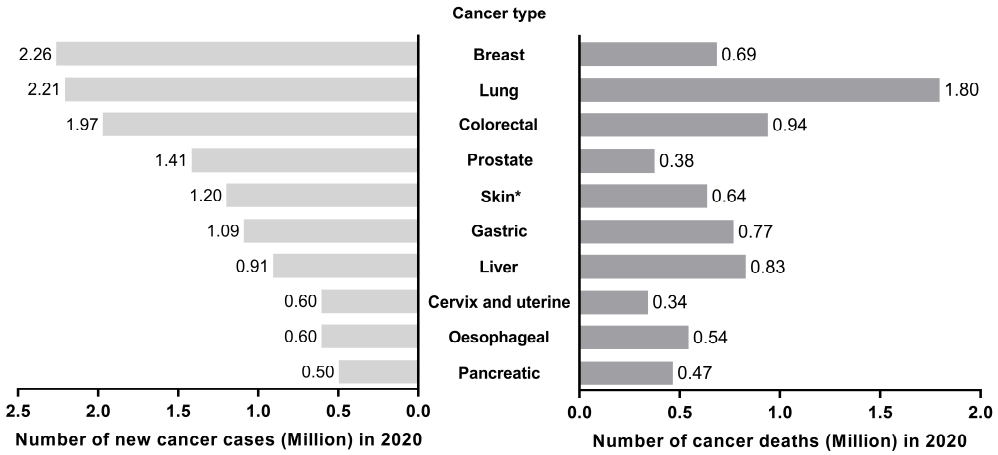


Figure 1. New cases and deaths for the ten most common cancers in 2020.

*: Non-melanoma skin cancer.

1.2 Gastrointestinal cancers

Gastrointestinal (GI) cancers, including oesophageal, gastric, colorectal, and pancreatic cancers, make up 21.1% of all new cancer cases and account for 27.1% of all cancer deaths in 2020, resulting in a major healthcare burden to both developed and less developed areas. Moreover, their survival rates are among the lowest of any cancer types, and late diagnosis of these cancers is associated with significantly worse overall survival. If diagnosed early (stage I), five-year survival rates of oesophageal and gastric cancer can reach 52% and 65.3%, respectively, as opposed to around 0% if diagnosed late (stage IV).[11,12] For colorectal cancer, five-year survival rates at stage I, stage II, and stage III are 91.7%, 84.1%, and 64.9%, while the number drops significantly to 10% when diagnosed at stage IV.[13] For pancreatic cancer, five-year survival rate can reach 75% if detection would be possible before clinical symptoms present.[15] However, as many as 80% to 85% of patients are diagnosed with unresctable or metastatic disease, resulting in overall survival of 7.3%.[14-16] The opportunity to provide early therapeutic intervention with curative intent, before the cancer reaches an advanced and incurable stage, is seen as the primary reason for

improved survival in early disease. Therefore, the earlier detection of gastrointestinal cancers is critical to improve the patient's prognosis and to relieve cancer-related economic burden.

The majority of gastrointestinal cancers are adenocarcinomas, arising from the gland cells in the mucosa from the lower oesophagus to rectum, as well as accessory digestive organs (e.g., pancreas).[17-20] Other histological subtypes include squamous cell carcinoma, lymphoma, neuroendocrine tumour, sarcoma, and melanoma.[17-20]

1.3 Gastrointestinal cancer symptoms

It is common for people with early-stage gastrointestinal cancers to experience no symptoms. With the growth and spread of tumours, they may experience symptoms such as unexplained weight loss, indigestion, pain in abdomen or back, weakness and fatigue, many of which are vague and are also the symptoms of common benign gastrointestinal conditions. This overlapping of symptoms is often associated with a delay in the diagnosis of gastrointestinal cancers. It is therefore common that many patients do not present to medical attention until the occurrence of "red flag" symptoms, including jaundice, dysphagia, bleeding, etc. These symptoms often suggest that tumours have grown to an advanced stage and have metastasised to other organs.

Current strategies to detect gastrointestinal cancers often result in delayed diagnosis. There is still an important unmet clinical need to improve early cancer diagnosis.

1.4 Methods for cancer diagnosis

A cancer diagnosis may occur in several ways, including: patient initiated presentation to medical services, usually as a result of either new or concerning symptoms; as a result of an established cancer screening programme, or; opportunistically at the time of investigation for an unrelated medical condition. In most circumstances, confirmation of a cancer diagnosis is made through acquisition of a tissue biopsy.

1.4.1 Screening

Cancer screening is a strategy that looks for early cancer in at risk individuals without symptoms.

1.4.1.1 Oesophageal cancer

Endoscopy is now widely recognised as the most effective procedure for oesophageal screening. Oesophageal adenocarcinoma screening is recommended by the current British Society of Gastroenterology and American College of Gastroenterology guidelines in patients with a history of gastroesophageal reflux disease lasting > 5 years and multiple other risk factors, including male sex, Caucasian race, central obesity, and current or previous history of smoking.[21,22] There is currently no screening guideline for oesophageal squamous cell carcinoma, although in 2014, Chinese experts established a consensus on oesophageal cancer screening.[23] In many Asian countries where the rate of upper gastrointestinal cancer is disproportionately high, including South Korea, Japan, and China, the implementation of screening programmes is more common. Several alternative non-endoscopic screening approaches have been developed in recent years and early findings have been published, including a novel test called Cytosponge, which can collect cells from the lining of the oesophagus coupled with an immunohistochemical test (Trefoil factor 3 / TFF3) for the screening of Barrett's oesophagus.[24,25]

1.4.1.2 Gastric cancer

Although the upper gastrointestinal series (UGI) was formerly a gold standard for identifying gastrointestinal disorders, it has a poor sensitivity of 38%.[26] UGI series is still recommended in Japan and South Korea's national gastric cancer screening

programmes because studies conducted in Japan showed that screening with the UGI series resulted in a 40% reduction in gastric cancer mortality.[27] In Japan and China, serum pepsinogen (PG) testing is gaining widespread acceptance due to its convenience, lack of discomfort or danger, efficiency, and economy, with the sensitivity and specificity reaching 84.6% and 73.5%, respectively.[28] Endoscopy is the principal approach for gastric cancer screening in Japan and South Korea, and it is also highly recommended in China. There is no general screening programme for gastric cancer using endoscopy in less prevalence countries due to its limitations. *Helicobacter pylori* serology is used to detect the antibody of *H. pylori*, which is associated with the presence of gastric cancer or pre-cancerous lesions. Serology is cheap hence is considered the best option for population-based screening. This test is advised in conjunction with others because of its limited sensitivity and specificity.

Only Japan and South Korea have a comprehensive gastric cancer screening system.[27,29] Chinese experts' consensus on gastric cancer screening are that people over the age of 40 years with at least one of the following risk factors should participate in screening to start the serological biopsy for five biomarkers (PGI, PGII, PGI/PGII ratio, *H. pylori* antibody, and gastrin-17): having *H. pylori* infection, originating from a high incidence rate area, having a family history, having pre-cancerous diseases, or having other lifestyle risk factors.

1.4.1.3 Colorectal cancer

Colorectal cancer is one of the cancers that might benefit the most from screening, as there is usually a wide window (can be 10 years) for apparent lesions to evolve into colorectal cancer. Major approaches for colorectal cancer screening include faecal occult blood test (FOBT) / faecal immunochemical test (FIT), colonoscopy, and other tests based on stool samples. FOBT or FIT has been suggested as a first-line population-screening method for colorectal cancer in multiple countries due to its lowest cost and ease of implementation. However, a positive FOBT or FIT test may result from bleeding in anywhere of the gastrointestinal tract therefore it cannot directly detect colon cancer. A colonoscopy must be performed to confirm the positive results. Colorectal cancer screening standards and programmes have been established in most European countries, the USA, and China. Most countries (China, Denmark, France, Norway, UK, etc.) recommend FIT to people over 50 years old and average risk population. Only the USA, China, Germany, and Austria recommend colonoscopy

as a screening test.[30,31] Most guidelines recommend that individuals over the age of 50 have a FIT test once a year and a colonoscopy once every ten years.[32,33]

1.4.1.4 Pancreatic cancer

There is no standard diagnostic tool or established early detection method for pancreatic cancer in the general population yet. People who have a high risk of developing pancreatic cancer, such as hereditary pancreatitis, familial pancreatic cancer, and BRCA2 gene mutation, can take part in a screening study consists of blood tests, a CT scan, an endoscopic ultrasound, and gene tests (K-Ras, p53 and p16).[34]

Table 1 summaries the approaches for screening gastrointestinal cancers and countries that have established guidelines or programmes for the screening of the GI cancers.

Table 1. Screening approaches for GI cancers in different countries.

Cancer Type	Screening Approach	Country that Has Guidelines or Programmes for Cancer Screening
Oesophageal	Endoscopy	USA, UK (Only for oesophageal adenocarcinoma)
Gastric	UGI; pepsinogen; *H. pylori* serology; endoscopy	Japan, South Korea
Colorectal	FOBT/FIT; DNA-FIT; colonoscopy	USA, China, Japan, most European countries, etc.
Pancreatic	Not established	-

UGI: Upper GI series; GI: gastrointestinal cancer; FOBT: faecal occult blood test; FIT: faecal immunochemical test.

1.4.2 Endoscopy

The diagnosis of gastrointestinal cancers (oesophageal, gastric, and colorectal cancer) relies on endoscopy with biopsies for histological confirmation.[17-19] Endoscopy can also provide information about local and sublocation extent of the tumour. Overall, the procedure is safe and effective, but it does have potential complications such as perforation, infection, bleeding, over sedation, persistent pain, and pancreatitis (after endoscopic retrograde cholangiopancreatography, ERCP). Its invasiveness makes it less acceptable to patients and unsuitable for large scale screening of asymptomatic population.

1.4.2.1 Oesophago-gastro-duodenoscopy (OGD)

In most countries, an oesophago-gastro-duodenoscopy (OGD) examination is the standard first-line investigation for upper gastrointestinal symptoms. There is however no international agreement on the best examination strategy for maximising diagnostic output of OGD. There are guidelines for standardised endoscopic examination and documentation, but they are complex and rarely followed. False negative rates of OGD examinations are anticipated to range between 10% and 20% in the absence of a widely recognised and acceptable protocol for a systemic assessment and objective determination of quality in upper gastrointestinal endoscopy.[35,36]

Attempts have been made to improve the diagnostic performance of OGD. A mandatory set of systemic images in endoscopy reports may improve their quality and reduce interpretive variabilities. The use of systemic alphanumeric coded endoscopy approach during endoscopy increases the detection of high-risk lesions. A longer examination time (at least 7 to 8 min) allows for higher likelihood of detecting neoplastic lesions. Implementation of image enhanced endoscopy (absence of iodine staining on chromoendoscopy, non-magnifying narrow band imaging (NBI), blue laser imaging (BLI), magnifying endoscopy (ME), linked colour imaging (LCI), etc.) can improve the detection of oesophageal and gastric lesions.[37]

1.4.2.2 Colonoscopy

Colonoscopy is the gold standard method for the diagnosis of colorectal cancer, with direct visualisation of the colonic mucosa and the ability to biopsy or excise polyps and

localised cancers. It can lower the incidence and mortality of colorectal cancer to around 50% among subjects who had accepted endoscopic removal of adenomas.[38] However, early colorectal cancers might appear as subtle mucosal lesions, such as an innocuous flat laterally spreading polyp. Therefore, there is a risk of developing colorectal cancer after colonoscopy due to incomplete mucosal inspection.

1.4.2.3 Sigmoidoscopy

Sigmoidoscopy enables the inspection of the inside of the large intestine from the rectum to the descending colon. With sufficient training, primary care doctors, nurse practitioners, and physician assistants can perform sigmoidoscopy. Since the technique is less invasive than colonoscopy or CT colonography, the procedure may be conducted in the office without sedation.

Compared to colonoscopy, sigmoidoscopy only covers the lower part (left side) of the colon, thus any proximal cancer or polyps cannot be detected. Very rarely it can cause complications such as perforation or bleeding. The evidence of benefits of sigmoidoscopy in detecting colorectal cancer is stronger than colonoscopy. Screening with flexible sigmoidoscopy lowers colon cancer related mortality, according to four major randomised controlled studies.[39-42] Studies also show that the benefit of inspecting the proximal colon is overshadowed by the higher risks associated with colonoscopy for average patients at risk.[43,44]

1.4.2.4 Endoscopic ultrasonography (EUS)

Endoscopic ultrasonography (EUS) has long been considered as a primary approach in diagnosing and staging oesophageal, gastric, and colorectal cancer.[17-19] For pancreatic cancer, EUS and endoscopic ultrasonography guided fine needle aspiration (EUS-FNA) are both excellent diagnostic tools. A meta-analysis of contrast-enhanced EUS reported a sensitivity of 94% and a specificity of 89% for pancreatic cancer diagnosis.[45] EUS-FNA has a high diagnostic accuracy of more than 85% to 90% for pancreatic cancer.[46]

On the other hand, EUS is invasive, highly operator dependent with risk of perforation, and it cannot detect distant metastases. In large or stenotic tumour, the malignant

stricture may prevent the passage of echoendoscope to visualise the entire tumour and surrounding lymph nodes completely, therefore resulting in incomplete cancer staging.

1.4.2.5 Endoscopic retrograde cholangiopancreatography (ERCP)

Endoscopic retrograde cholangiopancreatography (ERCP) combines the endoscopy and fluoroscopy to diagnose and treat disorders of the biliary or pancreatic ductal systems. ERCP continues to be an important modality to provide biliary drainage in patients with obstructive lesions in the head of pancreas due to cancer and can be used to perform biliary and pancreatic duct brushing cytology in patients with invasive pancreatic cancer. The pancreatogram acquired during the ERCP can show pancreatic duct stenosis; blockage, obstruction, narrowing and abnormal branching of the main pancreatic duct; as well as obstruction and encasement of the common bile duct.

ERCP is primarily performed by highly skilled and specialty trained gastroenterologists therefore is highly operator dependent. Because of its invasiveness, it is now rarely performed without therapeutic purpose, particularly when less invasive options (imaging techniques such as multidetector computed tomography (MDCT)) are available.

1.4.3 Imaging

Imaging techniques enable the visualisation of structures within human body. They have become an essential element not only for cancer early detection, but also for cancer diagnosis, staging, and introducing or evaluating cancer treatments.

1.4.3.1 Computed tomography (CT)

Computed tomography (CT) has become the most used modality in the initial staging of newly diagnosed intestinal cancer. High associated costs and exposure to ionising radiation has meant that CT has not found wider use as the initial modality for cancer detection.

In oesophagogastric cancer, CT is of principal use in detecting distant metastasis and helping to determine those patients who are not suitable for a curative treatment pathway.[17,18,47] A lack of sensitivity for identifying lymph node metastases and extraserosal invasion of gastric cancer is an important limitation of CT in respect to staging of oesophagogastric cancer.[47-49]

For the diagnosis of polyps and colorectal cancer, CT colonography is utilised as a complementary imaging modality, especially after incomplete or inadequate colonoscopy.[19] However, there is debate that it is no better than barium enema colonoscopy, and it cannot certainly determine the histology of most polyps it depicts.[50,51]

When a diagnosis of a pancreatic cancer is suspected, the recommended initial imaging method is a contrast enhanced (pancreatic protocol) CT, which has a sensitivity over 90%.[52,53] The use of early arterial phase, pancreatic phase, and portal venous phase images allows comprehensive visualisation of the lesion and reginal vasculature, and is the current gold standard to evaluate the resectability of any lesion.[16]

1.4.3.2 Magnetic resonance imaging (MRI)

MRI has high sensitivity (71% to 92%) for detecting oesophageal cancer, which shows potential for determining the response to neoadjuvant therapy and for detecting locally advanced disease in order to apply appropriate treatment. However, because MRI has a limited specificity (67% to 86%), it should be used in conjunction with EUS guided biopsy.[54]

In gastric cancer, MRI is currently used to refine preoperative staging and to evaluate treatment response, as well as to provide additional information for diagnosis and treatment, particularly in patients who cannot receive iodine contrast agents, have peritoneal implants, or have small hepatic metastatic lesions. EUS and CT remain the primary imaging modalities used in clinical decision making for gastric cancer. There is no standardisation or criteria for using MRI to define tumour invasion depth, reginal lymph nodes involvement, or distant metastases. There are also no clear criteria to precisely assess treatment response.[55]

MRI can be used as an imaging tool in the staging of colorectal cancer. MR colonography can be used as an alternative to CT colonography when the latter is contraindicated.[56] In rectal cancer, loco-regional staging is routinely carried out by magnetic resonance imaging (MRI) rather than CT.[57] MRI's role for determination of liver lesions is also increasing. However, when compared with other staging modalities, MRI is typically linked with higher prices, longer examination durations, less availability, and a lower robustness.

For pancreatic cancer diagnosis, MRI can provide excellent resolution of the pancreatic ducts and of any cysts that might be present. It might also be more sensitive than multidetector computed tomography (MDCT) in detecting and evaluating liver metastases. Since MRI has been found to have similar overall sensitivity and specificity as CT in detecting and staging pancreatic cancer, it is often used as a second-line test.[58]

1.4.3.3 Positron emission tomography and computed tomography (PET-CT)

Positron emission tomography and computed tomography (PET-CT) is a staging measure for more advanced oesophageal tumours, while both PET-CT and MRI are not routinely used for staging in gastric cancer especially in patients with mucinous tumours, as it might understage the disease.[59] The role of PET-CT in colorectal cancer staging is still controversial.[60] Although PET-CT can be used in selected situations, it is not a part of the routine pancreatic cancer diagnosis.[58]

1.4.4 Biomarkers

Given the limitations of endoscopy, biopsy, and imaging techniques, researchers have been attempting to develop sensitive and specific non-invasive biomarkers to detect cancer or pre-cancerous states when patients are asymptomatic. Currently, a number of cancer biomarkers have been approved for clinical use and are being applied to detect cancer in patients.

Table 2 summarises the gastrointestinal cancer biomarkers that have been approved by the US Food and Drug Administration (FDA) for clinical use. Despite years of research and a considerable number of publications proposing novel screening tools for gastrointestinal cancer diagnosis, there are limited single biomarkers with satisfactory sensitivity and specificity.

Faecal occult blood test (FOBT) and faecal immunochemical test (FIT) are both lateral flow immunoassays used for the screening of colorectal cancer. They have been discussed in Section 1.4.1.3.

AMDL-ELISA DR-70® (FDP) immunoassay is an informative test for monitoring disease progression in colorectal cancer patients. It is an enzyme-linked immunosorbent assay (ELISA) based serological test that can quantitatively measure the fibrin degradation products (FDP). Fibrin degradation products are reported to be elevated in the plasma of cancer patients as products of angiogenic, chemoattractant, and anti-inflammatory activities which are highly active in tumour growth, invasion, and metastasis.[61] It has been proven that the DR-70 test yields informative data regarding colon cancer progression when taken as a 15% or greater change from the previous follow-up visit, providing evidence to show that its performance is greater than the clinical diagnoses based on chance alone.[62]

CA19-9 is an established serum biomarker for pancreatic cancer, with a sensitivity of 79% to 81% and specificity of 82% to 90% for symptomatic patients.[63] Serial CA19-9 testing is useful in monitoring response to systemic treatment in the neoadjuvant or metastatic disease. Preoperative CA19-9 elevation can also assist in predicting long-term survival following resection and identifying individuals who are less likely to have

R0 (microscopic margin negative) resections.[63,64] CA19-9 concentrations might predict prognosis for patients with inoperable pancreatic cancer.[65,66] One major limitation of CA19-9 is that it can be elevated in patients with biliary obstruction.[67]

Carcinoembryonic antigen (CEA) is a set of highly related glycoproteins involved in cell adhesion. CEA are glycosyl phosphatidyl inositol (GPI) cell-surface-anchored glycoproteins that may play a critical role in the metastasis of colon carcinoma cells. In healthy adult, CEA is present only at low concentration (less than 2.5 ng/mL). Its concentration is increased in heavy smokers (up to 5 ng/mL), and can increase to significantly high level in patients with colorectal, gastric, pancreatic, non-small cell lung, and breast carcinomas.[68] Since increased concentration of CEA often occur several months before other clinical signs appear, it is used as a primary biomarker for staging colorectal cancer, and monitoring the recurrence or spread of colon cancer after surgical resection.[69]

Cell-free DNA, miRNA, and proteomics approaches are the most common targets to develop novel tests for cancer detection. Some examples are: Epi proColon® 2.0 CE (detecting Septin 9 methylated DNA in blood for colorectal cancer screening); single-nucleotide polymorphisms (SNPs), genome-wide association studies (GWASs), whole-exome sequencing (WES); noncoding RNAs (ncRNAs); cell-free DNAs (cfDNAs), etc.[70] In terms of biosample types, current potential blood-based biomarkers for GI cancers are far more than urinary biomarkers.

Table 2. List of FDA-approved GI cancer biomarkers.

Biomarker	Clinical Use	Cancer Type	Specimen	Methodology	Year First Approved
Fibrin / fibrinogen degradation product (DR-70)	Monitoring disease progression	Colorectal	Serum	Immunoassay	2008
c-Kit	Detection of tumours, aid in patient selection	GI stromal tumours	FFPE tissue	Immuno-histochemistry	2004
Cancer antigen 19-9 (CA19-9)	Monitoring disease status	Pancreatic	Serum, plasma	Immunoassay	2002
Carcinoembryonic antigen (CEA)	Monitoring therapy, detecting recurrence	Colon, gastric, pancreatic, lung, and breast	Serum, plasma	Immunoassay	1985
Human haemoglobin (faecal occult blood, FOBT/FIT)	Detection of faecal occult blood (home use)	Colorectal	Faeces	Lateral flow immunoassay	1976

GI: gastrointestinal cancer; FFPE: formalin-fixed paraffin-embedded; FOBT/FIT: faecal occult blood test / faecal immunochemical test.

1.5 Metabolism and cancer

More than 80 years ago, Otto Warburg observed that proliferating cancer cells would undergo a metabolic switch from oxidative to glycolytic metabolism. The cancer cells produce far less adenosine triphosphate (ATP) than expected from a full tricarboxylic acid (TCA) cycle coupled to oxidative phosphorylation. Instead, a significant amount of lactate is accumulated. This phenomenon is known as “Warburg Effect”.[71]

When the research on cancer metabolism advances, it was found that cancer cells of some tumours induce neighbouring stromal cells to undergo aerobic glycolysis (Warburg Effect) and then transfer the products back to feed the cancer cells for utilisation for mitochondrial oxidative phosphorylation. This phenomenon has been called ‘‘Reverse Warburg Effect”.[72] Cancer cells in some tumours are not entirely dependent on accelerated glycolysis, allowing tumours to adapt to changing micro-environmental conditions in order to maximise cellular proliferation and growth.

For decades, studies have been conducted trying to understand the underlying molecular mechanisms for these altered metabolic regulations. It is now widely accepted that cancer cells adjust their flux through various metabolic pathways automatically to satisfy increasing bioenergetic and biosynthetic demand, and to reduce oxidative stress, both of which are essential for cancer cell proliferation and survival. The flux through these metabolic pathways is controlled by two main factors: cancer driver mutations and environmental nutrient availability. Importantly, when metabolites accumulate abnormally, they might also contribute to tumorigenesis.[73]

Since most tumour cells are extremely metabolically active and hence undergo dramatic alterations in a variety of metabolic pathways, cancer is an excellent candidate for metabolomic profiling. Metabolic profiling (metabolomics/metabonomics) is the measurement of low molecular weight metabolites (less than around 1 kDa) and their intermediates in biological systems to determine how they respond dynamically to genetic alterations, as well as physiological, pathological, and/or developmental stimuli.[74-76] After a metabolic profiling analysis, a few hundred metabolites with changing abundance can be identified, plus a few hundred more that are not clearly identified (might be contaminants). A typical metabolomics experiment, when analysed

properly using statistical methods, can provide a metabolite "signature" that distinguishes cancer from a normal condition.[77] Unlike transcriptomics and proteomics, where substrates are frequently modified, metabolomics can also shed light on disease mechanisms by identifying compounds that are directly produced in response to cancer or toxicity, and can lead to druggable pathways that are altered during the disease process.

1.6 VOC analysis for cancer diagnosis

Volatile organic compounds (VOCs) are carbon-based compounds that have high vapor pressure therefore are in gaseous form at room temperature. VOCs have traditionally been measured in the assessment of environmental contaminants, the food and fragrance industry, and counterterrorism. There is now strong evidence linking different cancers to the dysregulated production of volatile organic compounds (VOCs).[78-83] As end products of metabolism, cancer-related VOCs may be produced by oxidative stress and peroxidation of cell-membranes, as well as due to gene or protein alterations in cancer cells. VOCs levels can reflect pathophysiological processes including inflammation, necrosis, and cancer development.[84,85] VOCs formed within cancer tissues pass to the systemic circulation, from where they can be released from the body through a number of routes, including within exhaled breath and urine. VOCs have been detected in a variety of biological matrices taken from humans, including breath, urine, blood, faeces, tissue, and skin.

Previous studies have reported the unique VOC signatures of breast, lung, colorectal, oesophagogastric, and pancreatic cancer. Peng, G. et al. suggested each of the cancers (lung, breast, colorectal, and prostate cancer) could have a unique pattern of breath VOCs, when compared with healthy states.[78] Li, J. et al. verified the diagnostic value of hexanal, heptanal, octanal, and nonanal as breath biomarkers in early diagnosis of breast cancer (Area under the receiver operating characteristic (ROC) curves were 0.816, 0.809, 0.731, and 0.830, respectively), and the diagnostic accuracy improved when in combined use (AUC 0.934).[79] 2-butanone, 1-propanol, isoprene, ethylbenzene, styrene, and hexanal have been suggested as VOC breath biomarkers of lung cancer by a number of studies.[80] Altomare, D. F. et al.'s study indicated a pattern of 15 compounds in exhaled breath that was able to discriminate patients with colorectal cancer at an accuracy of 85%.[86] Kumar, S. et al. found 12 compounds as candidate breath VOC biomarkers to distinguish oesophageal adenocarcinoma (AUC 0.97) and gastric adenocarcinoma (AUC 0.98) from non-cancer control.[82] Markar, S. R. et al. identified 12 compounds which were significantly different between breath from pancreatic cancer and non-cancer patients, and the AUC was 0.736 for differentiating cancer from non-cancer, and 0.744 for differentiating adenocarcinoma from non-cancer.[83]

1.7 Urinary VOCs

In comparison to other biological samples such as blood and tissue, urine can be collected and handled simply and non-invasively at minimum cost in large amounts. Additionally, since urine includes the effluent of renal haemofiltration, it may offer information not only on the urogenital tract's local environment, but also on systemic metabolism.

Profiling of VOCs within urine and exhaled breath has the advantage of being entirely non-invasive. However, unlike breath, urine is potentially easier to collect and store.

A number of studies have investigated urinary VOCs in cancer patients with promising findings. Hanai et al. discovered that nine VOCs that were found in greater amounts in the urine of lung cancer patients.[87] Nissinen, S. I. et al. evaluated the ability of field asymmetric waveform ion mobility spectrometry (FAIMS) as a non-invasive rapid urine test for the discrimination of pancreatic cancer patients from healthy controls by analysing urinary VOCs, with a sensitivity of 79% and a specificity of 79%.[88] Through detecting urinary VOCs, Arasaradnam et al. demonstrated for the first time the efficacy of urine specimens in discriminating healthy subjects from pancreatic ductal adenocarcinoma (PDAC) patients. Additionally, their results were able to differentiate between early and late stage PDAC.[89]

To thoroughly understand present progress and advancements of urinary VOC analysis for cancer detection, and to identify published urinary VOC biomarkers, a systematic literature review was conducted and is presented below in Section 2.

1.8 Methods for VOC detection

Concurrent technological advancements have helped to establish further understanding of the complex and often subtle variation in disease specific biomarkers including VOCs. Whilst nuclear magnetic resonance spectroscopy (NMR) has been used extensively for the purpose of fluid phase metabolic phenotyping, mass spectrometry (MS) based platforms are traditionally favoured for the detection of urinary VOCs. These platforms are now capable to accurately detect trace VOCs at levels equivalent to which they are found within human samples, typically parts-per-million (ppm) and parts-per-billion (ppb). Gas chromatography mass spectrometry (GC-MS) is the most widely reported analytical technique for urinary VOC profiling due to its sensitivity, selectivity, and identification capabilities as well as its compatibility with volatile analytes.[90]

1.9 Overview of gas chromatography mass spectrometry (GC-MS)

A GC-MS instrument is composed of two major components: the gas chromatograph (GC) and the mass spectrometer (MS) (Figure 2). In the GC, a capillary column coated with a stationary phase (liquid or solid) is used, with its separation characteristics determined by the column dimensions (length, diameter, and film thickness) as well as the phase properties (e.g., polysiloxanes, polyethylene glycols) (Figure 2, a). When the sample is effectively vaporised into the gas phase mixture and is introduced into GC, the mixture is propelled by an inert carrier gas, which can be helium, hydrogen, or nitrogen. As the sample travels the length of the column, due to the difference of boiling point, polarity, and relative affinity for the stationary phase of the column, each compound will be separated from the mixture and elute from the column at a different time. The time it takes for a compound to elute is referred to as its retention time (RT) (Figure 2, a). After leaving the GC, the compounds are captured, ionised, and fragmented by the mass spectrometer using electron or chemical ionisation (EI or CI) sources (Figure 2, b). EI is a hard (high fragmentation) ionisation method in which energetic electrons interact with gas phase molecules to produce ions. It is one of the first ionisation techniques developed for mass spectrometry and is still a popular method of ionisation. Ionised compounds and fragments are then accelerated through the mass analyser of the device (e.g., quadrupole or time of flight), where these ions are separated based on their distinct mass-to-charge (m/z) ratios. The analytical data

acquired from the instrument consist of mass spectra and their corresponding retention times (total ion chromatograms (TIC)) (Figure 2, c, d).

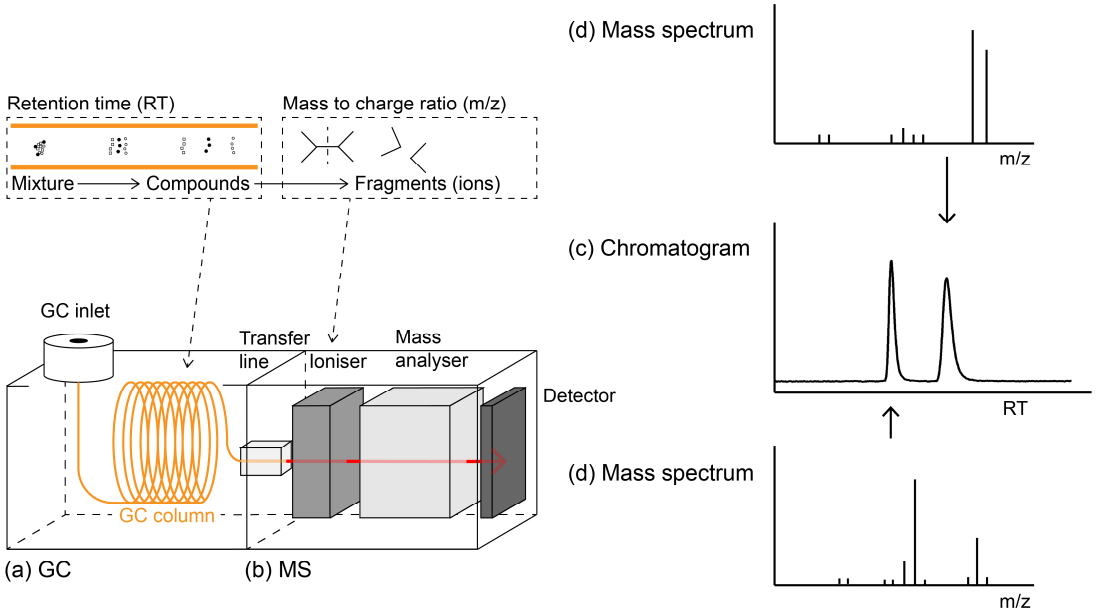


Figure 2. Schematic representation of GC-MS instrument.

(a) The gas phase sample is propelled by an inert carrier gas into the heated GC equipped with a capillary GC column for chromatographic separation. (b) Compounds that have been separated enter the MS through the transfer line and are ionised and fragmented by the ioniser. Ions and fragments are accelerated through the mass analyser and detected by the detector. The analytical data consists of (c) total ion chromatograms (TIC) and (d) the mass spectra of the separated compounds.

1.10 Thesis hypothesis and objectives

The hypothesis of this thesis was:

The urine of gastrointestinal cancer patients contains a unique VOC profile that can be used for the detection of cancer.

The research questions of this thesis were:

1. Is there a distinct VOC profile in the urine of gastrointestinal cancer patients?
2. Can cancer-related urinary VOCs be exploited for the purpose of cancer detection?
3. What techniques and strategies can be used to develop a high throughput and standardised non-invasive approach for the deep profiling of urinary VOCs?

Specific objectives of this thesis were:

1. To conduct a systematic review to summarise the existing literature on cancer-related urinary VOCs;
2. To compare solid-phase microextraction (SPME) versus HiSorb sorptive extraction, and select the technique that outperformed the others as the urinary VOC extraction method;
3. To determine the optimal conditions for urinary VOC extraction using HiSorb sorptive extraction;
4. To perform multiplatform method development for a standardised GC-MS urinary VOC analysis workflow;
5. To characterise the urinary VOC profile of patients with gastrointestinal cancer (pancreatic cancer) compared to controls.

SECTION 2

SYSTEMATIC LITERATURE REVIEW

2 SYSTEMATIC LITERATURE REVIEW

2.1 Introduction

While some encouraging findings have been reported (Section 1.6 and 1.7), there remains considerable uncertainties about the role of urine VOC analysis in cancer detection. In the absence of standardisation of methods, attempts to establish repeatability of results and their wider adoption in clinical practise may be limited. In order to offer a comprehensive figure of current knowledge and indications on urinary VOC analysis for cancer diagnosis, a systematic literature review and quality assessment was conducted.

2.2 Objectives

The primary objective of the systematic review was to summarise the existing literature on cancer-related urinary VOCs.

Specific objectives were:

1. To identify published urinary VOC markers of cancer;
2. To explore emerging metabolic pathways of cancer-specific VOCs;
3. To assess the methodological quality of published research studies.

2.3 Methods

2.3.1 Literature search

The Cochrane library and Meta-analysis of Observational Studies in Epidemiology (MOOSE) criteria were used to perform an online systematic literature review to identify all the studies that evaluated the difference of urinary VOCs between cancer patients and suitable controls.[91] Medline (1946 – 13th December 2019) database via OvidSP, Ovid Embase (1947 – 13th December 2019) database, and Cochrane Library were searched. The following keywords were used in the search strategy: urine, volatile organic compounds, biomarkers, metabolomics, metabolic profiling, magnetic resonance spectroscopy; mass spectrometry, and carcinoma. All possible spelling variations, including abbreviated search terms utilising wild card characters and the

"related articles" feature, were combined using the Boolean operators AND and OR. Reference lists from eligible publications were examined for research studies that could be relevant. The complete search strategies are shown in Table 3.

Table 3. Search strategy.

Search No.	Embase Classic + Embase	Ovid MEDLINE(R) ALL
1	Volatile Organic Compounds/ or volatile organic compound*.mp. (20445)	Volatile Organic Compounds/ or volatile organic compound*.mp. (14685)
2	biomarkers.mp. or BIOMARKERS/ (385783)	biomarkers.mp. or BIOMARKERS/ (491461)
3	Metabolomics/ or metabo*omics.mp. (35735)	Metabolomics/ or metabo*omics.mp. (25507)
4	Magnetic Resonance Spectroscopy/ or magnetic resonance spectrometry.mp. (100046)	Magnetic Resonance Spectroscopy/ or magnetic resonance spectrometry.mp. (147500)
5	mass spectrometry.mp. or Mass Spectrometry/ (463935)	mass spectrometry.mp. or Mass Spectrometry/ (309505)
6	metabolic profiling.mp. (4883)	metabolic profiling.mp. (3623)
7	metabotyping.mp. (71)	metabotyping.mp. (55)
8	urine.mp. or URINE/ (564820)	urine.mp. or URINE/ (357244)
9	1 or 2 (405092)	1 or 2 (505235)
10	3 or 4 or 5 or 6 or 7 (569457)	3 or 4 or 5 or 6 or 7 (454801)
11	8 and 9 and 10 (5080)	8 and 9 and 10 (4492)
12	prostate.mp. or PROSTATE/ (347922)	prostate.mp. or PROSTATE/ (189208)
13	prosta*.mp. (583298)	prosta*.mp. (364594)
14	gastric.mp. or GASTRIC/ (369992)	gastric.mp. or GASTRIC/ (287312)
15	gastro*.mp. (894775)	gastro*.mp. (522330)
16	esophageal.mp. or ESOPHAGEAL/ (149069)	esophageal.mp. or ESOPHAGEAL/ (137920)
17	esophago*.mp. (53403)	esophago*.mp. (37648)
18	colorectal.mp. or COLORECTAL/ (261334)	colorectal.mp. or COLORECTAL/ (154456)
19	rectal*.mp. (153592)	rectal*.mp. (114296)
20	bowel.mp. or BOWER/ (249315)	bowel.mp. or BOWER/ (151056)
21	intestinal.mp. or INTESTINAL/ (386822)	intestinal.mp. or INTESTINAL/ (403809)
22	intestin*.mp. (799633)	intestin*.mp. (499655)
23	pancreas.mp. or PANCREAS/ (394885)	pancreas.mp. or PANCREAS/ (137239)
24	pancrea*.mp. (520745)	pancrea*.mp. (325641)
25	carcinoma.mp. or CARCINOMA/ (1218118)	carcinoma.mp. or CARCINOMA/ (805969)
26	ca*.mp. (24378921)	ca*.mp. (18137476)
27	12 or 13 (583298)	12 or 13 (364594)
28	14 or 15 (1143350)	14 or 15 (714689)
29	16 or 17 (180363)	16 or 17 (150433)
30	18 or 19 or 20 or 21 or 22 (1231013)	18 or 19 or 20 or 21 or 22 (799204)
31	23 or 24 (520745)	23 or 24 (325641)
32	25 or 26 (24378921)	25 or 26 (18137476)
33	27 or 28 or 29 or 30 or 31 (3084057)	27 or 28 or 29 or 30 or 31 (2026101)
34	32 and 33 (2400354)	32 and 33 (1468790)
35	11 and 34 (689)	11 and 34 (449)
36	limit 35 to English language (676)	limit 35 to English language (435)
37	limit 36 to human (571)	limit 36 to human (315)

Q.W. and P.B. independently reviewed the titles and abstracts of all studies identified through database searching. The full text of relevant papers was evaluated for eligibility. Only original research articles published in the English language were considered. Studies that identified potential VOC biomarkers of cancers by profiling the urine of patients and relevant controls (patients without cancer) using mass spectrometry based technologies were included. Studies were excluded if they did not report named VOC biomarkers or if they reported results from mixed cancer cohorts where the results of each cancer subtype could not be clearly separated. Review articles, conference abstracts, articles not written in the English language, and animal and cell studies were excluded. When disagreements over study inclusion occurred, a third reviewer (G.H.) was consulted.

2.3.2 Outcome measures

The following information was extracted and summarised from included articles: year of publication, country of origin, study design, recruitment period, number of participants, cancer type, analytical platform used, sampling technique methodology, quality control method(s), normalisation method(s), number of VOCs identified, identity of VOCs increased/decreased in cancer, method of statistical analysis (including prediction model used), sensitivity and specificity, and area under the receiver operating characteristic (AU-ROC) curve.

2.3.3 Quality assessment

Three tools were used to evaluate the quality of all the included studies. The risk of bias and applicability of the study were assessed using the Quality Assessment of Diagnostic Accuracy Studies-2 (QUADAS-2) tool.[92] It was divided into risk of bias of patient selection, diagnostic test, reference standard and patient flow and timing. This test also investigated the applicability of patient selection, diagnostic test, and reference standard to the systematic review. Reporting quality of the study was assessed using the Standards for Reporting of Diagnostic Accuracy Studies (STARD) tool.[93] This tool evaluated all the sections including title, abstract, introduction, methods, results and discussion to provide a full picture of the completeness and transparency of reporting of diagnostic accuracy studies. The quality of metadata of the study was assessed using the Chemical Analysis Working Group (CAWG)-Metabolomics Standard Initiative (MSI) criteria.[94] CAWG-MSI proposed minimum

reporting standards associated with the chemical analysis aspects of metabolomics experiments, including sample preparation, experimental analysis, quality control, metabolite identification, and data pre-processing.

2.3.4 Metabolic analysis

All VOCs identified were checked and classed according to the Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathway and the Human Metabolome Database (HMDB). The pathway analysis module in MetaboAnalyst 4.0, which is based on the R programming language (version 3.5.3, The R Project for Statistical Computing, www.r-project.org), was used to perform statistical analysis. VOCs that were significantly increased or decreased in each study were selected. To match well-annotated compounds in KEGG pathway libraries, compound names were standardised against HMDB, KEGG, and PubChem in the pathway analysis module. A hypergeometric test for over representation analysis and a relative-betweeness centrality test for pathway topology analysis were used as the parameters to analyse data based on KEGG pathway libraries.[95-97] For weighted means of each identified VOC, an equation (Equation 1) was used to perform normalisation: the proportion of the total number of VOCs identified per study, divided by the total number of VOCs identified in each cancer type, then multiplied by the total number of studies in which this VOC was identified.

$$\frac{1/(Total\ No.of\ VOCs\ identified\ in\ each\ study)}{Total\ No.of\ VOCs\ identified\ in\ each\ cancer\ type} \times Total\ No.of\ studies\ that\ identifed\ this\ VOC \quad \text{Equation 1}$$

2.4 Results

2.4.1 Selected studies

There were 886 studies identified in the systematic literature search. A total of thirteen studies were included (Figure 3) following screening and eligibility evaluation.[98-110] Table 4 summarises the details of included studies. Included studies were from Europe (*n* = 10), North America (*n* = 2), and Asia (*n* = 1), reporting the results of 1266 patients of which 700 had been diagnosed with cancer. Studies reported the urinary VOC profiles of prostate cancer (*n* = 5); gastrointestinal cancer (*n* = 7); lung cancer (*n* = 1); haematological malignancies (*n* = 1); and bladder cancer (*n* = 1).

The most frequently used analytical technique was gas chromatography mass spectrometry (GC-MS) (*n* = 10). Three studies used selected ion flow tube mass spectrometry (SIFT-MS). Field asymmetric ion mobility spectrometry (FAIMS) was used in one single study. Two studies used more than one analytical technique.[99,107] The majority of studies (*n* = 9) analysed VOCs within urine headspace rather than the fluid phase. Other urinary VOC extraction techniques included solid-phase microextraction (SPME) and stir bar sorptive extraction with or without derivatisation.

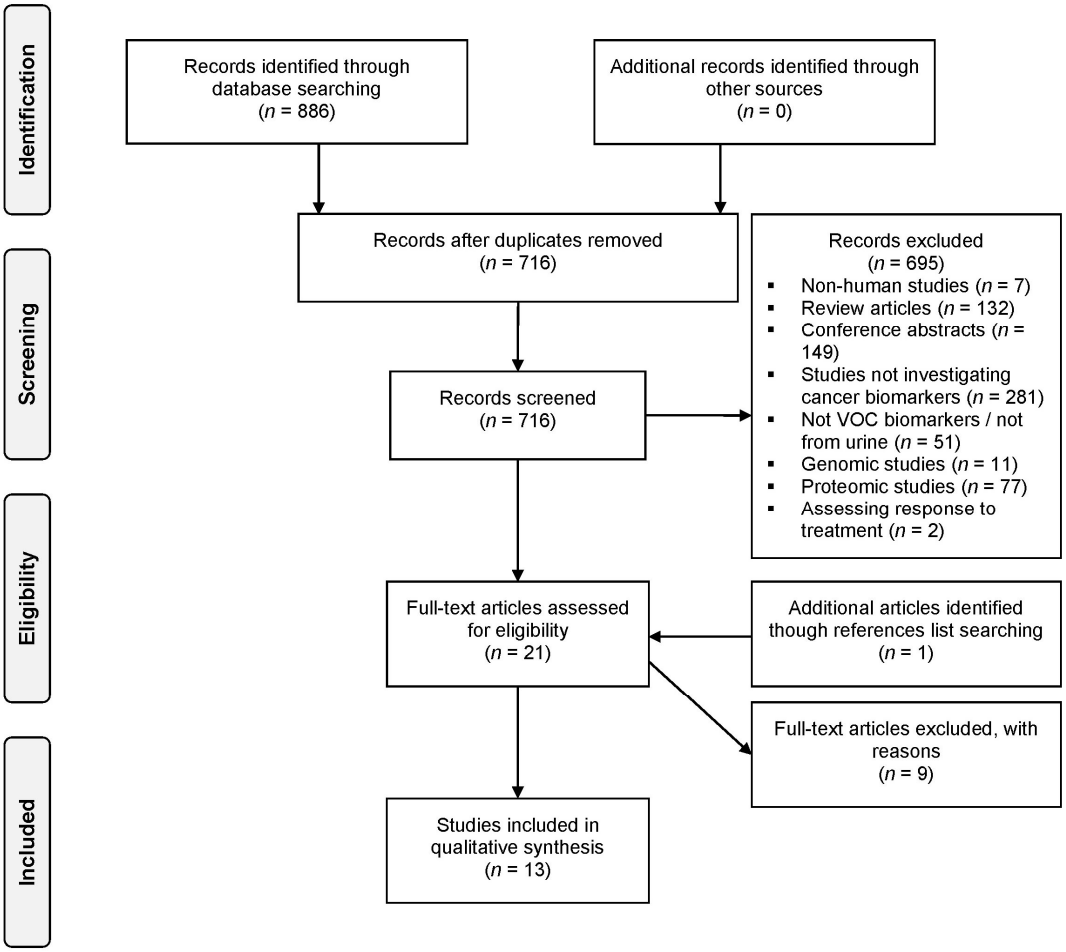


Figure 3. PRISMA flow diagram.

The systematic literature search identified 886 studies, with thirteen studies included after screening and assessing for eligibility. VOC: volatile organic compound; PRISMA: Preferred Reporting Items for Systematic Reviews and Meta-Analyses.

Table 4. Study characteristics.

Urinary volatile organic compound analysis for cancer diagnosis: analytical and biostatistical techniques for biomarker discovery.

Author	Year	Country	Cancer Type	Cancer Stage	No. Patients (cancer/all)	Sample Type	Analytical Platform	Sampling Technique	Prediction Model	Sensitivity (%)	Specificity (%)	AUC	Ref.
Lima	2019	Portugal	Prostate	I, II, IIa, IIb, III, IV	58/118	Headspace	GC-MS	SPME, PFBHA derivatisation	PLS-DA, ROC	72 (Training) 89 (Validation)	96 (Training) 83 (Validation)	0.856 (Training) 0.904 (Validation)	[98]
Struck-Lewicka	2014	Poland	Prostate	Not reported	32/64	Liquid phase	HPLC-ESI-TOF-MS; GC-MS	LC: Centrifugation; GC: BSTFA derivatisation	PCA, PLS-DA	-	-	-	[99]
Gao	2019	USA	Prostate	Not reported	108/183	Liquid phase	TD-GC-MS	Stir bar sorptive extraction	ROC	96 (Training), 87 (Validation)	80 (Training), 77 (Validation)	0.92 (Training), 0.86 (Validation)	[100]
Jimenez-Pacheco	2018	Spain	Prostate	Not reported	29/50	Headspace	GC-MS	Dynamic headspace SPME	-	-	-	-	[101]
Khalid	2015	UK	Prostate	Not reported	59/102	Headspace	GC-MS	SPME	Repeated 10-Fold CV, Repeated Double CV	-	-	-	[102]
Spanel	1999	Czech Republic	Prostate, Bladder	Not reported	38/52	Headspace	SIFT-MS	Direct headspace sampling (sampling needle connected to inlet port)	-	-	-	-	[103]
Chen	2016	China	GI (G)	Ia/b, IIa/b, IIIa/b/c, IV	159/293	Liquid phase	GC-MS	Derivatisation	OPLS-DA	77.4 (Validation)	85.1 (Validation)	0.893 (Validation)	[104]
Navaneethan	2015	USA	GI (HPB)	Not reported	15/54	Liquid phase	SIFT-MS	Centrifugation	Logistic regression model, ROC	93.3	61.5	0.83	[105]
Panebianco	2017	Italy	GI (G, C, HPB)	Not reported	23/38	Headspace	GC-TOF-MS; GC-qMS; GC/O	SPME	-	-	-	-	[106]

Arasaradnam	2014	UK	GI (CR)	Not reported	83/133	Headspace	FAIMS; GC-MS	FAIMS: Dynamic headspace sampling; GC: Direct headspace sampling (automated pre-concentration robot)	Fisher discriminant analysis	88	60	-	[107]
Huang	2013	UK	GI (O, G)	Not reported	17/44	Headspace	SIFT-MS	Direct headspace sampling (sampling needle connected to inlet port)	ROC	-	-	0.904	[108]
Rozhentsov	2014	Russia	Lung GI (O, G, C)	Not reported	46/81	Headspace	GC-MS	Headspace SPME (three phase micro-extraction (TPME))	Image analysis	100	90.62	-	[109]
Silva	2011	Portugal	Leukaemia; Lymphoma; GI (CR)	Not reported	33/54	Headspace	GC-qMS	dHS-SPME	PCA	-	-	-	[110]

GI: gastrointestinal cancer; O: oesophageal cancer; G: gastric cancer; HPB: Hepato-biliary cancer; C: colon cancer; CR: colorectal cancer; GC-MS: gas chromatography mass spectrometry; TD: thermal desorption; HPLC-ESI-TOF-MS: high-performance liquid chromatography electrospray ionisation time of flight mass spectrometry; TOF: time of flight; SIFT-MS: selected ion flow tube mass spectrometry; GC/O: gas chromatography-olfactometry; FAIMS: field asymmetric ion mobility spectrometry; qMS: quadrupole mass spectrometry; SPME: solid-phase microextraction; PFBHA: O-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine; BSTFA: N,O-Bis(trimethylsilyl)trifluoroacetamide; dHS-SPME: dynamic solid-phase microextraction in headspace mode; PCA: principal component analysis; PLS-DA: partial least squares-discriminant analysis; OPLS-DA: orthogonal partial least squares discriminant analysis; ROC: receiver operating characteristic; Repeated 10-Fold CV: repeated 10-fold cross-validation; Repeated Double CV: repeated double cross-validation.

2.4.2 Quality assessment

Table 5 summarises the outcomes of quality assessments. QUADAS-2 was used to analyse Bias and applicability of outcomes. The thirteen included studies were considered to have a low overall risk of bias and a high applicability of these studies to the review question.

The STARD checklist was used to assess the general reporting quality of the studies (Table 5). STARD scores ranged from 11 to 27 with a mean of 19.9 ± 4.6 where the largest score is 41, indicating that reporting standards were often inadequate.

The CAWG-MSI was used to assess the reporting of metadata in metabolomics datasets (Table S1, Appendix 1).[94] Only three studies reported more than 50% of the CAWG-MSI criteria.[98,99,104] Eight of the thirteen included studies used a relative quantification of compounds,[98-102,106,107,110] whilst five studies used an absolute quantification of compounds.[103-105,108,109] In general, the included studies provided an adequate description of sample preparation, experimental analysis, and instrumental performance. No study provided an acceptable description of method validation. Internal standards were used in three of the eight studies that analysed the relative quantification of metabolites.[98-100] Methods used for assessing instrument variation were described in five studies.[98,99,102,106,107] Of the five studies that used absolute quantification, four did not report accuracy or precision validation data for their method on the instrument,[103,105,108,109] while one study reported the limits of quantification and detection of the method.[104] Six studies provided a detailed description of data pre-processing.[98-100,105-107] Two studies reported level one metabolite identification.[103,104] Nine studies reported level two metabolite identification.[98-102,105,106,108,110]

Table 5. Quality assessment with STARD, QUADAS-2, and CAWG-MSI.

There was an overall low risk of bias and high applicability of the thirteen studies to the review question. The completeness and transparency of reporting was inadequate. There was an overall inadequate reporting of metadata of the studies.

	Study	Overall Diagnostic Quality	QUADAS							STARD Score	CAWG-MSI Metadata
			Risk of Bias				Applicability Concerns				
			Patient Selection	Index Test	Reference Standard	Flow and Timing	Patient Selection	Index	Reference Standard		
Prostate cancer (PCa)	Lima	Good	Low	Low	Low	Low	Low	Low	Low	26	23
	Struck-Lewicka	Fair	Unclear	Unclear	Unclear	Low	Low	Low	Low	14	20
	Gao	Good	Unclear	Unclear	Unclear	Low	Low	Low	Low	27	18
	Jimenez-Pacheco	Good	Low	Unclear	Low	Low	Low	Low	Low	19	14
	Khalid	Good	Low	Unclear	Low	Low	Low	Low	Low	23	15
PCa, bladder cancer	Spanel	Fair	Low	Unclear	Unclear	Low	Low	Low	Unclear	12	9
GI cancer	Chen	Good	Low	Unclear	Low	Low	Low	Low	Low	23	22
	Navaneethan	Good	Low	Unclear	Low	Low	Low	Low	Low	23	25
	Panebianco	Fair	Unclear	Unclear	Low	Low	Low	Low	Low	18	13
	Arasaradnam	Fair	Unclear	Unclear	Low	Low	Low	Low	Low	21	15
	Huang	Fair	Low	Low	Low	Low	Low	Low	Low	21	9
Lung, GI cancer	Rozhentsov	Fair	Unclear	Unclear	Low	Low	Low	Low	Low	16	6
Leukaemia, colorectal cancer, lymphoma	Silva	Fair	Unclear	Unclear	Low	Low	Low	Low	Low	16	15

STARD: Standards for Reporting of Diagnostic Accuracy Studies; QUADAS-2: Quality Assessment of Diagnostic Accuracy Studies-2; CAWG-MSI: Chemical Analysis Working Group Metabolomics Standard Initiative.

2.4.3 Reported urinary VOCs

Within the thirteen identified studies, a total of forty-eight cancer-associated urinary VOCs were reported, with significant variation observed between different cancer types. These VOCs were classified into to nine chemical classes (Figure 4). Five of them, namely 2,6-dimethyl-7-octen-2-ol, p-cresol, phenol, acetic acid, and dimethyl disulphide, were reported in more than one study (Table 6).

For prostate cancer, twenty-nine urinary VOCs from eight chemical classes were identified (Figure 4 and Table 6). The majority of identified VOCs were aromatic compounds, ketones, and organic acids (Figure 4). Most VOCs showed decreased concentrations in the urine of prostate cancer patients compared to the urine of non-cancer patients. The concentration of alcohols, organic acids, and ketones were reported to decrease the most in prostate cancer. In comparison, aldehydes and siloxanes were identified to be increased in the urine of prostate cancer patients (Figure 5).

For gastrointestinal cancers (gastroesophageal, colorectal, and hepato-biliary), twenty-one urinary VOCs from six chemical classes were identified, nineteen of which were not identified in prostate cancer urine (Figure 4 and Table 6). The majority of the gastrointestinal cancer related VOCs were aromatic compounds, alcohols, and ketones (Figure 4). Sulfur compounds were only detected in the urine of gastrointestinal cancer patients. Most of the gastrointestinal cancer related VOCs, particularly aromatic VOCs, showed increased concentrations when compared to prostate cancer. Alcohols and ketones were identified to be decreased in the urine of gastrointestinal cancer patients (Figure 5).

There was one single study that reported six leukaemia and lymphoma-related urinary VOC biomarkers.[110] Except for anisole, which was decreased in the urine of lymphoma patients, these VOCs were found to be increased in the urine of patients with these haematological malignancies. One study reported that formaldehyde were associated with bladder cancer.[103] No study reported lung cancer related urinary VOC biomarker (Table 6).

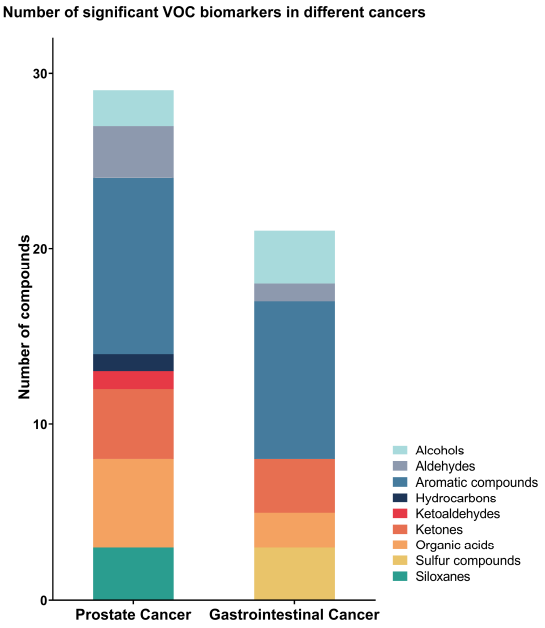


Figure 4. The number of VOCs identified in different cancers.

There were twenty-nine urinary VOCs from eight chemical classes identified for prostate cancer, with the majority of them being aromatic compounds, ketones, and organic acids. There were twenty-one VOCs from six chemical classes identified for gastrointestinal cancer, with the majority of them being aromatic compounds, alcohols, and ketones. Nineteen of the twenty-one VOCs were not identified in prostate cancer urine.

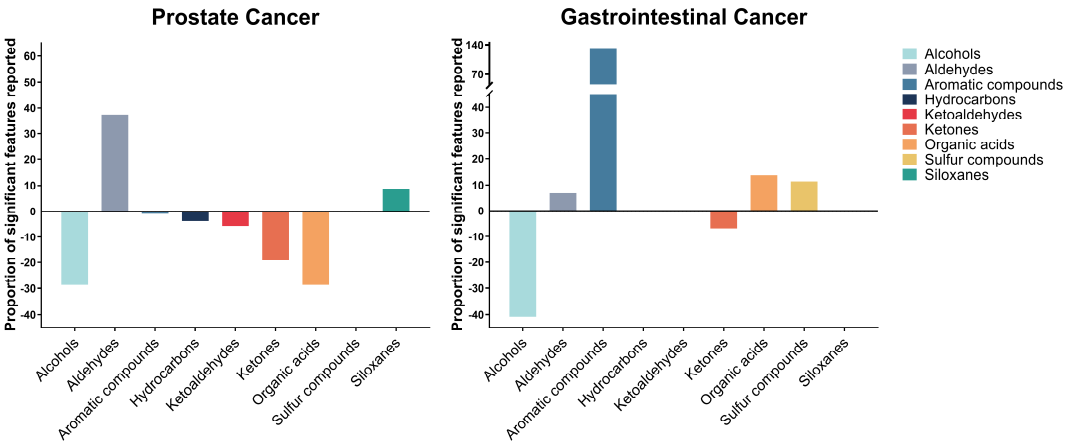


Figure 5. The proportion of identified compound chemical classes in different cancers.

When compared to the urine of non-cancer patients, most prostate cancer related VOCs showed decreased concentrations in the urine of prostate cancer patients. The majority of the gastrointestinal cancer related VOCs showed increased concentrations, particularly aromatic VOCs.

Table 6. List of identified VOCs.

Compound Name	Chemical Class	Study	Prostate Cancer	GI Cancer	Leukaemia Lymphoma		Bladder Cancer
			Increased/Decreased in a Cancer				
2,6-Dimethyl-7-octen-2-ol	Alcohols	Jimenez-Pacheco; Khalid	↓				
2-Propanol		Navaneethan		↓			
Ethanol		Navaneethan		↓			
Methanol		Huang		↑			
2-Ethylhexanol		Jimenez-Pacheco	↓				
Formaldehyde	Aldehydes	Spanel	↑				↑
Acetaldehyde		Huang		↑			
Hexanal		Lima	↓				
Pentanal		Khalid	↑				
1-(2,4-Dimethylphenyl)-3-(tetrahydrofuryl-2)propane	Aromatic compounds	Gao	↓				
1,2-Dihydro-1,1,6-trimethyl-naphthalene		Silva		↑	↑	↑	
Dihydroedulan IA		Lima	↓				
3-Phenylpropionaldehyde		Lima	↑				
Phenylacetic acid		Panebianco		↑			
2,5-Dimethylbenzaldehyde		Lima	↑				
3,5-Dimethylbenzaldehyde		Jimenez-Pacheco	↓				
p-Xylene		Jimenez-Pacheco	↑				
3-Methylphenol (m-Cresol)		Jimenez-Pacheco	↑				
p-Cresol		Chen; Silva		↑	↑	↑	
Phenol		Jimenez-Pacheco; Huang	↑	↓			
4-Ethyl guaiacol		Panebianco		↓			
Anisole		Silva		↑	↑	↓	
Furan		Jimenez-Pacheco	↑ ↓				
Thiophene		Panebianco		↓			
p-Cymene		Silva		↑	↑	↑	
Indole		Struck-Lewicka	↓				
2-Methyl3-phenyl-2-propenal		Silva		↑	↑	↑	
Santolina triene	Hydrocarbons	Jimenez-Pacheco	↓				
Methylglyoxal	Ketoaldehydes	Lima	↓				
2-Butanone	Ketones	Jimenez-Pacheco	↑				
2-Octanone		Khalid	↓				
3-Methyl-2-pentanone		Panebianco		↓			
3-Octanone		Khalid	↓				
4-(or 5-) Methyl-3-hexanone		Panebianco		↓			
4-Methylhexan-3-one		Lima	↓				
Acetone		Huang		↑			

Hexanoic acid	Organic acids	Huang		↑			
Butyric acid		Struck-Lewicka	↓				
Acetic acid		Struck-Lewicka; Huang	↓	↑			
Propenoic acid		Struck-Lewicka	↓				
Isobutyric acid		Struck-Lewicka	↓				
Propionic acid		Struck-Lewicka	↓				
Hydrogen sulfide	Sulfur compounds	Huang		↑			
2-Methoxythiophene		Panebianco		↑			
Dimethyl disulphide		Silva; Panebianco		↓	↓	↓	
1,1,1,5,5,5-Hexamethyl-3,3-bis[(trimethylsilyl)oxy]-trisiloxane	Siloxanes	Gao	↑				
1,1,3,3,5,5,7,7,9,9-Decamethyl-pentasiloxane		Gao	↑				
Ethyl à-hydroxymyristate trisiloxane		Gao	↓				

In Jimenez-Pacheco A. 2018, furan is increased before prostate massage and is decreased after prostate massage. GI: gastrointestinal.

2.4.4 Related metabolic pathways

Diverse levels of VOCs associated with KEGG metabolic pathways that belonged to carbohydrate, lipid, amino acid, and energy metabolism were identified by metabolic pathway analysis. Among metabolic pathways related to cancer-specific urinary VOCs, Glycolysis and the gluconeogenesis pathway showed the greatest impact. Prostate cancer has been linked to seven metabolic pathways categorised into carbohydrate, lipid, and amino acid metabolism. Gastrointestinal cancer has been linked to six metabolic pathways belonging to the three pathway categories as well as the energy metabolism. Compared to prostate cancer, gastrointestinal cancer had a stronger association with carbohydrate metabolism and energy metabolism, indicating that both cancers had different underlying metabolic profiles (Figure 6 and Table 7).

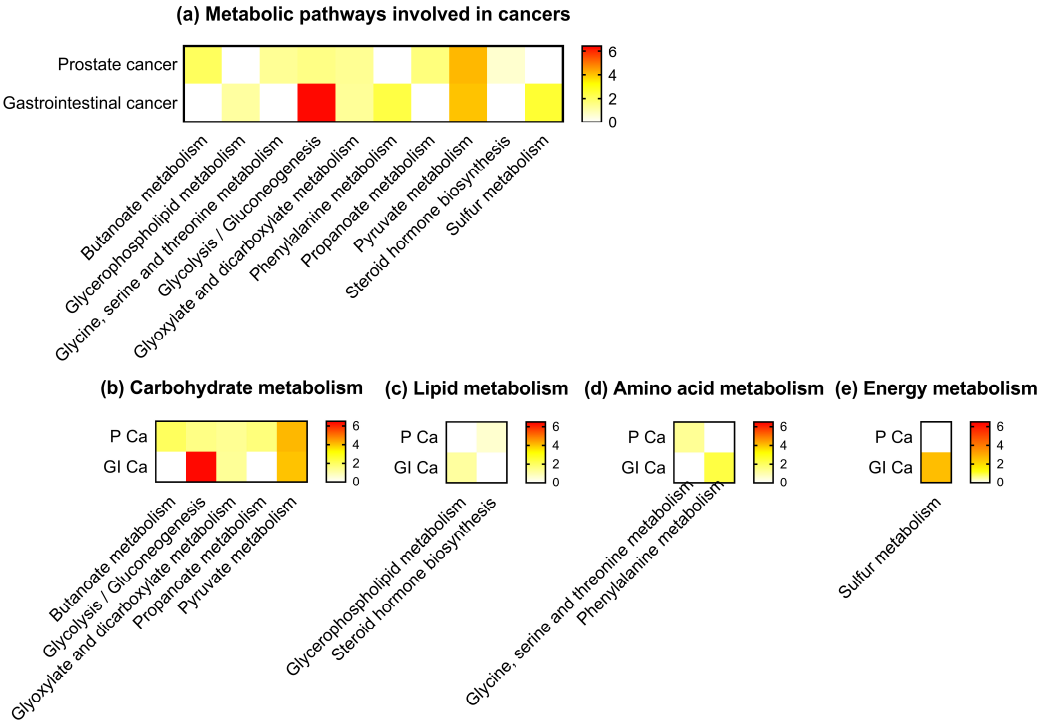


Figure 6. Metabolic pathways involved in cancers.

(a) All metabolic pathways (b) Carbohydrate metabolism (c) Lipid metabolism (d) Amino acid metabolism (e) Energy metabolism. P Ca: prostate cancer; GI Ca: gastrointestinal cancer. Compared to prostate cancer, gastrointestinal cancer had a stronger association with carbohydrate metabolism and energy metabolism, indicating that both cancers had different underlying metabolic profiles.

Table 7. List of identified VOCs and associated metabolic pathways.

Compound Name	Chemical Class	Metabolic Pathway	Prostate Cancer	Gastrointestinal Cancer
Ethanol	Alcohols	Glycolysis / Gluconeogenesis		↓
Acetaldehyde	Aldehydes	Pyruvate metabolism; Glycolysis / Gluconeogenesis; Glycerophospholipid metabolism		↑
Phenylacetic acid	Aromatic compounds	Phenylalanine metabolism		↑
Methylglyoxal	Ketoaldehydes	Glycine, serine, and threonine metabolism; Pyruvate metabolism	↓	
Acetic acid	Organic acids	Glyoxylate and dicarboxylate metabolism; Pyruvate metabolism; Glycolysis / Gluconeogenesis	↓	↑
Propionic acid		Propanoate metabolism	↓	
Butyric acid		Butanoate metabolism	↓	
Hydrogen sulfide	Sulfur compounds	Sulfur metabolism		↑

2.5 Discussion

An overview of the use of urinary VOCs for cancer diagnosis is provided by this systematic review. Major findings include: (i) a description of characteristic cancer-associated urinary VOC biomarkers; (ii) promising diagnostic performance of urinary VOCs for the detection of prostate and gastrointestinal cancers, and; (iii) and a lack of standardisation in reported practices for urinary VOCs analysis.

One of the most critical elements determining cancer survival is early detection. Many cancers, including those identified in this review, often have ambiguous symptoms resulting in delayed investigation and detection. Late diagnosis is associated with a worse overall survival. For example, patients with advanced (stage IV) prostate cancer had a 49% five-year survival rate, compared to nearly 100% for patients with early (stage I/II) disease.[111] As a result, developing accurate, acceptable, and affordable methods for cancer early detection is critical.

GC-MS was the most common analytical platform used for urinary VOC analysis, with multiple sampling techniques aiding pre-analysis VOC extraction. However, there was a lack of information on patient recruitment and study design, including strategies for mitigation of bias, in the majority of the included studies. There was no adequate validation of results in any of the studies. Three of the thirteen included studies verified their initial findings in independent cohorts.[98,100,104] Four studies normalised data using internal standards,[98-100,104] and two studies applied quality control measures.[98,99] Therefore, future studies are required to establish an “optimal” method for urinary VOC analysis. To ensure accuracy and reproducibility, these studies should acknowledge the importance of standardisation and adoption of quality control measures.

Most studies in this review investigated urinary VOCs of prostate and gastrointestinal cancer. The wide variation in reported cancer-associated VOCs is due to diversity in the underlying tumour metabolic profiles and/or methodological variability secondary to the lack of standardised practices for urinary VOCs analysis. There have been previously reported protocols for urinary metabolite analysis.[112-114] While the current evaluation was not intended to develop similar guidelines, Table 8 summarises important considerations for the specific analysis of urinary VOCs.

Table 8. Considerations for the analysis of urinary VOCs.

Workflow	Analytical Step	Considerations	Ref.
Experimental design		Patient selection	
		Testing and independent validation cohort setup	98,100,104
Sample preparation	Urine collection and storage	Mode of collection (e.g., spot collection or 24h collection)	
		Choice of receptacle (e.g., appropriate volume, ultra-low temperature friendly, no unwanted contaminants)	
		Sources of contamination	
		Sample filtration	
		Sample handling, aliquot, transfer, and storage	
		Impact of freeze-thaw cycles	
	Sampling technique	Analytical phase (e.g., headspace, liquid phase)	
		Selection of sample extraction techniques (e.g., SPME, HiSorb, direct injection)	98,100-103,106-110
		Sample extraction optimisation (e.g., pH, dilution, salting out, temperature, agitation)	
		Sample preparation (e.g., derivatisation)	98,99,104,105
	Internal standards and QC samples	Appropriate internal standards	98-100,104
		QCs (pooled sample QCs, synthetic urine, spiked urine. Monitor and correction of analytical variabilities)	98,99
MS analysis	Analytical platform	Selection of analytical platform (e.g., GC-MS, SIFT-MS, LC-MS)	
		Selection of column	
		Automatic or manual injection	
		Optimisation of separation parameters (e.g., column dimensions, gradients, temperatures, flow rates, etc.)	
		Selection of ionisation (e.g., EI, ESI) and mass analyser (high mass resolution MS (TOF, qTOF), low mass resolution MS (single and triple quadrupoles and quadrupole ion-traps))	
		Optimisation of MS parameters (e.g., m/z range, mass resolution)	
	MS data collection	Run order (e.g., use of randomised block design), pooled QCs	
Data analysis	Data pre-processing	Peak alignment	
		Peak detection, integration, and identification	
		Removal of non-reproducible, non-linear, and contamination-related compounds	
	Statistical analysis	Descriptive statistics	
		Univariate analysis	98-102,104-106,108,110
		Multivariate analysis (e.g., PCA, PLS-DA, OPLS-DA)	98-100,102,104,105,107,110
		Prediction model (e.g., ROC analysis)	98,100,104,105,108

VOC: volatile organic compound; SPME: solid-phase microextraction; QC: quality control; GC-MS: gas chromatography mass spectrometry; SIFT-MS: selected ion flow tube mass spectrometry; LC-MS: liquid chromatography mass spectrometry; EI: electron ionisation; ESI: electrospray ionisation; TOF: time of flight; PCA: principal component analysis; PLS-DA: partial least squares-discriminant analysis; OPLS-DA: orthogonal partial least squares discriminant analysis; ROC: receiver operating characteristic.

Prostate and gastrointestinal cancer related VOCs showed excellent sensitivity and specificity for cancer detection. As previously mentioned, a limited number of studies used an independent dataset to validate their findings. Most prostate cancer specific VOCs were decreased as compared to controls. In comparison, the concentration of gastrointestinal cancer associated VOCs were tended to be found increased. Identified VOCs derived from a wide range of metabolic pathways, including carbohydrate metabolism, lipid metabolism, amino acid metabolism, and energy metabolism. Metabolic analysis provided evidence that prostate and gastrointestinal cancer specific VOCs originated from different metabolic pathways, with the latter being associated more with carbohydrate metabolism and energy metabolism.

The origin of VOCs within the body, as well as the mechanism by which they enter the urine, remains incompletely understood. Cancer-specific VOCs are presumed to be endogenous, meaning that they are produced because of aberrant metabolism inside the tumour or related tissues. VOCs generated by tissues may travel in the systemic circulation and be excreted via the lung, skin, or renal tract. Local release directly from the prostate gland into urine may also occur in the case of prostate cancer. Additionally, the tumour-associated intestinal microbiome has been hypothesised to contribute to VOC generation in gastrointestinal cancers.[115,116] The fundamental mechanisms of VOC production in cancer and the kinetics of their release require further research.

This systematic review suffers from a number of limitations that principally concern the relatively small number of published studies within this field. Individual studies employed a broad variety of methodologies, making it difficult to draw strong conclusions. Limited studies implemented robust quality control strategies, and few studies validate findings within an independent patient cohort. It was difficult to assess the performance of urinary VOC analysis on diagnosing early-stage cancer due to insufficient reporting of clinical parameters, including cancer stage. Most enrolled patients in those studies that included cancer stage information had locally advanced cancer. Therefore, observed metabolic differences can not been interpreted as accurately representing “early” disease, which is the ultimate aim of developing the test. The fact that the vast majority of the articles in this review were from Europe means that they may not be representative of other population.

2.6 Conclusion

This systematic review summarises the advancements in the use of urinary VOC analysis for cancer diagnosis. Although study quality varied, urinary VOC analysis exhibited promising performance for developing non-invasive diagnostic tools for cancer diagnosis and demonstrating metabolic profiling of different types of cancer. The methodological weakness and limitations summarised in this review must be overcome in order to conduct future studies and apply their findings into large clinical practice.

SECTION 3

METHOD DEVELOPMENT FOR URINARY VOC ANALYSIS

3 METHOD DEVELOPMENT FOR URINARY VOC ANALYSIS

3.1 Introduction

Urine is rich in metabolites and poses multiple analytical challenges for metabolic profiling: There are large variations in urine ionic strength, pH and osmolality, particularly under conditions of physiological stress; There is a wide dynamic range of metabolite concentrations due to highly varied endogenous metabolic processes as well as variable and unpredictable dilution; There is a great diversity in chemical characteristics of urine metabolites due to age, health, diet, or other factors, encompassing microbial cometabolites as well as mammalian metabolites; Urine may also include drugs, pollutants and industrial chemicals. As a result, capturing complete metabolic information in urine sample to provide insight to both local urogenital tract environment and systemic metabolism is considerably challenging.

In order to obtain a comprehensive picture, complementary analytical platforms are required to provide deep profiling, and the choice of technique is dependent on the demand of the specific study. The systematic review in Section 2 showed that GC-MS has become the most common analytical platform for the measurement of urinary VOCs, with multiple different extraction techniques aiding pre-analysis VOC extraction. The general principles of GC-MS have been discussed in Section 1.9. However, there is no established “optimal” standardised method for urinary VOC analysis.

In this method development section, GC-MS was selected to overcome the analytical challenges for global metabolic profiling of urine, with attempt to characterise as many compounds as possible. Different GC columns were tested to achieve maximum VOC coverage and best separation. The more powerful electron impact ionisation (EI) was selected for better ionisation and fragmentation. Not only the conventional quadrupole GC-MS, but also GC coupled with time-of-flight mass spectrometry (TOF-MS) were used due to their high data acquisition speed, superior sensitivity compared to quadrupoles in full scan mode, reliable deconvolution of overlapping peaks, and ability to scan a broad mass range. GC-MS analytical conditions and parameters were investigated to facilitate high throughput and high performance data acquisition.

Due to the unique nature of urine, dedicated urinary VOC extraction tools were needed to effectively collect VOCs released from urine. Pre-analysis urine sample preparation, which can include adding salt, acid, or base to the sample, altering phase ratio (the ratio between sample volume and gas volume in a vial), was an important step to achieving maximum urinary VOC acquisition. Moreover, to minimise analytical variabilities in urinary VOC analysis, standardisation of the experimental process and adoption of quality control measures to ensure accuracy and reproducibility were key focuses of current section.

In metabolomics, there are three commonly used analytical strategies, including untargeted analysis, targeted analysis, and semi-targeted analysis. The objective of untargeted analysis is to reproducibly measure as many metabolites (typically low thousands) as possible. Untargeted analysis provides non-quantitative data with peak areas of chromatographic peaks being compared among groups rather than concentrations, and it does not necessarily provide robust identification of metabolites. Targeted analysis is suitable for studying a small, well-defined set (usually metabolic cycle or homologous series, e.g., glycolysis and aldehydes, respectively) of biologically important metabolites. The chemical identities of these metabolites are known before data acquisition, and absolute concentrations of each metabolite are determined by producing calibration curves for each metabolite using authentic standards and isotopically labelled internal standards. Semi-targeted analysis bridges the gap between untargeted and targeted analysis by focusing on low hundreds of metabolites whose identities are known prior to data acquisition. Semi-quantification is achieved by defining approximate concentrations (e.g., producing one calibration curve and internal standard for multiple metabolites).

This section consists of eight subsections. A comprehensive method development was conducted on urine samples from healthy volunteers with the aim of providing a complete methodology for untargeted urinary VOC analysis. This would generate initial data to confirm the ability of these advanced GC-MS platforms to profile urinary VOCs in a high throughput and standardised manner with the adoption of robust quality control measures.

3.2 Objectives

Specific objectives of this section were:

1. To compare solid-phase microextraction (SPME) versus HiSorb sorptive extraction, and select the technique that outperformed the others as the urinary VOC extraction method;
2. To determine the optimal conditions for urinary VOC extraction using HiSorb sorptive extraction;
3. To perform method development for TD-GC-quadrupole MS equipped with a mid-polar column, and to establish standardised urinary VOC analysis workflow;
4. To perform method development for TD-GC-TOF-MS equipped with a polar column;
5. To perform method development for TD-GC-TOF-MS equipped with a non-polar column;
6. To compare the developed multiplatform GC-MS methods for urinary VOC analysis;
7. To test TD recollection for multiple urinary VOC analysis;
8. To evaluate the applicability of osmolality normalisation in urinary VOC analysis.

3.3 Materials and reagents

Material and reagents used in Section 3 of this thesis are described in full below.

Reagents

The following commercial chemicals were used: Analytical grade sodium chloride, methanol, and hydrochloric acid (HCl) were purchased from Sigma-Aldrich (Gillingham, UK). Deionised nanopure water was produced by Millipore Direct-Q® 3 water purification system (Merck Millipore, Watford, UK). Alkane standards including C_8 to C_{20} and hexane were purchased from Sigma-Aldrich (Gillingham, UK). Isotope labelled analytical standards, including toluene-d_8, acetone-d_6, butyraldehyde-d_2, phenol-d_6, benzene-d_6, and acetophenone-d_8, were bought from Sigma-Aldrich (Gillingham, UK) and used as internal standards. Details of the reagents are summarised in Table 9.

Consumables

1.5 mL and 2 mL cryovials with safe lock were products of Scientific Laboratory Supplies Ltd. (Nottingham, UK). Falcon 15 mL and 50 mL centrifuge tubes were purchased from Scientific Laboratory Supplies Ltd. (Nottingham, UK). Clear glass 20 mL headspace vials with crimp top and round bottom, caps with HiSorb septa for 20 mL crimp-top headspace vials, HiSorb handle for manual probe extraction, and HiSorb septum plugs were supplied from Markes International (Llantrisant, UK). HiSorb probes coated with polydimethylsiloxane (PDMS) and empty inert-coated thermal desorption (TD) tubes were also products of Markes International (Llantrisant, UK). Cardboard cryoboxes and cryobox inserts for ultra-low lab freezer were products of VWR (Poole, UK) and Fisher Scientific UK Ltd. (Loughborough, UK), respectively. Laser cryo-tags were bought from Sigma-Aldrich (Gillingham, UK). Pipettes and pipette tips were purchased from Fisher Scientific UK Ltd. (Loughborough, UK). Table 10 provides information about consumables.

Equipment

A -80 °C biomedical freezer was the product of Haier (Qingdao, China). A 20 mm vial crimper and a decrimper were purchased from Agilent Technologies (Didcot, UK). A

OsmoPRO® Multi-Sample Micro-Osmometer was the product of Advanced Instruments (Horsham, UK). A HiSorb agitator and a TC-20 TD tube conditioner were both purchased from Markes International (Llantrisant, UK). A FiveGo™ pH meter was from Mettler Toledo (Columbus, USA).

Three GC-MS systems were used:

1. GC-MS instrument equipped with a PerkinElmer Clarus 580 GC with split/splitless inlet and a PerkinElmer SQ8S MSD (PerkinElmer, Inc., Waltham, USA);
2. TD-GC-MS instrument equipped with a Markes TD100 thermal desorber, an Agilent 7890B GC, and an Agilent 5977A MSD quadrupole MS (Markes International, Llantrisant, UK; Agilent Technologies, Santa Clara, USA);
3. TD-GC-TOF-MS instrument equipped with a Markes TD100-XR thermal desorber, an Agilent 7890B GC, and a SepSolve BenchTOF Select TOF-MS (Markes International, Llantrisant, UK; Agilent Technologies, Santa Clara, USA; SepSolve Analytical, Peterborough, UK);

The following GC columns were used in the chromatographic separation:

1. Stabilwax-DA, 30 m × 0.25 mm × 0.25 μm (Restek Corporation, Bellefonte, USA);
2. Mega WAX HT, 20 m × 0.18 mm × 0.18 μm (MEGA S.r.l., Legnano, Italy);
3. DB5-MS UI, 30 m × 0.25 mm × 1.00 μm (Agilent Technologies, Santa Clara, USA);
4. Zebron ZB-624UI, 60 m × 0.25 mm × 1.40 μm (Phenomenex, Torrance, USA).

The software packages used for the analyses were:

For data pre-processing: MassHunter Software (Agilent Technologies, Santa Clara, USA); ChromSpace (SepSolve Analytical, Peterborough, UK); MATLAB packages (Gavin 3)[117] (MathWorks, Natick, USA); MSHub/GNPS (University of California San Diego, San Diego, USA); NIST 17 Mass Spectral and Retention Index Libraries (NIST, Gaithersburg, USA); Microsoft Office apps (Microsoft Corporation, Redmond, USA).

For data analysis: SIMCA 15 (Sartorius, Gottingen, Germany); MetaboAnalyst 5.0 (Xia Lab @ McGill, Ste. Anne de Bellevue, Canada); SPSS 25 (SPSS Inc., Chicago, USA); Prism 8 (GraphPad Software Inc., La Jolla, USA).

Table 9. List of reagents.

Compound Name	Abbreviation	-R	MW	CAS	Supplier	Cat. No.
Sodium chloride	NaCl	NaCl	58	7647-14-5	Sigma-Aldrich	S7653-5KG
Methanol	MeOH	CH_3OH	32	67-56-1	Sigma-Aldrich	34860-2.5L-R
Hydrochloric acid	HCl	HCl	36	7647-01-0	Sigma-Aldrich	320331-2.5L
Deionised nanopure water	DI water	H_2O	18	7732-18-5	-	-
Hexane	C_6	C_6H_{14}	86	110-54-3	Sigma-Aldrich	270504-2.5L
Octane	C_8	C_8H_{18}	114	111-65-9	Sigma-Aldrich	74820-5ML
Nonane	C_9	C_9H_{20}	128	111-84-2	Sigma-Aldrich	74250-5ML
Decane	C_{10}	$C_{10}H_{22}$	142	124-18-5	Sigma-Aldrich	30540-5ML
Undecane	C_{11}	$C_{11}H_{24}$	156	1120-21-4	Sigma-Aldrich	94000-5ML
Dodecane	C_{12}	$C_{12}H_{26}$	170	112-40-3	Sigma-Aldrich	44010-5ML
Tridecane	C_{13}	$C_{13}H_{28}$	184	629-50-5	Sigma-Aldrich	91490-5ML
Tetradecane	C_{14}	$C_{14}H_{30}$	198	629-59-4	Sigma-Aldrich	87139-5ML
Pentadecane	C_{15}	$C_{15}H_{32}$	212	629-62-9	Sigma-Aldrich	76509-5ML
Hexadecane	C_{16}	$C_{16}H_{34}$	226	544-76-3	Sigma-Aldrich	52209-5ML
Heptadecane	C_{17}	$C_{17}H_{36}$	240	629-78-7	Sigma-Aldrich	51578-5ML
Octadecane	C_{18}	$C_{18}H_{38}$	254	593-45-3	Sigma-Aldrich	74691-5G
Nonadecane	C_{19}	$C_{19}H_{40}$	268	629-92-5	Sigma-Aldrich	74158-5G
Icosane	C_{20}	$C_{20}H_{42}$	282	112-95-8	Sigma-Aldrich	44818-5G
Toluene-d_8	-	C_7D_8	100	2037-26-5	Sigma-Aldrich	434388-10X0.75ML
Acetone-d_6	-	C_3D_6O	64	666-52-4	Sigma-Aldrich	444863-10X0.5ML
n-Butyraldehyde-2,2-d_2	-	$C_4H_6D_2O$	74	81214-92-8	Toronto Research Chemicals	TR-B689891
Phenol-d_6	-	C_6D_6O	100	13127-88-3	Sigma-Aldrich	176060-1G
Benzene-d_6	-	C_6D_6	84	1076-43-3	Sigma-Aldrich	175870-10X0.5ML
Acetophenone-d_8	-	C_8D_8O	128	19547-00-3	Sigma-Aldrich	296732-1G

Table 10. List of consumables.

Product Name	Supplier	Cat. No.
Eppendorf Safe-Lock micro test tubes 2.0 mL	Scientific Laboratory Supplies Ltd.	E0030120094
Eppendorf Safe-Lock micro test tubes 1.5 mL	Scientific Laboratory Supplies Ltd.	E0030120086
Falcon 15 mL Conical PP Centrifuge Tubes	Scientific Laboratory Supplies Ltd.	352096
Falcon 50 mL Conical Polypropylene Centrifuge Tube Sterile	Scientific Laboratory Supplies Ltd.	352070
Headspace vial, 20 mL, crimp-top, clear, round-bottomed, pk 100	Markes International	C-VCC20
Cap with HiSorb septum, for 10 mL/20 mL crimp-top vials, magnetic, pk 100	Markes International	C-HSPCCS
HiSorb septum plug, pk 6	Markes International	C-HSPVLP-6
HiSorb handle	Markes International	C-HSPH
HiSorb probe, PDMS, inert, standard length, pk 6	Markes International	H1-AXAAC
Empty inert-coated tubes with primary gauze pk 10	Markes International	C0-CXXX-0000
Cryobox cardboard 133 × 133 × 50 mm	VWR, Part of Avantor	HEATHS2860A
Fisher brand Cardboard Cryobox Inserts, 136mm, Height: 25 mm, Array: 10 × 10, Grid Inserts	Fisher Scientific UK Ltd.	11547673
Laser Cryo-babies and Cryo-tags	Sigma-Aldrich	Z378992-1PAK
Eppendorf Research plus EU-IVD Single Channel Variable Volume Pipette, Volume: 100 to 1000 µL	Fisher Scientific UK Ltd.	10234204
Tips 200 µL Gilson Type Bulk 1k	VWR, Part of Avantor	MEDL200016
Kartell Polypropylene Universal Tip, Volume: 100 to 1000 µL, Packaging: Rack, Autoclavable: Yes, Certifications/Compliance: CE/IVD	Fisher Scientific UK Ltd.	10459371

3.4 Urine sample collection and storage

A cohort of fifteen healthy volunteers (eight males and seven females, age 23 to 41 years) was recruited. Subjects were informed of the aim of the study and were invited to sign the consent before participating in the study (REC reference 04/Q0403/119, Appendix 2).

No special pre-test dietary requirements were applied. Subjects were asked to pass urine into standardised 50 mL urine specimen vials, which were then immediately sealed. All samples were immediately aliquoted into smaller 15 mL vials, labelled, and stored in the -80 °C biomedical freezer prior to further analysis. Stability studies have shown that storage at -80 °C does not impact upon the presence of urinary VOCs in headspace gases.[118,119] In addition, aliquoting samples for each usage ensures that the freeze-thaw cycles are minimised.[118]

3.5 Urinary VOC extraction techniques (SPME vs HiSorb)

3.5.1 Introduction

Sample preparation is a critical step in GC analysis. The sample should be introduced as an appropriate form into the flash vaporiser port at the head of the column. Sample preparation also determines the accuracy and precision of the urinary VOC analysis, as well as the amount of sample consumption, cost, possibility of method automation, analysing time, etc.

The basic concept of sample preparation is to convert an actual matrix into a sample format suitable for separation or other analytical procedures. For VOCs, the major sample preparation method is extraction, which can efficiently and selectively separate the compounds of interest from a sample matrix and concentrate the compounds in one phase. Such extraction tools are needed to extract and concentrate headspace VOCs released from urine and introduce these VOCs into the GC column.

Sample preparation can be categorised into online or offline sample preparation. Online sample preparation means that the tool used for sample preparation is also used for injecting the sample into the analyser and for starting data acquisition. Offline sample preparation entails removing the tool holding samples from the sample preparation platform and then injecting them into the analyser. In the case of aiming to develop a high throughput method suitable for clinical adoption, sample preparation should be a quicker process than analysis, allowing multiple samples to be prepared rapidly. This can be achieved in an offline system. Another important reason to choose offline sample preparation is that urine as a potentially infectious biosample cannot be prepared close to the GC due to biosafety. Urine samples can also contaminate the lab environment, affecting the performance of GC instruments. In addition, exposing to heat generated by the GC (e.g., prolonged stay in the autosampler when queuing for analysis) can cause unwanted loss of urinary VOCs.

Two offline urinary VOC extraction techniques, solid-phase microextraction (SPME) and HiSorb sorptive extraction, were compared in this subsection.

3.5.1.1 Solid-phase microextraction (SPME)

Six of the thirteen identified studies in the systematic review (Section 2) involved the use of solid-phase microextraction (SPME). SPME in combination with GC-MS is an established method for the extraction and detection of VOCs within biofluids.[110,120-123] SPME is a solvent free sampling technique that uses small-size sorbent to extract different kinds of analytes from liquid, gas, or solid samples (Figure 7). The extracting phase is mounted on supporting substrates or produced into monolithic fibres or thin films. In current method development section, SPME fibre were used (Figure 7). Analytes can partition from the sample to extraction phases, driven by chemical potential gradients between the sample and extraction phases. The quantities of analytes extracted by the extraction phases are maximised when the sample and extraction phases achieve partition equilibrium. SPME is a non-exhaustive technique because of the low absolute recoveries caused by the small amount of extracting phase is using. The fact that a high proportion of the extracted analytes can be effectively transported to the instrument for analysis allows for high sensitivity when SPME is coupled with GC-MS. As a fast and cost-efficient technique, SPME can efficiently extract VOCs from urine headspace. However, there is no widely accepted standardised protocol for the preparation of urine samples for SPME extraction.

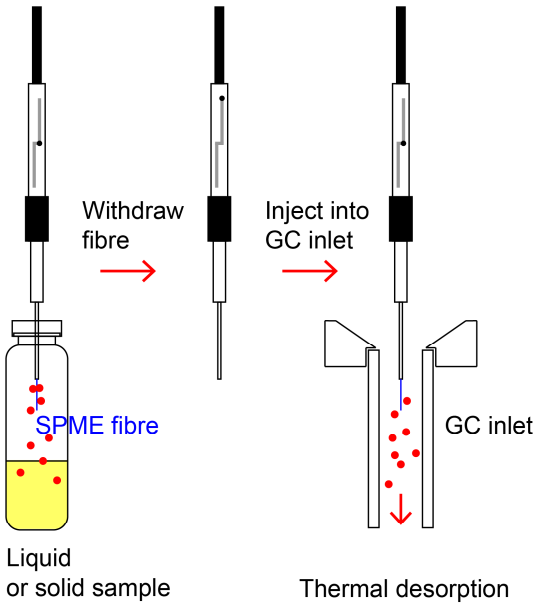


Figure 7. Schematic representation of SPME.

The analytes from liquid or solid sample are extracted by the SPME fibre (extracting phase). When equilibrium is reached, the fibre is withdrawn and injected into the heated GC inlet, allowing the analytes to enter the GC column. SPME: solid-phase microextraction.

3.5.1.2 HiSorb sorptive extraction

HiSorb is a novel sampling system for VOCs analysis in liquids or solids by TD-GC-MS that involves the use of a probe coated with an extracting phase. It integrates sampling, extraction, concentration, and sample introduction into one single step without the requirement for a solvent. A HiSorb probe is built using inert metal, with the extracting phase mounted at the tip of the probe (Figure 8). The extracting phase works following same principles as SPME, with analytes partitioning from the sample to extraction phases (absorption, not surface adsorption), driven by chemical potential gradients between the sample and extraction phases, until equilibrium is achieved. The workflow of HiSorb extraction includes inserting the probe into a glass 20 mL or 10 mL headspace vial for immersive or headspace sampling, agitating the vial on a HiSorb agitator for efficient mixing and heating of sample, drying the probe to remove residual matrix, and inserting the probe into a standard TD tube for analysis by TD-GC-MS. There is not yet a standardised protocol for preparing urine samples for HiSorb extraction.

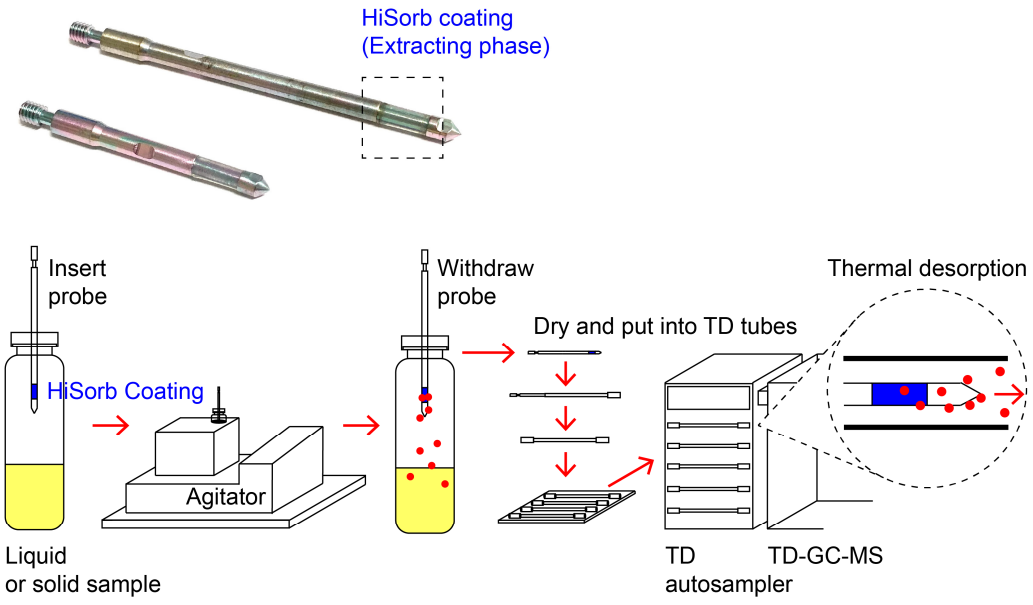


Figure 8. Schematic representation of HiSorb sorptive extraction.

The analytes from liquid or solid sample are extracted by the extracting phase on the HiSorb probe. The glass headspace vial is agitated on a HiSorb agitator for efficient mixing and heating of sample. When the extraction is complete, the probe is withdrawn, dried and inserted into a standard TD tube for TD-GC-MS analysis.

3.5.2 Objectives

The primary objective of this subsection was to compare SPME versus HiSorb and to select the most appropriate extraction method for urinary VOC analysis.

Secondary objectives were:

1. To compare the background levels of conditioned blank SPME fibres and HiSorb probes;
2. To establish calibration curves for representative urinary VOCs to evaluate and compare the extraction ability of SPME and HiSorb;
3. To compare the robustness, throughput, reliability, and automation capabilities of SPME and HiSorb.

3.5.3 Methods

3.5.3.1 Subjects

Experiments utilised urine from one healthy volunteer. Deionised nanopure water was used as blank control.

3.5.3.2 Sample preparation

Urine samples were removed from the -80 °C freezer and thawed in the 4 °C refrigerator overnight. Following this, 2 mL urine was transferred into glass 20 mL headspace vials. The urine was diluted with 6 mL deionised nanopure water. Sodium chloride was added into each urine sample for the resulting solution to have an NaCl concentration of 0.2 g/mL (0.4 g to a 2 mL sample). 5 M hydrochloric acid (HCl) was added into each urine sample to adjust the pH to 2.0 under the monitoring of the pH meter. The purpose of adding salt and acid was to drive the partitioning and enhance the realising of VOCs.[123,124]

Seven compound standards were spiked for the generation of calibration curves for the SPME extraction method: butanal, 1-butanol, dimethyl disulphide, 2-hexanone, 2-heptanone, octanal, and phenol. Serial dilutions of these compounds were added to the urine at following concentrations: 0.02 µg/mL, 0.2 µg/mL, 2 µg/mL, and 20 µg/mL.

Ten compound standards were spiked for the conducting of calibration curves for the HiSorb extraction method: butanal, 1-butanol, 1-pentanol, dimethyl disulphide, 2-butanone, 2-pentanone, 2-hexanone, 2-heptanone, octanal, and phenol. Serial dilutions of these compounds were added to the urine at following concentrations: 0.05 µg/mL, 0.1 µg/mL, 0.2 µg/mL, 0.4 µg/mL, 0.8 µg/mL, 1.6 µg/mL, 3.2 µg/mL, 6.4 µg/mL, 12.8 µg/mL, and 25.6 µg/mL.

Toluene-d_8 was added as an internal standard at 0.5 µg/mL.

3.5.3.3 Urinary VOC extraction

Urinary VOCs were extracted using SPME fibres and HiSorb probes. One urine sample was only extracted once using either SPME or HiSorb technique.

For SPME, the divinylbenzene (DVB) / carboxen (CAR) / polydimethylsiloxane (PDMS) fibre type was used. Before each extraction, the fibres were pre-conditioned according to supplier's recommendation at 250 °C for 5 min by inserting into the GC inlet. Urinary VOC extraction was conducted by inserting the SPME fibre extracting phase into the urine headspace without contacting the urine or the headspace vial wall. Meanwhile, the headspace vial was agitated in the agitator. The initial experimental parameters for both HiSorb and SPME extraction were set up referring to user instructions from manufacturer and previous studies.[110,114,123]

The extracting phase of HiSorb was polydimethylsiloxane (PDMS). Prior to the VOC extraction by HiSorb, TD tubes with HiSorb probes loaded were pre-conditioned using the TC-20 TD tube conditioner at 260 °C for 60 min under nitrogen stream at 20 psi head pressure according to the instructions for use. Urinary VOC extraction was conducted by inserting a HiSorb probe into the glass headspace vial through the vial septum, exposing the extracting phase in the urine headspace without touching the urine or the wall of the headspace vial, aiding with agitating in the heated agitator (60 °C) for 30 min (agitating temperature and time will be discussed in Section 3.6). After extraction, the HiSorb probe was withdrawn, dried, and placed into the TD tube for further thermal desorption and GC-MS analysis.

3.5.3.4 (TD-)GC-MS

Urinary VOCs extracted by SPME fibres were analysed using a Perkin Elmer Clarus 580 GC with SQ8S MSD. Desorption of VOC was performed by exposing the fibre coating in the GC inlet at 270 °C for 5 min. VOC mixture was separated on a Stabilwax-DA column (30 m × 0.25 mm × 0.25 μm) programmed at 1.0 mL/min constant Helium carrier. Oven temperature was set at 40 °C initially for 5 min, ramp to 240 °C at 10 °C/min with 5 min hold. The MS transfer line was maintained at 260 °C with 70 eV electron impact set at 180 °C. MS analyser was set to acquire over the range of 40 to 400 m/z with data acquisition rate approximately at 0.5 scan/sec.

Urinary VOCs extracted by HiSorb probes were analysed using the Agilent 7890B GC with 5977A quadrupole MSD coupled to the Markes TD100 instrument. TD tubes loaded with HiSorb probes were placed onto the TD autosampler and checked for leaks, and dry purged for 10 min under constant flow of 100 mL/min helium to remove excess moisture to ensure that VOCs were not oxidised during heating. The tube was then heated at 260 °C for 15 min, with the VOCs transferred on to a 10 °C cold trap for 15 min desorption (nitrogen flow 50ml/min) in splitless mode. Subsequently, the cold trap was heated to 290 °C at 99 °C/min and held for 4 min, transferring the VOCs to the ZB-624UI capillary column (60 m × 0.25 mm × 1.40 µm) with the flow path onto GC heated constantly at 200 °C. VOCs separation was performed on the ZB-624UI column programmed at 1.0 mL/min constant helium carrier flow. Oven temperature was set at 40 °C initially for 4 min, ramp to 150 °C (15 °C/min without hold), and ramp to 240 °C (10 °C/min with 15 min hold). The MS transfer line was maintained at 230 °C, and 70 eV electron impact was set at 240 °C with MS quadruple held at 150 °C. MS analyser was set to acquire over the range of 35 to 250 m/z with data acquisition approximated to 0.2 scan/sec.

3.5.3.5 Data processing

Data from the Perkin Elmer GC-MS system were processed using MSHub, while data from the Agilent TD-GC-MS system were processed using Agilent MassHunter Software, to perform targeted analysis to identify the spiked standards and to obtain their abundance. Standard deviations (SD) were calculated in multiple replicate samples. Compounds were identified by matching and reverse matching the spectra in NIST 17 Mass Spectral and Retention Index Libraries. GraphPad Prism 8 was used for statistical analysis and graphic creation.

3.5.4 Results

3.5.4.1 Background chromatogram comparison

The typical total ion chromatograms (TIC) of conditioned blank SPME fibres showed few background peaks (Figure 9, a). In comparison, TIC of conditioned blank HiSorb probes revealed a higher number of peaks. These peaks represented for four different chemical classes of compounds including siloxanes, acids, fluorides, and heterocyclic compounds (Figure 9, b). Additionally, analysis of empty TD tubes with no HiSorb probe loaded using the same TD-GC-MS programme obtained TICs with few background peaks.

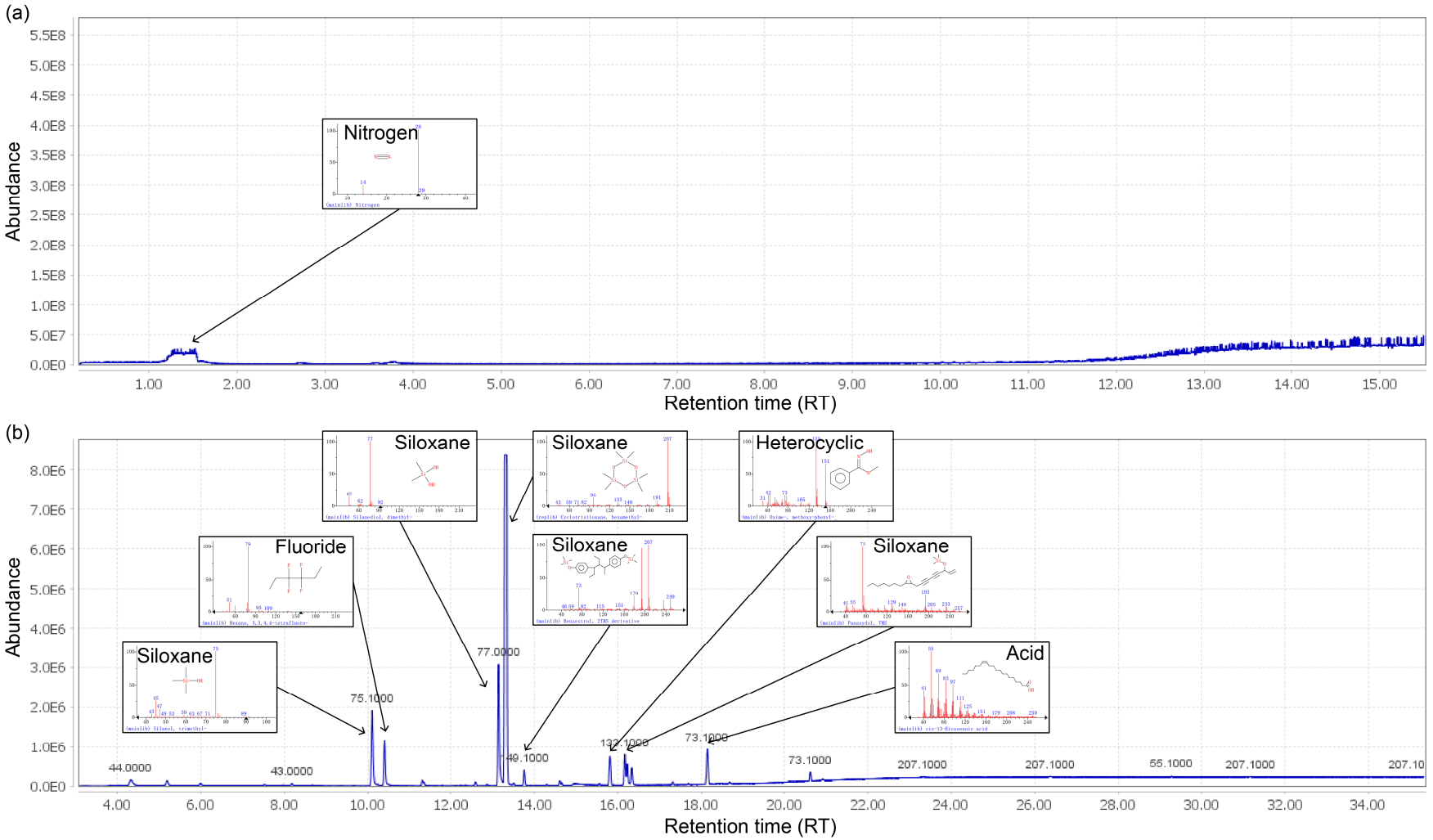


Figure 9. Typical chromatograms of blank SPME fibre and blank HiSorb probe.

Examples of total ion chromatograms (TIC) for (a) blank SPME fibre and (b) blank HiSorb probe. Few peaks appeared on the TIC for the blank SPME fibre, while several peaks were observed on the TIC for the blank HiSorb probe.

3.5.4.2 Calibration curves

When using SPME extraction method, phenol was not detected at every concentration level. For the remaining compounds, the curves showed good linearity between 0.02 µg/mL to 2 µg/mL although reached a plateau phase above this range (Figure 10).

When using HiSorb extraction method, the curves of butanal, 1-butanol, 2-butanone, and phenol showed good linearity from 0.05 µg/mL to 25.6 µg/mL. The curves of 1-pentanol, dimethyl disulphide, 2-pentanone, 2-hexanone, 2-heptanone, and octanal showed good linearity from 0.05 µg/mL to 1.6 µg/mL. However, when the concentration exceeded 1.6 µg/mL, the slope of the curves dropped significantly, indicating the limit of linearity was reached (Figure 11).

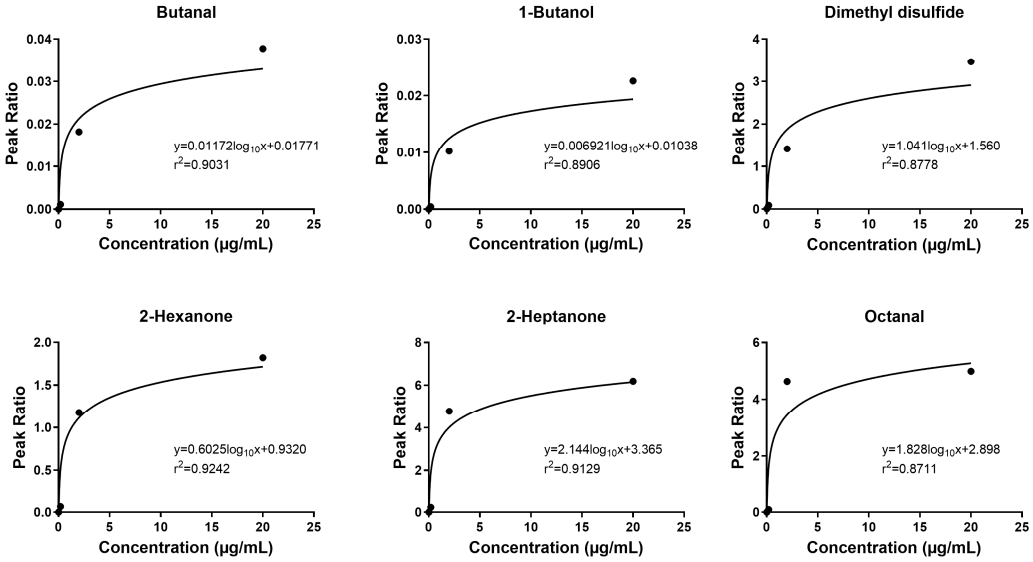


Figure 10. Calibration curves for the SPME extraction method.

All the curves showed good linearity between 0.02 µg/mL to 2 µg/mL. Phenol was not detected at every concentration level.

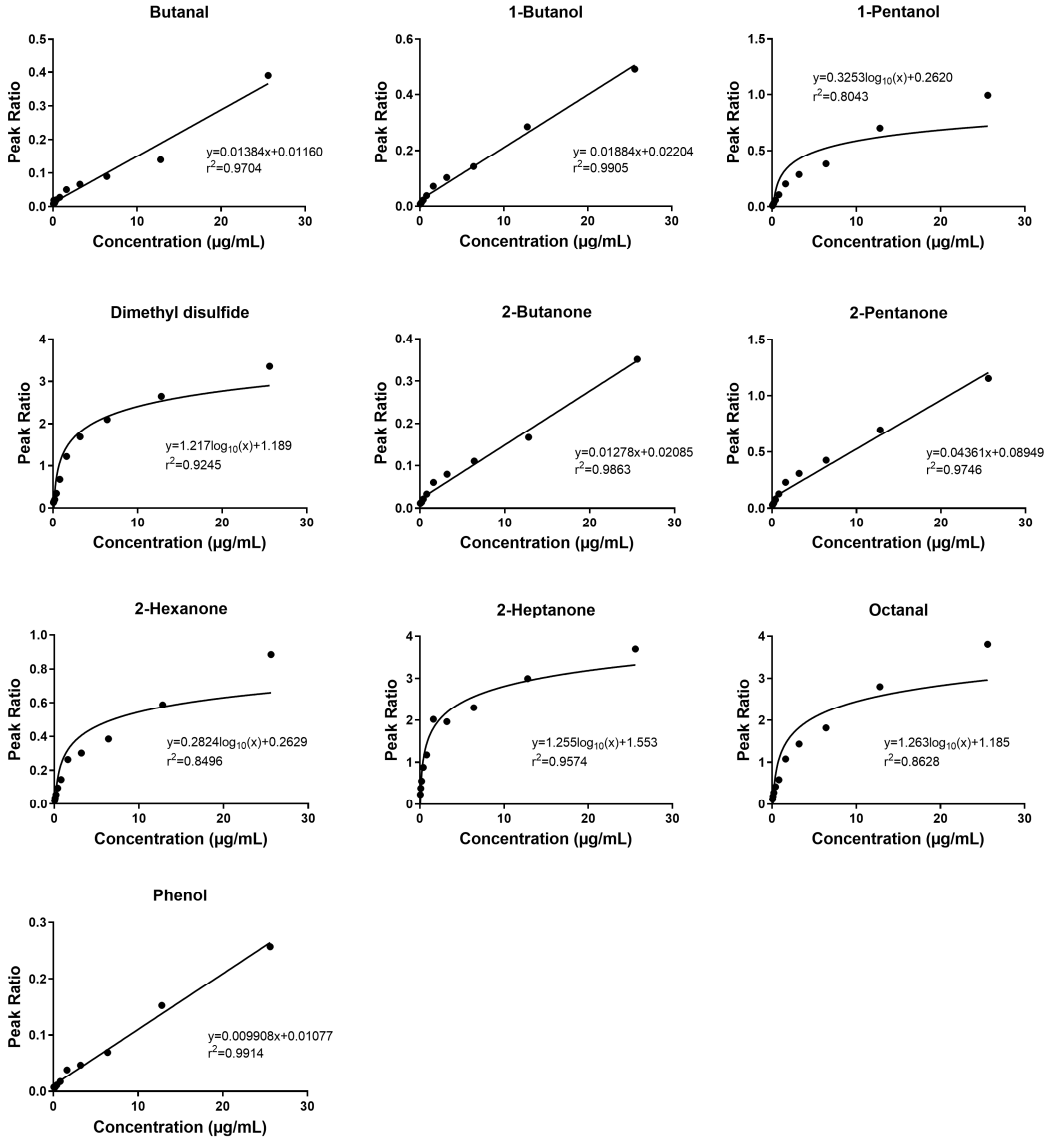


Figure 11. Calibration curves for the HiSorb extraction method.

The curves of butanal, 1-butanol, 2-butanone and phenol showed good linearity from 0.05 µg/mL to 25.6 µg/mL. The curves of 1-pentanol, dimethyl disulphide, 2-pentanone, 2-hexanone, 2-heptanone, and octanal showed good linearity from 0.05 µg/mL to 1.6 µg/mL, with the slope of the curves dropping significantly when exceeding 1.6 µg/mL.

3.5.5 Discussion

TIC of conditioned blank HiSorb probes revealed peaks of siloxanes, acids, and heterocyclic compounds, while the TIC of empty TD tubes with no HiSorb probe loaded showed few background peaks. This indicated that these peaks were from conditioned blank HiSorb probes themselves. Due to the usage of multibed sorbent in SPME, which is “dirtier” than the PDMS used in HiSorb, there should be more peaks observed on the TIC of conditioned SPME. However, few peaks were observed on the TIC for the blank SPME, and multiple peaks were visible on the TIC for the blank HiSorb. This could be explained by the higher amount of sorbent used in the HiSorb. It is reported by the HiSorb manufacturer that all PDMS sorptive extraction sampling devices have an intrinsic siloxane background, and this does not affect the analysis unless siloxanes are a key compound that requires quantitation, or the target peaks overlap with siloxane peaks. Therefore, deconvolution, which is the process of decomposing peaks that overlap with each other, is necessary when performing peak detection. In terms of the non-siloxane background compounds, they might be contaminants released by GC columns or the GC-MS instrument or were from lab environment. It is critical to reduce the instrumental and environmental contamination as much as possible. It is also critical to properly condition HiSorb probes to eliminate the contamination-related compounds within the HiSorb probes.

When building calibration curves, the same matrix (urine) as the real experiment was used. For both SPME and HiSorb, more than five calibration standards were used. These compounds were previously detected in human urine and were representatives of several common chemical classes (aldehydes, ketones, and alcohols, etc.). The middle point of the calibration was the expected average level for the samples and the concentration of calibration points covered four digits. With the exception of phenol, the detection limit of SPME method for the rest compounds could reach 10 ppb. Good linearity was observed between the 10 ppb and ppm level. The detection limit of HiSorb method was at 10 ppb level. The limit of linearity for butanal, 1-butanol, 2-butanone, and phenol was at 10 ppm level, significantly higher than that of SPME method. The limit of linearity for the remaining compounds, including 1-pentanol, dimethyl disulphide, 2-pentanone, 2-hexanone, 2-heptanone, and octanal, was at a ppm level, similar to that of the SPME method. This difference of extraction ability can be explained by the difference of sampling phase. The SPME fibres were equipped with 30/50 μm thickness DVB/CAR/PDMS fibre coating, covering polarity ranging from non-

polar to polar, therefore can extract non-polar, semipolar, and polar VOCs. While the HiSorb probe's sampling phase is 65 µL volume PDMS (around 100 times the amount of SPME coating), extracting mainly non-polar compounds. The much greater volume of sampling phase on HiSorb may have contributed to the better urinary VOC extraction that was observed within this method. This extra sorbent on HiSorb explained why phenol, which is a partially volatile and semipolar compound, was detected by HiSorb but not SPME.

The structural strength of HiSorb probe is much greater than that of SPME. It is common for a SPME fibre to break during analysis. The replacement of SPME fibre often results in a shift in the baseline of TIC, retention time, and/or peak abundance, etc., introducing batch effects and subsequently complicating data analysis, or sometimes even non-correctable batch effects. In comparison, using HiSorb probe usually does not have such batch effect problems. The number of usage cycles for HiSorb (50 times recommended by manufacturer) is also larger than SPME, not only because of its greater structural strength but also its much thicker extracting phase. These means that the obtained data can be more reliable, and costs are significantly reduced, which is extremely useful in clinical trials and epidemiological studies, enabling the ability to conduct large scale studies.

HiSorb also outperforms SPME for urinary VOC analysis with high throughput reliable automated sampling. HiSorb is easier and quicker to handle than SPME fibre. Multiple samples, up to sixteen, can be extracted by HiSorb on the agitator at the same time. The analysis in one day can be divided into two to three batches depending on the demand, maximum sixteen samples within each batch. The HiSorb agitator allows unattended sample preparation. Cryogen free pre-concentration by TD prior to GC-MS analysis improves the sensitivity. The use of auto TD sampler allows unattended GC-MS analysis. The sample extraction using HiSorb can also be completed fully automatically using Centri®, a sampling and concentration system for GC-MS, but is not discussed in this study.[125]

A limitation of this subsection is that different GC-MS platforms were used during the investigation of extraction performance and the establishment of calibration curves. This was because SPME requires a GC inlet for injection, whereas HiSorb is desorbed

on the TD, so that the experiments were conducted on two platforms, with the Agilent instrument's GC injection inlet closed and the GC connected to the TD. Another limitation is that different number and concentration of calibration points were used for SPME and HiSorb, although the middle point of the calibration was at the expected average level and the concentration of calibration points covered four digits.

3.5.6 Conclusion

SPME and HiSorb sorptive extraction were compared as two urinary VOC extraction techniques. Following observing the background total ion chromatograms and establishment of calibration curves for representative compounds, HiSorb outperformed SPME for urinary VOC analysis, providing broader VOC detection, reduced batch effects, and high throughput reliable automated sampling.

3.6 Optimisation of HiSorb method

3.6.1 Introduction

Once the HiSorb method was chosen as the preferred method for VOC extraction, conditions were optimised to maximise the extraction of urinary VOCs. It is important to find the optimal conditions at which the equilibrium occurs as quickly as possible so that the extraction recovery can be maximised. Parameters of optimisation included: extraction temperature, extraction time, urine acidification, sample volume, and urine dilution.

Extraction temperature can play an important role in headspace HiSorb extraction. Adjusting the temperature in HiSorb can affect recovery (e.g., headspace generation, time to equilibrium, possible thermal degradation) therefore requires thorough investigation.

The optimal extraction time represents the time required for the system to reach equilibrium. Longer extraction than the optimal extraction time does not improve the urinary VOC extraction results.

Adjusting the sample pH or its salt content can potentially improve the extraction recovery by changing extraction behaviour by driving partitioning due to protonation or deprotonation and by saturating the solution thus driving the VOCs into the headspace.[126]

Similar to SPME, the sensitivity of HiSorb method is proportional to the number of analyte molecules recovered from the sample. Analyte recovery rises as the sample volume increases. While larger sample volume may result in slower kinetics and higher analyte recovery in very dilute samples.

3.6.2 Objectives

The primary objective of this subsection was to determine the optimal conditions for urinary VOC extraction using HiSorb sorptive extraction.

Secondary objectives were:

1. To evaluate the effect of extraction temperature and time on urinary VOC extraction performance;
2. To evaluate the effect of urine acidification on urinary VOC extraction performance;
3. To evaluate the effect of urine sample volume on urinary VOC extraction performance;
4. To evaluate the effect of urine dilution on urinary VOC extraction performance.

3.6.3 Methods

3.6.3.1 Subjects

Urine from a single healthy volunteer was used in this subsection.

3.6.3.2 Sample preparation

Urine samples were removed from the -80 °C freezer and thawed in the 4 °C refrigerator overnight. Different volumes of urine (1 mL, 2 mL, 3 mL, and 5 mL) were transferred into glass 20 mL headspace vials when optimising sample volume. When testing the effect of dilution, urine samples were diluted at the ratio of 1:1 (2 mL urine : 2 mL deionised nanopure water), 1:2 (2 mL urine : 4 mL water), 1:3 (2 mL urine : 6 mL water), and 1:4 (2 mL urine : 8 mL water), and samples without dilution (2 mL urine) were used as controls. NaCl was added into each urine sample to reach the concentration of 0.2 g/mL. When evaluating the effect of acidification, urine samples (5 mL each) were acidified by HCl. HCl (5 M) was slowly added to urine samples at room temperature monitored by the pH meter, until a pH of 2.0. Un-acidified urine samples (5 mL each) served as a control. Toluene-d_8 dissolved in methanol was added as an internal standard (0.5 µg/mL).

3.6.3.3 Urinary VOC extraction

Urinary VOCs were extracted using HiSorb probes. HiSorb probes loaded onto TD tubes were pre-conditioned using the TC-20 TD tube conditioner at 260 °C for 60 min under nitrogen stream at 20 psi head pressure. Urinary VOC extraction was conducted by inserting a HiSorb probe into the glass headspace vial, exposing the PDMS extracting phase in the urine headspace, aiding with agitation at stirring speed of 300 rpm. Four combinations of extraction temperature and time were tested: 37 °C / 30 min; 37 °C / 120 min; 60 °C / 30 min; and 60 °C / 120 min. After extraction, the HiSorb probe was withdrawn, dried, and placed into the TD tube for TD-GC-MS analysis.

3.6.3.4 TD-GC-MS

The Agilent 7890B GC with 5977A quadrupole MSD coupled to the Markes TD100 instrument was used. TD tubes with HiSorb probes loaded were placed onto the TD and checked for leaks, and dry purged for 10 min under constant 100 mL/min helium

to remove excess moisture to avoid oxidation of VOCs during heating. The tube was then heated at 260 °C for 15 min, with the VOCs transferred on to a 10 °C cold trap for 15 min desorption (nitrogen flow 50 mL/min) in splitless mode. Subsequently, the cold trap was heated to 290 °C at 99 °C/min and held for 4 min, transferring the VOCs to the ZB-624UI capillary column (60 m × 0.25 mm × 1.40 µm) with the flow path onto GC heated constantly at 200 °C. VOC separation was performed on the ZB-624UI column programmed at 1.0 mL/min constant helium carrier flow. Oven temperature was set at 40 °C initially for 4 min, ramp to 150 °C (15 °C/min without hold), and ramp to 240 °C (10 °C/min with 15 min hold). The MS transfer line was maintained at 230 °C, and 70 eV electron impact was set at 240 °C with the MS quadruple held at 150 °C. MS analyser was set to acquire over the range of 35 to 250 m/z with data acquisition approximated to 0.2 scan/sec.

3.6.3.5 Data processing

Data were processed using Agilent MassHunter Software to perform targeted analysis to identify fifteen compounds, with their abundance obtained at the same time. Among them there were one heterocyclic organic compound (furan, 2-methyl), two organosulfur compounds (dimethyl sulfide and dimethyl disulphide), six aldehydes (butanal, pentanal, hexanal, heptanal, octanal, and nonanal), four ketones (acetone, 2-butanone, 2-pentanone, and 4-heptanone), and one alcohol (1-butanol). Total peak areas ± standard deviations (SD) were calculated for each chemical group. MetaboAnalyst 5.0 and SPSS 25 were used for statistical analysis. Univariate statistical analyses were used to distinguish groups with significant difference of total peak areas. All tests were considered statistically significant when p value < 0.05. Compounds were identified by matching and reverse matching the spectra in NIST 17 libraries. GraphPad Prism 8 was used for further statistical analysis and graphic creation.

3.6.4 Results

3.6.4.1 Extraction temperature and time

Four conditions were tested to evaluate the effect of extraction temperature and time: 37 °C / 30 min; 37 °C / 120 min; 60 °C / 30 min; and 60 °C / 120 min. The experiment consisted of five replicates (5 mL urine per sample). Figure 12, a shows total peak areas of the extracted compounds based on their chemical classes. With the exception of heterocyclic organic compounds and aldehydes (37 °C / 120 min) and organosulfur compounds and ketones (60 °C / 120 min), there was no statistically significant difference among the four conditions. Despite greater standard deviation, extracting by HiSorb probes at 60 °C provided higher total peak area in most chemical groups.

3.6.4.2 Urine acidification

To evaluate the effect of acidification, three urine samples (5 mL each) were acidified by HCl. Three un-acidified urine samples (5 mL each) were used as controls. As shown in Figure 12, b, for all chemical groups, acidified samples had significantly higher total peak area compared to un-acidified samples. The total peak area of acidified samples for organosulfur compounds was ten times higher than that of un-acidified samples.

3.6.4.3 Urine sample volume

Urine sample volume of 1 mL, 2 mL, 3 mL, and 5 mL were compared to evaluate the effect of different sample volumes on extracting performance. Three replicates were performed for each volume. Total peak area of the compounds grouped by chemical class and sample volume is presented in Figure 12, c. At volumes of 1 mL and 2 mL, the peak areas of ketones were significantly lower than that observed at higher volumes. For compounds of other chemical classes, samples volume did not appear to have a significant effect on total peak area. There was a high variation within the results of 1 mL samples.

3.6.4.4 Urine dilution

Figure 12, d demonstrates the total peak ratio (the peak area of the reference compound divided by the peak area of the internal standard (toluene-d_8, 0.5 µg/mL) of the extracted compounds grouped by chemical class and dilution factor. Both

organosulfur compounds and ketones had significantly lower total peak ratio for diluted compared to non-diluted samples. Observed variation tended to be lower of organosulfur compounds, ketones, heterocyclic organic compounds, aldehydes, and alcohols within diluted samples especially at the ratio of 1:3.

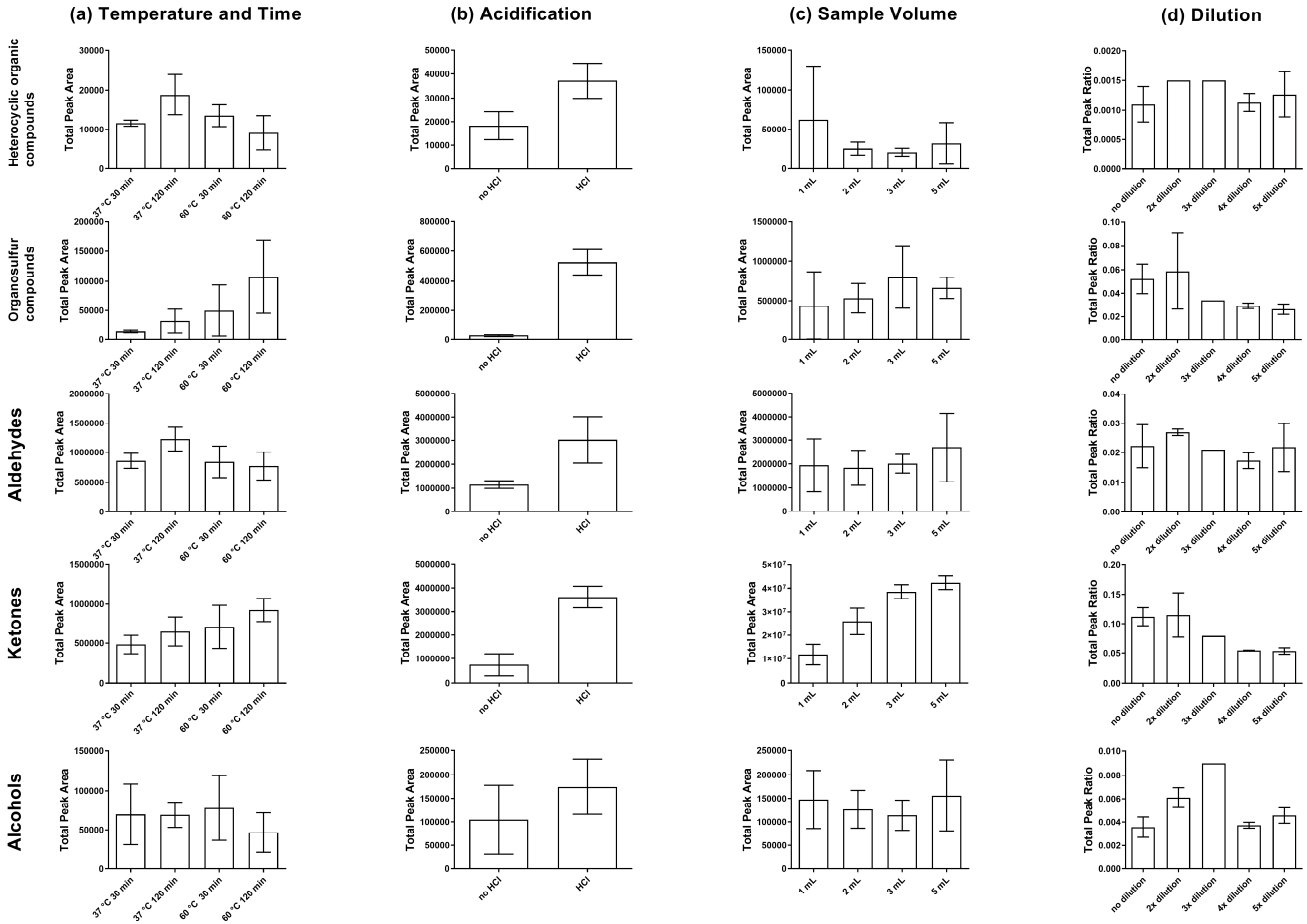


Figure 12. Optimisation of HiSorb extraction conditions.

The effect of (a) extraction temperature and time, (b) acidification, (c) sample volume, and (d) dilution on HiSorb extraction performance. Total peak areas (or internal standard normalised total peak ratios) of the extracted compounds are grouped based on their chemical groups (Heterocyclic organic compound, Organosulfur compound, Aldehyde, Ketone, and Alcohol); error bars show the SD of five replicates for Temperature and Time, three replicates for Acidification, three replicates for Sample Volume, and three replicates for Dilution.

3.6.5 Discussion

Experimental conditions for the HiSorb method, including extraction temperature and time, acidification, sample volume, and dilution, were evaluated. Selected VOCs are frequently detected in human urine, and are also representatives of different chemical classes, therefore allowing comprehensive assessment of the extraction performance of HiSorb.

With exception of heterocyclic organic compound and aldehydes (37 °C / 120 min) and organosulfur compounds and ketones (60 °C / 120 min), there was no statistically significant difference among the four temperature and time conditions. This indicated that for most compounds, extraction temperature and time did not have a significant impact on VOC detection when using HiSorb extraction method. Therefore, in the interest of time efficiency, 30 min is proposed to be a suitable extraction time for clinical adoption. In addition, extracting by HiSorb probes at 60 °C provided higher total peak areas in most chemical groups, despite sometimes having greater standard deviations. Therefore, HiSorb extraction at 60 °C for 30 min is advised to obtain excellent extraction outcomes in a relatively short amount of time.

For all chemical groups, samples acidified with HCl had significantly higher total peak area when compared to un-acidified samples. These results suggested that adding acid to urine samples significantly increases total peak area and should therefore be carried out routinely. This was also supported by other studies, which reported that more urinary VOCs can be detected with an acid environment than with a neutral or alkaline environment.[121,127] Although there is limited literature examining which acid to choose, HCl is the most common acid used for urine acidification. Changing the pH can alter the urine sample matrix due to several reasons. It may increase the decomposition or degradation of certain compounds present in the sample, especially when a powerful oxidising acid is used, which will almost surely modify the sample composition. It may cause more compounds transitioning from liquid to gas phase by increasing activity coefficients (how much a solution differs from an ideal solution) and decreasing the partition coefficients (the ratio of a sample's concentration in the liquid phase to the concentration of VOCs in the headspace).

For most chemical classes, sample volume did not appear to have a significant effect on total peak areas. There was a high variation within the results of 1 mL samples, raising doubts about the stability of the extraction performance. Thus, a sample volume of 2 mL is suitable for urinary VOC analysis to achieve optimal extraction efficiency with the least amount of sample.

As a matrix, urine is a rich source of endogenous components, which can cause matrix effects. The matrix effect is that the matrix components of a sample might have an impact on the analytical measurement of the primary compound, usually in a negative way.[128] The dilution of urine samples has the potential to compensate the matrix effects between urine samples and water. While in the current experiment, for all chemical groups, the total peak ratios for non-diluted samples were higher than diluted ones, although the variations of non-diluted samples were sometimes higher. As a result, no dilution of the sample is suggested to ensure optimal extraction efficiency.

This subsection is limited by the selection of the representative VOCs. Some other VOCs which are frequently detected in human urine and are potential biomarkers might have been missed. The extraction performance of HiSorb on these compounds required specific assessment if necessary. This subsection is also limited by the selection of the tested conditions for the extraction parameters, which were mainly based on manufacturer's recommendations and previous studies. The tested parameters (e.g., extraction temperature, time, and acidification) may have interactions on each other. Employing a multivariate optimisation strategy (e.g., central composite design (CCD)), which considers all the tested parameters and conditions, may allow the evaluation of those interactions with fewer experiments required.[129]

3.6.6 Conclusion

The evaluation of the effect of extraction temperature and time, urine acidification, sample volume, and urine dilution on urinary VOC extraction performance suggest that for the majority of compounds, a 2 mL urine sample, without dilution, acidified with 5 M HCl to pH 2.0, and then extracted at 60 °C for 30 min can deliver the best outcomes among all tested experimental conditions.

3.7 TD-GC-quadrupole MS method development and GC-MS analysis workflow establishment

3.7.1 Introduction

In this subsection, a conventional quadrupole-based TD-GC-MS with an all-around mid-polar column was used to establish a benchmark method for urinary VOC detection.

3.7.1.1 TD-GC-quadrupole MS

As discussed in Section 1.9, during GC-MS analysis, the gas phase sample is first propelled by an inert carrier gas into the heated GC equipped with a capillary GC column for chromatographic separation. Compounds that have been separated then enter the MS through the transfer line and are ionised and fragmented by the ioniser. Finally, ions and fragments are accelerated through the mass analyser to a detector.

TD-GC-quadrupole MS is a technique that combines a gas chromatograph with a mass spectrometer that includes one quadrupole as mass analyser. A quadrupole consists of four cylindrical rods, set in parallel to each other. Ions are separated in a quadrupole according to the stability of their trajectories in the oscillating electric fields that are applied to the rods (Figure 13). More specifically, after the sample is ionised, the sample is introduced into the ion guides and the quadrupole. A fluctuating field is created by applying a mix of radio frequency (RF) voltage and direct current (DC) voltage to the quadrupole. The alternating field directs ions based on their mass-to-charge (m/z) ratio. The TD-GC-quadrupole MS with electron ionisation as ion source is the most extensively used platform for GC-MS analysis. Using full scan mode, in which the RF and DC voltages are scanning transmitting ions in sequence, the TD-GC-quadrupole MS can identify as many compounds as possible with relatively good sensitivity and specificity, thus is ideal for untargeted metabolic profiling. For targeted analysis, where fewer m/z are followed (using selected ion monitoring (SIM) mode), the sensitivity of TD-GC-quadrupole MS is much better.

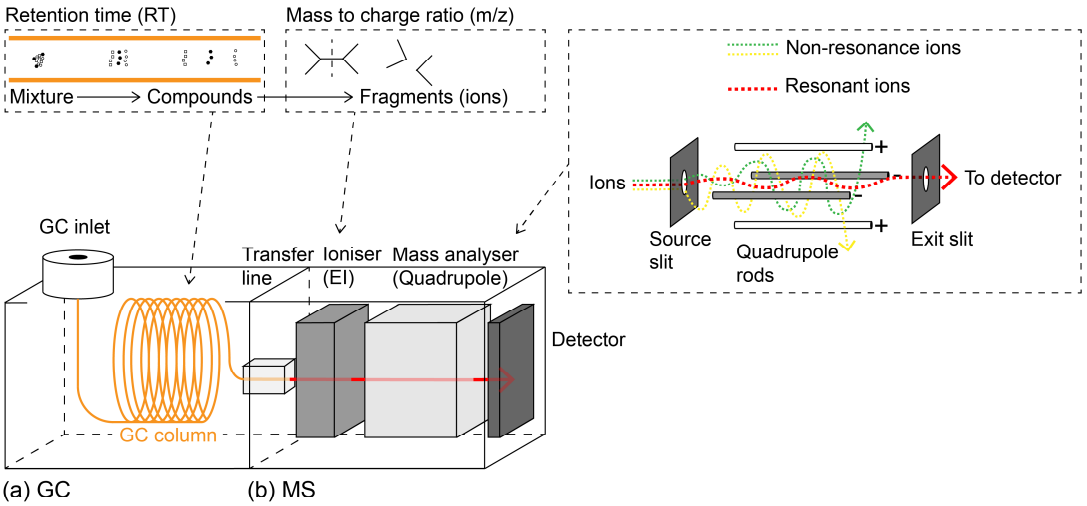


Figure 13. Schematic representation of TD-GC-quadrupole MS instrument.

The ions are separated based on their mass-to-charge ratio (m/z) by a quadrupole consisting of four cylindrical rods set parallel to each other.

3.7.1.2 GC-MS analysis workflow

The systematic review in Section 2 suggested that there is no established standardised method for urinary VOC analysis due to lack of standardised data acquisition, data pre-processing, and quality control (QC) measures. Therefore, QC procedures were examined and implemented in current subsection, including the analysis of pooled QC urine samples interspaced in the analytical sequence, the use of internal standards, and instrumental quality control. Data pre-processing was also assessed for quality control purposes.

3.7.1.2.1 Quality control (QC)

It is common that GC-MS metabolomic studies need significant sample preparation and analysis time, especially when the sample size is large (over 30 to 50). This can bring noise or variance in the data such as changes in retention time (RT), signal intensity and mass accuracy. Thus, to monitor the performance of data acquisition and to improve the reliability of downstream data analysis, randomising study samples and using QC samples are important. To avoid or correct for biases, the sample run order should ideally be orthogonal to the samples. Randomised block design is one way for randomising the samples. In such design, the samples are assigned into relatively

homogeneous subgroups and the desired experimental treatment are randomly allocated to the samples in each block.[130] Test mixes created from commercial standards (e.g., n-alkane standard mixture) can provide quick evaluation of gross performance characteristics including RT stability, peak shape, detector response, and mass accuracy. A typical QC sample, for a small sample set, would be a pooled urine sample, prepared by pooling aliquots of all the test samples. For larger, epidemiological, or large multicenter clinical studies with thousands of samples collected over many months, the approach of using a pooled sample made from all the test samples is impractical. For these larger studies, a small subset of representative samples from each group can be selected to create the QC samples, which are then sub-aliquoted and stored frozen at -80 °C until needed. It is worth noting that in studies where a large number of samples are analysed in multiple batches, a quality check of each batch requires identical QCs pooled from samples in all batches. The QCs are injected on a regular basis (i.e., every ten samples) throughout the analytical run to produce a set of data that can be used to assess repeatability.[112,114,131,132] A typical run is in the following order: Test mix (e.g., n-alkane standard mixture); five to ten conditioning QCs; QC; five to ten teat samples; QC; repeat QC, five to ten test samples, QC for necessary times; end with test mix.

3.7.1.2.2 Data pre-processing

GC-MS based metabolomics often includes several data pre-processing steps, including baseline correction, noise reduction, deconvolution, peak area calculation, and peak alignment (including RT alignment), to provide a consistent data matrix for further statistical analysis. Among these steps, alignment of peak information into a single data matrix is of foremost importance and is challenging to perform.

Various commercially available software packages have been developed for data pre-processing. Different GC-MS instrument from different companies will have different software solutions. For Agilent instruments, the idea is to generate a list of reliable compounds through untargeted analysis based on QC data, and then apply this compound list to perform semi-quantification on all the samples' data. Agilent MassHunter Unknown Analysis software is the vendor software for untargeted metabolic profiling. It performs data pre-processing steps to the raw QC data and outputs a peak list of detected compounds with information including RT, potential identities, quantifier ions, verification ions, and peak areas. Only reliable compounds

(e.g., reverse match factor (RMF) > 900) on the list will be input into Agilent MassHunter Quantitative Analysis and MATLAB package Gavin 3 for targeted analysis, followed by generating an aligned peak table. The performance of these software packages on data pre-processing were evaluated in this subsection.

3.7.1.2.3 Feature filtering during data pre-processing

It is possible that low-quality or artefact variables be introduced into the aligned peak table due to the complexity of the peak-picking process. Low-quality features are real metabolic features that cannot be correctly measured due to technical reasons including low signal-to-noise ratio, overlaid peaks, or detector saturation. Artefacts are gross peak integration errors, for example, baseline above peak threshold. These features may be large in number, making the analysis challenging, occupying enormous amounts of computer storage, requiring long processing time, and most importantly, may have negative impact during statistical analysis. Therefore, the aligned peak table should be filtered using proper feature filtering methods.

The following feature filterings are usually performed in metabolomic studies:

Blank filtering: To remove contaminants. Theoretically, urinary VOC should not be present in blanks (water).

Linearity filtering: To remove compounds respond non-linearly to the serial dilution. Serial dilution samples of pooled QC are usually run in the beginning of the experiment. Correlation coefficient tests are usually used to observe the trend of the dilution series and evaluate the model fit.

Coefficient of variation (CV) filtering: To remove compounds that show large variability, applied in the end. Theoretically, peak area of a compound should be consistent in all the QC samples.

3.7.2 Objectives

The primary objective of this subsection was to perform method development for TD-GC-quadrupole MS equipped with a mid-polar column and to establish standardised urinary VOC analysis workflow.

Secondary objectives were:

1. To apply n-alkane standard mixture for instrumental quality control;
2. To determine a TD-GC-quadrupole MS assay as benchmark method for urinary VOC analysis;
3. To apply pooled QC samples in the analytical sequence and evaluate its performance on improving data quality;
4. To evaluate the performance of internal standard normalisation;
5. To evaluate the performance of data pre-processing software packages;
6. To evaluate the effect of feature filterings on improving data quality;
7. To establish standardised workflow for GC-MS based urinary VOC analysis using HiSorb.

3.7.3 Methods

3.7.3.1 Subjects

Experiments utilised urine from nine healthy volunteers.

3.7.3.2 Sample preparation

Urine samples were removed from the -80 °C freezer and thawed in 4 °C refrigerator overnight. Pooled QC samples were generated by mixing equal amounts (0.8 mL) of each sample. These QC samples were mixtures of all urine samples, therefore were assumed to contain all the VOCs of each study sample. Deionised nanopure water was served as blank. 2 mL of urine or water was transferred into glass 20 mL headspace vials. NaCl (0.4 g) was added into each urine sample. HCl (5 M) was slowly added to urine samples at room temperature, adjusting the pH to 2.0 whilst monitoring with the pH meter. Toluene-d_8 was spiked as an internal standard to reach the concentration of 0.5 µg/mL in the 2 mL liquid. Caps with septa were placed on all the vials, and the vials were sealed using the 20 mm vial crimper. The sealed vials were left for 10-15 min to reach equilibrium.

3.7.3.3 Urinary VOC extraction

Extraction of urinary VOCs was conducted using the same HiSorb method at the optimal conditions determined in Section 3.6. TD tubes with HiSorb probes loaded were pre-conditioned at 260 °C for 60 min under 20 psi nitrogen stream. A HiSorb probe was inserted into the glass headspace vial with PDMS extracting phase exposing in the urine headspace. Once all the probes were inserted, all the vials were agitated for VOC extraction (temperature: 60 °C, speed: 300 rpm, time: 30 min). When extraction was complete, the HiSorb probe was removed from the vial, put back in the matching TD tube, and the tube was capped using the matching steel caps immediately. All loaded TD tubes were put onto the TD autosampler for TD-GC-MS analysis.

3.7.3.4 TD-GC-MS

The TD-GC-quadrupole MS instrument was quality controlled before every run by analysing TD tubes loaded with an n-alkane standard mixture (C_8 to C_{20} in Hexane, 4 µg/mL) and assessing reproducibility, mass resolution, peak area, shape and RT.

Primary desorption was performed on the Markes TD100 instrument by pre-purging the TD tubes with HiSorb probes inside for 1 minute with helium flow at 50 mL/min and heated to 250 °C for 5 min to desorb the VOCs onto a cold trap (Material emissions) in splitless mode. Secondary desorption initially cooled the cold trap to 25 °C and then heated to 250 °C for 3 min, with the flow path onto GC heated constantly at 200 °C.

The Agilent 7890B GC instrument was used. VOC separation was performed on the ZB-624UI capillary column (60 m × 0.25 mm × 1.40 µm) programmed at 1.0 mL/min constant helium carrier flow. Oven temperature was set at 40 °C initially for 4 min, ramp to 150 °C (15 °C/min without hold), and ramp to 240 °C (10 °C/min with 15 min hold). The GC cycle time of the programme was 55 min.

The MS analysis was performed on the Agilent 5977A MSD quadrupole MS. The MS transfer line was maintained at 230 °C, and 70 eV electron impact was set at 240 °C with MS quadruple held at 150 °C. MS analyser was set to acquire over the range of 35 to 250 m/z with data acquisition approximated to 0.2 scan/sec.

3.7.3.5 Sample analysis strategy

Table 11 presents the schematic of the sample analysis strategy. A total of twenty-seven samples were analysed in the following sequence: three blank samples (nanopure water instead of urine), a dilution series of the same pooled QC (0.625%, 1.25%, 2.5%, 5%, 10%, 25%, 50%, 75% of QC), seven pooled QC samples without dilution, and nine samples of healthy individuals (randomised).

Table 11. Run order for TD-GC-quadrupole MS method development.

Analytical sequence included: 3 blank, 7 QC samples, 8 serial dilution (SD) of QC samples, and 9 samples of healthy individual (Total 27 samples).

No. of Run	1-3	4	5	6	7	8	9	10	11	12-18	19-27
Sample	B1-3	SD1	SD2	SD3	SD4	SD5	SD6	SD7	SD8	QC1-7	S1-9
Dilution	0%	0.625%	1.25%	2.5%	5%	10%	25%	50%	75%	100%	-

B: blank; SD: serial dilution; QC: pooled quality control; S: study sample.

3.7.3.6 Data pre-processing

The representative QC sample data acquired from the TD-GC-quadrupole MS instrument were processed using the Agilent MassHunter Unknown Analysis software for deconvolution, which created a peak list of detected compounds with information including RT, potential identities, quantifier ions, and verification ions. The compound inclusive criterion was set as reverse match factor (RMF) with NIST > 900.

Two software packages, Agilent MassHunter Quantitative Analysis and Gavin 3, were evaluated for their data pre-processing capabilities. Compounds acquired by Unknown Analysis whose RMF > 900 along with all the information were input into Agilent MassHunter Quantitative Analysis and Gavin 3, respectively. Peak integration was performed in the two software packages in parallel. Each software package created one aligned peak table, which contained the peak area of each compound in each sample in one single excel table. The two aligned peak tables were used throughout the remaining data processing.

The performance of internal standard normalisation was evaluated by calculating the percentage of coefficient of variation (CV) of the internal standard (toluene-d_8, 0.5 µg/mL). Three feature filtering procedures were performed to the data successively to remove non-linear, contamination-related, and non-reproducible VOCs. In linearity filtering, the dilution series from 0% to 100% of QC were analysed with 1-tailed Spearman's rank correlation coefficient test to observe the trend. The Benjamini-Hochberg procedure was applied to adjust false discovery rate (FDR) to identify as many significant features as possible while incurring a relatively low proportion of false positives.[133-135] Compounds with Benjamini-Hochberg adjusted p value < 0.05 and correlation coefficient > 0.7 were considered significant. In blank filtering, the average

of the peak area of a compound in all the blanks was divided by the average of the peak area of this compound in all the QCs. This was performed to all the detected compounds. Compounds whose average peak area in the blanks was over 30% of that in the QCs were removed. CV filtering was performed to remove those compounds with a percentage of CV higher than 30%.

3.7.3.7 Statistical analysis

Principal component analysis (PCA) was performed to show the separation of the nine healthy urine samples and the pooled QCs in SIMCA 15, after the data was log transformed (y=10log(10x+1)) and Pareto scaled. The performance of data pre-processing measures in monitoring analytical variabilities and improving dataset quality was evaluated.

3.7.4 Results

3.7.4.1 Urinary VOC analysis workflow

Figure 14 demonstrates the established flow diagram of stages involved in urinary VOC analysis. Firstly, urine samples were collected according to all relevant institutional and government guidelines (Figure 14, a), and were stored in -80 °C lab freezer (Figure 14, b). To create pooled QC samples, equal volumes of urine were taken from each study sample and mixed (Figure 14, c). Urine sample preparation included adding salt, acid, and internal standards into urine in the glass headspace vials (Figure 14, d). Urinary VOC extraction was carried out using HiSorb sorptive extraction by agitating vials with HiSorb exposing to urine headspace on the agitator (Figure 14, e). When the extraction was complete, the probes were withdrawn, dried, and put into TD tubes which were placed on TD trays and loaded onto the TD autosampler for TD-GC-MS analysis (Figure 14, e, f). Samples were analysed following randomised block design with QC interspaced in the analytical run. GC instrumental QC was achieved by analysing test mix at the beginning of the analytical sequence (Figure 14, f). Data generated by the GC instrument were exported, followed by data pre-processing and generating of aligned peak tables (Figure 14, g). Feature filtering was an extra step in data pre-processing to remove low-quality features and artefacts in the aligned peak tables (Figure 14, h). Statistical analysis was performed to determine the potential urinary VOC biomarkers and to explore the separation between independent research groups (Figure 14, i). Finally, the identity of the potential biomarkers was determined, and their metabolic pathways were interpretated (Figure 14, j).

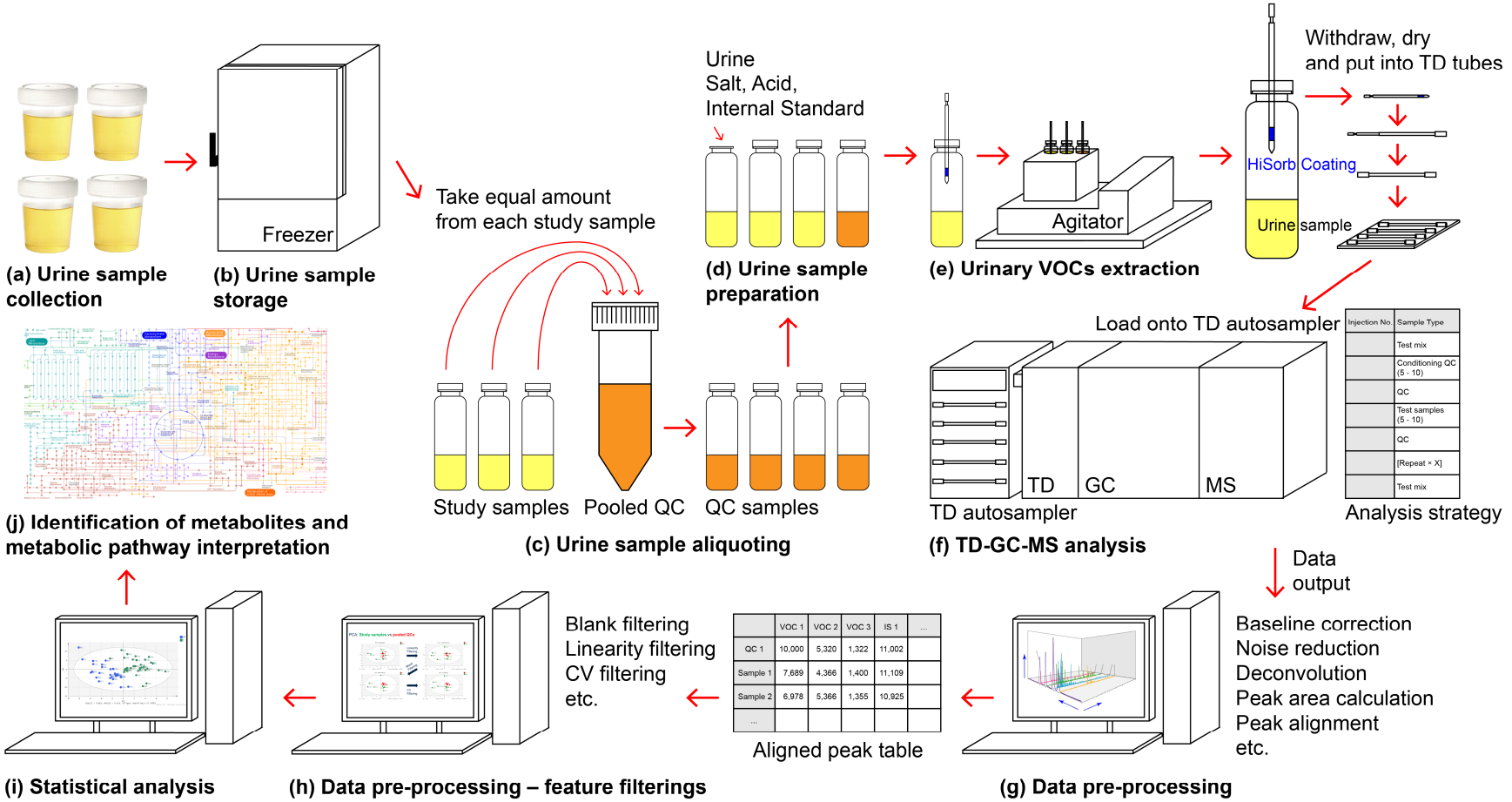


Figure 14. Flow diagram of stages involved in urinary VOC analysis workflow.

3.7.4.2 Reliable compounds and comparison of Quantitative Analysis and Gavin 3

Unknown Analysis software created a peak list consisted of 123 compounds whose RMF to NIST > 900. The list also included RT, potential compound identities, qualifier ions and verification ions of these compounds.

When processed using Quantitative Analysis, the percentage of CV for toluene-d_8 was 42.96%. This number was 42.92% when the data were processed using Gavin 3. The observed percentage of CV for toluene-d_8 were over 30%, indicating significant fluctuation in the amount of the toluene-d_8. When Quantitative Analysis was used, 64 of the 123 compounds were observed to respond monotonically to the decreasing trend of the serial dilution. When processed using Gavin 3, 67 of the 123 compounds passed the linearity filtering. 48 of the 64 compounds identified by Quantitative Analysis did not have unexpected higher concentrations in the blanks than in the QCs, while 43 of the 67 compounds identified by Gavin 3 passed the blank filtering. 41 of the 48 compounds identified by Quantitative Analysis showed promising repeatability with the percentage of CV < 30%, and 41 of the 43 compounds identified by Gavin 3 had a coefficient of variance lower than 30% in QC samples. Table 12 presents urinary VOCs identified from the data pre-processing procedures. When the final 41 compounds identified by the two software methods were compared, 35 of them overlapped (85%).

Table 12. List of urinary VOCs passing the data pre-processing procedures.

After removing non-linear, contamination-related, and non-reproducible metabolic features, 41 compounds were retained for both Quantitative Analysis and Gavin 3.

Retention Time (RT)	Compound Name	Quantitative Analysis	Gavin 3
8.86	2-Propanol, 2-methyl-	Y	N
10.02	Propane, 2-ethoxy-2-methyl-	N	Y
11.11	1-Propanol, 2-methyl-	Y	N
11.21	Amylene hydrate	Y	Y
12.78	1-Methoxy-3-methyl-2-butene	Y	Y
13.03	Methyl Isobutyl Ketone	Y	N
13.52	Furan, 2-ethyl-5-methyl-	Y	Y
13.75	3-Hexanone	Y	Y
14.02	Hexanal	Y	Y
14.40	Furan, 2-(methoxymethyl)-	Y	Y
14.57	3-Hexanone, 5-methyl-	Y	Y
14.69	3-Hexanone, 4-methyl-	Y	Y
15.18	4-Heptanone	Y	Y
15.41	3-Heptanone	Y	Y
16.68	Furan, 2-pentyl-	Y	Y
17.39	D-Limonene	N	Y
17.44	o-Cymene	Y	Y
17.67	1-Hexanol, 2-ethyl-	Y	N
17.77	.gamma.-Terpinene	Y	Y
18.24	7-Octen-2-ol, 2,6-dimethyl-	Y	Y
18.48	trans-Linalool oxide (furanoid)	Y	Y
18.55	Benzene, (2-methyl-1-propenyl)-	Y	Y
18.68	Linalool	Y	Y
18.73	Ethyl 2-(5-methyl-5-vinyltetrahydrofuran-2-yl)propan-2-yl carbonate	N	Y
18.88	1,5,7-Octatrien-3-ol, 3,7-dimethyl-	N	Y
19.06	Benzoic acid, hydrazide	N	Y
19.13	2(3H)-Furanone, 5-ethenyldihydro-5-methyl-	Y	Y
19.14	1,3,8-p-Menthatriene	Y	Y
19.22	Hexanoic acid, 2-ethyl-	Y	Y
19.27	Benzaldehyde, 4-methyl-	Y	Y
19.45	p-Cresol	N	Y
19.71	4-Acetyl-1-methylcyclohexene	Y	Y
20.10	l-Menthone	Y	Y
20.32	Cyclohexanone, 5-methyl-2-(1-methylethyl)-, trans-	Y	Y
20.50	L-.alpha.-Terpineol	Y	Y
20.73	p-Mentha-1,5-dien-8-ol	Y	Y
20.95	Benzofuran, 4,7-dimethyl-	Y	Y
21.61	Carvone	Y	Y
22.92	Ethanone, 1-(2-hydroxy-5-methylphenyl)-	Y	Y
23.04	1, 1, 5-Trimethyl-1, 2-dihydronaphthalene	Y	Y
23.62	m-Aminophenylacetylene	Y	N
23.68	Indole	Y	Y
23.75	2-Buten-1-one, 1-(2,6,6-trimethyl-1,3-cyclohexadien-1-yl)-, (E)-	Y	Y
26.51	Nerolidol	Y	Y
26.82	4-Isopropyl-6-methyl-1-methylene-1,2,3,4-tetrahydronaphthalene	Y	Y
30.68	Pentadecanal-	Y	N
31.12	Naphthalene, 1,6-dimethyl-4-(1-methylethyl)-	Y	Y

Y: yes; N: no.

3.7.4.3 Effect of feature filterings

PCA score plots for healthy urine samples and pooled QCs after each filtering are presented in Figure 15. In the PCA plot of the raw data, QCs were grouped, although the cluster was rather loose. After removing non-linear and contamination-related compounds, QCs became increasingly clustered. The elimination of non-reproducible compounds had limited effect on the cluster level.

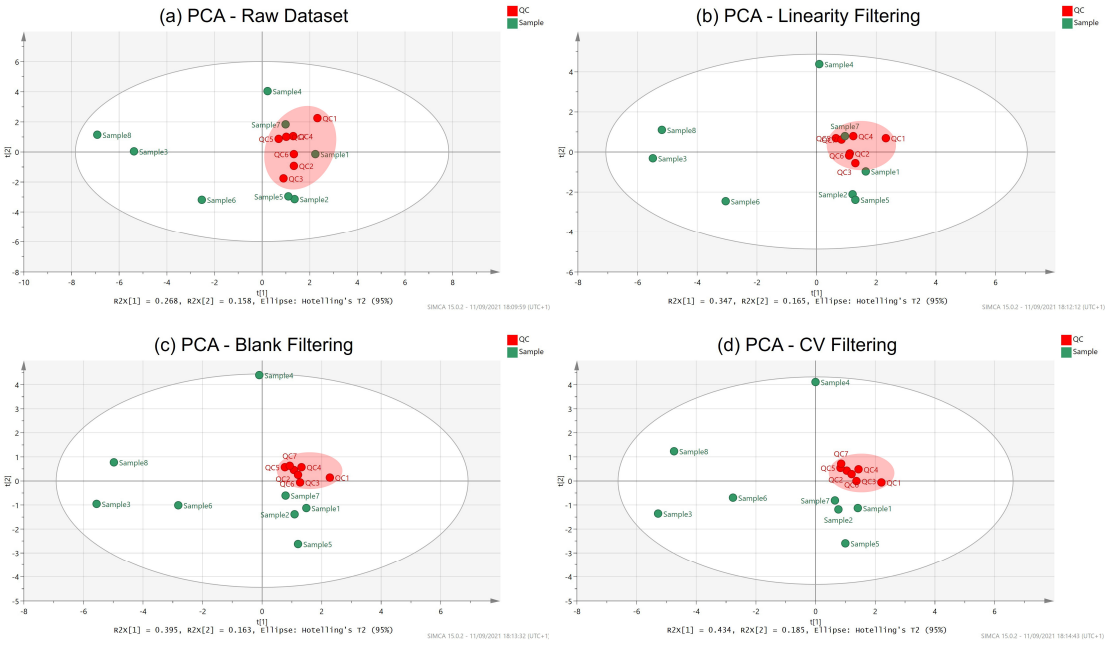


Figure 15. PCA based on urine of healthy individuals and pooled QC samples.

The PCA score plots showed that QCs were separated from the study samples, and after removing (b) non-linear, (c) contamination-related and (d) non-reproducible compounds, QCs became increasingly clustered compared to the (a) raw dataset. PCA: principal component analysis; QC: quality control.

3.7.5 Discussion

Through the data pre-processing procedure, non-linear, non-reproducible, and contamination-related metabolic features were removed. Therefore, the identification of 41 urinary VOCs, which belonged to chemical groups including ketone, aldehyde, lipids, phenol, indole, terpenes, etc., and related derivatives, could be defined as reliable identifications. These 41 compounds could be confirmed to be derived from the urine of healthy individuals. The reliable identification was also supported by the adoption of C_8 to C_{20} n-alkane standard mixture as test mix to assess the gross performance of GC-MS instrument, ensuring the stable RT, peak shape, detector response, and mass accuracy. In addition, adoption of QC measures improved the reliability of results by enabling the monitoring of data acquisition performance and the creation of the peak list which was used to perform targeted semi-quantification. When pooled QC samples were used in untargeted metabolic analysis, it was also an indirect way to equilibrate the analytical platform prior to running analytical batches by matrix coating of GC and MS instrument which can absorb metabolites, allowing higher data reproducibility.[136] Moreover, sample analysis followed randomised block design, eliminating the bias brought by run order.

The pre-processed dataset showed excellent analytical characteristics. PCA is an unsupervised statistical procedure for reducing the dimension of data by projecting each data point onto only the first few principal components to get lower-dimensional data while retaining as much variance as possible. It allowed the visualisation and summarisation of the difference between QCs and healthy urine samples. On the PCA score plots, areas occupied by QCs indicated analytical variabilities potentially caused by instrument, environment, operator or reagents, etc. Biological variations were represented by the areas occupied by study samples minus areas occupied by QCs. In an idealised scenario, where there are no analytical variabilities, all the QCs would cluster in the centre of the PCA plot with study samples distributed in surrounding area. In the raw dataset of current experiment, QCs were loosely clustered and were not in the centre, suggesting the existence of analytical variabilities (Figure 15, a). After data pre-processing, QCs became increasingly clustered compared to the raw dataset (Figure 15, b, c, d). This indicated a significant improvement in the quality of the GC dataset. Even after the pre-processing steps QCs were still not in the centre. This could be explained by the small sample size and small number of identified compounds. The analytical variabilities could potentially be adjusted by using internal standards.

A primary requirement for the use of an internal standard is that the variability in internal standard peak measurement is small. In current experiment, the obtained percentage of CV for toluene-d_8 by Quantitative Analysis and Gavin 3 were both over 30%, while the amount of toluene-d_8 added to each sample was the same. This indicated that there was significant fluctuation in the amount of the toluene-d_8, mainly due to the low number of samples. Therefore, toluene-d_8 was deemed unsuitable for use in the data pre-processing and analysis of current experiment. Suitable compounds needed to be chosen as internal standards, with the following characteristics being considered: An internal standard compound should be stable; It should never be found in the untreated urine samples; It should be structurally similar to important urinary VOCs; It should be extracted by HiSorb; It should have adequate detector response with excellent annotations; It should be available in pure form, and ideally should be inexpensive; The size of the analyte and internal standard peaks can be measured by GC-MS independently (native, or isotopically labelled, versions of the analyte); Ideally the internal standard is eluted after the analyte (can provide additional information on the quality of the separation). Based on these criteria, a series of isotopically labelled internal standards whose retention time (RT) were in the beginning, middle, and the end of the QC chromatogram and represented urinary VOCs in different chemical groups were determined as internal standards: acetone-d_6, butyraldehyde-d_2, phenol-d_6, benzene-d_6 and acetophenone-d_8.

Quantitative Analysis and Gavin 3 both showed satisfactory performance in data pre-processing. They both delivered the same amount of reliable urinary VOCs, with 86% of them being identical. This provided two approaches for the data pre-processing of GC-MS data. Importantly, Quantitative Analysis was developed by Agilent company and principally can only be used to process data generated by Agilent instruments. While Gavin 3 supports universal open-source data formats, such as .CDF.

The successful and reliable identification of urinary VOCs in current subsection also established TD-GC-quadrupole MS as benchmark against which to compare other methods.

A standardised urinary VOC analysis workflow was established (Figure 14). This workflow consisted of guided urine collection and storage, optimised sample preparation with optimal extraction conditions, adoption of internal standard and QC samples, optimised analytical platform, use of randomised block design for sample analysis with QC interspaced in the analytical sequence, and adoption of data pre-processing including feature filterings. Urinary VOCs could be profiled using this workflow, which complied with the important considerations outlined in Table 8 in Section 2.5, in a high throughput and standardised manner.

Main limitation for this subsection is the small sample size and small number of identified compounds.

3.7.6 Conclusion

A TD-GC-quadrupole MS assay was developed as benchmark method for urinary VOC analysis. A high throughput and standardised workflow was established for GC-MS based urinary VOC analysis using HiSorb, with the adoption of robust QC measures. In the next two subsections, TD-GC-time of flight (TOF)-MS was involved in the method development in order to improve the results even further.

3.8 TD-GC-TOF-MS (polar column) method development

3.8.1 Introduction

TD-GC-quadrupole MS is a relatively low-cost technique which can be used for qualitative and quantitative analyses in metabolomic studies, with its sensitivity increases when using selected ion monitoring (SIM) mode. However, the scanning nature of quadrupole limits the data acquisition rate and reduces sensitivity and chromatographic resolution due to slower collection of data points. Therefore, TD-GC-time of flight (TOF)-MS may be used in an attempt to overcome these limitations for improved results.

3.8.1.1 TD-GC-time of flight (TOF)-MS

TD-GC-time of flight (TOF)-MS is a technique that involves first separating a sample mixture using GC, then accelerating sample ions of various masses to the same (known) kinetic energy and measuring the time it takes for each ion to reach a detector at a known distance.

The TOF-MS uses time of flight analyser instead of quadrupole in the TD-GC-quadrupole MS (Figure 16). In a time of flight analyser, all ions enter the evacuated time of flight tube at the same time and are separated as they travel along the tube. Ions with the smallest mass-to-charge ratio (m/z) ions arrive first at the detector. The ion source must be pulsed to prevent the simultaneous arrival of ions with different m/z at the detector. In addition, a reflectron consisting of a series of high-voltage ring electrodes is frequently attached to the flight tube's end, in order to solve the problem that not all the ions with the same m/z at high masses reach their ideal velocities. When an ion enters the reflectron, it is reflected in the opposite direction due to high voltage. For ions with identical m/z, faster ions travel further into the reflectron than the slower ones. Thus, both fast and slow ions with the same m/z arrive at the detector simultaneously. By limiting the broadband range of flight time for a single m/z, the reflectron significantly increases the mass resolution of TOF-MS.

TOF-MS from Markes has a short flight tube which minimises the need for reflectrons. It generates spectra more similar to quadrupole ones with less mass discrimination effect. However, it has lower mass resolution than instruments with longer flight tubes.

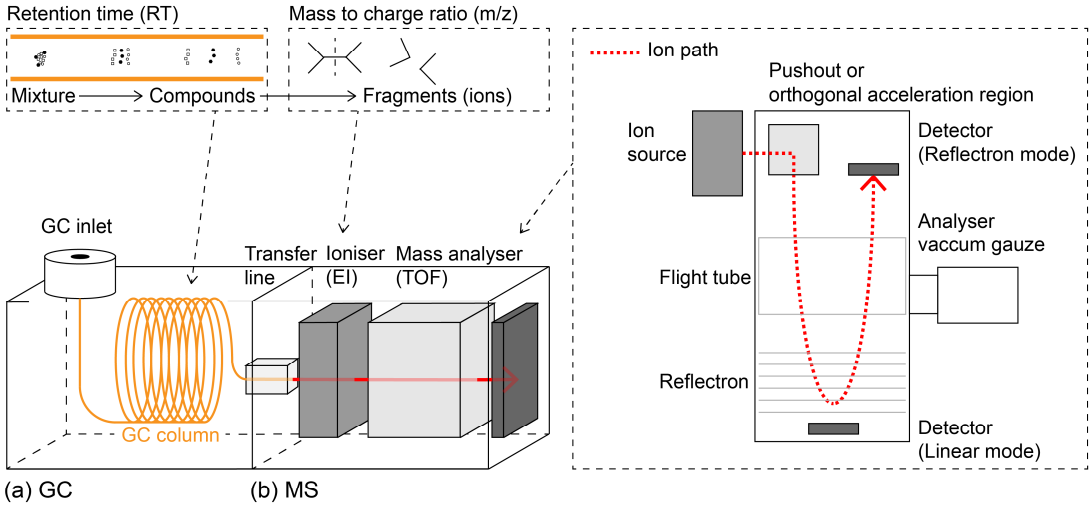


Figure 16. Schematic representation of TD-GC-TOF-MS instrument.

The ions are separated based on their mass-to-charge ratio by a time of flight analyser where sample ions are accelerated by an electric field of known strength.

3.8.1.2 Advantages of TOF-MS over quadrupole-MS

There are multiple advantages of TOF-MS compared to the conventional quadrupole MS. It is possible to accumulate several thousand independent mass spectra in one second due to the extremely short flight times for all the ions, leading to excellent reproducibility and better signal-to-noise characteristics, and maximal use of a small amount of sample. The continuous spectra acquisition provides increased selectivity of correct masses and no loss during ion separation in the flight tube. As a comparison, the maximum scanning rate for a quadrupole MS system is approximately 10,000 u/s. This means that the acquisition rate will be slower when the scan range is wider, and vice versa. Theoretically, the m/z range is unlimited for TOF-MS, while single quadrupole typically has a maximum range of up to 2,000-3,000 m/z. The whole mass range can be analysed in one scan with exceptionally high resolution and sensitivity. Constant ion abundance ratios across a chromatographic peak can be provided, with overlapping peaks being detected with trace level detection.

In summary, GC-TOF-MS is ideal for fast GC or GC×GC applications and targeted or untargeted metabolomic studies due to its fast acquisition rate, spectral continuity, high

resolution, exceptional dynamic range, high sensitivity, high selectivity, and less amount of sample required.

3.8.1.3 GC column phase

In GC-MS method development, there is no existing reference to provide guidance on phase selection. It is usually based on the characteristics of the compounds to be analysed.

Polar columns are usually recommended for the analysis of polar compounds mainly, including but not limited to alcohols, amines, carboxylic acids, diols, esters, ethers, ketones, and thiols. The separation of polar compounds is determined by the differences in the overall effects of the interactions including dispersive interactions governed by Van der Waals forces as well as interactions between polar compounds and the phase including dipole, π-π, and/or acid-base interactions.

Non-polar columns are frequently used to separate non-polar compounds such as alkanes, which generally contain only carbon and hydrogen atoms and are composed of carbon-carbon single bonds. These compounds are separated in the GC column based on the differences in the effects of dispersive interactions, which are intermolecular attractions that increase with the size of the compound.

This subsection established a panel of assays using TD-GC-TOF-MS equipped with a polar column. The next subsection (Section 3.9) created a panel of assays using a non-polar column. When combined, they were expected to be able to cover the analysis of polar, mid-polar, and non-polar compounds.

3.8.2 Objectives

The primary objective of this subsection was to perform method development for TD-GC-TOF-MS equipped with a polar column.

Secondary objectives were:

1. To determine the optimal urine sample volume for urinary VOC analysis;
2. To determine the optimal ramp gradients of TD-GC-TOF-MS;
3. To determine the optimal concentration of internal standards;
4. To determine a penal of TD-GC-TOF-MS (polar column) assays for urinary VOC analysis.

3.8.3 Methods

3.8.3.1 Subjects

Experiments utilised urine from fifteen healthy volunteers.

3.8.3.2 Sample preparation

Sample preparation followed the same principles in Section 3.7.3.2. Urine samples were thawed in 4 °C refrigerator overnight. Equal amounts (0.8 mL) of each sample were pooled to create QC samples. Blanks were deionised nanopure water.

To determine the optimal urine sample volume, different volume of urine (0.1 mL, 0.25 mL, 0.5 mL, 1 mL, and 2 mL) or water were transferred into glass 20 mL headspace vials. Deionised nanopure water was added to top up liquid in all the vials to 2 mL (e.g., adding 1.9 mL water to 0.1 mL urine). NaCl (0.4 g) and 5 M HCl (adjusting pH to 2.0) were added into each sample for increased VOC releasing.

As discussed in Section 3.7.5, candidate internal standards, namely acetone-d_6, butyraldehyde-d_2, phenol-d_6, benzene-d_6 and acetophenone-d_8, were further evaluated in current subsection. Samples were spiked with a mixture of these isotopically labelled internal standards to reach different concentrations (0.2 μg/mL, 0.5 μg/mL, 1 μg/mL, and 2 μg/mL) in the 2 mL liquid. All the vials were sealed and left for 10-15 min to reach equilibrium.

3.8.3.3 Urinary VOC extraction

Extraction of urinary VOCs was conducted using HiSorb at the same optimal procedures and conditions determined in Section 3.6. Pre-conditioned HiSorb probes were inserted into the headspace vials with PDMS extracting phase exposing in the urine headspace. All the vials were agitated for VOC extraction (temperature: 60 °C, speed: 300 rpm, time: 30 min). All the probes were removed and put back in the matching TD tube when extraction was complete. The capped TD tubes were placed onto the TD autosampler for TD-GC-MS analysis.

3.8.3.4 TD-GC-MS

The same as Section 3.7.3.4, the TD-GC-TOF-MS instrument was quality controlled by assessing peak shapes and retention times before every run by analysing TD tubes loaded with an n-alkane standard mixture (C_8 to C_{20} in Hexane, 4 µg/mL). Furthermore, the alkane retention times were used to generate retention indices (RI) to facilitate VOC annotation.

The TD method was common for all the developed GC-MS assays. The Markes TD100-XR was used for primary desorption. The TD tubes with HiSorb probes inside were pre-purged for 1 min at 50 mL/min helium flow and heated to 250 °C for 5 min to desorb the VOCs onto a cold trap (Material emissions) in split mode (1:10). Following this, the cold trap was cooled to 25 °C and then heated to 250 °C for 3 min, with the flow path onto GC heated constantly at 200 °C.

The Agilent 7890B GC instrument was involved. VOC separation was performed on the polar Mega WAX HT capillary column (20 m × 0.18 mm × 0.18 µm). Different initial oven temperature (35 °C, 55 °C, 60 °C) and different oven ramp rates (4 °C/min, 6 °C/min, 15 °C/min, 20 °C/min, 25 °C/min, and 30 °C/min) were tested to determine the best settings for a long and a short GC programme. The highest oven temperature was 240 °C.

The SepSolve BenchTOF Select TOF-MS was used for MS analysis. The MS transfer line was maintained at 260 °C, and 70 eV electron impact was set at 260 °C. MS analyser was set to acquire over the range of 30 to 597 m/z with data rate at 10.

3.8.3.5 Sample analysis strategy

Sample analysis followed the same principles in Section 3.7.3.5. A total of thirty-three samples were analysed in the following sequence: three blank samples (nanopure water), a dilution series of the same pooled QC (0.625%, 1.25%, 2.5%, 5%, 10%, 25%, 50%, 75% of QC), seven pooled QC samples, and fifteen samples of the healthy individuals (randomised) (Table 13).

Table 13. Run order for TD-GC-TOF-MS (polar column) method development.

Analytical sequence included: 3 blank, 7 QC samples, 8 serial dilution (SD) of QC samples, and 15 samples of healthy individual (Total 33 samples).

No. of Run	1-3	4	5	6	7	8	9	10	11	12-18	19-33
Sample	B1-3	SD1	SD2	SD3	SD4	SD5	SD6	SD7	SD8	QC1-7	S1-15
Dilution	0%	0.625%	1.25%	2.5%	5%	10%	25%	50%	75%	100%	-

B: blank; SD: serial dilution; QC: pooled quality control; S: study sample.

3.8.3.6 Data pre-processing

The representative QC sample data acquired from the TD-GC-TOF-MS instrument were processed in ChromSpace. Data were initially dynamically baseline corrected (DBC), then deconvolution to the peaks was performed, and a peak list of detected compounds with RT, potential identities, quantifier ions, and verification ions was created. Potential identities were obtained by comparing the NIST library with RI penalty based on the n-alkane pattern (C_8 to C_{20}). Features with RMF < 800, siloxanes, gases and with S/N < 3 were removed. In urine sample volume testing, internal standard testing, and ramp gradient testing within current subsection, the performance was evaluated by comparing the number of compounds in the peak list after non-valid features were removed.

Data processed by ChromSpace were input into Gavin 3 for peak integration. After integration, an aligned peak table which contained the peak area of each compound in each sample was created in one single excel table, which also included the peak areas for all the spiked internal standards. The data were filtered three times to remove non-linear, contamination-related, and non-reproducible VOCs. The 1-tailed Spearman's rank correlation coefficient test was used to observe the trend of the dilution series. The Benjamini-Hochberg procedure was used to reduce false positives.[133-135] Compounds having an Benjamini-Hochberg adjusted p value < 0.05 and correlation coefficient > 0.7 were considered significant. In blank filtering, compounds whose average peak area in the blanks was over 30% of that in the QCs were removed. Finally, CV filtering was applied to remove compounds with a CV greater than 30%.

3.8.4 Results

3.8.4.1 Urine sample volume

Urine sample volume of 0.1 mL, 0.25 mL, 0.5 mL, 1 mL, and 2 mL were compared to evaluate the effect of different sample volumes on the number of detected peaks (Figure 17, a). Three replicates were performed for each sample volume. A short GC programme was used: Initial temperature was at 60 °C, held for 0.2 min, ramped to 240 °C at 30 °C/min, held for 0.2 min. When urine sample volume was 2 mL, an average of 224 compounds were reliably detected. With 1 mL urine, an average of 217 compounds were reliably detected. In 0.5 mL urine sample, 194 compounds were detected. When the sample volume was reduced to 0.25 mL and 0.1 mL, the average numbers stabilised (182, 181, respectively) at around 80% of the detected compound number when urine volume was 2 mL. There was a higher variation within the results of 0.1 mL samples, suggesting a lower level of stability when using 0.1 mL urine.

3.8.4.2 Ramp gradients

Different GC oven ramp rates (15 °C/min, 20 °C/min, 25 °C/min, and 30 °C/min) were evaluated by comparing the number of detected peaks (Figure 17, b). Two replicates were performed for each ramp rate. 0.25 mL urine was used in the analysis. The initial temperature of the GC oven was set to 35 °C. When the ramp rate was set to 15 °C/min, an average of 173 compounds were identified, which was the highest among the tested conditions. When the ramp rate was 20 °C/min, 159 compounds were detected. When the ramp rate was increased to 25 °C/min and 30 °C/min, an average of 146 and 148 compounds were detected, respectively, which was around 82% of the compound number detected at the 15 °C/min ramp rate.

3.8.4.3 Comparison of long and short assay

Two long polar assays and two short polar assays were compared by investigating the number of detected compounds (Figure 17, c). Experiments were conducted using 0.25 mL urine for each sample. Key details of the four assays were:

Long assay 1: Initial oven temperature 55 °C, ramp rate 4 °C/min;
Long assay 2: Initial oven temperature 35 °C, ramp rate 6 °C/min;
Short assay 1: Initial oven temperature 35 °C, ramp rate 20 °C/min;
Short assay 2: Initial oven temperature 60 °C, ramp rate 30 °C/min.

In general, the two short assays produced better results by detecting more compounds. 157 compounds were reliably detected when the short assay 1 was used for analysis. When the short assay 2 was used, the result was 182, but peak over saturation was recorded for a large proportion of the detected compounds. When the long assay 1 was used, 158 compounds were reliably detected. In the long assay 2, 132 compounds were identified when the initial oven temperature was reduced to 35 °C and the ramp rate was increased to 6 °C/min. In summary, the long assay 1 (Initial oven temperature 55 °C, ramp rate 4 °C/min) and the short assay 1 (Initial oven temperature 35 °C, ramp rate 20 °C/min) were recommended as two complementary methods for obtaining the greatest number of compounds in a long and a short length of time, depending on demands. The short assay 2 was not selected due to peak over saturation.

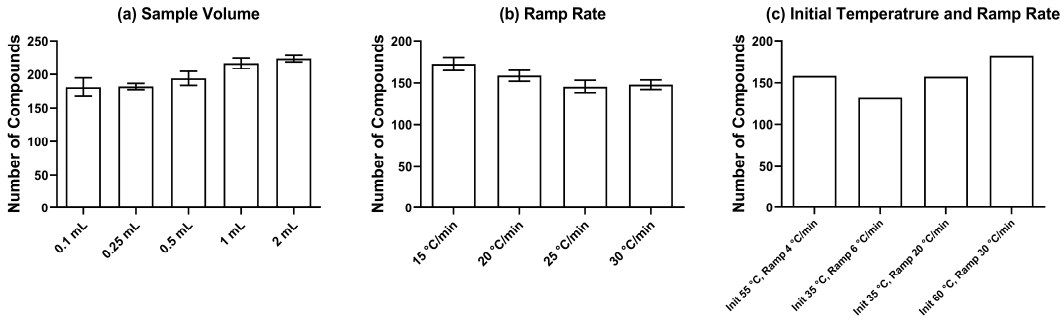


Figure 17. Optimisation of sample volume, oven ramp rate, and initial temperature.

The effect of (a) urine sample volume, (b) GC oven ramp rate, and (c) initial GC oven temperature and ramp rate on the number of detected urinary VOCs. The number of compounds decreased as the urine sample volume decreased; The number of compounds found with 0.25 mL urine was 80% of the number detected with 2 mL urine, with good stability. The number of compounds decreased as the oven ramp rate increased; The number of compounds found with 30 °C/min was 82% of the number detected with 15 °C/min. Two complementary assays, a long (initial temperature 55 °C, ramp rate 4 °C/min) and a short (initial temperature 35 °C, ramp rate 20 °C/min) were recommended. The short assay 2 (initial temperature 60 °C, ramp rate 30 °C/min) was not selected due to peak over saturation.

3.8.4.4 Internal standards

Four concentrations were tested to explore the optimal concentration of the five internal standards: 0.2 µg/mL, 0.5 µg/mL, 1 µg/mL, and 2 µg/mL. Peak areas of acetone-d_6, butyraldehyde-d_2, phenol-d_6, benzene-d_6 and acetophenone-d_8 obtained using the short assay 1 (initial temperature 35 °C, ramp rate 20 °C/min) are presented in Figure 18, a. The analysis was conducted using 2 mL urine. The peak areas of all internal standards increased significantly in proportion to the amount of internal standards spiked. However, when benzene-d_6 was spiked at the concentration of 2 µg/mL, the peak acquired exceeded the detector's saturation limit. The peaks for Acetophenone-d_8 were over saturated at the concentrations of 1 µg/mL and 2 µg/mL.

The difference between spiking internal standards in urine and spiking in water was compared. Figure 18, b demonstrates the detected peak areas of the internal standards in urine and deionised nanopure water when the spiked concentration was 0.2 µg/mL. Three replicates were performed for each internal standard. The average peak areas of acetophenone-d_8 and benzene-d_6 were the highest. The peak area of butyraldehyde-d_2 was the lowest. The average peak areas of acetophenone-d_8 and phenol-d_6 in urine were higher than in water. The average peak areas of butyraldehyde-d_2 in urine was lower than in water. In urine and water, the average peak areas of acetone-d_6 and benzene-d_6 were similar. At the spiking concentration of 0.2 µg/mL, there was no peak over saturation. Overall, spiking urine or water had no significant effect on the peak areas of the internal standards.

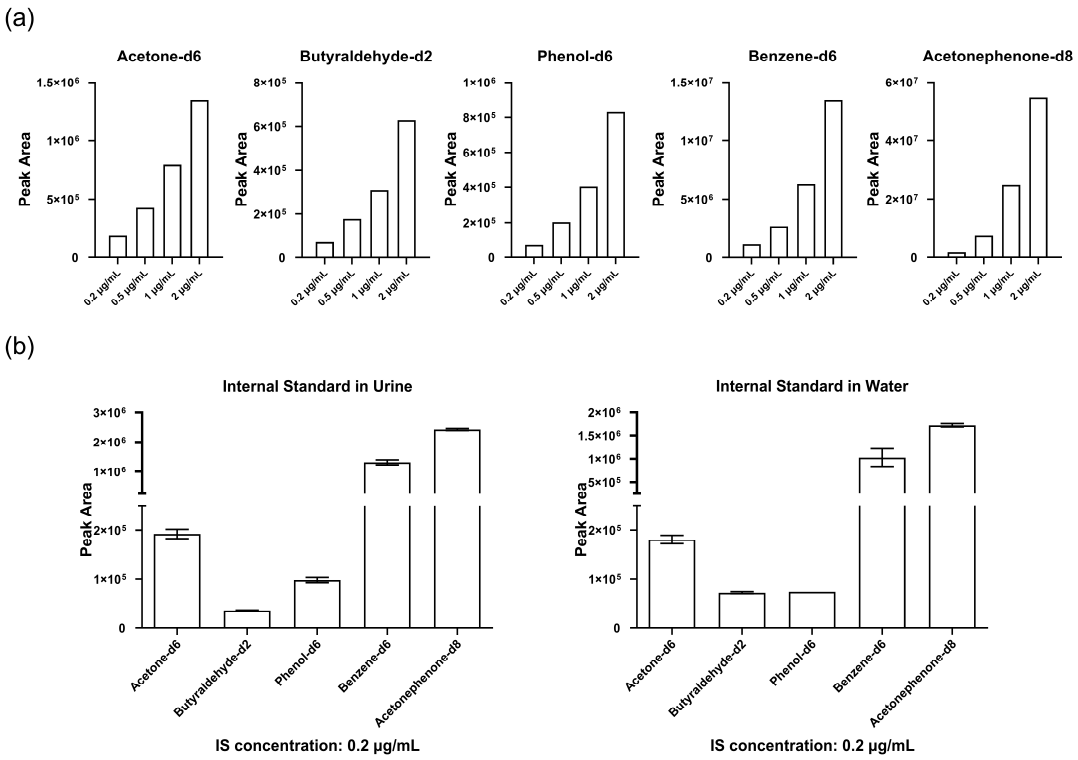


Figure 18. Evaluation of internal standards.

(a) The effect of spiking concentration on the peak areas of the IS. The peak areas of all IS increased significantly in proportion to the amount of IS spiked, with Benzene-d_6 being over saturated at 2 μg/mL and Acetophenone-d_8 being over saturated at 1 μg/mL and 2 μg/mL, respectively. (b) The difference of peak areas between spiking IS in urine and spiking in water. In general, spiking in urine or in water had no significant effect on the peak areas of IS. IS: internal standard.

3.8.4.5 Optimal long and short assay (Polar column)

Four different assays using the polar GC column were compared based on the number of identified, reproducible, linear and non-contamination-related VOCs to determine the optimal long and short assay based on polar column (Table 14). The following were the key details of the four assays:

2 mL urine long assay: Urine volume 2 mL, initial oven temperature 35 °C hold for 2 min, ramp to 240 °C at 4 °C/min hold for 2 min;

0.25 mL urine long assay: Urine volume 0.25 mL, initial oven temperature 35 °C hold for 2 min, ramp to 240 °C at 4 °C/min hold for 2 min;

2 mL urine short assay: Urine volume 2 mL, initial oven temperature 35 °C hold for 1.9 min, ramp to 240 °C at 20 °C/min hold for 0.2 min;

0.25 mL urine short assay: Urine volume 0.25 mL, initial oven temperature 35 °C hold for 1.9 min, ramp to 240 °C at 20 °C/min hold for 0.2 min.

The 2 mL urine long assay produced the highest number of reliable compounds (121) after removing invalid features, while the 0.25 mL urine long assay output the lowest number of compounds (33). In the 2 mL urine short assay, 96 compounds passed all the three filterings as reliable compounds. In the 0.25 mL urine short assay, 85 reliable compounds were identified.

Table 14. Four-assay panel of GC methods (polar column).

Instrument	TD-GC-TOF-MS (Polar Column)			
Urine volume	2 mL	0.25 mL	2 mL	0.25 mL
GC column	Mega WAX HT (20 m × 0.18 mm × 0.18 µm)			
Split	1:10	1:10	1:10	1:10
GC column flow	0.7 mL/min	0.7 mL/min	1.2 mL/min	1.2 mL/min
GC oven gradients	Initial temperature at 35 °C hold for 2 min, ramp to 240 °C at 4 °C/min hold for 2 min		Initial temperature at 35 °C hold for 1.9 min, ramp to 240 °C at 20 °C/min hold for 0.2 min	
GC cycle time	57 min	57 min	17 min	17 min
MS	MS range: 30 to 597 Data rate: 10 TF line: 260 Ion source: 260 -70eV		MS range: 30 to 597 Data rate: 10 TF line: 260 Ion source: 260 -70eV	
Deconvolved NIST matched peaks (S/N > 3), siloxanes and gases removed	220	103	188	209
VOCs passing blank filtering	166	48	114	124
VOCs passing linearity filtering	160	59	111	140
VOCs passing CV filtering	147	55	168	132
VOCs passing all filterings	121	33	96	85

3.8.5 Discussion

When the urine volume was 2 mL, the highest number of reliable compounds were detected with a polar column. Decreased urine volume resulted in a lower number of compounds. The number stabilised when the urine volume reduced to 0.25 mL and 0.1 mL, with the number detected being 80% of the number detected with 2 ml urine and a higher variation within the results of 0.1 mL samples. These results supported the use of 2 mL urine to achieve maximum VOC acquisition in urinary VOC analysis when the sample availability is relatively unrestricted, and the use of 0.25 mL as the least possible urine volume (higher stability than 0.1 mL) in studies where urine samples are limited to identify as many VOCs as possible without making excessive sacrifices. Both 2 mL and 0.25 mL are small volumes, which is crucial, as the available sample volume is commonly limited in clinical studies.

The peak areas of internal standards increased significantly in proportion to the amount of internal standards spiked, with two of them exceeded the detector's saturation limit (benzene-d_6 at 2 µg/mL and acetophenone-d_8 at 1 µg/mL and 2 µg/mL). To avoid over saturation, the concentration of 0.5 µg/mL was considered the optimal concentration for the internal standard spiking mixture.

Two complementary methods were developed for identifying a considerable number of compounds in a long and a short length of time, with the optimal long method obtaining around 30% more VOCs than the optimal short one. There is a trade-off between the number of identified VOCs and the overall throughput. Although long methods might offer better results, they are also more time consuming, making them less attractive for clinical adoption, particularly in large clinical studies requiring high throughput.

Three of the four assays using the TD-GC-TOF-MS with polar column identified over 80 urinary VOCs, more than double the number identified using quadrupole instrument in Section 3.7. This demonstrated the superiority of TOF for identifying urinary VOCs, owing to its rapid acquisition rate, spectral continuity, high resolution, outstanding dynamic range, high sensitivity and selectivity.

The polar assays developed in this subsection were suitable for the analysis of mainly polar compounds as well as a small proportion of mid-polar or non-polar compounds. In order to cover non-polar compounds, method development was required and was performed in the next subsection (Section 3.9).

This subsection has one major limitation, which is the selection of the tested conditions for the evaluation of urine sample volume, GC oven parameters, and internal standard concentrations. Employing a multivariate optimisation strategy (e.g., central composite design (CCD)) in future studies may allow the evaluation of the interactions among these tested conditions with fewer experiments.[129]

3.8.6 Conclusion

A panel of assays using TD-GC-TOF-MS equipped with a polar column were developed for urinary VOC analysis, with an optimal long assay and an optimal short assay determined. The optimal urine sample volume and the optimal concentration of internal standards were also determined.

3.9 TD-GC-TOF-MS (non-polar column) method development

3.9.1 Introduction

For the purpose of providing methods covering the analysis of non-polar compounds, a TD-GC-TOF-MS equipped with a non-polar column were tested with a comprehensive method development being carried out.

For the analysis of non-polar compounds, a non-polar GC column is the preferred starting point. Non-polar columns are made with the least selective of the GC stationary phases. They are frequently used to separate non-polar compounds such as alkanes, which generally contain only carbon and hydrogen atoms and are composed of carbon-carbon single bonds. These compounds are separated in the GC column based on the differences in the effects of dispersive interactions, which are intermolecular attractions that increase with the size of the compound. Thus, larger compounds with higher boiling points have longer retention. Elution order is usually determined by the boiling points of the compounds.

3.9.2 Objectives

The primary objective of this subsection was to perform method development for TD-GC-TOF-MS equipped with a non-polar column.

Secondary objectives were:

1. To determine the optimal ramp gradients for TD-GC-TOF-MS (non-polar column);
2. To determine a penal of TD-GC-TOF-MS (non-polar column) assays for urinary VOC analysis.

3.9.3 Methods

3.9.3.1 Subjects

Experiments utilised the same urine from fifteen healthy volunteers as the urine used Section 3.8.

3.9.3.2 Sample preparation

Sample preparation followed the same principles in Section 3.7.3.2 and 3.8.3.2. Urine samples were thawed in 4 °C refrigerator overnight. Equal amounts (0.8 mL) of each sample were pooled to create QC samples.

2 mL of urine or water was transferred into glass 20 mL headspace vials. NaCl (0.4 g) and 5 M HCl (adjusting pH to 2.0) were added into each sample for increased VOC releasing.

A mixture of internal standards, namely acetone-d_6, butyraldehyde-d_2, phenol-d_6, benzene-d_6, acetophenone-d_8 and toluene-d_8, were spiked into samples to reach 0.2 µg/mL in the 2 mL liquid. The vials were sealed and left for 10-15 min to equilibrate.

3.9.3.3 Urinary VOC extraction

Extraction of urinary VOCs was conducted using the same HiSorb extraction method optimised in Section 3.6. Pre-conditioned HiSorb probes were inserted into the headspace vials with PDMS extracting phase exposing in the urine headspace. All the vials were agitated for VOC extraction (temperature: 60 °C, speed: 300 rpm, time: 30 min). All the probes were removed, capped and put in the matching TD tube when extraction was complete. The TD tubes were placed onto the TD autosampler for TD-GC-MS analysis.

3.9.3.4 TD-GC-MS

The same as Section 3.7.3.4 and 3.8.3.4, the TD-GC-TOF-MS instrument was quality controlled before every run by analysing TD tubes loaded with an n-alkane standard

mixture (C_8 to C_{20} in Hexane, 4 µg/mL), with the alkane retention times being used to generate RI to facilitate VOC annotation.

The common TD method was used. The TD tubes were pre-purged for 1 minute at 50 mL/min helium flow and heated to 250 °C for 5 min to desorb the VOCs onto a cold trap in split mode (1:10). The cold trap was cooled to 25 °C and then heated to 250 °C for 3 min, with the flow path onto GC heated constantly at 200 °C.

The Agilent 7890B GC instrument was involved. VOC separation was performed on the non-polar DB5-MS UI capillary column (30 m × 0.25 mm × 1.00 µm). The initial oven temperature was 35 °C. Different GC column flow rates (1.4 mL/min, 1.6 mL/min, 1.8 mL/min, and 2 mL/min) and different GC oven ramp rates (6 °C/min, 8 °C/min, and 10 °C/min) were tested to determine the best settings for a long and a short GC programme. The end oven temperature was 300 °C.

The SepSolve BenchTOF Select TOF-MS was used for MS analysis. The MS transfer line was maintained at 250 °C, and 70 eV electron impact was set at 250 °C. MS analyser was set to acquire over the range of 30 to 350 m/z with data rate at 4.

3.9.3.5 Sample analysis strategy

Sample analysis followed the same principle in previous Section 3.7.3.5 and 3.8.3.5, which is presented in Table 15. A total of thirty-three samples were analysed in a sequence consisting of: three blank samples (nanopure water), a dilution series of the same pooled QC (0.625%, 1.25%, 2.5%, 5%, 10%, 25%, 50%, 75% of QC), seven pooled QC samples, and fifteen samples of the healthy individuals (randomised).

Table 15. Run order for TD-GC-TOF-MS (non-polar column) method development.

Analytical sequence included: 3 blank, 7 QC samples, 8 serial dilution (SD) of QC samples, and 15 samples of healthy individual (Total 33 samples).

No. of Run	1-3	4	5	6	7	8	9	10	11	12-18	19-33
Sample	B1-3	SD1	SD2	SD3	SD4	SD5	SD6	SD7	SD8	QC1-7	S1-15
Dilution	0%	0.625%	1.25%	2.5%	5%	10%	25%	50%	75%	100%	-

B: blank; SD: serial dilution; QC: pooled quality control; S: study sample.

3.9.3.6 Data pre-processing

Data pre-processing also followed the same principle as earlier polar column method development Section 3.8.3.6. The representative QC sample data acquired from the TD-GC-TOF-MS instrument were processed in ChromSpace. After DBC and deconvolution, a peak list of detected compounds with RT, potential identities, quantifier ions, and verification ions was created. Potential identities were obtained by comparing the NIST library with RI penalty based on the n-alkane pattern (C_8 to C_{20}). Features with RMF < 800, siloxanes, gases and with S/N < 3 were removed. To evaluate column flow rate and oven ramp rate the number of compounds in the peak list after removing non-valid features was compared.

Data processed by ChromSpace were input into Gavin 3 for peak integration. After integration, an aligned peak table which contained the peak area of each compound in each sample was created in one single excel table, with the peak areas for all the spiked internal standards included. The data were filtered three times to remove non-linear, contamination-related, and non-reproducible VOCs. The 1-tailed Spearman's rank correlation coefficient test was used to observe the trend of the dilution series. The Benjamini-Hochberg procedure was used to reduce false positives.[133-135] Compounds having an adjusted p value < 0.05 and correlation coefficient > 0.7 were considered significant. In blank filtering, compounds whose average peak area in the blanks was over 30% of that in the QCs were removed. Finally, CV filtering was applied to remove compounds with a CV greater than 30%.

3.9.4 Results

3.9.4.1 Column flow rate and ramp gradients

Different GC column flow rates (1.4 mL/min, 1.6 mL/min, 1.8 mL/min, and 2 mL/min) and different GC oven ramp rates (6 °C/min, 8 °C/min, and 10 °C/min) were evaluated by comparing the number of detected peaks (Figure 19, a, b). Three replicates were performed for each flow rate and ramp rate. 2 mL urine was used in the analysis. The initial temperature of the GC oven was set to 35 °C.

When testing the flow rate, the ramp rate was set to 10 °C/min. An average of 389 compounds were identified when the flow rate was 1.4 mL/min. The number of compounds decreased as the column flow rate increased. An average of 375 and 355 compounds were detected when the flow rate increased 1.6 mL/min and 1.8 mL/min, respectively. The compound number was approximately 86% of the number detected with 1.4 mL/min flow when the flow rate was 2 mL/min.

When testing the ramp rate, the column flow rate was set to 2 mL/min. When the ramp rate was set to 6 °C/min, an average of 359 compounds were identified. When the ramp rate was 8 °C/min, an average of 385 compounds were detected, which was the highest among the tested conditions. When the ramp rate was increased to 10 °C/min, an average of 333 compounds were detected, which was around 86% of the compound number detected at the 6 °C/min ramp rate.

Combining both column flow rate and ramp rate based on above results, 5 non-column assays were compared by investigating the number of detected compounds (Figure 19, c). 2 mL urine was used for each sample. Key details of the four assays were:

Long assay 1: Flow rate 2 mL/min, ramp rate 6 °C/min;
Long assay 2: Flow rate 2 mL/min, ramp rate 8 °C/min;
Short assay 1: Flow rate 2 mL/min, ramp rate 10 °C/min;
Short assay 2: Flow rate 1.8 mL/min, ramp rate 10 °C/min;
Short assay 3: Flow rate 1.6 mL/min, ramp rate 10 °C/min;
Short assay 4: Flow rate 1.4 mL/min, ramp rate 10 °C/min.

In general, the five assays delivered similar number (around 300 to 400) of compounds. The long assay 2 (Flow rate 2 mL/min, ramp rate 8 °C/min) and the short assay 4 (Flow rate 1.4 mL/min, ramp rate 10 °C/min) delivered highest number of compounds (385 and 389, respectively) and were recommended as two complementary methods for obtaining a considerable number of compounds in a long and a short length of time.

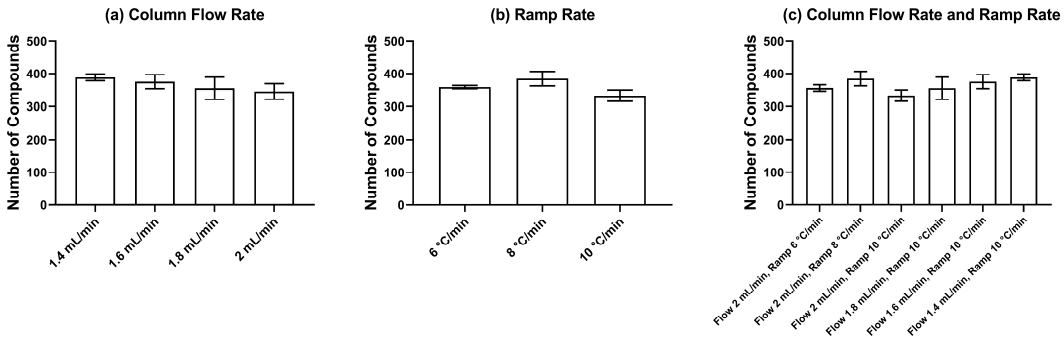


Figure 19. Optimisation of column flow rate and oven ramp rate.

The effect of (a) column flow rate, (b) GC oven ramp rate, and (c) column flow rate and ramp rate on the number of detected urinary VOCs. The number of compounds decreased as the column flow rate increased; The number of compounds found with 2 mL/min column flow was approximately 86% of the number detected with 1.4 mL/min flow. The number of compounds detected was the highest when ramp rate was set to 8 °C/min (around 10% more). Two assays provided more identified compounds: flow rate at 2 mL/min, ramp rate 8 °C/min; flow rate at 1.4 mL/min, ramp rate 10 °C/min.

3.9.4.2 Optimal long assay and short assay (non-polar column)

Four different assays using the non-polar GC column were compared based on the number of identified, reproducible, linear and non-contamination-related VOCs to determine the optimal long and short assay based on non-polar column (Table 16):

2 mL urine long assay: Urine volume 2 mL, initial oven temperature 35 °C hold for 4.5 min, ramp to 300 °C at 8 °C/min hold for 4 min;

0.25 mL urine long assay: Urine volume 0.25 mL, initial oven temperature 35 °C hold for 4.5 min, ramp to 300 °C at 8 °C/min hold for 4 min;

2 mL urine short assay: Urine volume 2 mL, initial oven temperature 35 °C hold for 4.5 min, ramp to 300 °C at 10 °C/min hold for 4 min;

0.25 mL urine short assay: Urine volume 0.25 mL, initial oven temperature 35 °C hold for 4.5 min, ramp to 300 °C at 10 °C/min hold for 4 min.

The 2 mL urine long assay detected the second highest number of reliable compounds (154), while the 0.25 mL urine long assay output the lowest number of compounds (85). The 2 mL urine short assay produced the highest number of compounds, with 167 compounds passed all the three filterings. In the 0.25 mL urine short assay, 103 reliable compounds were identified.

Table 16. Four-assay panel of GC methods (non-polar column).

Instrument	TD-GC-TOF-MS (Non-Polar Column)			
Urine volume	2 mL	0.25 mL	2 mL	0.25 mL
GC column	DB5-MS UI (30 m × 0.25 mm × 1.00 µm)			
Split	1:10	1:10	1:10	1:10
GC column flow	1.6 mL/min	1.6 mL/min	2 mL/min	2 mL/min
GC oven gradients	Initial temperature at 35 °C hold for 4.5 min, ramp to 300 °C at 8 °C/min hold for 4 min		Initial temperature at 35 °C hold for 4.5 min, ramp to 300 °C at 10 °C/min hold for 4 min	
GC cycle time	44 min	44 min	37 min	37 min
MS	MS range: 30 to 350 Data rate: 4 TF line: 250 Ion source: 250 -70eV		MS range: 30 to 350 Data rate: 4 TF line: 250 Ion source: 250 -70eV	
Deconvolved NIST matched peaks (S/N > 3), siloxanes and gases removed	382	385	354	357
VOCs passing blank filtering	186	113	191	117
VOCs passing linearity filtering	203	131	206	161
VOCs passing CV filtering	255	227	255	260
VOCs passing all filterings	154	85	167	103

3.9.5 Discussion

Two complementary non-polar methods were developed for identifying a considerable number of compounds in a long and a short length of time, with the optimal short method obtaining around 8% more VOCs than the optimal long method. The short assays produced better results in a shorter time frame, making them more attractive for clinical adoption especially in large clinical studies.

All the four assays using the TD-GC-TOF-MS with non-polar column identified over 80 urinary VOCs, more than double the number identified using quadrupole instrument in Section 3.7. The best assay identified almost 4 times the number identified using quadrupole This demonstrated again the superiority of TOF for the identification of urinary VOCs.

In principle, the urinary VOCs identified by TD-GC-TOF-MS non-polar assays are different from the ones identified by polar assays in terms of polarity due to different column phase selected. The non-polar assays should identify mainly non-polar compounds with a small proportion of mid-polar and polar compounds, whereas the polar assays should mainly identify polar compounds with a small proportion of mid-polar and non-polar compounds. How the identified compounds overlap between the non-polar and polar assays and how different between the compounds identified by TOF-MS and quadrupole MS required deep investigation, which was conducted in Section 3.10.

This subsection has the same limitation as Section 3.8, which is the selection of the tested conditions for the evaluation of GC oven parameters. Employing a multivariate optimisation strategy in future studies may allow the evaluation of the interactions among these tested conditions.[129]

3.9.6 Conclusion

A panel of assays using TD-GC-TOF-MS equipped with a non-polar column were developed for urinary VOC analysis, with an optimal long assay and an optimal short assay determined.

3.10 Comparison of the developed multiplatform GC-MS assays

3.10.1 Introduction

This subsection brought together the results shown in Section 3.7, 3.8 and 3.9 to compare the developed assays using quadrupole-based TD-GC-MS (mid-polar column), TD-GC-TOF-MS (non-polar column), and TD-GC-TOF-MS (polar column).

3.10.2 Objectives

The primary objective of this subsection was to compare the developed multiplatform GC-MS assays for urinary VOC analysis.

Secondary objectives were:

1. To compare the reliable VOCs identified by the developed assays;
2. To investigate the overlap of identified VOCs between the developed assays.

3.10.3 Methods

Four polar column based TOF assays were developed in Section 3.8:

2 mL urine long polar assay;
0.25 mL urine long polar assay;
2 mL urine short polar assay;
0.25 mL urine short polar assay.

Four non-polar column based TOF assays were developed in Section 3.9:

2 mL urine long non-polar assay;
0.25 mL urine long non-polar assay;
2 mL urine short non-polar assay;
0.25 mL urine short non-polar assay.

As a reference, an assay using quadrupole GC-MS was developed in Section 3.7. All these nine assays were compared.

3.10.4 Results

Table 17 summarises the details of the nine assays and the number of compounds passing the feature filterings. The 2 mL urine long polar assay delivered the best results among the polar assays, with 121 reliable urinary VOCs detected. The 2 mL urine short non-polar assay detected the highest number of 167 reliable VOCs among the non-polar assays, around 38% more than the VOCs identified by the best polar assay and 288% more than those identified by the quadrupole assay.

Figure 20 shows the number of reliable VOCs identified by the best assays (the 2 mL urine quadrupole assay, the 2 mL urine long polar assay, and the 2 mL urine short non-polar assay), and the overlap of the identified VOCs. There were 27 VOCs identified by both the best polar and non-polar TOF assays; 7 VOCs were identified by the quadrupole assay and the best polar assay; 5 VOCs were identified by the quadrupole assay and the best non-polar assay; 2 compounds were identified by the three best assays.

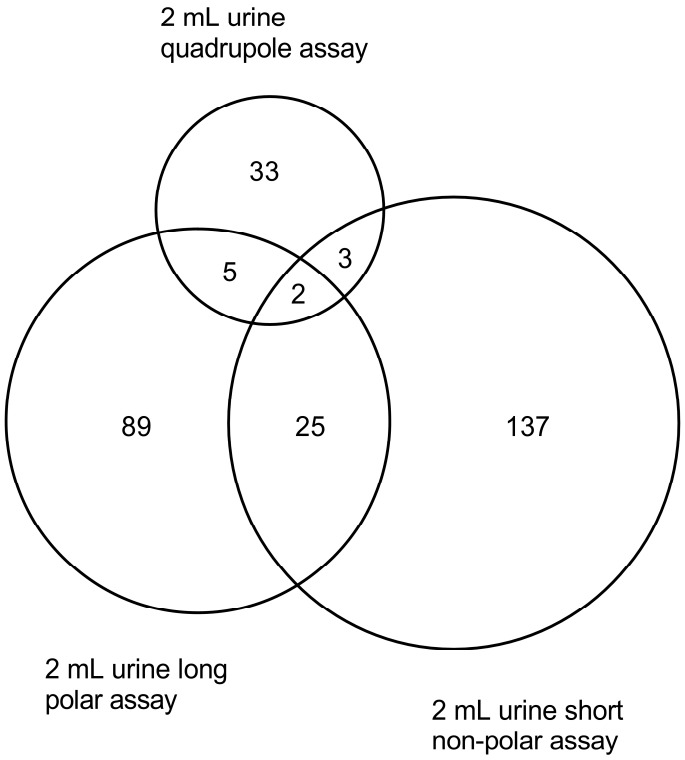


Figure 20. Venn diagram for the number of VOCs identified by the best assays.

Table 17. Nine-assay panel of GC methods.

Instrument	TD-GC-quadrupole MS	TD-GC-TOF-MS (Polar Column)				TD-GC-TOF-MS (Non-Polar Column)			
Urine volume	2 mL	2 mL	0.25 mL	2 mL	0.25 mL	2 mL	0.25 mL	2 mL	0.25 mL
GC column	Zebron ZB-624UI (60 m × 0.25 mm, 1.40 µm)	Mega WAX HT (20 m × 0.18 mm × 0.18 µm)				DB5-MS UI (30 m × 0.25 mm × 1.00 µm)			
Split	Splitless	1:10	1:10	1:10	1:10	1:10	1:10	1:10	1:10
GC column flow	1 mL/min	0.7 mL/min	0.7 mL/min	1.2 mL/min	1.2 mL/min	1.6 mL/min	1.6 mL/min	2 mL/min	2 mL/min
GC oven gradients	Initial temperature at 40 °C hold for 4 min, ramp to 150 °C at 15 °C/min without hold, ramp to 240 °C at 10 °C/min hold for 5 min	Initial temperature at 35 °C hold for 2 min, ramp to 240 °C at 4 °C/min hold for 2 min		Initial temperature at 35 °C hold for 1.9 min, ramp to 240 °C at 20 °C/min hold for 0.2 min		Initial temperature at 35 °C hold for 4.5 min, ramp to 300 °C at 8 °C/min hold for 4 min		Initial temperature at 35 °C hold for 4.5 min, ramp to 300 °C at 10 °C/min hold for 4 min	
GC cycle time	55 min	57 min	57 min	17 min	17 min	44 min	44 min	37 min	37 min
MS	MS range: 35 to 250 Data rate: 6.2 TF line: 230 Ion source: 240 -70eV	MS range: 30 to 597 Data rate: 10 TF line: 260 Ion source: 260 -70eV		MS range: 30 to 597 Data rate: 10 TF line: 260 Ion source: 260 -70eV		MS range: 30 to 350 Data rate: 4 TF line: 250 Ion source: 250 -70eV		MS range: 30 to 350 Data rate: 4 TF line: 250 Ion source: 250 -70eV	
Deconvolved NIST matched peaks (S/N > 3), siloxanes and gases removed	123	220	103	188	209	382	385	354	357
VOCs passing blank filtering	43	166	48	114	124	186	113	191	117
VOCs passing linearity filtering	67	160	59	111	140	203	131	206	161
VOCs passing CV filtering	41	147	55	168	132	255	227	255	260
VOCs passing all filterings	41	121	33	96	85	154	85	167	103

3.10.5 Discussion

Three of the four polar assays identified over 80 VOCs, more than double the number identified using the quadrupole assay. All the four non-polar assays identified over 80 VOCs, more than double the number identified using the quadrupole assay, with the best assay identified almost 4 times the number identified using quadrupole. This demonstrated the superiority of TOF over quadrupole GC-MS in identifying larger number of urinary VOCs. This is due to TOF's fast acquisition rate, great spectral continuity, high resolution, exceptional dynamic range, as well as high sensitivity and selectivity, as explained in Section 3.8.1.

The overlap of identified VOCs between different platforms was low (less than 23%). Although some of the VOCs might be misannotated, since as an untargeted analysis, no authentic standards were analysed in this section thus no robust identification was provided, it was clear that the VOCs detected in quadrupole, TOF with polar column, and TOF with non-polar column were highly different and complementary. This highlighted the necessity of analysing samples on multiple GC-MS platforms using different assays.

Overall, the non-polar assays identified more reliable VOCs than the polar assays. The major reason was the usage of PDMS extracting phase on the HiSorb, which extracted mainly non-polar compounds, as well as a proportion of mid-polar and polar compounds.

This subsection was limited by the mis-annotation of identified VOCs which might influence the investigation of shared VOCs.

3.10.6 Conclusion

The developed multiplatform GC-MS assays for urinary VOC analysis were compared, highlighting the superiority of TOF over quadrupole GC-MS and the necessity of analysing samples on multiple platforms using different assays.

3.11 TD recollection for multiple analysis

3.11.1 Introduction

The Markes TD100-XR features a sample recollection function which can quantitatively recollect all the split flows to a conditioned TD tube, enabling repeat analysis of critical samples, easy method validation, overcoming the “one-shot limitation" of conventional TD systems. The setup of the recollection procedure is presented in Figure 21. During either tube desorption and focusing trap desorption stage, the flow of analytes can be split and recollected onto a clean sorbent TD tube. This function can minimise the need to extract urine samples multiple times, which is important especially when the sample volume is limited.

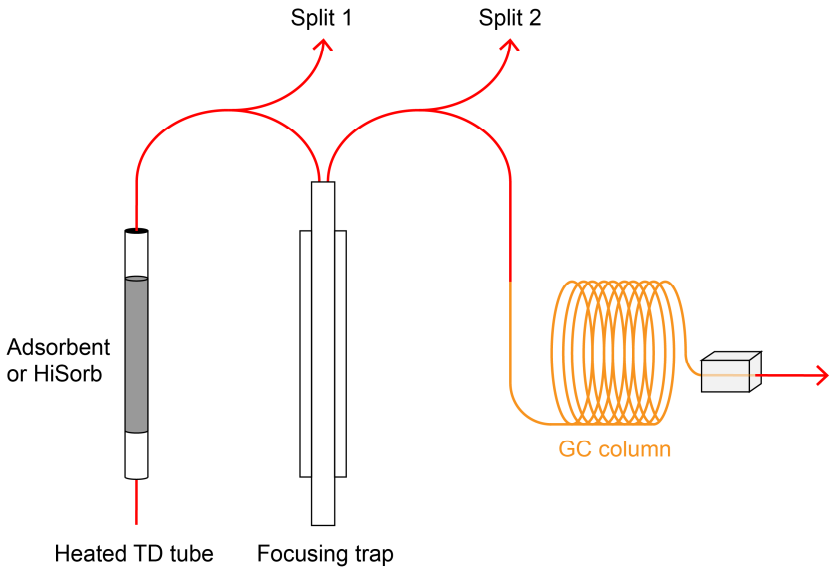


Figure 21. Schematic representation of the TD recollection function setup.

The split 1 flow during tube desorption and split 2 flow during focusing trap desorption can be recollected onto a clean sorbent tube.

3.11.2 Objectives

The objective of this subsection was to test TD recollection for multiple urinary VOC analysis.

3.11.3 Methods

3.11.3.1 Subjects

Experiments utilised urine from a single healthy volunteer.

3.11.3.2 Sample preparation

Recollection performance was evaluated over multiple recollection cycles from HiSorbs to Tenax/Carbograph-5 TD tubes and from the TD tubes to themselves. Sample preparation followed the same principles in Section 3.7.3.2, 3.8.3.2 and 3.9.3.2. NaCl (0.4 g) and 5 M HCl (adjusting pH to 2.0) were added to 2 mL urine from one healthy individual.

3.11.3.3 Urinary VOC extraction

Urine samples were extracted using HiSorb (Agitating temperature: 60 °C, speed: 300 rpm, time: 30 min) as determined in Section 3.6.

3.11.3.4 TD-GC-MS

Extracted urinary VOCs were analysed on the TD-GC-TOF-MS with the non-polar column. The common TD method was used (Section 3.7.3.4, 3.8.3.4, and 3.9.3.4). The TD tubes were pre-purged for 1 minute at 50 mL/min helium flow and heated to 250 °C for 5 min to desorb the VOCs onto a cold trap in split mode (1:10). The split flows were recollected completely and reload onto a Tenax/Carbograph-5 TD tube for the next test cycle. The cold trap was cooled to 25 °C and then heated to 250 °C for 3 min, with the flow path onto GC heated constantly at 200 °C.

The Agilent 7890B GC coupled with SepSolve BenchTOF Select TOF-MS was used for GC-MS analysis. GC programme was set to: Initial temperature at 35 °C hold for 4.5 min, ramp to 300 °C at 10 °C/min, hold for 4 min. The MS transfer line was maintained at 260 °C, and 70 eV electron impact was set at 260 °C. MS analyser was set to acquire over the range of 30 to 597 m/z.

3.11.3.5 Data processing

The data acquired from the TD-GC-TOF-MS instrument were processed in ChromSpace. After DBC and deconvolution, a peak list of detected compounds with RT, potential identities, quantifier ions, and verification ions was created. Potential identities were obtained by comparing the NIST library with RI penalty based on the n-alkane pattern (C_8 to C_{20}). Features with RMF < 800, siloxanes, gases and with S/N < 3 were removed. Details of methods were demonstrated in Table 18.

3.11.4 Results

The number of compounds in the peak list after invalid features were removed was compared (Table 18). The original run identified 167 compounds from healthy human urine. The first recollection identified 158 compounds, which was 94.6% of the number identified in the original run. The second and third recollection both identified 147 compounds, which was 88.0% of the original run. In the fourth recollection, 133 compounds were detected, which was 79.6% of the original run.

Table 18. TD recollection function test.

Four recollection cycles of one sample were performed. For up to four recollection cycles, recollection was found satisfactory in terms of repeatability, linearity, contamination, and sample loss. The number of compounds identified in one recollection cycle was 94.6% of the number identified in the original run.

Cycle	Original Run	Recollection 1	Recollection 2	Recollection 3	Recollection 4
Instrument	TD-GC-TOF-MS (Non-polar column)				
Urine volume	2 mL	-	-	-	-
GC column	DB5-MS UI (30 m × 0.25 mm × 1.00 μm)				
Split	1:10				
GC column flow	2 mL/min				
GC oven gradients	Initial temperature at 35 °C hold for 4.5 min, ramp to 300 °C at 10 °C/min, hold for 4 min				
GC cycle time	37 min				
MS	MS range: 30 to 597, Data rate: 10, TF line: 260, Ion source: 260, -70eV				
Deconvolved NIST matched peaks (S/N > 3), siloxanes and gases removed	354	Compound list same as that of original run	Compound list same as that of original run	Compound list same as that of original run	Compound list same as that of original run
VOCs passing blank filtering	191	178	175	178	168
VOCs passing linearity filtering	206	185	180	181	165
VOCs passing CV filtering	255	265	250	237	222
VOCs passing all filterings	167	158	147	147	133
% of VOC recovered	-	94.6%	88.0%	88.0%	79.6%

3.11.5 Discussion

Recollection was found to be satisfactory in terms of reproducibility, linearity, contamination, and sample loss for up to four recollection cycles. Particularly, for one recollection cycle, from HiSorb to TD tube, the number of reliable VOCs (identified, reproducible, linear and non-contamination-related) was 94.6% of the initial HiSorb sample.

Thermal desorption was traditionally considered to be a 'one-shot' process, with any sample split going to waste. With TD recollection, the split which is a portion of sample that is not sent to the column is directed to a clean sorbent-packed TD tube. This allows the sample to be analysed again because it is quantitatively retained, enabling the potential of deep profiling one sample on multiple platforms, such as GC×GC-MS, GC with different columns, and TD-selected ion flow tube mass spectrometry (SIFT)-MS, etc. Moreover, it enables the use of one sample for both targeted and untargeted analysis. It is recommended starting with a quantitative assay for targeted analysis and then run the recollected samples for untargeted analysis, because sample loss during each recollection results in dropping of chromatographic peaks which makes accurate quantification unachievable.

Main limitation for this subsection is the small sample size.

3.11.6 Conclusion

TD recollection was found satisfactory in terms of reproducibility, linearity, contamination, and sample loss, allowing for the possibility of profiling a single sample on multiple platforms in parallel or using a single sample for both targeted and untargeted analyses.

3.12 Urine concentration correction

3.12.1 Introduction

Urine concentration varies greatly, primarily due to unpredictable hydration levels of subjects caused by conditions including but not limited to dehydration, higher fluid intake, excess vomiting, kidney diseases, diabetes, etc. Under normal healthy physiological conditions, urine volume has been found to fluctuate by up to 15 folds, resulting in a significant fluctuation in metabolite concentration through urine dilution or concentration.[137] This complicates the task of generating high quality metabolomic datasets and detecting disease related biomarkers.

Urine concentration can be measured by testing urine osmolality. Urine osmolality is used to assess hydration status via measuring the number of dissolved particles per unit of water in the urine. It is a more accurate indicator of urine concentration than urine specific gravity (USG), which is calculated by comparing the weight of the solution to that of an equal volume of distilled water and is thus determined by both the number and size of particles in the solution.[138]

When first morning urine samples cannot be obtained, applying an osmolality correction to the data is advantageous in minimising the effect of variable urine concentration. Although urinary creatinine is also widely used for normalisation, it is altered in some pathologies and its concentration is also dependent on the individual's muscle mass, making it less effective than osmolality normalisation. Due to the absence of robust data of osmolality normalisation in VOC research, a validation experiment was conducted in this subsection to examine the applicability of osmolality normalisation in urinary volatolomics.

3.12.2 Objectives

The objective of this subsection was to evaluate the applicability of osmolality normalisation in urinary VOC analysis.

3.12.3 Methods

3.12.3.1 Subjects

Experiments utilised urine from eleven healthy volunteers.

3.12.3.2 Sample preparation

The eleven healthy subjects provided early morning (first micturition after waking) urine samples. Urine samples were aliquoted in duplicates, with the second aliquot diluted 1:1 with deionised nanopure water. Sample preparation followed the same principles in Section 3.7.3.2, 3.8.3.2 and 3.9.3.2, with NaCl (0.4 g) and 5 M HCl (adjusting pH to 2.0) added to 2 mL urine.

3.12.3.3 Urinary VOC extraction

Urine samples were extracted using HiSorb (Agitating temperature: 60 °C, speed: 300 rpm, time: 30 min) as determined in Section 3.6.

3.12.3.4 TD-GC-MS

Urinary VOC profiling was performed on the TD-GC-TOF-MS with the non-polar column. The common TD method was used. The TD tubes were pre-purged for 1 minute at 50 mL/min helium flow and heated to 250 °C for 5 min to desorb the VOCs onto a cold trap in split mode (1:10). The cold trap was cooled to 25 °C and then heated to 250 °C for 3 min, with the flow path onto GC heated constantly at 200 °C.

GC programme was set to: Initial temperature at 35 °C hold for 4.5 min, ramp to 300 °C at 10 °C/min, hold for 4 min. The MS transfer line was maintained at 260 °C, and 70 eV electron impact was set at 260 °C. MS analyser was set to acquire over the range of 30 to 597 m/z.

3.12.3.5 Sample analysis strategy

All twenty-two samples along with three blanks, seven pooled QC urine samples and a dilution series (0.625%, 1.25%, 2.5%, 5%, 10%, 25%, 50%, 75% of QC) were

analysed (Table 19). The osmolality of all these samples was measured using the OsmoPRO® Multi-Sample Micro-Osmometer.

Table 19. Run order for osmolality test.

Analytical sequence included: 3 blank, 7 QC samples, 8 serial dilution (SD) of QC samples, and 22 samples of undiluted or diluted healthy samples (Total 40 samples).

No. of Run	1-3	4	5	6	7	8	9	10	11	12-18	19-40
Sample	B1-3	SD1	SD2	SD3	SD4	SD5	SD6	SD7	SD8	QC1-7	S1-22
Dilution	0%	0.625%	1.25%	2.5%	5%	10%	25%	50%	75%	100%	-

B: blank; SD: serial dilution; QC: pooled quality control; S: study sample.

3.12.3.6 Data processing

The acquired data were processed in ChromSpace. After DBC and deconvolution, a peak list of detected compounds with RT, potential identities (obtained by comparing the NIST library with RI penalty based on the n-alkane pattern (C_8 to C_{20})), quantifier ions, and verification ions was created. Features with RMF < 800, siloxanes, gases and with S/N < 3 were removed.

Data processed by ChromSpace were input into Gavin 3 for peak integration. After integration, an aligned peak table which contained the peak area of each compound in each sample was created in one single excel table. The data were filtered to remove non-linear, contamination-related, and non-reproducible VOCs. The 1-tailed Spearman's rank correlation coefficient test was used to observe the trend of the dilution series. The Benjamini-Hochberg procedure was used to correct false positives.[133-135] Compounds with adjusted p value < 0.05 and correlation coefficient > 0.7 were considered significant. In blank filtering, compounds whose average peak area in the blanks was over 30% of that in the QCs were removed. Finally, CV filtering was applied to remove compounds with a CV greater than 30%.

The volatolomic profiles were compared with supervised multivariate statistics (orthogonal projections to latent structures discriminant analysis, OPLS-DA) before and after osmolality correction in SIMCA 15 (data not transformed, but UV scaled). The effect of osmolality normalisation on the dataset was evaluated.

3.12.4 Results

Figure 22 shows the separation between undiluted and diluted samples in OPLS-DA plot. Before osmolality correction, the difference between the undiluted and diluted samples was obvious (Figure 22, a). After normalising for osmolality, it became less clustered (Figure 22, b).

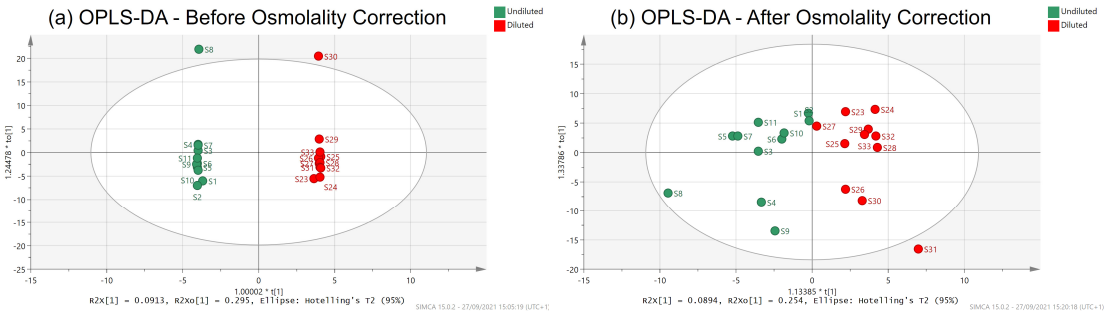


Figure 22. Osmolality test.

Undiluted versus diluted samples compared with OPLS-DA (a) before and (b) after osmolality correction.

3.12.5 Discussion

OPLS-DA is a supervised discriminant analysis suitable for diagnosing differences between two groups or systems. It is especially adept at forcing scores-space separation. When provided high-dimensional spectral data, OPLS almost invariably produces scores-space separation based on the assigned group memberships. Due to these powerful modelling capabilities, OPLS is fully capable of producing results only based on noise, if so requested.[139] Even though the OPLS-DA is powerful, the undiluted and diluted sample groups were less separated after osmolality normalisation, suggesting that the effect of variable urine concentration was partially corrected.

This subsection is mainly limited by the small sample size.

3.12.6 Conclusion

The application of osmolality normalisation can partially correct the effect of variable urine concentration, allowing the improvement of the ability to identify important metabolome components that differentiate across sample groups, and avoiding false discoveries that reflect different urine concentrations rather than other characteristics under investigation. Osmolality normalisation was therefore advised as a regular post-analysis procedure of data pre-processing in urinary VOC analysis.

SECTION 4

PILOT STUDY OF OPTIMISED URINARY VOC ANALYTICAL METHOD FOR THE DETECTION OF PANCREATIC CANCER

4 PILOT STUDY OF OPTIMISED URINARY VOC ANALYTICAL METHOD FOR THE DETECTION OF PANCREATIC CANCER

4.1 Introduction

Studies in Section 3 provided a complete optimised methodology for untargeted urinary VOC analysis in a high throughput and standardised manner with the adoption of robust quality control measures. In order to showcase its applicability in clinical studies and to generate data to support its use in larger clinical trials, urine samples from a cohort consisting of pancreatic cancer patients and controls were analysed.

As described in Section 1, pancreatic cancer is one of the most common cancers and is leading cause of cancer-related death. More than 80% of patients with pancreatic cancer are diagnosed late with unresectable or metastatic disease, resulting in low overall survival of 7.3%.[14-16] There is continuous quest for a simple, inexpensive, and non-invasive test to detect pancreatic cancer early, while it is still amenable to surgical resection. This provides the rationale for the implementation of the developed optimal methodology in this section for the purpose of urine metabolic profiling, trying to identify urinary VOCs that distinguishing pancreatic cancer from controls.

4.2 Objectives

The objective of this section was to apply the developed urinary VOC analysis methods for the discovery of urinary VOC biomarkers of gastrointestinal cancer (pancreatic cancer) using advanced multiplatform MS profiling techniques.

Specific objectives were:

1. To demonstrate the applicability and evaluate the performance of the urinary VOC analysis methods developed in Section 3 for the detection of pancreatic cancer;
2. To identify urinary VOC biomarkers that can discriminate pancreatic cancer from controls;
3. To establish a diagnostic model for the detection of pancreatic cancer based on urinary VOCs.

4.3 Methods

4.3.1 Materials and reagents

The chemical reagents, consumables, and equipment used in Section 4 were the same as those reported in Section 3.3.

4.3.2 Patients

Patients attending Hammersmith Hospital with histologically confirmed pancreatic cancer between 7 March 2016 and 7 March 2020 were approached to participate in this study. Patients with or without upper gastrointestinal symptoms but with normal upper gastrointestinal tract on endoscopy were recruited as control subjects. Where available, any additional investigations (e.g., imaging studies) will be used to verify the absence of upper gastrointestinal and pancreatic cancer. Patients' medical records were also reviewed at least one year after the time of recruitment to ensure no diagnosis of cancer had been made during this period.

All patients were informed of the aim of the study and their written consent was obtained prior to enrolment. The study was approved by NHS Research Ethics Committee (REC) and Health Research Authority (HRA) (REC reference 17/WA/016; REC reference 14/LO/1136, IRAS project ID 142097) and was performed in accordance with the Declaration of Helsinki (Appendix 2).

The inclusion criteria were as follows:

1. Pancreatic cancer: Patients aged > 18 years with biopsy confirmed pancreatic adenocarcinoma;
2. Controls: Patients aged > 18 years presenting with or without upper gastrointestinal symptoms but with normal upper gastrointestinal tract on endoscopy.

The exclusion criteria were:

1. Patients with non-adenocarcinoma pancreatic cancers (e.g., neuroendocrine tumours);
2. Patients with benign gastrointestinal diseases;

3. Patients diagnosed with other synchronous cancers;
4. Patients with either a suspected or confirmed active infection, liver failure and/or renal failure;
5. Patients who were unable to provide informed written consent or who were not able to provide a > 5 mL urine sample.

4.3.3 Urine sample collection and storage

Patients were asked to provide a sample of urine into a universal 50 mL sterile urine container which was then immediately sealed and delivered to the laboratory on ice to avoid loss of volatile compounds. All the containers were stored at -80 °C.

4.3.4 Urine sample aliquoting

Aliquoting of urine samples was completed in one day to prevent batch effects. Before aliquoting, all the safe-lock cryovials were pre-emptively labelled with ultra-low temperature friendly cryo-tags. During aliquoting, all the samples were kept on ice to minimise the loss of VOCs.

The pancreatic cancer urine samples and the control urine samples were aliquoted into two series, for the analysis using the optimal non-polar 2 mL urine short assay developed in Section 3.9.4.2 and the analysis using the optimal polar 2 mL urine long assay developed in Section 3.8.4.5, respectively. For each sample, 2 mL of urine was aliquoted into a 2 mL safe-lock cryovial. Internal standards were spiked at the sample aliquoting stage to avoid variations brought by multiple batches of spiking. The mixture of internal standards included: acetone-d_6, butyraldehyde-d_2, phenol-d_6, benzene-d_6, acetophenone-d_8 and toluene-d_8. They were spiked into samples to reach 0.2 µg/mL in the 2 mL liquid.

Two series of pooled QC samples were also generated at the sample aliquoting stage for the analysis using the optimal non-polar 2 mL urine short assay and the optimal polar 2 mL urine long assay. Equal amounts of each study sample (0.8 mL) were mixed in Falcon 50 mL tubes to create a big, pooled QC containing both pancreatic cancer urine and control urine. Then, the pooled QC was aliquoted into multiple 2 mL small

aliquots in 2 mL safe-lock cryovials, with the same internal standards added (acetone-d_6, butyraldehyde-d_2, phenol-d_6, benzene-d_6, acetophenone-d_8 and toluene-d_8, 0.2 µg/mL in 2 mL liquid).

Following the generation of QC samples, two series of serial dilution (SD) samples (0.625%, 1.25%, 2.5%, 5%, 10%, 25%, 50%, 75% of QC) were produced, with deionised nanopure water being used to top up to 2 mL. The same internal standards were also spiked to achieve the same concentration (0.2 µg/mL in 2 mL liquid).

Two series of blanks were generated by adding equal amounts (2 mL) deionised nanopure water into multiple 2 mL safe-lock cryovials. The same internal standards were spiked into each vial to reach the same concentration of 0.2 µg/mL in 2 mL liquid.

In addition, the osmolality of all the pancreatic cancer urine samples, controls, QC samples and blanks was determined by testing 20 µL of urine using the OsmoPRO® Multi-Sample Micro-Osmometer.

All the cryovials were then placed in ultra-low lab freezer cardboard cryoboxes and stored at -80 °C prior to further analysis. On each day of analysis, only relevant vials were taken from the freezer, minimising the number of repeated freeze-thaw cycles.

In summary, two identical series of samples were generated when sample aliquoting was completed:

Series 1, for the analysis using the optimal non-polar 2 mL urine short assay developed in Section 3.9.4.2:

- Pancreatic cancer urine samples in 2 mL aliquots;
- Control urine samples in 2 mL aliquots;
- QC samples in 2 mL aliquots;
- Serial dilution (SD) of QC samples in 2 mL aliquots;
- Blank samples in 2 mL aliquots.

Series 2, for the analysis using the optimal polar 2 mL urine long assay developed in Section 3.8.4.5:

Pancreatic cancer urine samples in 2 mL aliquots;
Control urine samples in 2 mL aliquots;
QC samples in 2 mL aliquots;
Serial dilution (SD) of QC samples in 2 mL aliquots;
Blank samples in 2 mL aliquots.

All the QC samples were identical throughout the analysis in this section. All the samples were spiked with the same types and concentration of internal standards.

4.3.5 Sample preparation

On evening before each analytical day, thirty-two safe-lock cryovials containing urine or blank samples were removed from the -80 °C freezer and thawed in 4 °C refrigerator overnight. On each day of analysis, 2 mL of urine or water was transferred into glass 20 mL headspace vials. NaCl (0.4 g) was added into each urine sample to enhance the realising of VOCs. 5 M HCl (60 µL) was added to all the samples at room temperature for sample acidification. The reason for using 60 µL HCl rather than pH meter monitoring was because it was impractical to measure large number of samples due to loss of urinary VOCs. 60 µL HCl was found satisfactory to achieve adequate sample acidification. Internal standards had been added at the sample aliquoting stage. The vials were sealed and left for 10-15 min to equilibrate.

4.3.6 Urinary VOC extraction

Extraction of urinary VOCs was conducted using HiSorb sorptive extraction at the optimal conditions determined in Section 3.6. In the early morning of each analytical day, TD tubes loaded with HiSorb probes were pre-conditioned using the TC-20 TD tube conditioner at 260 °C for 180 min under nitrogen stream at 20 psi head pressure to ensure the contamination of HiSorb was at lowest level. A HiSorb probe was inserted into the glass headspace vial, with the extracting phase exposed in the urine headspace. Once all the HiSorb probes were inserted, all the vials were placed in the HiSorb agitator for VOC extraction. The HiSorb agitator allowed sixteen glass headspace vials to be agitated at the same time. This required the urinary VOC extraction of thirty-two samples to be completed in two batches per day. Agitating parameters were set to: temperature: 60 °C, speed: 300 rpm, time: 30 min. When

extraction was complete, the HiSorb probe was removed from the vial and put back in the corresponding TD tube, which was capped using the matching steel caps immediately. Subsequently, TD tubes were put onto the TD autosampler for TD-GC-TOF-MS analysis.

4.3.7 Urinary VOC profiling

4.3.7.1 TD

Primary desorption was performed on the Markes TD100 instrument by pre-purging the TD tubes with HiSorb probes inside for 1 minute with helium flow at 50 mL/min and heated to 250 °C for 5 min to desorb the VOCs onto a cold trap (Material emissions) in split mode (1:10). Secondary desorption initially cooled the cold trap to 25 °C and heat to 250 °C for 3 min, with the flow path onto GC heated constantly at 200 °C.

4.3.7.2 GC

For the analysis using the optimal non-polar 2 mL urine short assay, the Agilent 7890B GC instrument was involved. VOC separation was performed on the non-polar DB5-MS UI capillary column (30 m × 0.25 mm × 1.00 µm). Oven temperature was set at 35 °C initially for 4.5 min and ramp to 300 °C (10 °C/min with 4 min hold). The end oven temperature was 300 °C.

The analysis using the optimal polar 2 mL urine long assay was also carried out on the Agilent 7890B GC instrument. VOC separation was performed on the polar Mega WAX HT capillary column (20 m × 0.18 mm × 0.18 µm). Oven temperature was set at 35 °C initially for 2 min and ramp to 240 °C (4 °C/min with 2 min hold). The end oven temperature was 240 °C.

4.3.7.3 MS

The SepSolve BenchTOF Select TOF-MS was used for MS analysis.

For the analysis using the optimal non-polar 2 mL urine short assay, the MS transfer line was maintained at 250 °C, and 70 eV electron impact was set at 250 °C. MS analyser was set to acquire over the range of 30 to 350 m/z with data rate at 4 Hz.

For the analysis using the optimal polar 2 mL urine long assay, the MS transfer line was maintained at 260 °C, and 70 eV electron impact was set at 260 °C. MS analyser was set to acquire over the range of 30 to 597 m/z with data rate at 10 Hz.

4.3.8 Sample analysis strategy

In order to evaluate gross performance characteristics of the instruments (RT stability, peak shape, detector response and mass accuracy), and also for data pre-processing purpose, a batch of samples including: three blank, seven QC samples, and eight serial dilution (SD) of QC samples were analysed at the beginning of an analytical sequence (Table 20).

Table 20. Run order for instrumental performance evaluation.

Evaluation batch included: 3 blank, 7 QC samples, 8 serial dilution (SD) of QC samples (Total 18 samples).

No. of Run	1-3	4	5	6	7	8	9	10	11	12-18
Sample	B1-3	SD1	SD2	SD3	SD4	SD5	SD6	SD7	SD8	QC1-7
Dilution	0%	0.625%	1.25%	2.5%	5%	10%	25%	50%	75%	100%

B: blank; SD: serial dilution; QC: pooled quality control.

In the analytical sequence for the analysis using the optimal non-polar 2 mL urine short assay, series 1 samples were used. Following the initial eighteen samples summarised in Table 20, samples were analysed in multiple batches. A typical batch included a total of thirty-two samples: one blank, six QC samples, and twenty-five clinical samples (pancreatic cancer samples or control samples) (Table 21). In such batches, every five clinical samples belonged to one block, with clinical samples assigned at random to each block. Therefore, a randomised block design was conducted. Each batch was analysed in one single day.

Following the same principle, series 2 samples were used to establish the analytical sequence for the analysis using the optimal polar 2 mL urine long assay. The sequence also began with eighteen evaluation samples and continued with multiple typical batches of samples in a randomised block design (Table 21). Each batch was analysed in one single day.

Table 21. Run order for TD-GC-TOF-MS non-polar and polar analysis.

A typical analytical batch included: 1 blank, 6 QC samples, and 25 clinical samples (Total 32 samples).

No. of Run	1	2	3-7	8	9-13	14	15-19	20	21-25	26	27-31	32
Sample	B	QC1	S1-5	QC2	S6-10	QC3	S11-15	QC4	S16-20	QC5	S21-25	QC6
Dilution	0%	100%	-	100%	-	100%	-	100%	-	100%	-	100%

B: blank; QC: pooled quality control; S: clinical sample (pancreatic cancer sample or control sample).

4.3.9 Data pre-processing

Two datasets were acquired from the TD-GC-TOF-MS instrument: a non-polar dataset and a polar dataset. Both datasets were initially processed by two software packages: ChromSpace and MSHub/GNPS; and the performance of the two software packages was evaluated.

When the ChromSpace was used, a representative pooled QC sample data was processed. After dynamically baseline correction (DBC) and deconvolution, a peak list of detected compounds with RT, potential identities, quantifier ions, and verification ions was created. Potential identities were obtained by comparing the NIST library with RI penalty based on the n-alkane pattern (C_8 to C_{20}). Features with RMF < 800 were removed. In order to preserve as much information as possible, NIST reported siloxanes, gases, and peaks whose S/N < 3 were kept at this stage and were checked and moved during further data pre-processing.

When the MSHub/GNPS was used, all the raw non-polar and polar data acquired from the TD-GC-TOF-MS were converted into the general .CDF format. Then the data were uploaded to the MassIVE resource library using an FTP client. In GNPS, a job was

created with all the converted files chosen for analysis. Parameters were set as MSHub/GNPS recommended. Metadata containing patient details were also uploaded. The spectral alignment, deconvolution, integration, and identification were all completed automatically online on MSHub/GNPS webpage (GNPS – Analyse, Connect, and Network with your Mass Spectrometry Data (ucsd.edu), https://gnps.ucsd.edu/ProteoSAFe/static/gnps-splash.jsp).[140,141]

Two software packages were used for further data pre-processing: Gavin 3 and MSHub/GNPS. Both the non-polar and polar data were processed and the performance of the two software packages was evaluated.

Data processed by ChromSpace were input into Gavin 3 for peak integration. Following integration, a single excel table was generated with an aligned peak table including the peak area of each compound in each sample (NIST reported compound name attached), which also included the peak areas for all the spiked internal standards.

MSHub/GNPS automatically generated aligned feature tables from the input data which comprised aligned peak areas for each compound in each sample. Peak annotation results were presented in a separate webpage where the compound name, input spectra, and the spectra from the library were visible. The main spectral library used was WILEY. Other spectral libraries involved were: NIST, FIEHN, MONA, BORISOV-COMPILATION, HMDB, AROMA-UNIV-CORSICA, and MASS SPECTROSCOPY SOC. OF JAPAN (MSSJ).[140]

The following feature filterings and normalisations were applied independently to both aligned peak tables created by Gavin 3 and MSHub/GNPS:

Blank filtering: To remove contaminants. Compounds whose average peak area in the blanks over 30% of that in the QCs were removed.

Linearity filtering: To remove compounds respond non-linearly to the serial dilution. The 1-tailed Spearman's rank correlation coefficient test was used to observe the trend of the dilution series. The Benjamini-Hochberg procedure was used to adjust

p values to correct false positives. Compounds having an adjusted p value < 0.05 and correlation coefficient > 0.7 were considered significant.

Best-matched internal standard normalisation: Selecting the internal standard that minimised the CV of replicate QCs from all the internal standards as the best matched internal standard for a compound, using this internal standard to adjust peak areas of that compound in all samples.[142]

Total peak area normalisation: Dividing the area of each peak in a profile by the total sum of all peaks within that profile.

QC normalisation:

2 QC normalisation: Dividing the area of each peak in a preparation day batch by the average of peak areas of that peak in the first and the last QC samples within that batch. Two QC samples were used because the rest QCs were used to observe if they were clustered better after the normalisation.

All QC normalisation: Dividing the area of each peak in a preparation day batch by the average of peak areas of that peak in all the QC samples within that batch. If the 2 QC normalisation improved data quality, it was assumed that using all the QC samples would produce even better results.

Osmolality normalisation: Peak areas of compounds were divided by the measured osmolality of the sample.

CV filtering: To remove non-reproducible compounds, applied in the end. Compounds with the percentage of CV higher than 30% were removed.

The number of reliable compounds obtained after each filtering was compared to determine the optimal filtering and normalisation approach that produced the highest number of compounds.

Datasets pre-processed using the optimal filterings and normalisations were included for further statistical analysis (Figure 23), including:

A non-polar dataset processed by ChromSpace/Gavin 3;

A non-polar dataset processed by MSHub/GNPS;

A polar dataset processed by ChromSpace/Gavin 3;

A polar dataset processed by MSHub/GNPS.

The entire procedure of data pre-processing is presented in Figure 23.

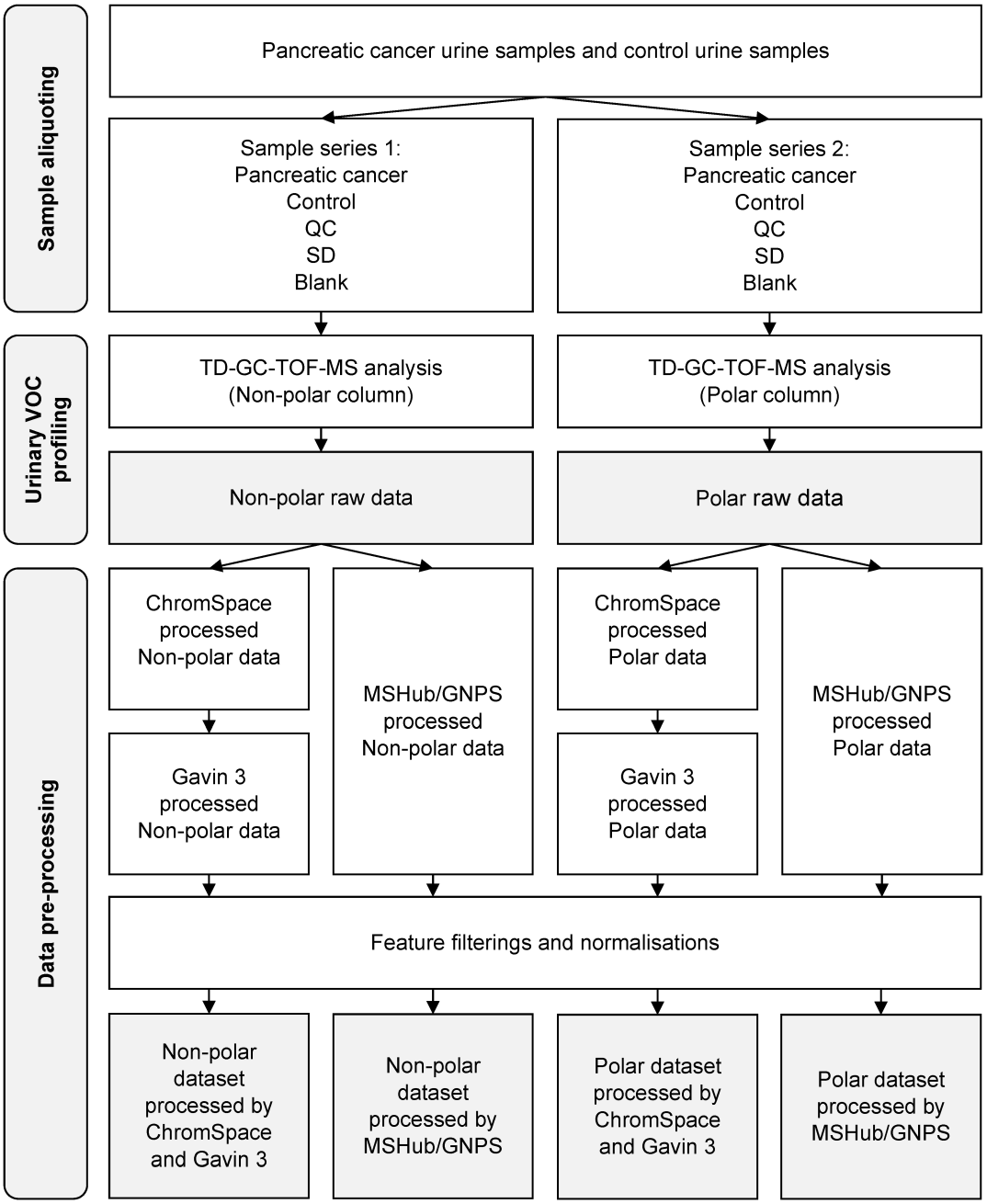


Figure 23. Flow diagram of data pre-processing steps.

The non-polar and polar raw data acquired from urinary VOC profiling were processed by ChromSpace/Gavin 3 and MSHub/GNPS, respectively, and followed by feature filterings and normalisations. QC: quality control; SD: serial dilution.

4.3.10 Statistical analysis

The datasets pre-processed using the optimal normalisations and the filterings were analysed with univariate analyses and multivariate analyses.

4.3.10.1 Univariate analysis

The nonparametric Mann-Whitney U test was used to compare differences between two independent groups (e.g., cancer and control, chemotherapy and non-chemotherapy, antibiotics and non-antibiotics). The procedure was performed in SPSS 25. The p values were input to MATLAB to perform the Benjamini-Hochberg procedure, which is a milder correction than Bonferroni correction and is widely used in metabolomics, to correct false positives.[133-135] The significant threshold were set to Benjamini-Hochberg adjusted p value < 0.25.

4.3.10.2 Multivariate analysis

The supervised partial least-squares discriminant analysis (PLS-DA) was performed in SIMCA 15 to show the separation of the samples between independent groups.[143,144] The data was log transformed (y=10log(10000x+1)) and UV scaled. PLS-DA models were constructed to observe the separation between cancer and control, chemotherapy and non-chemotherapy, antibiotics and non-antibiotics groups. The influence of confounding factors was also evaluated.

4.3.10.3 Receiver operating characteristic analysis

Receiver operating characteristic curves (ROC curves) were plotted in MetaboAnalyst 5.0 to illustrates the diagnostic ability of the potential pancreatic cancer related compounds. ROC curves were generated for multiple combined compounds. Number of features was less than 5000 thus no data filtering was applied. No sample normalisation was applied because there were no obvious systematic differences among samples. A mathematical transformation on individual peak areas themselves was performed by Log transformation (y=10log(x)). Peak areas were auto scaled (mean-centred and divided by the standard deviation of each compound).

Multivariate ROC curve based exploratory analysis tool was used to perform automated important feature identification and performance evaluation. Monte-Carlo cross validation (MCCV) with balanced subsampling was used to create ROC curves. Two-thirds (2/3) of the samples in each MCCV were used to determine the feature's significance. The top 2, 3, 5, 10...100 (max) significant characteristics were then utilised to construct classification models, which were verified using the remaining 1/3 of samples. The procedure was repeated several times to calculate each model's performance and confidence interval. Linear support vector machines (SVM) and SVM were set as the classification method and the feature ranking method, respectively.

4.4 Results

4.4.1 Patient details

A total of 131 patients were initially recruited, including 36 pancreatic cancer patients and 95 control patients. Among the 36 pancreatic cancer patients, there were 28 pancreatic adenocarcinoma (AD) patients and 8 pancreatic neuroendocrine tumour (NET) patients. Among the 95 controls, 54 were diagnosed with other gastrointestinal disorders and 8 had other synchronous cancer, therefore these patients were excluded. The remaining 33 patients were included in the final cohort. Clinico-demographic features of the final cohort are presented in Table 22.

The adenocarcinoma patient group and the control group were similar in terms of group size and were age-sex matched. Most subjects (82%) in the adenocarcinoma group had received chemotherapy at the time of urine collection. A small proportion (around 10%) of subjects in both adenocarcinoma and control group had received antibiotic treatment when their urine samples were collected. The proportion of subjects with diabetes in the pancreatic adenocarcinoma group (46%) was significantly higher than in the control group (9%).

Table 22. Characteristics of the study cohort.

All p values were calculated with Pearson Chi-Squared test.

	Pancreatic Adenocarcinoma (AD) (*n* = 28)	**Control (*n* = 33)**	**p**
Demographics			
Age (Mean ± SD)	67.9 ± 12.2	52.7 ± 17.9	0.571
Gender (M : F)	18 : 10	18 : 15	0.441
Primary tumour (T)			
T1	0 (0%)	-	
T2	0 (0%)	-	
T3	12 (43%)	-	
T4	12 (43%)	-	
Unknown	4 (14%)	-	
Reginal lymph node (N)			
N0	5 (18%)	-	
N1	16 (57%)	-	
N2	3 (11%)	-	
Unknown	4 (14%)	-	
Distant metastasis (M)			
M0	9 (32%)	-	
M1	9 (32%)	-	
Mx	6 (22%)	-	
Unknown	4 (14%)	-	
Chemotherapy	23 (82%)	0 (0%)	< 0.001
Antibiotics	4 (14%)	3 (9%)	0.526
Diabetes	13 (46%)	3 (9%)	< 0.001

4.4.2 Typical chromatograms

Examples of total ion chromatograms (TIC) for pancreatic adenocarcinoma, control, and QC samples analysed using the optimal non-polar assay are shown in Figure 24. In Figure 25, TICs represent samples that were analysed using the optimal polar assay.

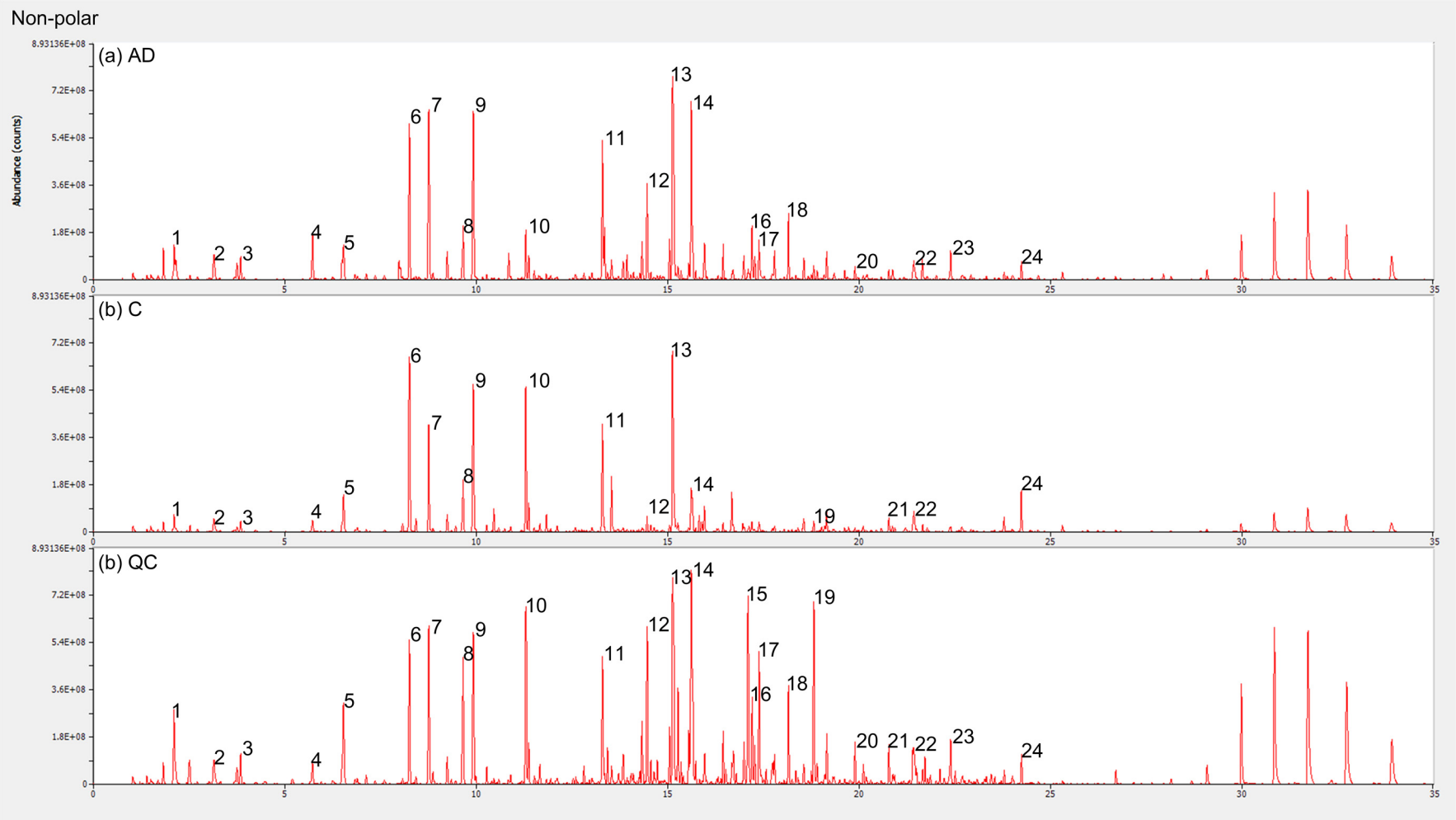


Figure 24. Typical chromatograms of non-polar assay.

Examples of total ion chromatograms for (a) pancreatic adenocarcinoma (AD), (b) control (C), and (c) QC samples analysed using the optimal non-polar assay. 1: diisobutyl cellosolve; 2: 2H-oxireno[3,4]cyclopenta[1,2-c]furan-2-one, 1a,1b,4,4a,5,5a-hexahydro-4-(dimethoxymethyl)-, (1bR,1a-cis,4-trans,4a-cis,5a-cis)-; 3: 2-ethyl-oxetane; 4: benzene-d_6; 5: 3-oxetanol, 2,2,3-trimethyl-; 6: disulfide, dimethyl; 7: toluene-d_8; 8: hexanal; 9:

cyclotrisiloxane, hexamethyl-; 10: 4-heptanone; 11: cyclotetrasiloxane, octamethyl-; 12: benzene, 1-methyl-3-(1-methylethyl)-; 13: 1H-imidazole-2-carboxaldehyde, 1-methyl-; 14: benzene, 1-methyl-4-(1-methylethenyl)-; 15: levomenthol; 16: terpinen-4-ol; 17: α-terpineol; 18: (-)-carvone; 19: 2(1H)-naphthalenone, 3,4,4a,5,6,7-hexahydro-1,1,4a-trimethyl-; 20: naphthalene, 1,2-dihydro-1,5,8-trimethyl-; 21: 4-(2,4,4-trimethyl-cyclohexa-1,5-dienyl)-but-3-en-2-one; 22: 4-(2,6,6-trimethylcyclohexa-1,3-dienyl)but-3-en-2-one; 23: α-calacorene; 24: furan, 2-[(2-ethoxy-3,4-dimethyl-2-cyclohexen-1-ylidene)methyl]-.

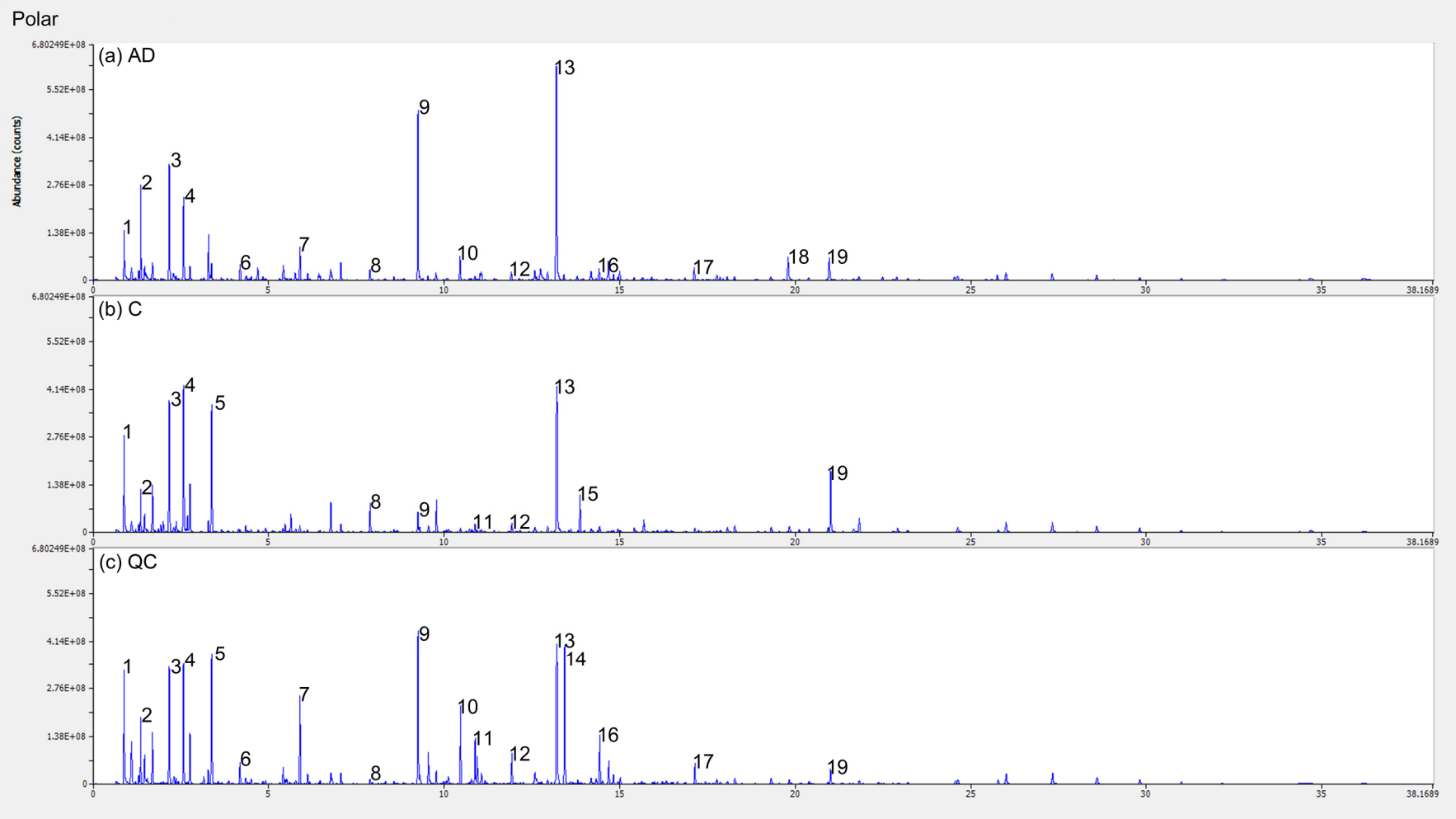


Figure 25. Typical chromatograms of polar assay.

Examples of total ion chromatograms for (a) pancreatic adenocarcinoma (AD), (b) control (C), and (c) QC samples analysed using the optimal polar assay. 1: 3-thiophenamine, tetrahydro-N-methyl-N-nitroso-, 1,1-dioxide; 2: cyclotrisiloxane, hexamethyl-; 3: toluene-d_8; 4: disulfide, dimethyl; 5: 3-hexanone, 2-methyl-; 6: 2-carene; 7: benzene, 1-methyl-3-(1-methylethyl)-; 8: dimethyl trisulfide; 9: o-isopropenyltoluene; 10: 7-octen-2-ol, 2,6-dimethyl-

; 11: 1h-2-Indenone,2,4,5,6,7,7a-hexahydro-3-(1-methylethyl)-7a-methyl; 12: linalool; 13: 3-isoxazolecarboxylic acid, 4-methyl-, ethyl ester; 14: cyclohexanol, 5-methyl-2-(1-methylethyl)-; 15: benzene, (2-methoxyethenyl)-; 16: α-terpineol; 17: benzenemethanol,α,α,4-trimethyl-; 18: phenol; 19: p-cresol.

4.4.3 Feature filtering and data normalisation

The number of reliable compounds obtained for all the datasets after each filtering and/or normalisation (details of them were discussed in Section 4.3.9) is presented in Table 23. Overall, sufficient numbers of reliable compounds were obtained. When using ChromSpace/Gavin 3, the polar data delivered more reliable compounds than the non-polar data. When using MSHub/GNPS, the non-polar data delivered more compounds than the polar data.

For the non-polar data processed by ChromSpace/Gavin 3, the largest number of reliable compounds (239) was recorded when the data was filtered by blank and linearity filtering as well as applied with the best-matched internal standard normalisation.

For the non-polar data processed by MSHub/GNPS, the largest number of 284 reliable compounds were obtained when the same blank and linearity filtering and best-matched internal standard normalisation were applied.

For the polar data processed by ChromSpace/Gavin 3, the highest number of compounds was 294 when the data were filtered by blank and linearity filtering, applied with the best-matched internal standard normalisation, plus QC normalisation.

For the polar data processed by MSHub/GNPS, 238 compounds were considered reliable after the data were filtered by the same blank and linearity filtering, applied with the best-matched internal standard normalisation and the QC normalisation.

These findings suggested that the optimal normalisation approaches that produced the highest number of compounds, regardless the processing programmes, were:

Non-polar data: Blank filtering and linearity filtering, plus best-matched internal standard normalisation;
Polar data: Blank filtering and linearity filtering, plus best-matched internal standard and all QC normalisation.

Therefore, four optimal datasets were determined and were used for data analysis, including:

A non-polar dataset processed by ChromSpace/Gavin 3 filtered by blank filtering and linearity filtering, plus best-matched internal standard normalisation, eventually filtered by CV filtering (abbreviated as non-polar ChromSpace/Gavin 3);

A non-polar dataset processed by MSHub/GNPS filtered by blank filtering and linearity filtering, plus best-matched internal standard normalisation, eventually filtered by CV filtering (non-polar MSHub/GNPS);

A polar dataset processed by ChromSpace/Gavin 3 filtered by blank filtering and linearity filtering, plus best-matched internal standard and all QC normalisation, eventually filtered by CV filtering (polar ChromSpace/Gavin 3);

A polar dataset processed by MSHub/GNPS filtered by blank filtering and linearity filtering, plus best-matched internal standard and all QC normalisation, eventually filtered by CV filtering (polar MSHub/GNPS).

Table 23. The number of reliable compounds.

The number of reliable compounds acquired after different normalisations and filterings using the two different data processing pipelines.

Normalisation and Filtering	Data Processing Pipeline			
	ChromSpace / Gavin 3		MSHUB / GNPS	
	Non-polar	Polar	Non-polar	Polar
Raw data	272	324	312	203
Best-matched IS normalised	294	324	352	193
B L filtered raw data	219	261	264	183
B L filtered O normalised	219	261	264	183
B L filtered TA normalised	232	268	276	198
B L filtered 2 QC normalised	224	294	261	246
B L filtered all QC normalised	-	-	-	-
B L filtered best-matched IS normalised	239	264	284	173
B L filtered best-matched IS 2 QC normalised	228	294	274	238
B L filtered best-matched IS all QC normalised	-	> 294	-	> 238
B L filtered best-matched IS O normalised CV filtered	232	264	284	173

IS: internal standards; B: blank filtering; L: linearity filtering; O: osmolality normalisation; TA: total area normalisation; QC: QC normalisation; CV: coefficient of variation filtering.

4.4.4 Data quality assessment

An assessment of data quality was conducted using the QC samples. The four optimal datasets determined in Section 4.4.3 were used to generate PCA scores plots in SIMCA 15. The PCA plots revealed highly close grouping of QCs which were presented along with the study samples (Figure 26). There were fewer QC samples shown in the PCA for polar data because some of the QC samples were used to conduct QC normalisation.

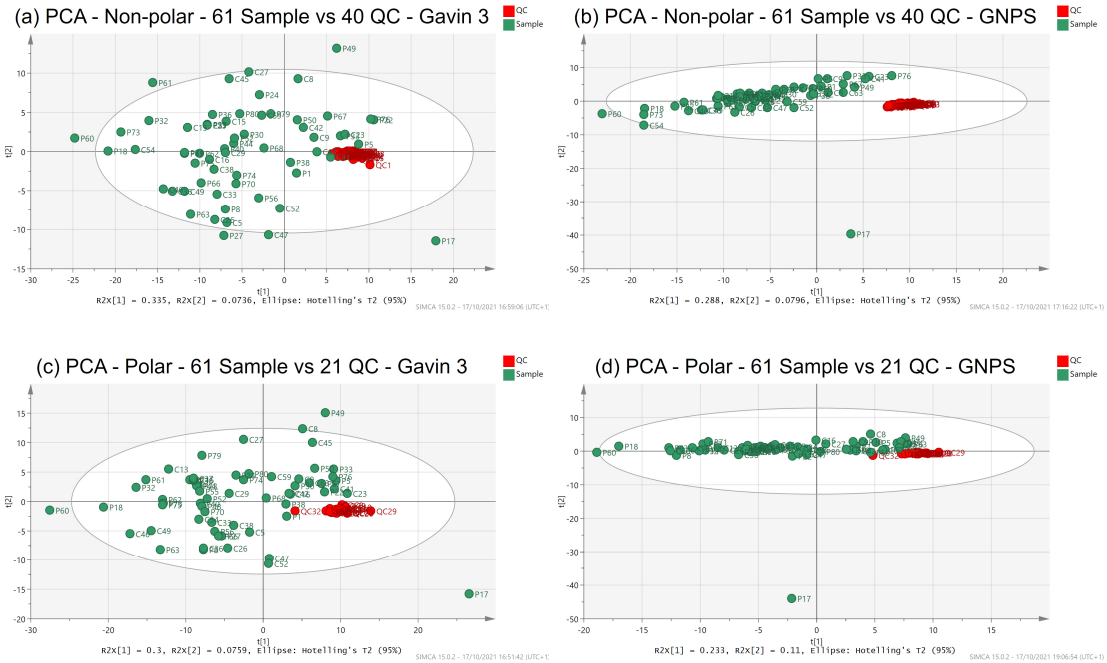


Figure 26. Data quality assessment using QC samples.

Principal component analysis (PCA) score plots were based on study samples and QC samples. The PCA score plots showed that QCs were presented along with the study samples and highly clustered in (a) non-polar Gavin 3, (b) non-polar GNPS, (c) polar Gavin 3, and (d) polar GNPS datasets, indicating good data quality.

4.4.5 Univariate analysis

The Mann-Whitney U test results were presented for the four optimal datasets to show the urinary VOCs with significantly changed abundance in pancreatic cancer, with the comparison of the ChromSpace/Gavin 3 pipeline and the MSHub/GNPS pipeline.

4.4.5.1 Non-polar results

4.4.5.1.1 Non-polar ChromSpace/Gavin 3

For the optimal non-polar dataset processed by ChromSpace/Gavin 3, 9 out of 239 compounds, namely phosphonic acid, (p-hydroxyphenyl)- (adjusted p value = 0.0946); 6-[4-chlorophenyl]-N,N-diethyl-1,2,4,5-tetrazin-3-amine (adjusted p value = 0.0946); Ethane, 1-isothiocyanato-2-methoxy- (adjusted p value = 0.117); N-CBZ-l-dihydrotryptophanyl-l-alanine (adjusted p value = 0.126); 2-buten-1-one, 1-(2,6,6-trimethyl-1,3-cyclohexadien-1-yl)-, (E)- (adjusted p value = 0.142; 1,3-dioxolane, 2-(bromomethyl)-2-(2-methylphenyl)- (adjusted p value = 0.158); 2,3,6-trifluorobenzyl alcohol, methyl ether (adjusted p value = 0.158); didodecyl phosphate (adjusted p value = 0.0946), and; ethyl 2 - (2 - chloroacetamido) - 3,3,3 - trifluoro - 2 - (4 - fluorobenzylamino) propionate (adjusted p value = 0.126) were significantly different (adjusted p value < 0.25) in patients with pancreatic adenocarcinoma compared to controls (Figure 27). Details of the compounds including retention time (RT), top ions, and p values were summarised in Table 24.

The abundance of the majority of the compounds were higher in adenocarcinoma compared to controls, whereas the abundance of didodecyl phosphate and ethyl 2 - (2 - chloroacetamido) - 3,3,3 - trifluoro - 2 - (4 - fluorobenzylamino) propionate were higher in controls.

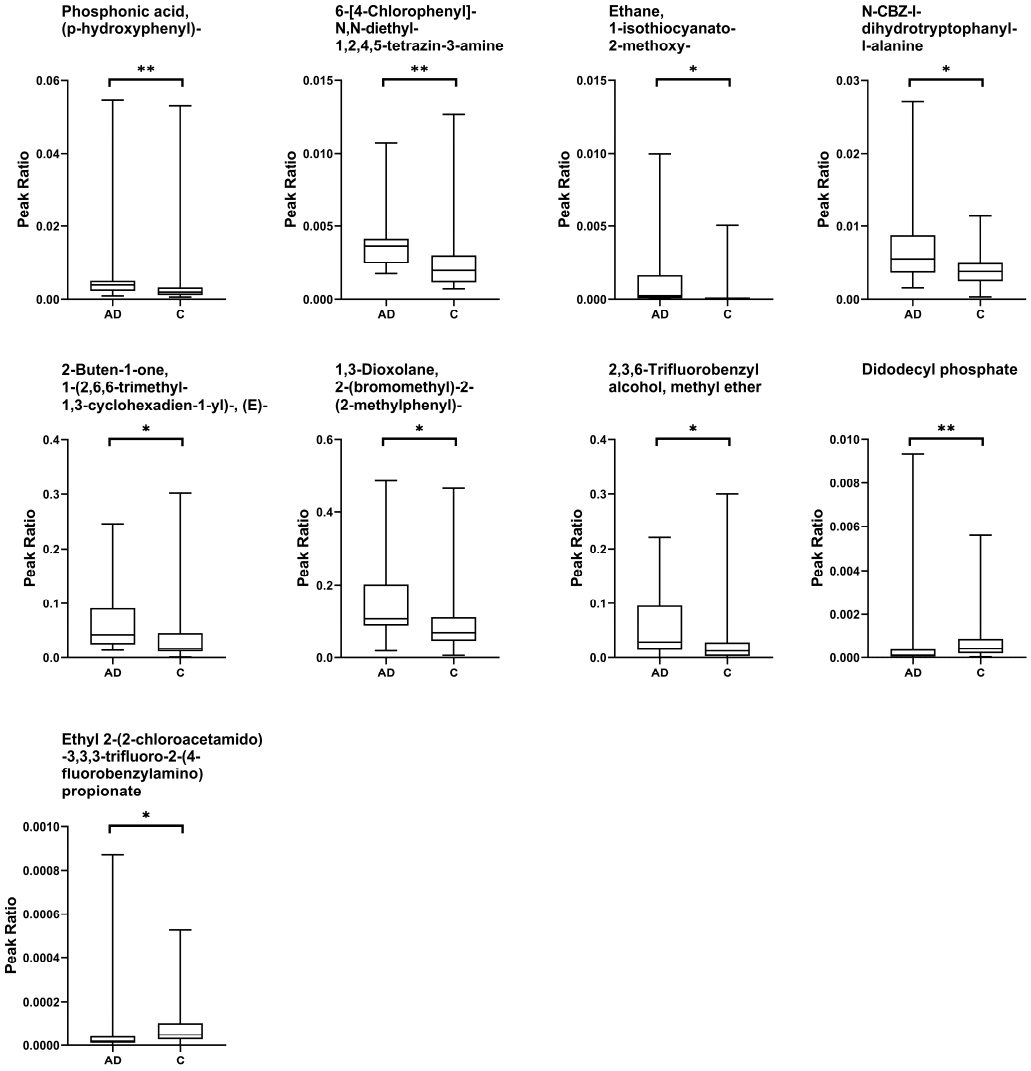


Figure 27. Abundance of significant urinary VOCs (non-polar, ChromSpace/Gavin 3).

Abundance of urinary VOCs that were significantly different in patients with pancreatic adenocarcinoma (AD) (*n* = 28) compared to controls (C) (*n* = 33). The optimal non-polar dataset processed by ChromSpace/Gavin 3 was used. *: FDR corrected p value < 0.25; **: FDR corrected p value < 0.1.

4.4.5.1.2 Non-polar MSHub/GNPS

For the optimal non-polar dataset processed by MSHub/GNPS, 13 out of 284 compounds were significantly different (adjusted p value < 0.25) in patients with pancreatic adenocarcinoma compared with controls (Figure 28). The 13 compounds were: 2-butanol, 2-methyl- (adjusted p value = 0.0798); butanal, 3-methyl- (adjusted p value = 0.0235); 2-pentanone (adjusted p value = 0.154); dimethylphosphinic fluoride (adjusted p value = 0.131); 2-vinylfuran (adjusted p value = 0.0652); 4-(bicyclo[2.2.1]heptan-2-ylmethyl)morpholine (adjusted p value = 0.131); furan, 2-methoxy- (adjusted p value = 0.0356); (3S,4S)-2-methyl-4-(methylamino)-3-phenyl-2-pentanol (adjusted p value = 0.200); phenylacetaldehyde (adjusted p value = 0.237); (2E)-1-(2,6,6-trimethyl-1,3-cyclohexadien-1-yl)-2-buten-1-one (adjusted p value = 0.154); 2,6-Di-tert-butyl-4-methyl-phenol (adjusted p value = 0.0652); benzene, 1-(1,5-dimethyl-4-hexenyl)-4-methyl- (adjusted p value = 0.131), and; 2-cyclopenten-1-one, 2,3,4-trimethyl- (adjusted p value = 0.0798). Details of the compounds were summarised in Table 24.

The abundance of the majority of the compounds were higher in adenocarcinoma compared to controls, whereas the abundance of 2-butanol, 2-methyl-; 2-pentanone; benzene, 1-(1,5-dimethyl-4-hexenyl)-4-methyl-, and; 2-cyclopenten-1-one, 2,3,4-trimethyl- were higher in controls.

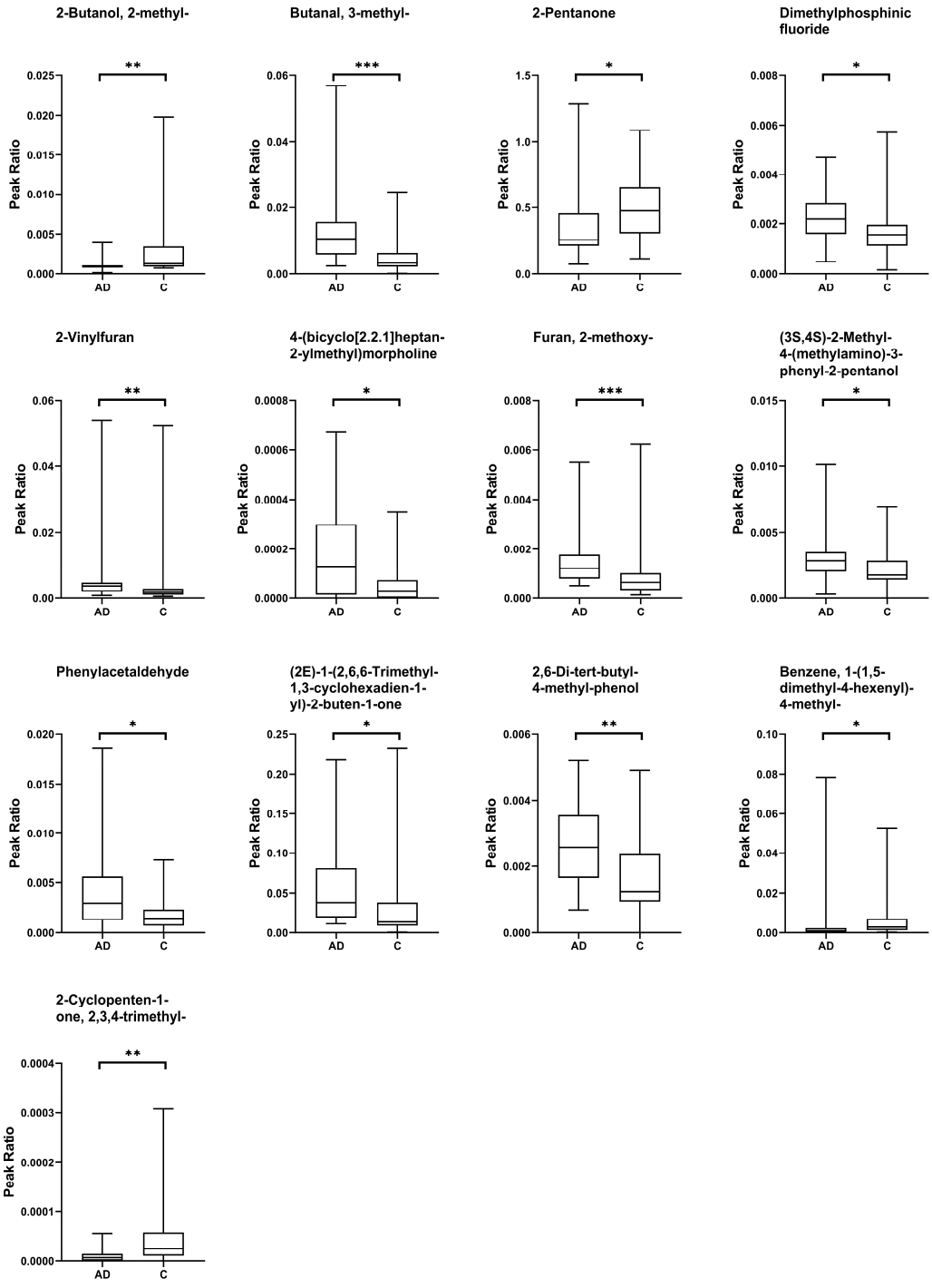


Figure 28. Abundance of significant urinary VOCs (non-polar, MSHub/GNPS).

Abundance of urinary VOCs that were significantly different in patients with pancreatic adenocarcinoma (AD) (*n* = 28) compared to controls (C) (*n* = 33). The optimal non-polar dataset processed by MSHub/GNPS was used. *: FDR corrected p value < 0.25; **: FDR corrected p value < 0.1; ***: FDR corrected p value < 0.05.

4.4.5.1.3 Non-polar ChromSpace/Gavin 3 and MSHub/GNPS comparison

Table 24 compared the outcomes of the two data processing pipelines. Five of the significant compounds were identified by both ChromSpace/Gavin 3 and MSHUB/GNPS (RT 7.63, top ions 94, 65, 66; RT 9.57, top ions 83, 98, 59; RT 14.80, top ions 91, 130, 115; RT 20.11, top ions 69, 121, 41; and RT 25.76, top ions 109, 124, 269). One compound (RT 13.64, top ions 58, 118, 117) was found to be significant when processed using MSHUB/GNPS but were not significant when using ChromSpace/Gavin 3. Two compounds (RT 20.42, top ions 163, 119, 93 and RT 20.67, top ions 145, 149, 176) were found to be significant only when processed using ChromSpace/Gavin 3. For above compounds, different compound names were provided for the same peak by the two different data processing pipelines. The remaining compounds were identified by one of the two pipelines only.

Table 24. Summary of significant urinary VOCs (non-polar).

The retention time, top ions, compound name, p values and false discovery rate (FDR) corrected p values of the urinary VOCs that were significantly different in patients with pancreatic adenocarcinoma (AD) (*n* = 28) compared to controls (C) (*n* = 33). The optimal non-polar datasets processed by ChromSpace/Gavin 3, as well as MSHub/GNPS were used. The p values were adjusted using the Benjamini-Hochberg procedure for FDR correction.

Retention Time (RT)	Top Ions	Data Processing Pipeline					
		ChromSpace / Gavin 3			MSHUB / GNPS		
		Compound Name	p value	FDR Corrected p value	Compound Name	p value	FDR Corrected p value
5.20	59, 55, 73	-	-	-	2-Butanol, 2-methyl-	0.00169	0.0798**
5.66	41, 44, 58	-	-	-	Butanal, 3-methyl-	0.0000826	0.0235***
6.53	43, 86, 41	-	-	-	2-Pentanone	0.00596	0.154*
6.95	81, 96, 53	-	-	-	Dimethylphosphinic fluoride	0.00362	0.131*
7.63	94, 65, 66	Phosphonic acid, (p-hydroxyphenyl)-	0.000542	0.0946**	2-Vinylfuran	0.000919	0.0652**
9.50	100, 69, 109	-	-	-	4-(bicyclo[2.2.1]heptan-2-ylmethyl)morpholine	0.00372	0.131*
9.57	83, 98, 59	6-[4-Chlorophenyl]-N,N-diethyl-1,2,4,5-tetrazin-3-amine	0.00119	0.0946**	Furan, 2-methoxy-	0.000250	0.0356***
11.88	45, 72, 123	Ethane, 1-isothiocyanato-2-methoxy-	0.00195	0.117*	-	-	-
13.64	58, 118, 117	2-Pentanone, 5-bromo-	0.0240	0.350	(3S,4S)-2-Methyl-4-(methylamino)-3-phenyl-2-pentanol	0.00844	0.200*
14.80	91, 130, 115	N-CBZ-l-dihydrotryptophanyl-l-alanine	0.00315	0.126*	Phenylacetaldehyde	0.0108	0.237*
20.11	69, 121, 41	2-Buten-1-one, 1-(2,6,6-trimethyl-1,3-cyclohexadien-1-yl)-, (E)-	0.00416	0.142*	(2E)-1-(2,6,6-Trimethyl-1,3-cyclohexadien-1-yl)-2-buten-1-one	0.00570	0.154*
20.42	163, 119, 93	1,3-Dioxolane, 2-(bromomethyl)-2-(2-methylphenyl)-	0.00596	0.158*	(7,7-Dimethyl-1-oxo-2,3,4,5,6,7-hexahydro-1H-inden-2-yl)acetic acid, ethyl ester	0.0774	0.390
20.67	145, 149, 176	2,3,6-Trifluorobenzyl alcohol, methyl ether	0.00557	0.158*	Methyl trans-4-methylcinnamate	-	-
20.69	99, 42, 110	Didodecyl phosphate	0.00102	0.0946**	-	-	-
21.06	58, 43, 93	-	-	-	2,6-Di-tert-butyl-4-methyl-phenol	0.000746	0.0652**
23.08	119, 132, 202	-	-	-	Benzene, 1-(1,5-dimethyl-4-hexenyl)-4-methyl-	0.00416	0.131*
25.76	109, 124, 269	Ethyl 2-(2-chloroacetamido)-3,3,3-trifluoro-2-(4-fluorobenzylamino)propionate	0.00267	0.126*	2-Cyclopenten-1-one, 2,3,4-trimethyl-	0.00145	0.0798**

*: FDR corrected p value < 0.25; **: FDR corrected p value < 0.1; ***: FDR corrected p value < 0.05.

4.4.5.2 Polar results

4.4.5.2.1 Polar ChromSpace/Gavin 3

For the optimal polar dataset processed by ChromSpace/Gavin 3, 11 out of 294 compounds, namely 2-pentanone (adjusted p value = 0.190); 2-buten-1-amine, N-butyl-, (E)- (adjusted p value = 0.190); 5-(2-thienoyl)butyric acid (adjusted p value = 0.00413); 7-octen-2-ol, 2,6-dimethyl- (adjusted p value = 0.190); 1-hydroxy-3-phenylacetone (adjusted p value = 0.190); 1,1,4,5,6-pentamethyl-2,3-dihydro-1H-indene (adjusted p value = 0.190); 1,3-benzenediamine, 4-methoxy- (adjusted p value = 0.190); 1,1,1,3,5,5,5-heptamethyltrisiloxane (adjusted p value = 0.190); benzoic acid, 4-ethoxy-, ethyl ester (adjusted p value = 0.174); 2(3H)-furanone, 5-heptyldihydro- (adjusted p value = 0.0986), and; 4'-tert-butyl-2',6'-dimethylacetophenone (adjusted p value = 0.190) were significantly different (adjusted p value < 0.25) in patients with pancreatic adenocarcinoma compared to controls (Figure 29). Details of the compounds including retention time (RT), top ions, and p values were presented in Table 25.

The abundance of 5-(2-thienoyl)butyric acid; 1-hydroxy-3-phenylacetone; 1,3-benzenediamine, 4-methoxy-; benzoic acid, 4-ethoxy-, ethyl ester, and; 2(3H)-furanone, 5-heptyldihydro- were higher in adenocarcinoma compared to controls, whereas the abundance of 2-pentanone; 2-buten-1-amine, N-butyl-, (E)-; 7-octen-2-ol, 2,6-dimethyl-; 1,1,4,5,6-pentamethyl-2,3-dihydro-1H-indene; 1,1,1,3,5,5,5-heptamethyltrisiloxane, and; 4'-tert-butyl-2',6'-dimethylacetophenone were higher in controls.

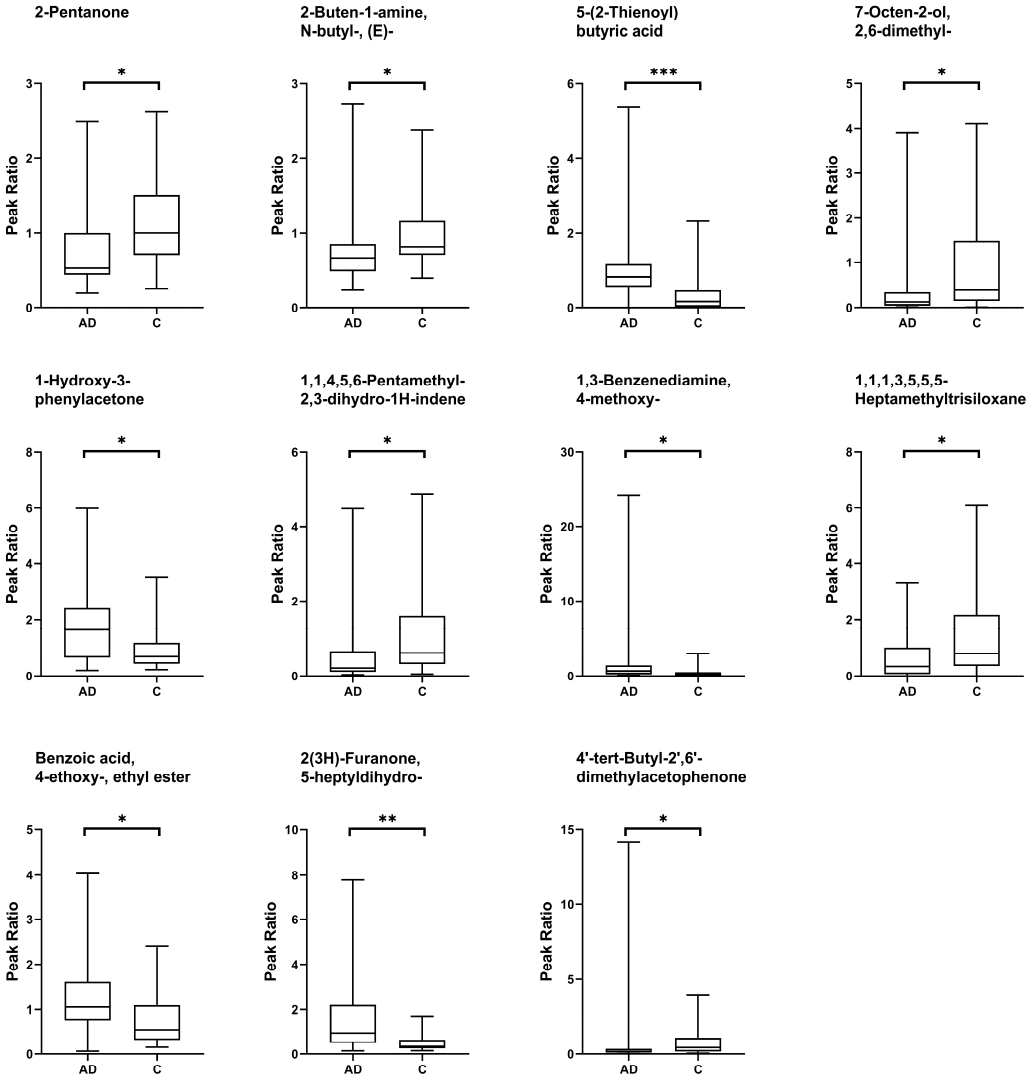


Figure 29. Abundance of significant urinary VOCs (polar, ChromSpace/Gavin 3).

Abundance of urinary VOCs that were significantly different in patients with pancreatic adenocarcinoma (AD) (*n* = 28) compared to controls (C) (*n* = 33). The optimal polar dataset processed by ChromSpace/Gavin 3 was used. *: FDR corrected p value < 0.25; **: FDR corrected p value < 0.1; ***: FDR corrected p value < 0.05.

4.4.5.2.2 Polar MSHub/GNPS

For the optimal polar dataset processed by MSHub/GNPS, 15 out of 238 compounds were significantly different (adjusted p value < 0.25) in patients with pancreatic adenocarcinoma compared with controls (Figure 30). The 15 compounds were: 2-butanone, 3-methyl- (adjusted p value = 0.0549); 2-pentanone (adjusted p value = 0.133); cyclohexanone, 4R-acetamido-2,3-cis-epoxy- (adjusted p value = 0.0400); dodecyl fluoroacetate (adjusted p value = 0.133); 3-nonanone (adjusted p value = 0.177); isobutyl-2-heptenone (adjusted p value = 0.133); myrcenol-dihydro (adjusted p value = 0.133); cyclopentanone, 3,4-bis(2-oxopropyl)-, trans- (adjusted p value = 0.145); taylorione (adjusted p value = 0.133); 1,1,5,6-tetramethyl-1,2,3,4-tetrahydronaphthalene (adjusted p value = 0.133); benzene, 2-(1-decylundecyl)-1,4-dimethyl- (adjusted p value = 0.189); thiourea, 1-(2,4,6-trimethylphenyl)-3-(2-propynyl)- (adjusted p value = 0.133); alpha bisabolol (adjusted p value = 0.220); curcumenol (adjusted p value = 0.133), and; 10,13-dimethyl-2,3,4,7,8,9,11,12,14,15-decahydro-1H-cyclopenta[a]phenanthren-3-ol (adjusted p value = 0.160). Details of the compounds were summarised in Table 25.

The abundance of the majority of the compounds were higher in controls compared to adenocarcinoma, whereas the abundance of dodecyl fluoroacetate and cyclopentanone, 3,4-bis(2-oxopropyl)-, trans- were higher in adenocarcinoma.

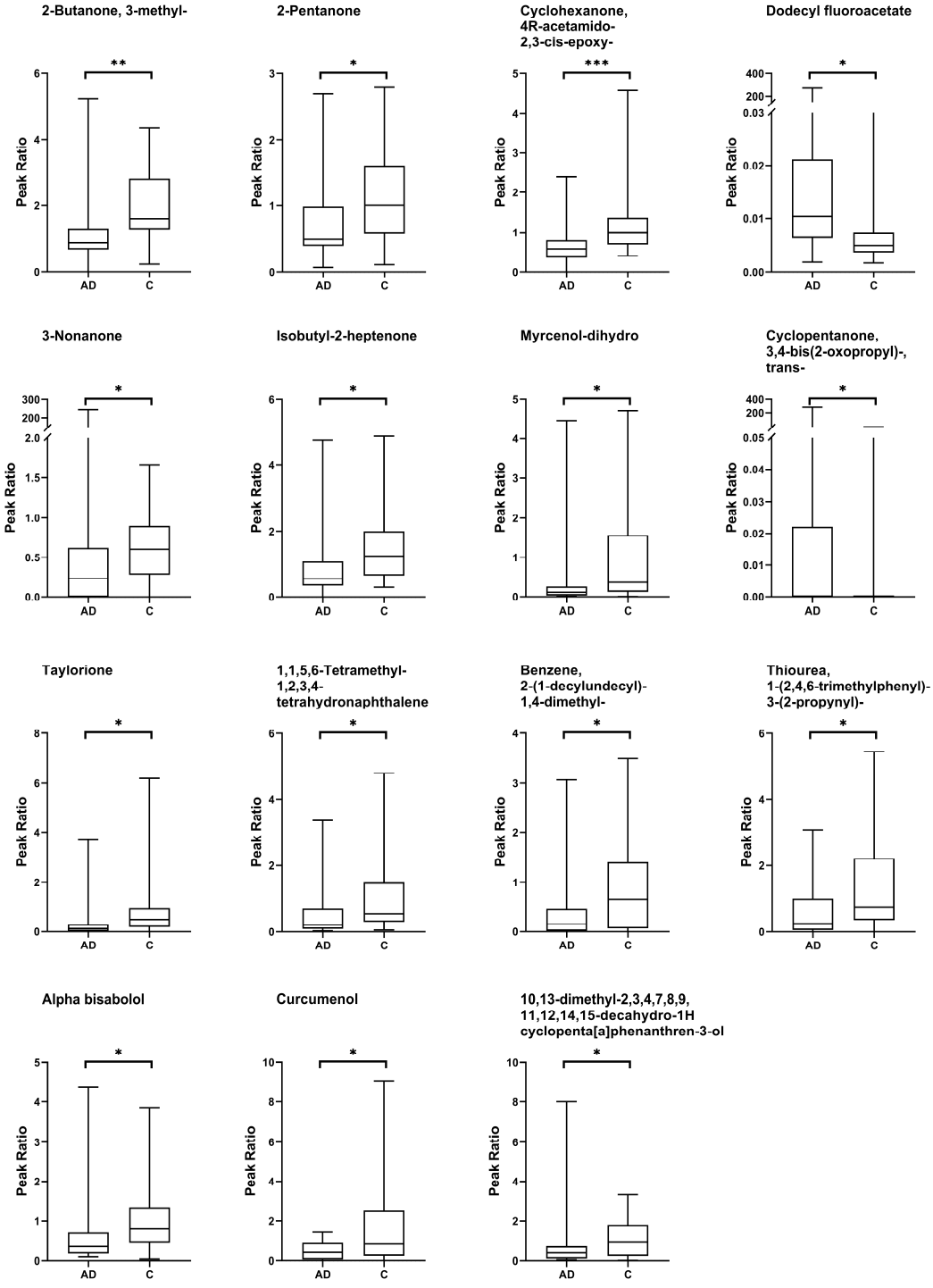


Figure 30. Abundance of significant urinary VOCs (polar, MSHub/GNPS).

Abundance of urinary VOCs that were significantly different in patients with pancreatic adenocarcinoma (AD) (*n* = 28) compared to controls (C) (*n* = 33). The optimal polar dataset processed by MSHub/GNPS was used. *: FDR corrected p value < 0.25; **: FDR corrected p value < 0.1; ***: FDR corrected p value < 0.05.

4.4.5.2.3 Polar ChromSpace/Gavin 3 and MSHUB/GNPS comparison

Table 25 compared the outcomes of the two data processing pipelines. Four of the significant compounds were identified at the same time by both ChromSpace/Gavin 3 and MSHUB/GNPS. Three compounds (RT 15.41, top ions 91, 150, 119; RT 24.77, top ions 85, 57, 55; RT 26.75, top ions 189, 147, 204) were found to be significant when processed using ChromSpace/Gavin 3 pipeline but were not significant when using MSHUB/GNPS. For above compounds, different compound names were provided for the same peak by the two different data processing pipelines. The remaining compounds were identified by one of the two pipelines only.

Table 25. Summary of significant urinary VOCs (polar).

The retention time, top ions, compound name, p values and false discovery rate (FDR) adjusted p values of the urinary VOCs that were significantly different in patients with pancreatic adenocarcinoma (AD) (*n* = 28) compared to controls (C) (*n* = 33). The optimal polar datasets processed by ChromSpace/Gavin 3, as well as MSHub/GNPS were used. The p values were adjusted using the Benjamini-Hochberg procedure for FDR correction.

Retention Time (RT)	Top Ions	Data Processing Pipeline					
		ChromSpace / Gavin 3			MSHUB / GNPS		
		Compound Name	p value	FDR Corrected p value	Compound Name	p value	FDR Corrected p value
1.43	43, 86, 41	Acetic acid ethenyl ester	-	-	2-Butanone, 3-methyl-	0.000461	0.0549**
1.68	43, 86, 41	2-Pentanone	0.00315	0.190*	2-Pentanone	0.00522	0.133*
2.83	43, 84, 55	-	-	-	Cyclohexanone, 4R-acetamido-2,3-cis-epoxy-	0.000168	0.0400***
2.84	84, 55, 32	2-Buten-1-amine, N-butyl-, (E)-	0.00623	0.190*	-	-	-
4.26	56, 98, 61	-	-	-	Dodecyl fluoroacetate	0.00477	0.133*
6.61	113, 81, 72	-	-	-	3-Nonanone	0.00966	0.177*
6.67	111, 126, 148	5-(2-Thienoyl)butyric acid	0.0000141	0.00413***	-	-	-
7.61	43, 95, 110	-	-	-	Isobutyl-2-heptenone	0.00226	0.133*
10.45	59, 55, 41	7-Octen-2-ol, 2,6-dimethyl-	0.00710	0.190*	Myrcenol-dihydro	0.00546	0.133*
11.55	138, 152, 140	-	-	-	Cyclopentanone, 3,4-bis(2-oxopropyl)-, trans-	0.00670	0.145*
15.41	91, 150, 119	1-Hydroxy-3-phenylacetone	0.00623	0.190*	Ethanone, 1-(2-methylphenyl)-	0.0442	0.363
16.04	145, 160, 147	-	-	-	Taylorione	0.00300	0.133*
16.17	173, 174, 188	1,1,4,5,6-Pentamethyl-2,3-dihydro-1H-indene	0.00546	0.190*	1,1,5,6-Tetramethyl-1,2,3,4-tetrahydronaphthalene	0.00522	0.133*
18.80	123, 138, 95	1,3-Benzenediamine, 4-methoxy-	0.00570	0.190*	-	-	-
20.18	119, 189, 173	-	-	-	Benzene, 2-(1-decylundecyl)-1,4-dimethyl-	0.0111	0.189*
20.63	159, 73, 207	1,1,1,3,5,5,5-Heptamethyltrisiloxane	0.00680	0.190*	Thiourea, 1-(2,4,6-trimethylphenyl)-3-(2-propynyl)-	0.00557	0.133*
21.89	121, 149, 194	Benzoic acid, 4-ethoxy-, ethyl ester	0.00177	0.174*	-	-	-
22.78	69, 93, 43	-	-	-	Alpha bisabolol	0.0139	0.220*

24.77	85, 57, 55	2(3H)-Furanone, 5-heptyldihydro-	0.000671	0.0986**	.gamma.-Dodecalactone	-	-
25.00	133, 201, 148	-	-	-	Curcumenol	0.00346	0.133*
25.58	254, 91, 146	-	-	-	10,13-dimethyl-2,3,4,7,8,9,11,12,14,15-decahydro-1Hcyclopenta[a]phenanthren-3-o	0.00808	0.160*
26.75	189, 147, 204	4'-tert-Butyl-2',6'-dimethylacetophenone	0.00499	0.190*	Benzenepropanal, 3-(1,1-dimethylethyl)-.alpha.-methyl-	-	-

*: FDR corrected p value < 0.25; **: FDR corrected p value < 0.1; ***: FDR corrected p value < 0.05.

4.4.5.3 Summary of non-polar and polar results.

Table 26 summarises all identified significant VOCs ranked by their FDR corrected p values, with information including chemical abstracts service (CAS) registry number or NIST number, chemical classes, as well as possible origin, application and related diseases.

Table 26. Summary of significant urinary VOCs ranked by FDR corrected p value.

Top Ions	Compound Name	Common Synonym	CAS No. or NIST No.	Chemical Class	Possible Origin, Applications and Related Diseases	FDR Corrected p value	Non-polar or Polar Assay	Data Pre-processing Pipeline
111, 126, 148	5-(2-Thienoyl)butyric acid	-	22971-62-6	Sulfur Compounds; Organic acids	-	0.00413***	Polar	ChromSpace /Gavin 3
41, 44, 58	Butanal, 3-methyl-	Isovaleraldehyde	590-86-3	Aldehydes	Used as chemical raw materials, rubber accelerators, and food fragrances; Food; Related to hepatic encephalopathy, ulcerative colitis, non-alcoholic fatty liver disease, celiac disease, Crohn's disease, clostridium difficile infection, and perillyl alcohol administration for cancer treatment	0.0235***	Non-polar	MSHub/GNPS
83, 98, 59	Furan, 2-methoxy-	2-Methoxyfuran	25414-22-6	Aromatic compounds; Furans; Ethers	-	0.0356***	Non-polar	MSHub/GNPS
43, 84, 55	Cyclohexanone, 4R-acetamido-2,3-cis-epoxy-	-	NIST#: 153727	Ketones	-	0.0400***	Polar	MSHub/GNPS
43, 86, 41	2-Butanone, 3-methyl-	Methyl isopropyl ketone (MIPK)	563-80-4	Ketones	Mainly used as dye intermediates, and can be used in medicine, pesticide, textile, paint, mineral processing, and other industries; Used as a solvent, also used in plastics and organic synthesis; dewaxing agent	0.0549**	Polar	MSHub/GNPS
94, 65, 66	2-Vinylfuran	-	1487-18-9	Aromatic compounds; Furans	-	0.0652**	Non-polar	MSHub/GNPS
58, 43, 93	2,6-Di-tert-butyl-4-methyl-phenol	Butylated hydroxytoluene (BHT)	128-37-0	Aromatic compounds; Phenylpropanes; Benzenoids	Pharmaceutic aid (antioxidant); Food; Environmental pollution	0.0652**	Non-polar	MSHub/GNPS
59, 55, 73	2-Butanol, 2-methyl-	tert-Pentanol	75-85-4	Alcohols	Synthetic fragrance, pesticide, medicine, and colour film	0.0798**	Non-polar	MSHub/GNPS
109, 124, 269	2-Cyclopenten-1-one, 2,3,4-trimethyl-	2,3,4-Trimethylcyclopent-2-enone	83321-16-8	Ketenes	-	0.0798**	Non-polar	MSHub/GNPS
99, 42, 110	Didodecyl phosphate	-	7057-92-3	Phosphorus compounds; Esters	-	0.0946**	Non-polar	ChromSpace /Gavin 3

85, 57, 55	2(3H)-Furanone, 5-heptyldihydro-	Peach aldehyde; Peach lactone; gamma-Undecalatone	104-67-6	Organoheterocyclic compounds; Lactones	Food additive	0.0986**	Polar	ChromSpace /Gavin 3
45, 72, 123	Ethane, 1-isothiocyanato-2-methoxy-	-	38663-85-3	Sulfur Compounds; Isocyanates; Esters	-	0.117*	Non-polar	ChromSpace /Gavin 3
91, 130, 115	N-CBZ-l-dihydrotryptophanyl-l-alanine	-	NIST#: 133102	Aromatic compounds; Nitrogen compounds	-	0.126*	Non-polar	ChromSpace /Gavin 3
109, 124, 269	Ethyl 2-(2-chloroacetamido)-3,3,3-trifluoro-2-(4-fluorobenzylamino)propionate	-	339352-65-7	Aromatic compounds; Fluorine compounds; Esters	-	0.126*	Non-polar	ChromSpace /Gavin 3
81, 96, 53	Dimethylphosphinic fluoride	-	753-70-8	Phosphorus compounds; Fluorine compounds	-	0.131*	Non-polar	MSHub/GNPS
100, 69, 109	4-(bicyclo[2.2.1]heptan-2-ylmethyl)morpholine	-	-	Bicyclic monoterpenoids	-	0.131*	Non-polar	MSHub/GNPS
119, 132, 202	Benzene, 1-(1,5-dimethyl-4-hexenyl)-4-methyl-	alpha-Curcumene	644-30-4	Aromatic compounds; Prenol lipids	Food; Antioxidant and antimicrobial effects	0.131*	Non-polar	MSHub/GNPS
43, 86, 41	2-Pentanone	-	107-87-9	Ketones	Food; Related to ulcerative colitis, celiac disease, non-alcoholic fatty liver disease, and Crohn's disease	0.133*	Polar	MSHub/GNPS
56, 98, 61	Dodecyl fluoroacetate	Fluoro-acetic acid dodecyl ester	408-14-0	Fluorine compounds; Esters	-	0.133*	Polar	MSHub/GNPS
43, 95, 110	Isobutyl-2-heptenone	-	NIST#: 132080	Ketenes	-	0.133*	Polar	MSHub/GNPS
59, 55, 41	Myrcenol-dihydro	(R)-2,6-Dimethyl-7-octen-2-ol; Dihydromyrcenol	53219-21-9	Alcohols; Enols	Used in white lemon, cologne, and citrus flavours; Used in essence and soap detergent fragrance; Related to prostate cancer	0.133*	Polar	MSHub/GNPS
145, 160, 147	Taylorione	4-[(1S,3R)-2,2-dimethyl-3-(5-methylidenecyclopenten-1-yl)cyclopropyl]butan-2-one	55304-00-2	Ketones	-	0.133*	Polar	MSHub/GNPS
173, 174, 188	1,1,5,6-Tetramethyl-1,2,3,4-tetrahydronaphthalene	4,4,7,8-tetramethyl-2,3-dihydro-1H-naphthalene	31197-54-3	Aromatic compounds; Naphthalenes	-	0.133*	Polar	MSHub/GNPS
159, 59, 145	Thiourea, 1-(2,4,6-trimethylphenyl)-3-(2-propynyl)-	-	NIST#: 267727	Aromatic compounds; Sulfur compounds; Thiourea;	-	0.133*	Polar	MSHub/GNPS

				Nitrogen compounds; Alkynes				
133, 201, 148	Curcumenol	-	19431-84-6	Prenol lipids	Efficient CYP3A4 inhibitor; An important extract of Curcuma zedoaria; Neuroprotective, anti-inflammatory, anti-tumour, anti-early pregnancy, anti-coagulation, anti-oxidation, and hepatoprotective effects	0.133*	Polar	MSHub/GNPS
69, 121, 41	2-Buten-1-one, 1-(2,6,6-trimethyl-1,3-cyclohexadien-1-yl)-, (E)-	beta-Damascenone	23726-93-4	Ketones; Enones	Food; Perillyl alcohol administration for cancer treatment	0.142*	Non-polar	ChromSpace /Gavin 3
138, 152, 140	Cyclopentanone, 3,4-bis(2-oxopropyl)-, trans-	-	NIST#: 310481	Ketones	-	0.145*	Polar	MSHub/GNPS
43, 86, 41	2-Pentanone	-	107-87-9	Ketones	Food; Related to ulcerative colitis, celiac disease, non-alcoholic fatty liver disease, and Crohn's disease	0.154*	Non-polar	MSHub/GNPS
163, 119, 93	1,3-Dioxolane, 2-(bromomethyl)-2-(2-methylphenyl)-	-	24169-43-5	Aromatic compounds; Acetals; Bromine compounds	-	0.158*	Non-polar	ChromSpace /Gavin 3
145, 149, 176	2,3,6-Trifluorobenzyl alcohol, methyl ether	-	NIST#: 364213	Aromatic compounds; Fluorine compounds; Esters	-	0.158*	Non-polar	ChromSpace /Gavin 3
254, 91, 146	10,13-dimethyl-2,3,4,7,8,9,11,12,14,15-decahydro-1H-cyclopenta[a]phenanthren-3-ol	Androst-5,16-diene-3β-ol	1224-94-8	Lipids; Androstane steroids	Endogenous steroid, pheromone, and chemical intermediate to several other pheromones that is found in the sweat of both men and women	0.160*	Polar	MSHub/GNPS
121, 149, 194	Benzoic acid, 4-ethoxy-, ethyl ester	ethyl 4-ethoxybenzoate	23676-09-7	Aromatic compounds; Esters	Pharmaceutical intermediates; Used in organic synthesis	0.174*	Polar	ChromSpace /Gavin 3
113, 81, 72	3-Nonanone	-	925-78-0	Ketones	Food; Used as solvent, organic synthesis intermediate; Flavour and fragrance intermediate	0.177*	Polar	MSHub/GNPS
119, 189, 173	Benzene, 2-(1-decylundecyl)-1,4-dimethyl-	11-(2,5-Dimethylphenyl)heneicosane	55373-91-6	Aromatic compounds; Benzenes	-	0.189*	Polar	MSHub/GNPS
84, 55, 32	2-Buten-1-amine, N-butyl-, (E)-	N-crotyl butylamine	62337-86-4	Amines	-	0.190*	Polar	ChromSpace /Gavin 3

91, 150, 119	1-Hydroxy-3-phenylacetone	-	4982-08-5	Aromatic compounds; Alcohols	-	0.190*	Polar	ChromSpace /Gavin 3
123, 138, 95	1,3-Benzenediamine, 4-methoxy-	2,4-Diaminoanisole	615-05-4	Aromatic compounds; Aminophenyl ethers; Anisoles; Nitrogen compounds	Azo dyes; Part of the human exposome	0.190*	Polar	ChromSpace /Gavin 3
189, 147, 204	4'-tert-Butyl-2',6'-dimethylacetophenone	-	2040-10-0	Aromatic compounds; Ketones	Organic photoinitiators; Polymerization Initiators	0.190*	Polar	ChromSpace /Gavin 3
58, 118, 117	(3S,4S)-2-Methyl-4-(methylamino)-3-phenyl-2-pentanol	-	-	Aromatic compounds	-	0.200*	Non-polar	MSHub/GNPS
69, 93, 43	Alpha bisabolol	-	515-69-5	Prenol lipids	Anti-inflammatory, wound-healing, anti-bacterial, anti-mycotic, and soothing properties; Part of the human exposome	0.220*	Polar	MSHub/GNPS

CYP3A4: Cytochrome P450 3A4 is an important enzyme mainly found in the liver and in the intestine which oxidizes small foreign organic molecules (toxins or drugs) so that they can be removed from the body. *: FDR corrected p value < 0.25; **: FDR corrected p value < 0.1; ***: FDR corrected p value < 0.05.

4.4.6 Multivariate analysis

The PLS-DA results were presented for the four optimal datasets with the comparison of the ChromSpace/Gavin 3 and the MSHub/GNPS pipeline. The data of the pancreatic adenocarcinoma patients (*n* = 28) and the controls (*n* = 33) were used.

4.4.6.1 Non-polar results

4.4.6.1.1 Non-polar ChromSpace/Gavin 3

PLS-DA was performed on the optimal non-polar dataset processed by ChromSpace/Gavin 3. The PLS-DA scores plot showed weak discrimination power between the pancreatic adenocarcinoma group and the control group (Figure 31). The predictive capability of the model was assessed by the internal validation (Component 1, R^2Y = 0.371, Q^2 = -0.037; Component 2, R^2Y = 0.644, Q^2 = 0.252), indicating model overfitting and poor predictive capability of the model.

Table S2 in Appendix 3 shows the variable importance for the projection (VIP) compounds for PLS-DA model responsible for differentiating between adenocarcinoma and controls.

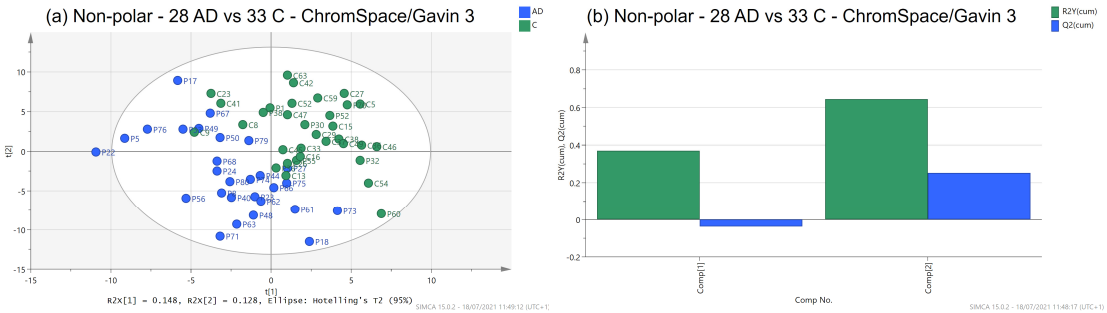


Figure 31. PLS-DA for pancreatic cancer vs control (non-polar, ChromSpace/Gavin 3).

PLS-DA score plot of the optimal non-polar dataset for pancreatic adenocarcinoma (AD) (*n* = 28) and controls (C) (*n* = 33) processed by ChromSpace/Gavin 3. Two-dimensional view showing the distribution of the groups according to the first two components of the PLS-DA model.

4.4.6.1.2 Non-polar MSHub/GNPS

PLS-DA was performed on the optimal non-polar dataset processed by MSHub/GNPS. The PLS-DA scores plot showed weak discrimination power between the pancreatic adenocarcinoma and control groups (Figure 32). The internal validation indicated model overfitting and poor predictive capability (Component 1, R^2Y = 0.625, Q^2 = 0.135; Component 2, R^2Y = 0.755, Q^2 = 0.215; Component 3, R^2Y = 0.850, Q^2 = 0.263).

Compared to non-polar ChromSpace/Gavin 3 data, the PLS-DA model of non-polar MSHub/GNPS data showed better tendency of separation.

Table S3 in Appendix 3 shows the VIP compounds for PLS-DA model responsible for differentiating between adenocarcinoma and controls.

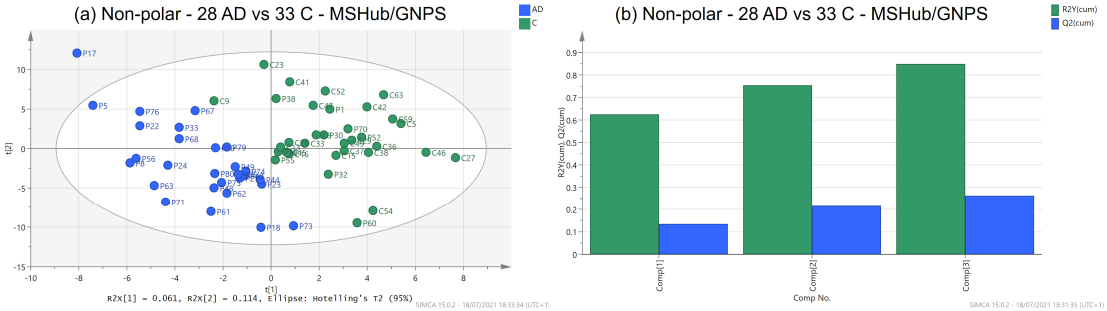


Figure 32. PLS-DA for pancreatic cancer vs control (non-polar, MSHub/GNPS).

PLS-DA score plot of the optimal non-polar dataset for pancreatic adenocarcinoma (AD) (*n* = 28) and controls (C) (*n* = 33) processed by MSHub/GNPS. Two-dimensional view showing the distribution of the groups according to the first three components of the PLS-DA model.

4.4.6.2 Polar results

4.4.6.2.1 Polar ChromSpace/Gavin 3

PLS-DA was performed on the optimal polar dataset processed by ChromSpace/Gavin 3. The PLS-DA scores plot showed weak discrimination power between the adenocarcinoma and control groups (Figure 33). The internal validation indicated model overfitting and poor predictive capability (Component 1, $R^2Y = 0.611$, $Q^2 = -0.1$; Component 2, $R^2Y = 0.685$, $Q^2 = -0.040$).

Table S4 in Appendix 3 shows the VIP compounds for PLS-DA model responsible for differentiating between adenocarcinoma and controls.

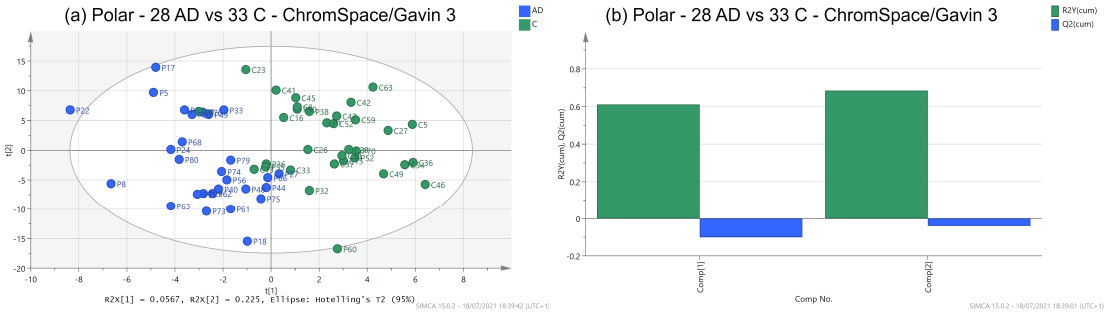


Figure 33. PLS-DA for pancreatic cancer vs control (polar, ChromSpace/Gavin 3).

PLS-DA score plot of the optimal polar dataset for pancreatic adenocarcinoma (AD) (*n* = 28) and controls (C) (*n* = 33) processed by ChromSpace/Gavin 3. Two-dimensional view showing the distribution of the groups according to the first two components of the PLS-DA model.

4.4.6.2.2 Polar MSHub/GNPS

PLS-DA was performed on the optimal polar dataset processed by MSHub/GNPS. The PLS-DA scores plot showed weak discrimination power between the adenocarcinoma and control groups (Figure 34). The internal validation suggested model overfitting and poor predictive capability (Component 1, $R^2Y = 0.421$, $Q^2 = 0.045$; Component 2, $R^2Y = 0.660$, $Q^2 = 0.140$).

Compared to polar ChromSpace/Gavin 3 data, the PLS-DA model of polar MSHub/GNPS data showed better tendency of separation.

Table S5 in Appendix 3 shows the VIP compounds for PLS-DA model responsible for differentiating between adenocarcinoma and controls.

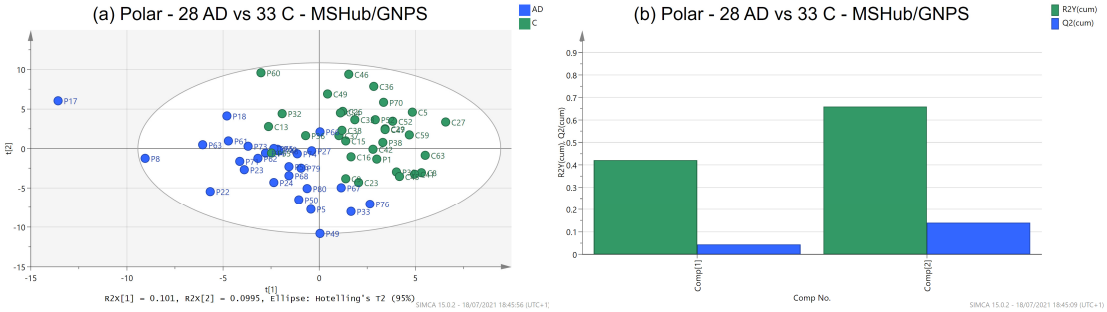


Figure 34. PLS-DA for pancreatic cancer vs control (polar, MSHub/GNPS).

PLS-DA score plot of the optimal polar dataset for pancreatic adenocarcinoma (AD) (*n* = 28) and controls (C) (*n* = 33) processed by MSHub/GNPS. Two-dimensional view showing the distribution of the groups according to the first two components of the PLS-DA model.

4.4.6.3 Non-polar and polar results

4.4.6.3.1 Non-polar and polar ChromSpace/Gavin 3

To further investigate the separation between the pancreatic adenocarcinoma group and the control group, PLS-DA model was built using the combination of the optimal non-polar and polar dataset processed by ChromSpace/Gavin 3.

The PLS-DA scores plot showed weak discrimination power between the pancreatic adenocarcinoma and control groups (Figure 35). The internal validation indicated model overfitting and poor predictive capability (Component 1, R^2Y = 0.521, Q^2 = -0.055; Component 2, R^2Y = 0.698, Q^2 = 0.215).

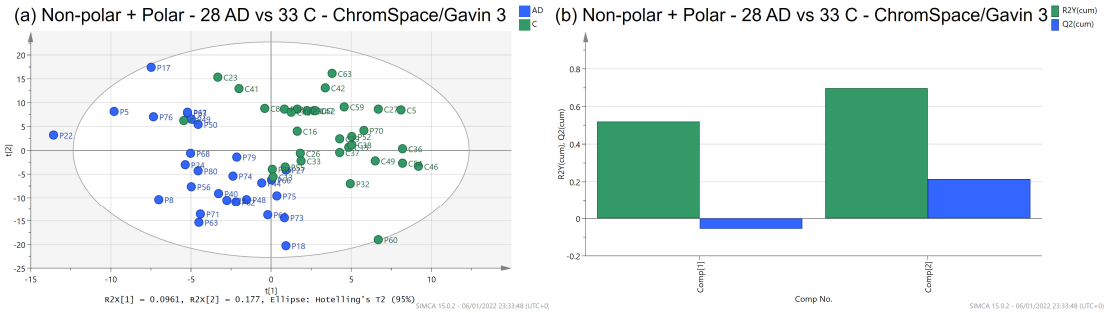


Figure 35. PLS-DA for pancreatic cancer vs control (non-polar + polar, ChromSpace/Gavin 3).

PLS-DA score plot of the optimal non-polar and polar dataset for pancreatic adenocarcinoma (AD) (*n* = 28) and controls (C) (*n* = 33) processed by ChromSpace/Gavin 3. Two-dimensional view showing the distribution of the groups according to the first two components of the PLS-DA model.

4.4.6.3.2 Non-polar and polar MSHub/GNPS

PLS-DA model was also built using the combination of the optimal non-polar and polar dataset processed by MSHub/GNPS to further investigate the separation between the adenocarcinoma and control groups.

The PLS-DA scores plot showed weak discrimination power between the pancreatic adenocarcinoma and control groups (Figure 36). The internal validation indicated model overfitting and poor predictive capability (Component 1, R^2Y = 0.628, Q^2 = 0.117; Component 2, R^2Y = 0.768, Q^2 = 0.105).

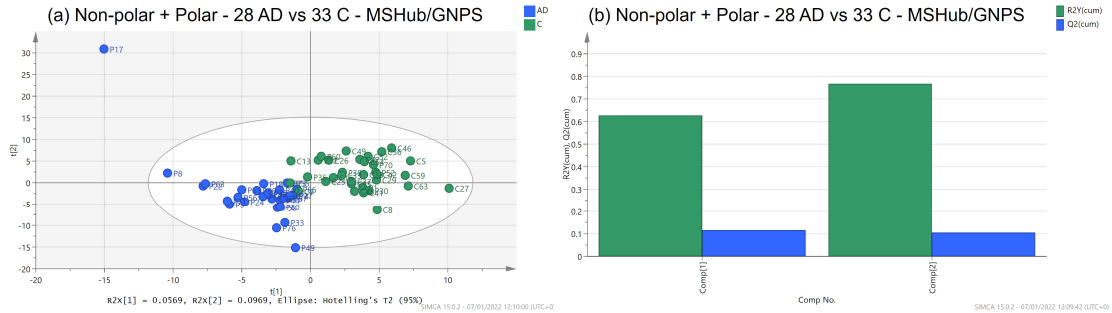


Figure 36. PLS-DA for pancreatic cancer vs control (non-polar + polar, MSHub/GNPS).

PLS-DA score plot of the optimal non-polar and polar dataset for pancreatic adenocarcinoma (AD) (*n* = 28) and controls (C) (*n* = 33) processed by MSHub/GNPS. Two-dimensional view showing the distribution of the groups according to the first two components of the PLS-DA model.

4.4.7 Investigation for confounding factors

The influence of confounding factors, including chemotherapy, diabetes, and antibiotics, on the separation of the pancreatic adenocarcinoma and control groups were investigated via PLS-DA.

4.4.7.1 Influence of chemotherapy

To evaluate the effect of chemotherapy on metabolic profiling results, the data of pancreatic adenocarcinoma patients who had chemotherapy (Chemo) (*n* = 23) were compared to the data of the chemotherapy naïve adenocarcinoma patients (Non-Chemo) (*n* = 5).

For the optimal non-polar and polar datasets processed by ChromSpace/Gavin 3 and MSHub/GNPS, all the four PLS-DA scores plots showed weak discrimination power between Chemo and Non-Chemo groups (Figure S1 and S2, Appendix 4). For the non-polar ChromSpace/Gavin 3 model: Component 1, $R^2Y = 0.458$, $Q^2 = -0.1$; Component 2, $R^2Y = 0.763$, $Q^2 = 0.232$. For the non-polar MSHub/GNPS model: Component 1, $R^2Y = 0.502$, $Q^2 = -0.048$; Component 2, $R^2Y = 0.892$, $Q^2 = 0.362$. For the polar ChromSpace/Gavin 3 model: Component 1, $R^2Y = 0.355$, $Q^2 = -0.019$; Component 2, $R^2Y = 0.777$, $Q^2 = 0.261$. For the polar MSHub/GNPS model: Component 1, $R^2Y = 0.419$, $Q^2 = -0.009$; Component 2, $R^2Y = 0.802$, $Q^2 = 0.072$. These internal validation values were low, indicating model overfitting and poor predictive capability of the models.

4.4.7.2 Influence of diabetes

To evaluate the effect of diabetes on metabolic profiling results, the data of pancreatic adenocarcinoma patients who had diabetes (DM) (*n* = 13) were compared to the data of the non-diabetic adenocarcinoma patients (Non-DM) (*n* = 15).

For the optimal non-polar and polar datasets processed by ChromSpace/Gavin 3 and MSHub/GNPS, all the four PLS-DA scores plots showed weak discrimination power between diabetes and non-diabetes groups (Figure S3 and S4, Appendix 4). The internal validation values were: For the non-polar ChromSpace/Gavin 3 model: Component 1, $R^2Y = 0.485$, $Q^2 = -0.018$; Component 2, $R^2Y = 0.760$, $Q^2 = 0.324$. For the non-polar MSHub/GNPS model: Component 1, $R^2Y = 0.575$, $Q^2 = 0.140$; Component 2, $R^2Y = 0.882$, $Q^2 = 0.412$. For the polar ChromSpace/Gavin 3 model: Component 1, $R^2Y = 0.513$, $Q^2 = -0.1$; Component 2, $R^2Y = 0.784$, $Q^2 = 0.356$. For the polar MSHub/GNPS model: Component 1, $R^2Y = 0.456$, $Q^2 = 0.025$; Component 2, $R^2Y = 0.780$, $Q^2 = 0.129$. The low internal validation values suggested model overfitting and poor predictive capability.

4.4.7.3 Influence of antibiotics

To evaluate the effect of antibiotics on metabolic profiling results, the data of pancreatic adenocarcinoma patients who had antibiotic treatment (ABX) at urine collection (n = 4) were compared to the data of the antibiotics naïve adenocarcinoma patients (Non-ABX) (n = 24).

For the optimal non-polar and polar datasets processed by ChromSpace/Gavin 3 and MSHub/GNPS, all the four PLS-DA scores plots showed weak discrimination power between antibiotic and non-antibiotic groups (Figure S5 and S6, Appendix 4). The internal validation values were: For the non-polar ChromSpace/Gavin 3 model: Component 1, R^2Y = 0.489, Q^2 = 0.154; Component 2, R^2Y = 0.670, Q^2 = 0.208. For the non-polar MSHub/GNPS model: Component 1, R^2Y = 0.464, Q^2 = 0.008; Component 2, R^2Y = 0.807, Q^2 = -0.092. For the polar ChromSpace/Gavin 3 model: Component 1, R^2Y = 0.438, Q^2 = 0.160; Component 2, R^2Y = 0.660, Q^2 = 0.284. For the polar MSHub/GNPS model: Component 1, R^2Y = 0.493, Q^2 = -0.004; Component 2, R^2Y = 0.826, Q^2 = -0.004. The low internal validation values indicated model overfitting and poor predictive capability.

4.4.8 Receiver operating characteristic analysis

The applicability of determining VOCs levels in the urine for discrimination between pancreatic cancer patients and controls was assessed using receiver operating characteristic (ROC) analysis.

4.4.8.1 Non-polar results

4.4.8.1.1 Non-polar ChromSpace/Gavin 3

The ROC curve was created using the 9 significant urinary VOCs identified based on the optimal non-polar dataset processed by ChromSpace/Gavin 3 summarised in Table 24. The area under the curve (AUC) was 0.908, indicating excellent predictive ability (Figure 37).

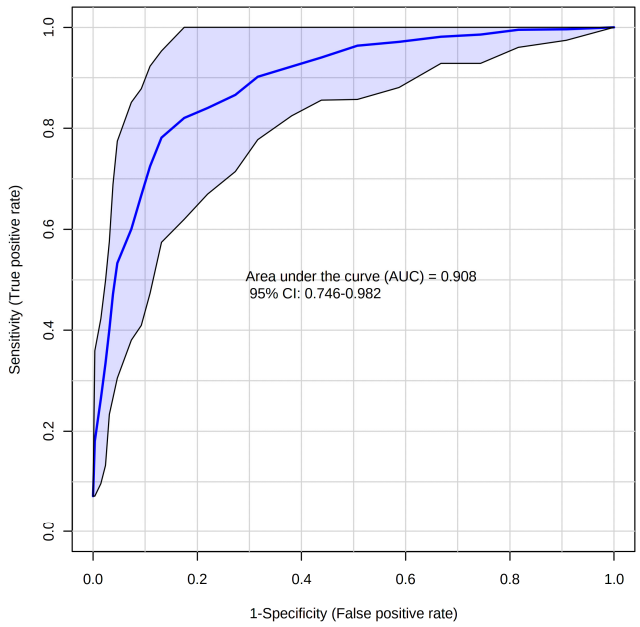


Figure 37. ROC curve for pancreatic cancer vs control (non-polar, ChromSpace/Gavin 3).

ROC curve for classification of pancreatic adenocarcinoma versus controls using urinary VOC biomarkers identified based on optimal non-polar dataset processed by ChromSpace/Gavin 3 (*n* = 9), with an AUC of 0.908.

4.4.8.1.2 Non-polar MSHub/GNPS

The ROC curve was created using the 13 significant urinary VOCs identified based on the optimal non-polar dataset processed by MSHub/GNPS summarised in Table 24. The AUC was 0.919, indicating excellent predictive ability (Figure 38).

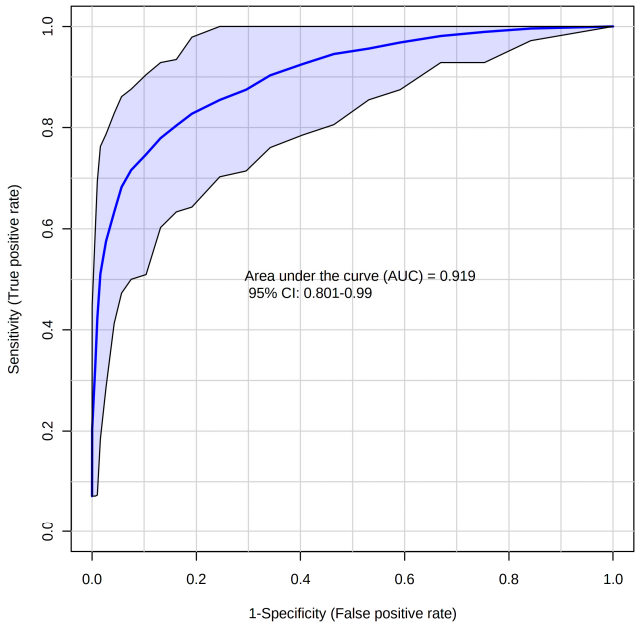


Figure 38. ROC curve for pancreatic cancer vs control (non-polar, MSHub/GNPS).

ROC curve for classification of pancreatic adenocarcinoma versus controls using urinary VOC biomarkers identified based on optimal non-polar dataset processed by MSHub/GNPS (*n* = 13), with an AUC of 0.919.

4.4.8.2 Polar results

4.4.8.2.1 Polar ChromSpace/Gavin 3

The ROC curve was created using the 11 significant urinary VOCs identified based on the optimal polar dataset processed by ChromSpace/Gavin 3 summarised in Table 25. The AUC was 0.845, indicating good predictive ability (Figure 39).

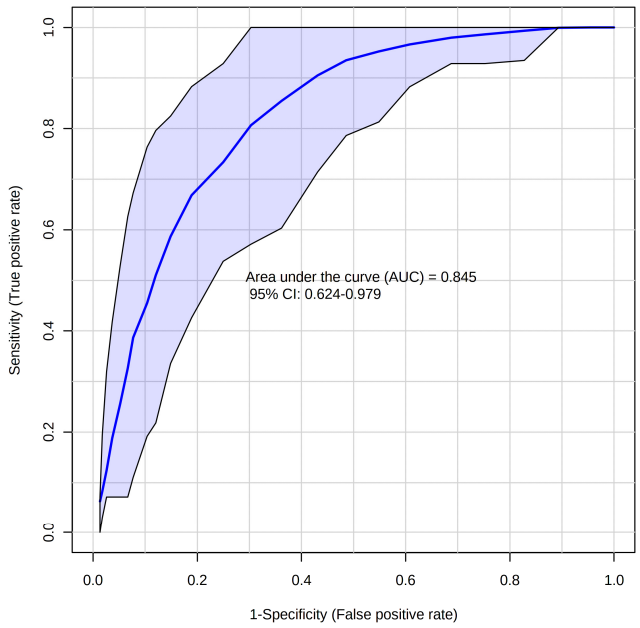


Figure 39. ROC curve for pancreatic cancer vs control (polar, ChromSpace/Gavin 3).

ROC curve for classification of pancreatic adenocarcinoma versus controls using urinary VOC biomarkers identified based on optimal polar dataset processed by ChromSpace/Gavin 3 (*n* = 11), with an AUC of 0.845.

4.4.8.2.2 Polar MSHub/GNPS

The ROC curve was created using the 15 significant urinary VOCs identified based on the optimal polar dataset processed by MSHub/GNPS summarised in Table 25. The AUC was 0.794, indicating fair predictive ability Figure 40).

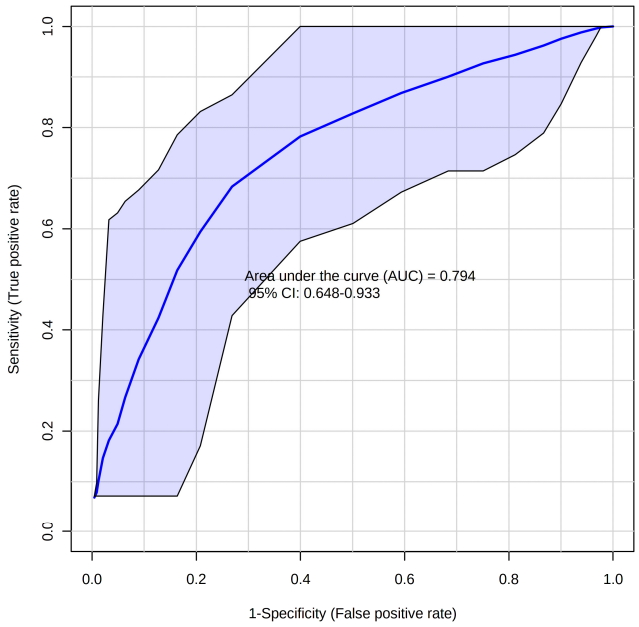


Figure 40. ROC curve for pancreatic cancer vs control (polar, MSHub/GNPS).

ROC curve for classification of pancreatic adenocarcinoma versus controls using urinary VOC biomarkers identified based on optimal polar dataset processed by MSHub/GNPS (*n* = 15), with an AUC of 0.794.

4.4.8.3 Non-polar and polar ChromSpace/Gavin 3

To further investigate the diagnostic applicability of the identified urinary VOC biomarkers, a ROC curve was created using the combination of the significant urinary VOCs identified based on both the optimal non-polar and polar dataset processed by ChromSpace/Gavin 3.

A total of 20 compounds were used: 9 non-polar compounds and 11 polar compounds. The AUC was 0.937, indicating excellent predictive ability (Figure 41).

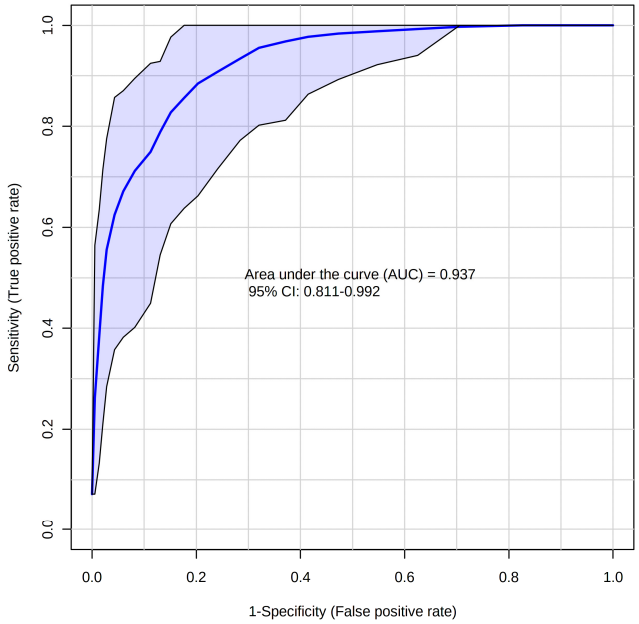


Figure 41. ROC curve for pancreatic cancer vs control (non-polar + polar, ChromSpace/Gavin 3).

ROC curve for classification of pancreatic adenocarcinoma versus controls using urinary VOC biomarkers identified based on optimal non-polar dataset (*n* = 9) and polar dataset (*n* = 11) processed by ChromSpace/Gavin 3, with an AUC of 0.937.

4.4.8.4 Non-polar and polar MSHub/GNPS

To further investigate the diagnostic applicability of the identified urinary VOC biomarkers, a ROC curve was created using the combination of the significant urinary VOCs identified based on both the optimal non-polar and polar dataset processed by MSHub/GNPS.

A total of 28 compounds were used: 13 non-polar compounds and 15 polar compounds. The AUC was 0.922, indicating excellent predictive ability (Figure 42).

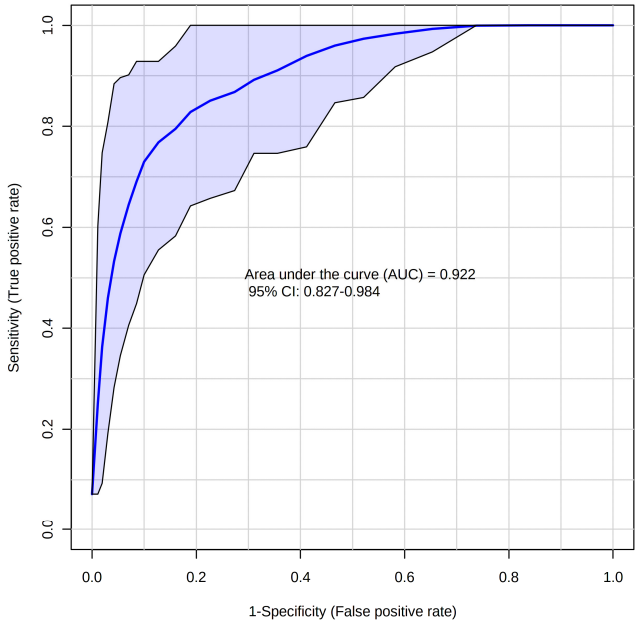


Figure 42. ROC curve for pancreatic cancer vs control (non-polar + polar, MSHub/GNPS).

ROC curve for classification of pancreatic adenocarcinoma versus controls using urinary VOC biomarkers identified based on optimal non-polar dataset (*n* = 13) and polar dataset (*n* = 15) processed by MSHub/GNPS, with an AUC of 0.922.

4.4.8.5 Non-polar and polar ChromSpace/Gavin 3 and MSHub/GNPS

To further investigate the diagnostic applicability of the identified urinary VOC biomarkers, ROC curves were created using the combination of the significant urinary VOCs identified based on both the optimal non-polar and polar dataset processed by ChromSpace/Gavin 3 and MSHub/GNPS.

All the significant compounds summarised in Table 24 and Table 25 were ranked according to their FDR corrected p values. Two ROC curves were generated. The first model used the four compounds with the lowest adjusted p values which were below 0.05 (Table 27). The AUC was at 0.914, indicating excellent predictive ability (Figure 43).

The second curve used the eleven compounds whose adjusted p values were lower than 0.1 (Table 28). The obtained AUC was at 0.942, suggesting excellent predictive ability (Figure 44).

Table 27. Summary of significant urinary VOCs (FDR corrected p value < 0.05).

Retention Time (RT)	Top Ions	Compound Name	FDR Corrected p value	Non-polar or Polar Assay	Data Pre-processing Pipeline
6.67	111, 126, 148	5-(2-Thienoyl)butyric acid	0.00413***	Polar	ChromSpace /Gavin 3
5.66	41, 44, 58	Butanal, 3-methyl-	0.0235***	Non-polar	MSHub/GNPS
9.57	83, 98, 59	Furan, 2-methoxy-	0.0356***	Non-polar	MSHub/GNPS
2.83	43, 84, 55	Cyclohexanone, 4R-acetamido-2,3-cis-epoxy-	0.0400***	Polar	MSHub/GNPS

***: FDR corrected p value < 0.05.

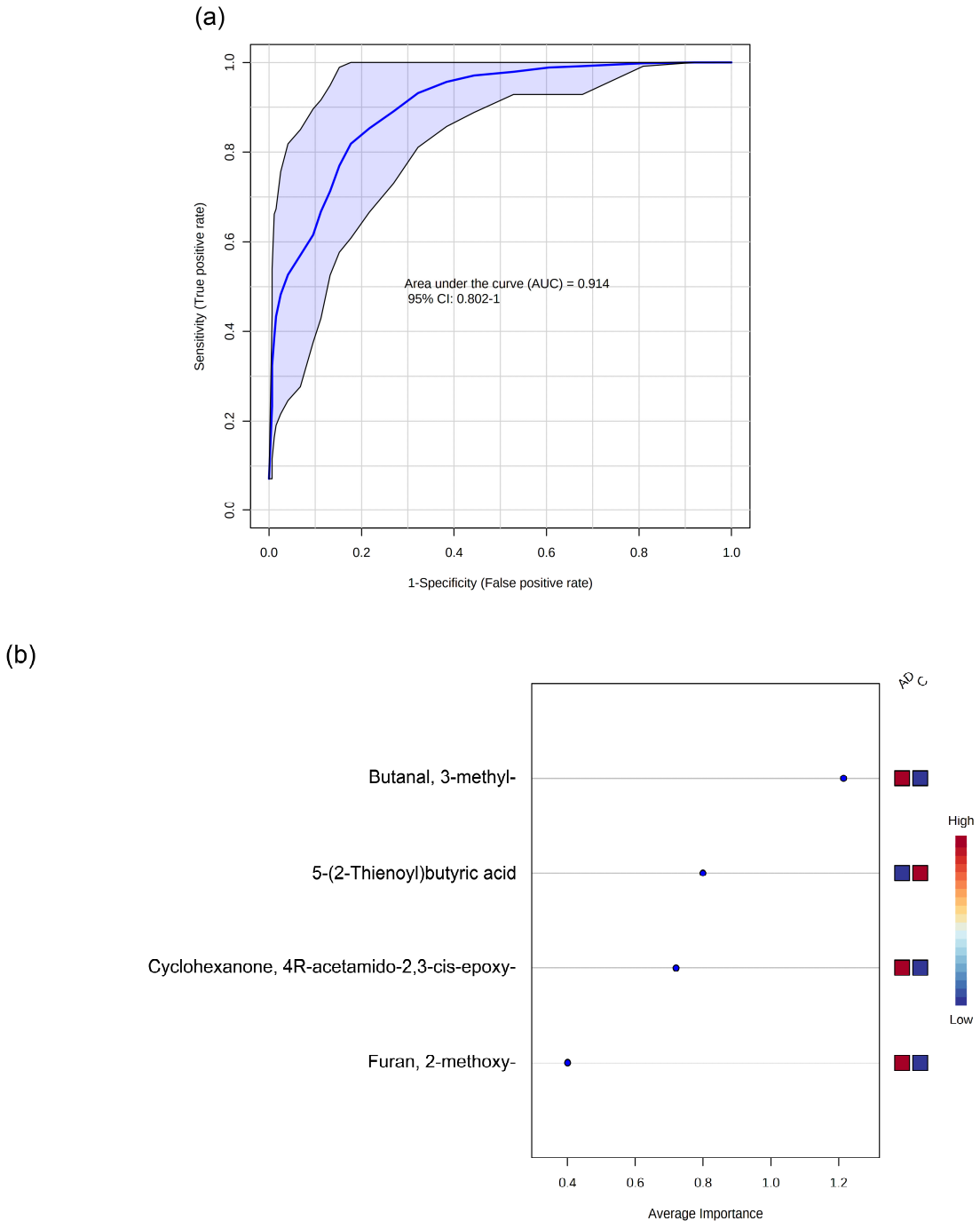


Figure 43. ROC curve for pancreatic cancer vs control (FDR corrected p value < 0.05).

(a) ROC curve for classification of pancreatic adenocarcinoma versus controls using urinary VOC biomarkers whose FDR corrected p value < 0.05 (*n* = 4), with an AUC of 0.912. (b) Features ranked by average importance.

Table 28. Summary of significant urinary VOCs (FDR corrected p value < 0.1).

Retention Time (RT)	Top Ions	Compound Name	FDR Corrected p value	Non-polar or Polar Assay	Data Pre-processing Pipeline
6.67	111, 126, 148	5-(2-Thienoyl)butyric acid	0.00413***	Polar	ChromSpace /Gavin 3
5.66	41, 44, 58	Butanal, 3-methyl-	0.0235***	Non-polar	MSHub/GNPS
9.57	83, 98, 59	Furan, 2-methoxy-	0.0356***	Non-polar	MSHub/GNPS
2.83	43, 84, 55	Cyclohexanone, 4R-acetamido-2,3-cis-epoxy-	0.0400***	Polar	MSHub/GNPS
1.43	43, 86, 41	2-Butanone, 3-methyl-	0.0549**	Polar	MSHub/GNPS
7.63	94, 65, 66	2-Vinylfuran	0.0652**	Non-polar	MSHub/GNPS
21.06	58, 43, 93	2,6-Di-tert-butyl-4-methyl-phenol	0.0652**	Non-polar	MSHub/GNPS
5.20	59, 55, 73	2-Butanol, 2-methyl-	0.0798**	Non-polar	MSHub/GNPS
25.76	109, 124, 269	2-Cyclopenten-1-one, 2,3,4-trimethyl-	0.0798**	Non-polar	MSHub/GNPS
20.69	99, 42, 110	Didodecyl phosphate	0.0946**	Non-polar	ChromSpace /Gavin 3
24.77	85, 57, 55	2(3H)-Furanone, 5-heptyldihydro-	0.0986**	Polar	ChromSpace /Gavin 3

: FDR corrected p value < 0.1; *: FDR corrected p value < 0.05.

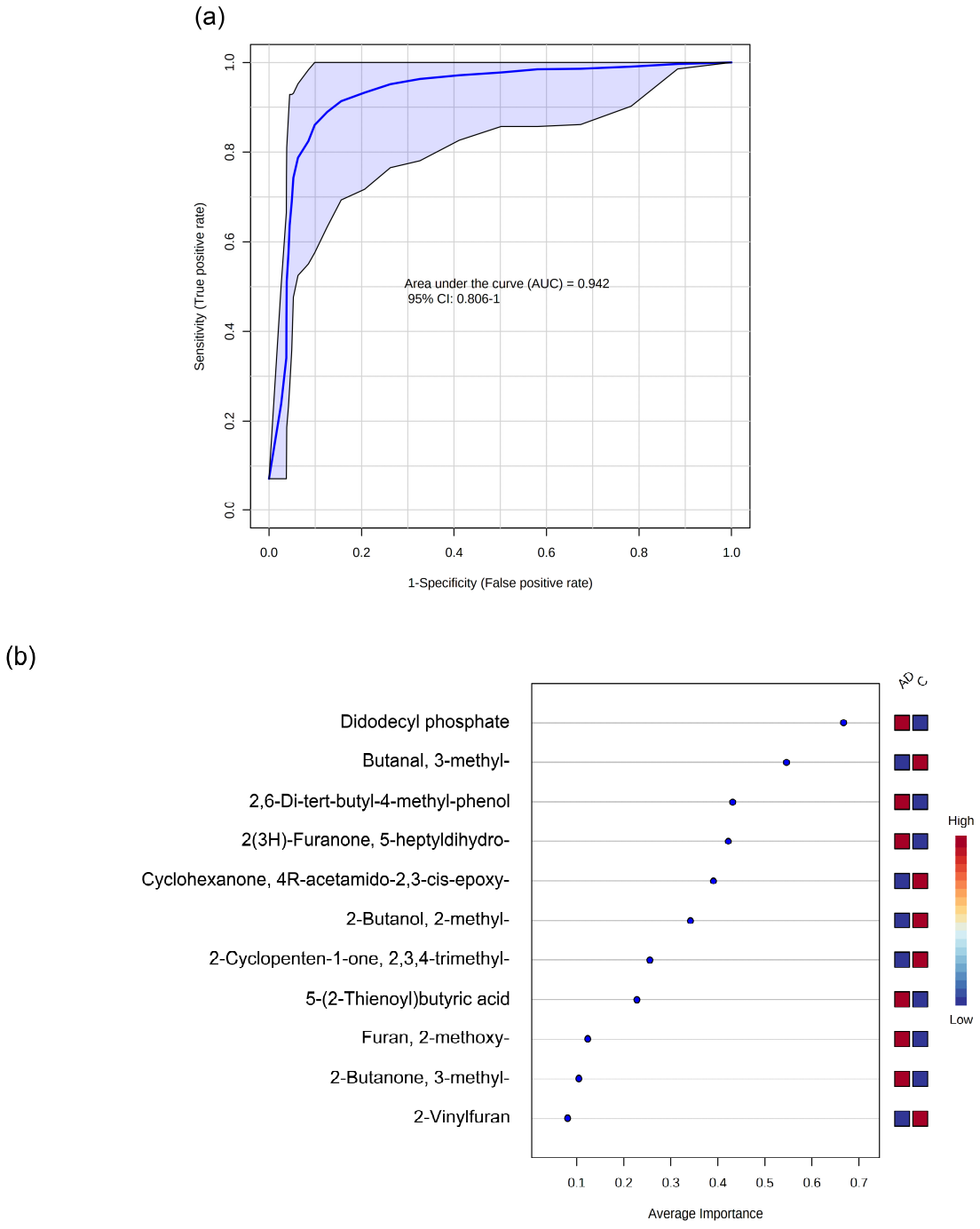


Figure 44. ROC curve for pancreatic cancer vs control (FDR corrected p value < 0.1).

(a) ROC curve for classification of pancreatic adenocarcinoma versus controls using urinary VOC biomarkers whose FDR corrected p value < 0.1 (*n* = 11), with an AUC of 0.942. (b) Features ranked by average importance.

4.5 Discussion

The principal outcomes of this study were: (i) optimised and quality controlled non-polar and polar GC-MS analysis of urinary VOCs; (ii) dual data processing pipelines for characterisation of discriminant VOCs; (iii) excellent discrimination of pancreatic adenocarcinoma and controls using VOCs (principally aromatic compounds and ketones) identified through univariate analysis; (iv) poor separation of cancer and controls by multivariate analysis, and; (v) results were unaffected by confounding variables such as chemotherapy, diabetes, and antibiotics.

In general, GC-MS chromatograms of urinary VOCs within the study exhibited clear baseline separation between peaks and symmetric peak shapes, indicating their high-quality. Chromatograms of pancreatic adenocarcinoma patients, controls, and QC samples showed visually distinct differences (Figure 24, 25). QC chromatograms typically exhibited more peaks than on adenocarcinoma and control chromatograms, as they were from both patient cohorts.

Feature filtering and data normalisation successfully identified sufficient numbers of reliable urinary VOCs. In Section 4.4.3, minimum over 230, maximum over 290 reproducible and linear-responding urinary VOCs were included as reliable detections, allowing unambiguous and reliable statistical analysis. The number of identified compounds at around 200 to 300 was satisfactory for an untargeted metabolomic analysis (explained in Section 3.1). Optimal VOC identification for polar and non-polar GC-MS analysis was achieved using ChromSpace/Gavin 3 and MSHub/GNPS data pre-processing platforms, respectively. In principle, more compounds should be obtained from non-polar data using the non-polar assay, because HiSorb has a PDMS coating that extracts mainly non-polar compounds. The MSHub/GNPS results supported this theory by identifying around 20% more reliable compounds. However, the ChromSpace/Gavin 3 results did not. This was likely due to the fact that algorithms used by these software packages for spectral alignment, deconvolution, and integration were different. This is discussed further below.

The pre-processed dataset showed excellent analytical characteristics. PCA, as explained in Section 3.7.5, was used to assess data quality. The PCA plots generated

using the four optimal datasets revealed tightly packed grouping of QCs with study samples surrounded, indicating that analytical variability was low. This was a positive indication of the reproducibility of the method and instrumental stability, and an indication of good data quality.

When conducting multiple comparisons in statistics, it is common that some will have p values less than 0.05 purely by chance, even if all the null hypotheses are true. This is called false positive. False positives were a significant concern in the current pilot study since hundreds of compounds were assessed using the Mann-Whitney U test. Whilst Bonferroni correction is one simple way to consider false positives during multiple comparison,[145] it is generally considered to be strict in its evaluations and hence may lead to a high rate of false negatives when large numbers of multiple comparisons are made. An alternative approach was therefore applied in the current study, which was to correct the FDR. FDR is a method of conceptualising the proportion of significant results that are actually false positives. Benjamini and Hochberg reported the method to solve false positive problems by adjusting raw p values.[133,135] The adjusted p value for a test is either the raw p value multiplied by m/i or the adjusted p value for the next higher raw p value, whichever is smaller (m: the number of tests; i: rank of each test, with i = 1 the rank of the smallest p value). The test is significant if the adjusted p value is smaller than the FDR. In the current study, the FDR was corrected by adjusting the raw p values using the Benjamini-Hochberg procedure. The acceptable FDR was set at 0.25, mainly because the current study, due to its untargeted exploratory nature, had a limited number of samples and therefore potentially lower statistical power. This helped to measure as many metabolites as possible with fewer false negatives.

MSHub/GNPS outperforms ChromSpace/Gavin 3 in providing a greater number of significant compounds with lower p values and better compound identifications. Table 26 summarises significant compounds ranked by their FDR corrected p values, with information including CAS registry number or NIST number, chemical classes, as well as possible origin, applications and related diseases. MSHub/GNPS findings were kept for compounds identified by both data pre-processing pipelines, since they offered more reliable deconvolution and identification. The main reason for MSHub/GNPS outperforming ChromSpace/Gavin 3 was because of the different deconvolution algorithms. In MSHub/GNPS, auto-deconvolution of compound fragmentation patterns

is performed via unsupervised non-negative matrix factorisation (NMF), and the reproducibility of fragmentation patterns across samples is quantified.[140,141] NMF is an unsupervised algorithm that lowers the dimension of the data and reduces the number of features while keeping the basis information required to recreate the original data. It converts a matrix with only non-negative coefficients into the product of two non-negative matrices with lower ranks.[146] During this auto-deconvolution process, all the input study samples are processed by the NMF algorithm, and the least input sample number is 10.[141] In comparison, when using ChromSpace/Gavin 3 pipeline, only one representative QC sample data are processed.

The most common VOCs that discriminated pancreatic adenocarcinoma and controls subjects were aromatic compounds and ketones (Table 26). Other chemical classes included lipids, phosphorus compounds, sulfur compounds, aldehydes, and alcohols. When profiled using non-polar TOF, the abundance of the majority of the significant VOCs were higher in adenocarcinoma compared to controls (Section 4.4.5.1), but when profiled using polar TOF, most VOCs had higher abundance in the urine of controls (Section 4.4.5.2). For significant VOCs (adjusted $p < 0.25$), diagnostic utility for pancreatic adenocarcinoma was assessed using ROC models. The results of ROC analysis indicated that when using VOCs identified with ChromSpace/Gavin 3 pipeline, higher AUCs were obtained than using those identified with MSHub/GNPS (Section 4.4.8.1 to 4.4.8.4). Importantly, when using compounds with the lowest FDR corrected p values which were below 0.05, the AUC was 0.914; when adjusted p values were below 0.1, the AUC was 0.942 (Section 4.4.8.5). These results demonstrated that pancreatic adenocarcinoma may be diagnosed with high accuracy and sensitivity utilising these urinary VOCs.

The biological significance of the majority of the identified compounds is uncertain (Table 26). Due to a paucity of publications focusing on urinary VOCs for the diagnosis of pancreatic cancer (also see Section 1.7 and Section 2), only one of the identified VOCs, 2-pentanone, were reported by previous literature. Daulton, E. et al. reported that urinary 2-pentanone is able to separate urine of pancreatic ductal adenocarcinoma (PDAC) versus chronic pancreatitis (CP) patient and CP versus healthy subjects, with AUC of ROC curve at around 0.7.[147] 2-pentanone has also been found to be associated with ulcerative colitis,[148,149] celiac disease,[150] non-alcoholic fatty liver disease,[151] and

Crohn's disease,[149] suggesting that it may serve as a more general marker of inflammation.

Another significant VOCs identified in current study was previously identified as urinary biomarker for prostate cancer. myrcenol-dihydro (2,6-dimethyl-7-octen-2-ol, dihydromyrcenol) has been reported to have lower concentrations in urine from prostate cancer patients.[101,102] Some of the VOCs identified in current study have been proposed as potential biomarkers in blood, breath, or stool for other diseases. For example, butanal, 3-methyl- (isovaleraldehyde) is reported to be associated with hepatic encephalopathy,[152,153] ulcerative colitis,[148,154] Crohn's disease,[154] non-alcoholic fatty liver disease,[151] celiac disease,[150] and clostridium difficile infection.[155] It is also related to perillyl alcohol administration for breast cancer treatment.[156]

Most of the VOCs identified in this study were different from previous reports. This might be due to patient variability, differences in the VOC extraction techniques and analytical platforms used, the adoption of QC, differences in data processing procedures, and differences in the biological samples (urine, breath, blood, stool, etc.).

It is worth noting that some of the VOCs identified in this study may reflex exogenous agents such as pharmacological agents (Table 26). For example, butanal, 3-methyl- (isovaleraldehyde) and 2-buten-1-one, 1-(2,6,6-trimethyl-1,3-cyclohexadien-1-yl)-, (E)- (beta-damascenone) are reported to be related to perillyl alcohol administration for cancer treatment.[156] 2-butanone, 3-methyl- (methyl isopropyl ketone (MIPK)) and benzoic acid, 4-ethoxy-, ethyl ester (ethyl 4-ethoxybenzoate) are other important components of medicines.[157,158] 2-butanol, 2-methyl- (tert-pentanol), also known as tert-amyl alcohol (TAA), is used as a solvent for tribromoethanol (TBE) forming Avertin which is an anaesthetic, and more recently it has been used as a recreational drug.[159,160] 2,6-Di-tert-butyl-4-methyl- phenol (butylated hydroxytoluene (BHT)) is used as an antioxidant for vitamins and pharmaceuticals.[161] Benzene, 1-(1,5-dimethyl-4-hexenyl)-4-methyl- (alpha-curcumene), which is one of the main active compounds in ginger, has antioxidant and antimicrobial effects.[162] Curcumenol can be used as efficient CYP3A4 inhibitor, and has neuroprotective, anti-inflammatory, anti-tumour, anti-early pregnancy, anti-coagulation, anti-oxidation, and hepatoprotective

effects.[163,164] Alpha bisabolol has anti-inflammatory, wound-healing, anti-bacterial, and anti-mycotic properties, and is widely used in skin-care products.[165]

Food consumption is another important source of VOCs identified by this study (Table 26). Butanal, 3-methyl- (isovaleraldehyde) is found in different foods (e.g., cow's milk, beer, and tacos) and use of food fragrance.[166] 2,6-Di-tert-butyl-4-methyl- phenol (butylated hydroxytoluene (BHT)) can be detected in human urine due to consumption of soft-necked garlic.[166] 2(3H)-furanone, 5-heptyldihydro- (peach aldehyde), a peach flavoured compound, is widely used as food additive.[166] Benzene, 1-(1,5-dimethyl-4-hexenyl)-4-methyl- (alpha-curcumene) is the main active compounds in ginger.[162] 2-pentanone, in addition to its role as cancer and disease biomarker, has a role as food consumption biomarker for cow's milk, fats and oils, and corns.[166] 2-buten-1-one, 1-(2,6,6-trimethyl-1,3-cyclohexadien-1-yl)-, (E)- (beta-damascenone) is associated with red wines, grapes, and apples.[166] 3-nonanone is related to the consumption of duck, chickens, domestic pigs, fruits, and cow's milk.[166]

Some of the identified VOCs belonged to human exposome (Table 26), which is the collection of all the exposures of an individual from environmental and occupational sources. These compounds can be found in individuals exposed to themselves or their derivatives. As mentioned above, 2,6-Di-tert-butyl-4-methyl-phenol (butylated hydroxytoluene (BHT)) is widely used as an antioxidant for vitamins and pharmaceuticals, and is related to consumption of garlic.[161,166] It is also widely used in foods, polymers, and cosmetics.[167] Myrcenol-dihydro (dihydromyrcenol) is not only associated with prostate cancer, but can be a human exposome due to its predominantly use in soap fragrances and in citrus and lime-type perfumes.[101,102,168] 1,3-benzenediamine, 4-methoxy- (2,4-diaminoanisole) and alpha bisabolol are also reported to be part of human exposome and can be detected in human blood.[167,169]

Multivariate analysis conducted using PLS-DA showed weak separation between adenocarcinoma and control patients (Section 4.4.6). The major reason for this observation was small sample size. PLS-DA models generated using MSHub/GNPS showed greater tendency for separation and internal validation values than those using ChromSpace/Gavin 3, even if the number of metabolites of MSHub/GNPS processed dataset is not consistently higher than that of ChromSpace/Gavin 3. This reflected the

consequence of the difference in the algorithms used by the two software packages for spectral alignment, deconvolution, and integration, as discussed above.[140,141]

The investigation of confounding factors was an essential step for this study. Chemotherapy is one of the main treatments for pancreatic cancer, which involves the use of chemotherapy drugs such as FOLFIRINOX, Gemcitabine and Cisplatin.[170] These medications are capable of killing not just cancer cells but also healthy cells and may affect metabolic profiling. Diabetes is one of the most common symptoms of pancreatic adenocarcinoma. Pancreatic adenocarcinoma can result in the development of insulin resistance and can impair the ability of pancreas' insulin-producing cells to respond adequately to this insulin resistance. Long-standing diabetes itself is also a modest risk factor for pancreatic adenocarcinoma.[171] Taking antibiotics may affect urinary VOC profiles as a response of metabolic changes of microbiome within human body due to antibiotic exposure.[172] Evaluation of these confounding factors conducted using PLS-DA showed weak separation between groups, indicating that the results comparing adenocarcinoma versus controls were unaffected by these confounding variables.

The optimised urinary VOC analytical method in this study worked in a high throughput, automated, and reliable manner. As described in Section 4.3.8, twenty-five clinical samples were analysed in one analytical day. This analysis capability could be increased on demand if an adequate supply of HiSorb probes and GC-MS instruments is available.

A limitation of this study is that observations were made from only a relatively small number of pancreatic adenocarcinoma and control subjects. A follow-up study enrolling appropriate number of patients is needed, which will enable the conducting of a cohort for VOC biomarker discovery and an independent validation cohort to verify the results. Another limitation is the HiSorb probes coated with PDMS, which extracted mainly non-polar compounds. PDMS HiSorb was the only commercially available product when this pilot study was conducted. Future usage of multibed HiSorb will cover polarity ranging from non-polar to polar.

4.6 Conclusion

The developed urinary VOC analysis methods were applied for the discovery of urinary VOC biomarkers of pancreatic cancer, with high quality non-polar and polar data acquired. The adoption of data pre-processing steps ensured the maximal detection of reliable VOCs. VOCs identified by univariate analysis were able to discriminate pancreatic cancer patients from controls with a high degree of accuracy. Multivariate analysis showed weak separation between adenocarcinoma and controls, therefore suggesting limited predictive value of the PLS-DA models. The results were unaffected by confounding variables such as chemotherapy, diabetes, and antibiotics. Preliminary analysis of urine from pancreatic cancer patients supports its application in future large scale clinical trials.

SECTION 5

THESIS CONCLUSION AND FUTURE WORK

5 THESIS CONCLUSION AND FUTURE WORK

5.1 Thesis conclusion

This thesis has initially presented a systematic review of the existing literature relating to cancer-specific urinary VOCs. The advancements in the use of urinary VOC analysis for cancer diagnosis has been summarised, with the majority of existing studies investigated urinary VOCs of prostate and gastrointestinal cancer. Cancer-associated urinary VOC biomarkers, mainly belonging to aromatic compounds, ketones, alcohols, and related derivatives exhibited promising diagnostic performance, although the origin of VOCs within the body and the mechanism by which they enter the urine remains incompletely understood. GC-MS was the most commonly employed analytical platform for urinary VOC analysis. Multiple sampling techniques were frequently used to assist pre-analysis VOC extraction. One critical finding was the lack of standardised practices for urinary VOCs analysis. There was a lack of information on patient recruitment and study design, no guidelines on sampling techniques and analytical platforms, no standardised workflow, no adequate validation of results, and limited adoption of quality control measures. This is where the contribution of this thesis comes in, attempting to overcome these methodological weaknesses for a broader application of urinary VOC analysis in large clinical practices.

The work in the method development section of this thesis commenced with the comparison of two offline VOC extraction techniques, SPME and HiSorb sorptive extraction. HiSorb outperformed SPME in providing broader VOC detection, reduced batch effects, and high throughput reliable automated sampling, after observing the background total ion chromatograms and establishment of calibration curves. Following the selection of HiSorb as the preferred method for VOC extraction, the effects of extraction conditions including: temperature and time, urine acidification, sample volume, and urine dilution on urinary VOC extraction performance were evaluated by comparing the total peak areas ± standard deviations of selected chemical groups consisting of fifteen representative urinary VOCs. For the majority compounds, a 2 mL urine sample, without dilution, acidified with 5 M HCl to pH 2.0, and then extracted at 60 °C for 30 min delivered the best outcomes among all tested experimental conditions.

A comprehensive method development has been conducted on urine samples from healthy volunteers using multiple advanced GC-MS instruments with the aim of providing a complete methodology for untargeted urinary VOC analysis in a high throughput and standardised manner with the adoption of robust quality control measures.

A TD-GC-quadrupole MS assay has been developed as benchmark method for urinary VOC analysis, with 41 reliable VOCs identified after removing non-reproducible, non-linear, and contamination-related features. A high throughput and standardised workflow has been established for GC-MS based urinary VOC analysis using HiSorb, with the adoption of robust QC measures such as assessing the gross performance of GC-MS instrument with C_8 to C_{20} n-alkane standard mixture pre-analysis, use of randomised block design for sample analysis, analysis of pooled QC samples, the use of internal standards, and standardised data processing procedures. A series of isotopically labelled internal standards whose retention time (RT) were in the beginning, middle, and the end of the QC chromatogram and represented urinary VOCs in different chemical groups have been determined as appropriate internal standards: acetone-d_6, butyraldehyde-d_2, phenol-d_6, benzene-d_6 and acetophenone-d_8.

Furthermore, TD-GC-time of flight (TOF)-MS has been used in an attempt to obtain improved results with its rapid acquisition rate, spectral continuity, high resolution, outstanding dynamic range, as well as high sensitivity and selectivity.

A panel of assays using TD-GC-TOF-MS equipped with a polar column have been developed, with the optimal long assay identified 121 reliable VOCs and the optimal short assay identified 96 reliable VOCs. In terms of the urine sample volume, 2 mL has been confirmed to achieve maximum VOC acquisition when the sample availability is relatively unrestricted, and 0.25 mL as the least possible urine volume to identify as many VOCs as possible without making excessive sacrifices. Taking into consideration of the sensor over saturation, the concentration of 0.5 μg/mL has been considered the optimal concentration for the internal standard spiking mixture.

A panel of assays using TD-GC-TOF-MS equipped with a non-polar column have been developed for urinary VOC analysis, with the optimal long assay identified 154 reliable VOCs and the optimal short assay identified 167 reliable VOCs. The GC column flow rates and oven ramp gradients have also been optimised.

The developed multiplatform GC-MS assays for urinary VOC analysis have been compared by bring together the results. The 2 mL urine long polar assay delivered the best results among the polar column based assays, with 121 reliable urinary VOCs detected. The 2 mL urine short non-polar assay detected the highest number of 167 reliable VOCs among all the assays, around 38% more than the VOCs identified by the best polar assay and around 288% more than those identified by the quadrupole assay. However, the overlap of identified VOCs between different platforms was low (less than 23%). These results highlighted the superiority of TOF over quadrupole GC-MS and the necessity of analysing samples on multiple platforms using different assays due to highly complementary outcomes.

In addition, TD recollection has been found satisfactory in terms of reproducibility, linearity, contamination, and sample loss, allowing for the possibility of profiling a single sample on multiple platforms in parallel or using a single sample for both targeted and untargeted analyses. The application of osmolality normalisation can partially correct the effect of variable urine concentration, allowing the improvement of the ability to identify important metabolome components that differentiate across sample groups, and avoiding false discoveries that reflect different urine concentrations rather than other characteristics under investigation.

In order to showcase the applicability of optimised urinary VOC analytical method in clinical studies and to generate data to support its use in larger clinical trials, this thesis involved a pilot study analysed urine samples from a cohort consisting of pancreatic adenocarcinoma patients (n = 28) and controls (n = 33). High quality non-polar and polar data have been acquired, with the adoption of data pre-processing steps ensuring the maximal detection of reliable VOCs. The univariate analysis successfully delivered a total of 20 significant VOCs for the ChromSpace/Gavin 3 pipeline, and a total of 28 for the MSHub/GNPS pipeline, with 8 of them being identified by both pipelines, all with FDR corrected p value < 0.25. Most of these VOCs were aromatic

compounds, ketones, and related derivatives. All ROC models showed excellent discrimination, with AUC at 0.914 and 0.942 when compounds with the lowest FDR corrected p values which were below 0.05 (5-(2-thienoyl)butyric acid; butanal, 3-methyl-; furan, 2-methoxy-, and; cyclohexanone, 4R-acetamido-2,3-cis-epoxy-) and below 0.1 (5-(2-thienoyl)butyric acid; butanal, 3-methyl-; furan, 2-methoxy-; cyclohexanone, 4R-acetamido-2,3-cis-epoxy-; 2-butanone, 3-methyl-; 2-vinylfuran; 2,6-di-tert-butyl-4-methyl-phenol; 2-butanol, 2-methyl-; 2-cyclopenten-1-one, 2,3,4-trimethyl-; didodecyl phosphate, and; 2(3h)-furanone, 5-heptyldihydro-) were used, respectively. Multivariate analysis showed weak separation between adenocarcinoma and control group, therefore suggesting limited predictive value of the PLS-DA models. It has been confirmed that the results were not affected by confounding variables such as chemotherapy, diabetes, and antibiotics.

In conclusion, findings within this thesis represent current advancements of urinary VOC analysis for cancer diagnosis. This thesis also provides standardised high throughput and reliable HiSorb-TD-GC-MS methods for the analysis of urinary VOCs for the diagnosis of gastrointestinal cancers, particularly pancreatic cancer. Preliminary analysis of urine from pancreatic cancer patients supports its application in future large scale clinical trials.

5.2 Future research

The work in this thesis has identified potential urinary VOC biomarkers of pancreatic adenocarcinoma. Validation of the identification of these possible biomarkers requires the analysis of authentic chemical standards with the optimised urinary VOC analytical methods.

It will be necessary for any future study to enrol an appropriate number of patients with sample sizes being predetermined by power calculation. Large enough sample size will be required in order to establish a cohort for VOC biomarker discovery and an independent validation cohort for result verification. Potential confounding factors, including and not limited to: chemotherapy, use of antibiotics, and diabetes, should be considered.

Future work should also include the optimisation of multibed HiSorb, and the comparison of the extraction performance for HiSorb with different extracting phases. There will be four types of HiSorb available, including: polydimethylsiloxane (PDMS), PDMS / carbon wide range (CWR), PDMS / divinylbenzene (DVB), and DVB/CWR/PDMS. The evaluation of extraction performance will include: number of identified reliable VOCs; the chemical classes of identified compounds, etc. Suitable HiSorb materials will be used depending on the urine matrices.

There will be a need to include GC×GC-TOF-MS in the discovery of potential urinary VOC biomarkers. GC×GC employs two GC columns. The second column is frequently much shorter than the first with a different stationary phase and is operated at a higher temperature. The metabolites coeluting from the first column can be separated again in the second column due to different stationary phase and temperature. With its higher peak capacity, selectivity, and better detection limit, GC×GC can serve as a powerful tool for broad-spectrum cancer-related biomarker discovery. In addition, other metabolomic analytical platforms, such as LC-MS and NMR, can be involved in the biomarker discovery phase. Together, these platforms will generate comprehensive and multidimensional data, allowing for the compilation of a list of potential biomarkers.

Once the potential biomarker list for pancreatic cancer has been obtained, targeted analysis will be conducted for the validation of these biomarkers on the diagnosis of pancreatic cancer, with targeted methods for different instruments created based on the findings of the discovery phase. It will be possible to conduct multistage study to evaluate the diagnostic capability of the biomarkers in differentiating different cancer stages, aiming to establish diagnostic model for early diagnosis of pancreatic cancer.

In order to further interpret the biological significance, metabolic pathway analysis will be necessary to explore the possible relationship between the identified biomarkers and metabolic pathways within humans. The underlying metabolic pathways can be further investigated on cell, protein, and genomic levels. For example, comparing the amount of the discovered VOC biomarker in cancer cells to that in control cells by performing targeted analyses, and investigation of related enzymes or genes.

Furthermore, the aforementioned strategies can be used on the analysis of urine samples from other cancers, such as oesophageal cancer, gastric cancer and prostate cancer, to show feasibility of the methods developed within this thesis, and to discover urinary VOC biomarkers associated with these cancers.

In the future, urinary VOC analysis may help to improve the earlier detection of pancreatic, and other cancers. It is anticipated that the non-invasive detection of VOC in both urine and other biological matrices (e.g., breath) will offer an accessible, accurate, and affordable method for identifying ‘at risk’ of cancer who present to their general practitioner with often vague and non-specific symptoms that could represent an early cancer. Such patients often do not meet existing criteria for further investigation with either endoscopy or imaging studies. A VOC test may therefore be able triage these patients in primary care, identifying those patients who would most benefit from onward referral for further investigation. The test could also be used to provide reassurance to the majority of patients who do not have a cancer. It may be possible to combine the VOC test with other biomarkers (e.g., CA19-9) to improve the test performance. There may be other applications of a VOC base testing in future clinical practice including in population base screening and disease monitoring.

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APPENDIX 1 Systematic review

PRISMA Checklist

Section/topic	#	Checklist item	Reported on page #
TITLE			
Title	1	Identify the report as a systematic review, meta-analysis, or both.	42
ABSTRACT			
Structured summary	2	Provide a structured summary including, as applicable: background; objectives; data sources; study eligibility criteria, participants, and interventions; study appraisal and synthesis methods; results; limitations; conclusions and implications of key findings; systematic review registration number.	N/A, no registration number
INTRODUCTION			
Rationale	3	Describe the rationale for the review in the context of what is already known.	42
Objectives	4	Provide an explicit statement of questions being addressed with reference to participants, interventions, comparisons, outcomes, and study design (PICOS).	42
METHODS			
Protocol and registration	5	Indicate if a review protocol exists, if and where it can be accessed (e.g., Web address), and, if available, provide registration information including registration number.	42-43
Eligibility criteria	6	Specify study characteristics (e.g., PICOS, length of follow-up) and report characteristics (e.g., years considered, language, publication status) used as criteria for eligibility, giving rationale.	42-43
Information sources	7	Describe all information sources (e.g., databases with dates of coverage, contact with study authors to identify additional studies) in the search and date last searched.	42-43
Search	8	Present full electronic search strategy for at least one database, including any limits used, such that it could be repeated.	42-43, Table 3
Study selection	9	State the process for selecting studies (i.e., screening, eligibility, included in systematic review, and, if applicable, included in the meta-analysis).	44

Data collection process	10	Describe method of data extraction from reports (e.g., piloted forms, independently, in duplicate) and any processes for obtaining and confirming data from investigators.	44-45
Data items	11	List and define all variables for which data were sought (e.g., PICOS, funding sources) and any assumptions and simplifications made.	44-45
Risk of bias in individual studies	12	Describe methods used for assessing risk of bias of individual studies (including specification of whether this was done at the study or outcome level), and how this information is to be used in any data synthesis.	44-45
Summary measures	13	State the principal summary measures (e.g., risk ratio, difference in means).	44-45
Synthesis of results	14	Describe the methods of handling data and combining results of studies, if done, including measures of consistency (e.g., I2) for each meta-analysis.	N/A
Risk of bias across studies	15	Specify any assessment of risk of bias that may affect the cumulative evidence (e.g., publication bias, selective reporting within studies).	44-45
Additional analyses	16	Describe methods of additional analyses (e.g., sensitivity or subgroup analyses, meta-regression), if done, indicating which were pre-specified.	N/A
RESULTS			
Study selection	17	Give numbers of studies screened, assessed for eligibility, and included in the review, with reasons for exclusions at each stage, ideally with a flow diagram.	46, Figure 3
Study characteristics	18	For each study, present characteristics for which data were extracted (e.g., study size, PICOS, follow-up period) and provide the citations.	46, Table 4
Risk of bias within studies	19	Present data on risk of bias of each study and, if available, any outcome level assessment (see item 12).	50, Table 5, Table S1
Results of individual studies	20	For all outcomes considered (benefits or harms), present, for each study: (a) simple summary data for each intervention group (b) effect estimates and confidence intervals, ideally with a forest plot.	52-56, Figure 4, 5 and 6, Table 6, Table 7
Synthesis of results	21	Present results of each meta-analysis done, including confidence intervals and measures of consistency.	N/A
Risk of bias across studies	22	Present results of any assessment of risk of bias across studies (see Item 15).	50, Table 5, Table S1
Additional analysis	23	Give results of additional analyses, if done (e.g., sensitivity or subgroup analyses, meta-regression [see Item 16]).	N/A

DISCUSSION			
Summary of evidence	24	Summarize the main findings including the strength of evidence for each main outcome; consider their relevance to key groups (e.g., healthcare providers, users, and policy makers).	58-60, Table 8
Limitations	25	Discuss limitations at study and outcome level (e.g., risk of bias), and at review-level (e.g., incomplete retrieval of identified research, reporting bias).	60
Conclusions	26	Provide a general interpretation of the results in the context of other evidence, and implications for future research.	60-61
FUNDING			
Funding	27	Describe sources of funding for the systematic review and other support (e.g., supply of data); role of funders for the systematic review.	N/A

Table S1. Quality assessment of metabolic metadata based on CAWG-MSI guidelines.

Author	
Biological replicates	Sample preparation
Biofluid harvesting	
Storage condition	
Extraction method	
Extract concentration, dilution, resolubilisation process	
Extract enrichment	
Extract cleanup	
Extract storage and/or relocation	
Instrument description	Experimental analysis
Auto-injector	
Separation column	
Technique specific sample preparation	
Separation parameters	
Instrument description	Instrumental performance
Sample introduction & delivery	
Ionisation source	
Mass analyser description and acquisition mode	
Data acquisition parameters	
m/z calibration standard	Method validation
Accuracy of m/z calibration	
Mass resolution	
Ion source optimisation parameters	
Chromatographic resolution	
QC samples	
Internal Standards used	
Accuracy of Internal standard	
Precision of internal standard	
Retention time markers	
Accuracy and precision for replicated analyses	
Accuracy and precision for validation samples	
Cycles per column/injector/septum/blank	
Relative quantification	Metabolite identification
Absolute quantification	
Post-acquisition data pre-processing	Data pre-processing
	Metabolite identification

																																Description, quantifier of IS metabolite	Method for assessing instrument response	Replicate analyses	Calibration curves for each metabolite	Range of standards used	Quantification of method accuracy	Quantification of method precision	LLOQ	LLOD	Recovery/ stability	Data file format/ conversion methods	Data pre-processing methods	Level
Lima A.R. 2019	Y	Y	Y	Y	N	Y	N	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	N	NA	N	Y	N	Y	Y	N	N	N	N	Y	N	Y	Y	N								N	Y	2
Struck-Lewicka W. 2014	Y	Y	Y	Y	Y	N	N	N	Y	N	Y	Y	Y	Y	N	Y	Y	Y	N	NA	N	Y	N	Y	Y	N	N	Y	N	N	N	Y	Y	N								N	Y	2
Gao Q. 2019	Y	Y	Y	Y	N	Y	N	Y	Y	N	Y	Y	Y	Y	N	Y	Y	Y	N	NA	N	N	N	N	Y	N	N	N	N	Y	N	Y	N	N								N	Y	2
Jimenez-Pacheco A. 2018	Y	Y	Y	Y	N	Y	N	Y	Y	Y	Y	N	Y	Y	N	Y	Y	Y	N	NA	N	N	N	N	N	NA	NA	N	N	N	N	N	N	N								N	N	2
Khalid T. 2015	Y	Y	Y	Y	N	Y	N	Y	Y	N	Y	N	Y	Y	N	Y	Y	Y	N	NA	N	N	N	N	N	NA	NA	N	N	N	N	N	Y	Y								N	N	2
Spanel P. 1999	Y	Y	Y	N	N	N	N	N	Y	N	N	N	N	Y	Y	Y	Y	Y	N	NA	N	N	N	N	N	NA	NA	N	N	N	N				N	N	N	N	N	N	N	N	N	1
Chen Y. 2016	Y	Y	Y	Y	Y	N	N	N	Y	N	Y	Y	Y	Y	N	Y	Y	Y	N	NA	N	N	N	N	Y	N	N	N	N	Y	N				Y	Y	Y	Y	Y	Y	Y	N	N	1
Navaneethan U. 2015	Y	Y	Y	Y	N	N	N	N	N	N	N	N	N	Y	Y	Y	Y	Y	N	NA	N	N	N	N	N	NA	NA	N	Y	N	N				N	N	N	N	N	N	Y	N	Y	2

Panebianco C 2017	Y	Y	Y	Y	N	Y	N	N	Y	Y	Y	N	Y	Y	Y	N	N	N	N	NA	N	N	N	N	N	NA	NA	N	N	N	N	N	Y	N								N	Y	2
Arasaradnam RP 2014	Y	Y	Y	Y	N	N	N	N	Y	Y	Y	N	Y	Y	N	Y	Y	Y	N	NA	N	Y	N	N	N	NA	NA	N	N	N	N	N	Y	N								N	Y	NA
Huang J. 2013	Y	Y	Y	N	N	N	N	N	N	N	N	N	N	Y	Y	Y	Y	Y	N	NA	N	N	N	N	N	NA	NA	N	N	N	N				N	N	N	Y	N	N	N	N	N	2
Rozhentsov A.A. 2014	Y	N	Y	Y	N	Y	N	Y	N	N	N	N	Y	N	N	N	N	N	N	NA	N	N	N	N	N	NA	NA	N	N	N	N				N	N	N	N	N	N	N	N	N	NA
Silva C.L. 2011	Y	Y	Y	Y	N	Y	N	Y	Y	N	Y	N	Y	Y	Y	Y	Y	Y	N	NA	N	Y	N	N	N	NA	NA	N	N	N	N	N	N	N								N	N	2

APPENDIX 2 Ethical approvals for use of human biomaterial

St Mary's Research Ethics Committee
Internal Mail Box 121
St Marys Hospital
Paddington
London
W2 1NY

Telephone: 020 7886 6514
Facsimile: 020 7886 1529

05 December 2005

Mr George B Hanna
Senior Lecturer & Honorary Upper GI Consultant Surgeon
Imperial College London
Department of Surgical Oncology & Technology
10th Floor, QEQM Wing, St Mary's Hospital
South Wharf Road, London
W2 1NY

Dear Mr Hanna

Full title of study: **Establishment of a Tissue Bank for Oesophageal and Gastric Cancer**
REC reference number: **04/Q0403/119**

The REC gave a favourable ethical opinion to this study on 31 March 2005.

Further notification(s) have been received from local site assessor(s) following site-specific assessment. On behalf of the Committee, I am pleased to confirm the extension of the favourable opinion to the new site(s). I attach an updated version of the site approval form, listing all sites with a favourable ethical opinion to conduct the research.

Research governance approval

The Chief Investigator or sponsor should inform the local Principal Investigator at each site of the favourable opinion by sending a copy of this letter and the attached form. The research should not commence at any NHS site until research governance approval from the relevant NHS care organisation has been confirmed.

Statement of compliance

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

04/Q0403/119	**Please quote this number on all correspondence**

Yours sincerely

Mrs Rosalind Cooke
Committee Co-ordinator

An advisory committee to North West London Strategic Health Authority

St Mary's Research Ethics Committee

LIST OF SITES WITH A FAVOURABLE ETHICAL OPINION

For all studies requiring site-specific assessment, this form is issued by the main REC to the Chief Investigator and sponsor with the favourable opinion letter and following subsequent notifications from site assessors. For issue 2 onwards, all sites with a favourable opinion are listed, adding the new sites approved.

REC reference number:	04/Q0403/119	**Issue number:**	4	**Date of issue:**	05 December 2005
Chief Investigator:	Mr George B Hanna				
Full title of study:	Establishment of a Tissue Bank for Oesophageal and Gastric Cancer				

This study was given a favourable ethical opinion by St Mary's Research Ethics Committee on 31 March 2005. The favourable opinion is extended to each of the sites listed below. The research may commence at each NHS site when management approval from the relevant NHS care organisation has been confirmed.

Principal Investigator	*Post*	*Research site*	*Site assessor*	*Date of favourable opinion for this site*	*Notes* [1]
Mr George Hanna		St Mary's NHS Trust Academic Surgical UnitSt Mary's Hospital.	St Mary's Research Ethics Committee	27/09/2005	
Dr Margaret Burke		Harefield Hospital Hillend Road Harefield Middlesex UB9 6JH	Brompton, Harefield & NHLI Ethics Committee	27/09/2005	
Mr T C Holme	Consultant Surgeon	Lister Hospital Coreys Mill Lane Stevenage Herts SG1 4AB	Hertfordshire 2 REC	28/04/2005	
Mr Jeremy Livingstone	Consultant Surgeon, Watford General Hospital	Watford General HospitalWest Hertfordshire Hospitals NHS trust	Hertfordshire 1 Research Ethics Committee	05/12/2005	

Approved by the Chair on behalf of the REC:

..... (Signature of Administrator)

Rosalind Cooke

[1] *The notes column may be used by the main REC to record the early closure or withdrawal of a site (where notified by the Chief Investigator or sponsor), the suspension of termination of the favourable opinion for an individual site, or any other relevant development. The date should be recorded.*

Imperial College Healthcare Tissue Bank
Department of Surgery and Cancer
Charing Cross Hospital
Fulham Palace Road
London W6 8RF
Tel: 0203 311 7173
Email: tissuebank@imperial.ac.uk

ICHTB HTA licence: 12275
REC Wales approval: 17/WA/0161

Professor George Hanna
Academic Surgical Department
10th Floor, QEQM Building
St Mary's Hospital
London
W2 1NY

30th January 2019

Dear Professor Hanna,

Re: Tissue Bank application number; R18068

Project title; Profiling of Urinary Volatile Organic Compounds for the Diagnosis of Gastro-Instestinal Adenocarcinoma

I am pleased to confirm that the Imperial College Healthcare Tissue Bank Review Committee has received and approved the above mentioned project.

In order to satisfy HTA tracking requirements, would you please ensure the samples you use are reported to the tissue bank via the online database and sub-collection, along with any publications you produce, that should contain the acknowledgment wording as per the signed MTA.

Yours sincerely,

Professor Gerry Thomas
DI, HTA Research Licence.

Human Cancer Studies Group Imperial College London

NHS
Health Research Authority

Professor George Hanna
Head of Division of Surgery
Imperial College London
10th Floor QEQM Wing
St Mary's Hospital
London
W2 1NY

Email: hra.approval@nhs.net

6 December 2016

Dear Professor Hanna,

Letter of HRA Approval for a study processed through pre-HRA Approval systems

Study title:	**Non-invasive testing for the diagnosis and assessment of Gastro-intestinal disease**
IRAS project ID:	**142097**
Sponsor:	**Imperial College London**

Thank you for your request for HRA Approval to be issued for the above referenced study.

I am pleased to confirm that the study has been given **HRA Approval.** This has been issued on the basis of an existing assessment of regulatory compliance, which has confirmed that the study is compliant with the UK wide standards for research in the NHS.

The extension of HRA Approval to this study on this basis allows the sponsor and participating NHS organisations in England to set-up the study in accordance with HRA Approval processes, with decisions on study set-up being taken on the basis of capacity and capability alone.

If you have submitted an amendment to the HRA between 23 March 2016 and the date of this letter, this letter incorporates the HRA Approval for that amendment, which may be implemented in accordance with the amendment categorisation email (e.g. not prior to REC Favourable Opinion, MHRA Clinical Trial Authorisation etc., as applicable). If the submitted amendment included the addition of a new NHS organisation in England, the addition of the new NHS organisation is also approved and should be set up in accordance with HRA Approval processes (e.g. the organisation should be invited to assess and arrange its capacity and capability to deliver the study and confirm once it is ready to do so).

Participation of NHS Organisations in England

Please note that full information to enable set up of participating NHS organisations in England is not provided in this letter, on the basis that activities to set up these NHS organisations is likely to be underway already.

The sponsor should provide a copy of this letter, together with the local document package and a list of the documents provided, to participating NHS organisations in England that are being set up in accordance with HRA Approval Processes. It is for the sponsor to ensure that any documents provided to participating organisations are the current, approved documents.

For non-commercial studies the local document package should include an appropriate Statement of Activities and HRA Schedule of Events. The sponsor should also provide the template agreement to be used in the study, where the sponsor is using an agreement in addition to the Statement of Activities. Participating NHS organisations in England should be aware that the Statement of Activities and HRA Schedule of Events for this study have not been assessed and validated by the HRA. Any changes that are appropriate to the content of the Statement of Activities and HRA Schedule of Events should be agreed in a pragmatic fashion as part of the process of assessing, arranging and confirming capacity and capability to deliver the study. If subsequent NHS organisations in England are added, an amendment should be submitted to the HRA..

For commercial studies the local document package should include a validated industry costing template and the template agreement to be used with participating NHS organisations in England.

It is critical that you involve both the research management function (e.g. R&D office and, if the study is on the NIHR portfolio, the LCRN) supporting each organisation and the local research team (where there is one) in setting up your study. Contact details and further information about working with the research management function for each organisation can be accessed from www.hra.nhs.uk/hra-approval.

After HRA Approval

In addition to the document, *"After Ethical Review – guidance for sponsors and investigators"*, issued with your REC Favourable Opinion, please note the following:

- HRA Approval applies for the duration of your REC favourable opinion, unless otherwise notified in writing by the HRA.
- Substantial amendments should be submitted directly to the Research Ethics Committee, as detailed in the *After Ethical Review* document. Non-substantial amendments should be submitted for review by the HRA using the form provided on the HRA website, and emailed to hra.amendments@nhs.net.
- The HRA will categorise amendments (substantial and non-substantial) and issue confirmation of continued HRA Approval. Further details can be found on the HRA website.

The HRA website also provides guidance on these topics and is updated in the light of changes in reporting expectations or procedures.

Scope

HRA Approval provides an approval for research involving patients or staff in NHS organisations in England.

If your study involves NHS organisations in other countries in the UK, please contact the relevant national coordinating functions for support and advice. Further information can be found at http://www.hra.nhs.uk/resources/applying-for-reviews/nhs-hsc-rd-review/.

If there are participating non-NHS organisations, local agreement should be obtained in accordance with the procedures of the local participating non-NHS organisation.

User Feedback

The Health Research Authority is continually striving to provide a high quality service to all applicants and sponsors. You are invited to give your view of the service you have received and the application procedure. If you wish to make your views known please email the HRA at hra.approval@nhs.net. Additionally, one of our staff would be happy to call and discuss your experience of HRA Approval.

HRA Training

We are pleased to welcome researchers and research management staff at our training days – see details at http://www.hra.nhs.uk/hra-training/.

If you have any queries about the issue of this letter please, in the first instance, see the further information provided in the question and answer document on the HRA website.

Your IRAS project ID is **142097.** Please quote this on all correspondence.

Yours sincerely,

David Williams
Application Administrator

Email: hra.approval@nhs.net

Copy to: *Ms Christine Buicke*

APPENDIX 3 Multivariate analysis

Table S2. The variable importance for the projection (VIP) list of the optimal non-polar dataset for pancreatic adenocarcinoma (AD) (*n* = 28) and controls (C) (*n* = 33) processed by ChromSpace/Gavin 3.

Compound Name	VIP Value
6-[4-Chlorophenyl]-N,N-diethyl-1,2,4,5-tetrazin-3-amine	2.224
N-CBZ-l-dihydrotryptophanyl-l-alanine	1.956
Didodecyl phosphate	1.927
Phosphonic acid, (p-hydroxyphenyl)-	1.882
2-Buten-1-one, 1-(2,6,6-trimethyl-1,3-cyclohexadien-1-yl)-, (E)-	1.731
dl-5-Methyltryptophan	1.647
Cyclopentanemethanol, α,α-dimethyl-	1.609
Ethane, 1-isothiocyanato-2-methoxy-	1.604
Phenol	1.581
Carbamic acid, p-tolyl ester	1.581
Cyclohexene, 1,5,5-trimethyl-3-methylene-	1.579
1,3-Dioxolane, 2-(bromomethyl)-2-(2-methylphenyl)-	1.529
2-Trichloroacetylpyrrolidine	1.519
Levomenol	1.512
2,3,6-Trifluorobenzyl alcohol, methyl ether	1.508
Methyl 2-furoate	1.506
2-Octen-4-one	1.498
R(-)3,7-Dimethyl-1,6-octadiene	1.497
Cyclohexanemethanol, α,α,4-trimethyl-	1.492
Naphthalene, 1,2,3,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-, (1S-cis)-	1.466
Benzene, 1-methyl-4-(1-methylethenyl)-	1.461
Ethyl 2-(2-chloroacetamido)-3,3,3-trifluoro-2-(4-fluorobenzylamino)propionate	1.456
3,5-Di-tert-butylbenzoic acid	1.450
1,3,8-p-Menthatriene	1.450
2-Pentanone, 5-bromo-	1.439
Cyclobutaneacetonitrile, 1-methyl-2-(1-methylethenyl)-	1.419
Bicyclo[3.1.0]hex-2-ene, 4-methylene-1-(1-methylethyl)-	1.401
4-Methyl-4-vinylbutyrolactone	1.397
4-Isopropyl-6-methyl-1-methylene-1,2,3,4-tetrahydronaphthalene	1.392
Silane, 1,3-butadiynyltrimethyl-	1.383
5-Aminoindan	1.382
Benzene, 1-methyl-3-(1-methylethyl)-	1.373
3-Chloro-4-methoxybenzaldehyde	1.363
1,3-Cyclopentadiene, 5,5-dimethyl-1-ethyl-	1.339
2-(6-Chloro-3-nitro-4-phenyl-quinolin-2-ylsulfanyl)-1-(2,3-dihydro-benzo[1,4]dioxin-6-yl)- ethanone	1.336
m-Cymene, 5-tert-butyl-	1.333
2-Furancarboxaldehyde, 5-methyl-	1.311
2,3-Dimethyl-5-(2,6,10-trimethylundecyl) furan	1.300
5-Hexyn-3-ol	1.297
2-Acetyl-4-methylbenzo(b)thiophene	1.293
3-Methyl-4-propenyl-oxetan-2-one	1.292
2-Acetyl-6-methoxynaphthalene	1.290
l-Alanine, N-(4-butylbenzoyl)-, tetradecyl ester	1.283
1.2-diacetylhydrazine	1.279
1-(3,5-Bis-trifluoromethylphenyl)ethanol	1.269
3-Hexanone	1.261
1,4-Bis(trimethylsilyl)-1,3-butadiyne	1.257
p-(1-Propenyl)-toluene	1.255
Bicyclo[2.2.2]oct-7-en-2-one, 5-methylene-	1.249
Biphenylene	1.241
1,3,8-p-Menthatriene	1.240
2-Cyclopenten-1-one, 3,5,5-trimethyl-	1.237
Benzoic acid, 4-(trimethylsilyl)-	1.235
p-Cymene	1.232
2H-Pyran, 3,6-dihydro-4-methyl-2-(2-methyl-1-propenyl)-	1.232
Dodecane, 2,6,11-trimethyl-	1.224
Butylphosphonic acid, heptyl neopentyl ester	1.218
1-Cyclohexene-1-carboxaldehyde, 4-(1-methylethyl)-	1.215
1-Propanesulfonyl chloride	1.210
Bicyclo[4.2.0]octa-1,3,5-triene, 2,4-dimethyl-	1.204

1,6,10-Dodecatrien-3-ol, 3,7,11-trimethyl-	1.199
2-Pentanone, 3-ethyl-	1.198
Styrene	1.196
Benzofuran, 4,7-dimethyl-	1.188
Difenoxin	1.178
Carveol	1.169
Furan, 2-propyl-	1.169
Cyclohexene, 3,3,5-trimethyl-	1.152
3-Octanol, 3,7-dimethyl-	1.148
2-Butanone, 4-(2,6,6-trimethyl-1,3-cyclohexadien-1-yl)-	1.148
α-Bromomesitylene	1.148
3,7,7-Trimethyl-1-penta-1,3-dienyl-2-oxabicyclo[3.2.0]hept-3-ene	1.138
Naphthalene, 1,2,3,4-tetrahydro-1,1,6-trimethyl-	1.138
Limonene	1.120
Methyl ethyl cyclopentene	1.120
γ-Terpinene	1.116
α-Terpineol	1.115
Hexanal	1.112
5-Isopropyl-2-methylphenyl pentanoate	1.106
3-Pentenoic acid, 4-methyl-	1.102
Ethanone, 1-(2-hydroxy-5-methylphenyl)-	1.096
Linalool	1.082
β-Bisabolene	1.075
Cyclohexene, 1-methyl-4-(1-methylethylidene)-	1.075
Benzylamine, N-tert-butyldimethylsilyl-	1.066
4-[2-Dimethylaminopropyl]thiosemicarbazide	1.065
Benzene, 4-ethenyl-1,2-dimethoxy-	1.063
Naphthalene, 2-methyl-	1.061
α-Phellandrene	1.057
1,3-Cyclohexadiene, 1-methyl-4-(1-methylethyl)-	1.053
α-Campholenal	1.049
Camphene	1.046
1H-Indene, 4,7-dimethyl-	1.036
Bicyclo[3.1.1]hept-2-ene-2-carboxaldehyde, 6,6-dimethyl-	1.029
1-Pentanone, 1-(1H-imidazol-4-yl)-	1.026
Naphthalene, 1,2-dihydro-4-methyl-	1.024
2-Furanmethanol	1.017
Anthracene, 9,9-(1,2-ethanediyl)bis[10-methyl-	1.014
Silane, methylvinyl(2,4-dimethylpent-3-yloxy)(methylvinyldodecyloxysilyloxy)-	1.011
benzenesulfonamide, N-(4-hydroxyphenyl)-2,4,6-tris(1-methylethyl)-	1.011
Naphthalene, 1,2-dihydro-1,5,8-trimethyl-	1.005
2-Norpinanol, 3,6,6-trimethyl-	1.001

Table S3. The variable importance for the projection (VIP) list of the optimal non-polar dataset for pancreatic adenocarcinoma (AD) (*n* = 28) and controls (C) (*n* = 33) processed by MSHub/GNPS.

Compound Name	VIP Value
Butanal, 3-methyl-	2.643
Furan, 2-methoxy-	2.521
2-Butanol, 2-methyl-	2.265
2,6-Di-tert-butyl-4-methyl-phenol	2.250
4-(bicyclo[2.2.1]heptan-2-ylmethyl)morpholine	2.096
2-Cyclopenten-1-one, 2,3,4-trimethyl-	2.071
2-Vinylfuran	1.915
(2E)-1-(2,6,6-Trimethyl-1,3-cyclohexadien-1-yl)-2-buten-1-one	1.841
Dimethylphosphinic fluoride	1.838
7-isopropylcyclohepta-1,3,5-triene	1.794
Benzene, 1-(1,5-dimethyl-4-hexenyl)-4-methyl-	1.790
1-Propene, 3-ethoxy-	1.780
2-Pentanone	1.763
3-Hexanone, 2-methyl-	1.761
2-(chloromethyl)-5-methyl-7-benzofurancarboxylic acid methyl ester	1.739
Azulene, 1,4-dimethyl-7-(1-methylethyl)-	1.696
Vitispirane	1.664
5,8-Dihydroxy-4a-methyl-4,4a,4b,5,6,7,8,8a,9,10-decahydro-2(3H)-phenanthrenone	1.663
2-tert-Butoxy-2-methylpropane	1.600
1-Hexene, 3-methyl-6-phenyl-4-(1-phenylethoxy)-	1.598
Thiophene, 2-(methylthio)-	1.584
Beta citronellene	1.577
2-((2R,4aR,8aS)-4a-Methyl-8-methylenedecahydronaphthalen-2-yl)prop-2-en-1-ol	1.565
Octanoic acid, 3,5-dimethylphenyl ester	1.554
4-Hydroxy-.beta.-ionone	1.551
1-(4-tert-Butylphenyl)propan-2-one	1.542
Silane, diethyldifluoro-	1.526
Cyercene I	1.525
Cycloprop[e]indene-1a,2(1H)-dicarboxaldehyde, 3a,4,5,6,6a,6b-hexahydro-5,5,6b-trimethyl-, (1a.alpha.,3a.beta.,6a.beta.,6b.alpha.)-(+)-	1.514
(3S,4S)-2-Methyl-4-(methylamino)-3-phenyl-2-pentanol	1.513
3-Octenoic acid, methyl ester, (E)-	1.467
(E)-1-ethylidene-4,5,8-trimethyl-1,2,3,4-tetrahydronaphthalene	1.441
1,7-Octanediol, 3,7-dimethyl-	1.437
Phenylacetaldehyde	1.423
2(3H)-Furanone, 5-ethenyldihydro-5-methyl-	1.422
2-Ethyl-5,5-dimethyl-1,3-cyclopentadiene	1.419
Phenol	1.415
Alpha cadinol	1.414
(5E)-3,7-dimethyl-3-octa-1,5,7-trienol	1.391
Spathulenol	1.387
Modhephene	1.380
Benzenepropanal, 3-(1,1-dimethylethyl)-.alpha.-methyl-	1.372
Pentalene, octahydro-2,5-dimethyl-	1.361
1-[1-(2,6-dimethylphenyl)-1,2,3,4-tetrazol-5-yl]-N-[2-(2-methyl-1H-indol-3-yl)ethyl]cyclopentan-1-amine	1.354
2,4-Hexadiene	1.350
Furfuryl alcohol	1.319
Hexan-3-one	1.314
1-(3,5-Bis-trifluoromethylphenyl)ethanol	1.312
1,5-Dimethyl-6-oxa-bicyclo[3.1.0]hexane	1.307
4H,5H-Pyrano(4,3-b)pyran-4,5-dione, 2,3-dihydro-3-.alpha.-hydroxy-2-.beta.-methyl-7-propenyl-	1.295
2H-Pyran, 3,6-dihydro-4-methyl-2-(2-methyl-1-propenyl)-	1.289
6-Octen-1-ol, 3,7-dimethyl-	1.286
1,1,5,6-Tetramethylindane	1.258
Beta ionone	1.245
2-Pentenal, 2-methyl-	1.233
8-hydroxymenthol	1.224
2-Butanone, 4-(2,6,6-trimethyl-1,3-cyclohexadien-1-yl)-	1.215
Benzene, methyl-	1.211
3-Methylbutanol	1.208
tert-Butyldimethylsilylamine	1.192
2-Allyl-6-tert-butylphenol	1.183
Naphthalene, 1,2-dihydro-1,5,8-trimethyl-	1.162

Cyclopentene, 3-methyl-	1.149
5-Methyl-2-(1-methyl-1-phenylethyl)cyclohexanol	1.145
Benzene, 1,1'-(methoxymethylene)bis-	1.139
Geranyl alpha terpinene	1.133
Carvone	1.131
2-Propen-1-ol, 2-[(trimethylsilyl)methyl]-	1.127
P-cresol	1.122
2-Heptanone, 6-methyl-6-(5-methyl-2-furanyl)-	1.116
1-Isopropyl-2,3,4,5-tetramethylbenzene	1.112
1H-Indene, 2,3-dihydro-1,1,5,6-tetramethyl-	1.102
Dimethyl 2-methyl-2,3-oxiranedicarboxylate	1.095
6-fluoro-2,3,4,9-tetrahydro-1H-carbazol-3-amine	1.093
Limonene	1.093
2,5-Heptadien-4-one, 2,6-dimethyl-	1.090
(E)-1-esylprop-1-ene	1.089
2H-1-Benzopyran, 3,4,4a,5,6,8a-hexahydro-2,5,5,8a-tetramethyl-, (2.alpha.,4a.alpha.,8a.alpha.)-	1.086
3-Isopropyl-6-methyltetrahydro-2H-pyran-2-one	1.085
2-(2-methylnorbornan-2-yl)oxytetrahydropyran	1.082
(2E)-7-(Tetrahydro-2H-pyran-2-yloxy)-2-octen-1-ol	1.071
2H-Pyran-4-carboxylic acid, 2-ethoxytetrahydro-, methyl ester	1.058
2,3-Dimethyl-undec-1-en-3-ol	1.058
3-Isopropylamino-6-methyl-6,7-dihydro-9H-5-oxa-9-azabenzocyclohepten-8-one	1.044
Acetic acid, methyl ester	1.038
Alpha bisabolol	1.038
p-Mentha-8-dehydeo-1,3-diene	1.035
Isobutyraldehyde	1.028
Ethanone, 1-(4-methoxyphenyl)-	1.027
10,13-dimethyl-2,3,4,7,8,9,11,12,14,15-decahydro-1H-cyclopenta[a]phenanthren-3-ol	1.019
Cyclopenta[1,3]cyclopropa[1,2]cyclohepten-3(3ah)-one, 1,2,3b,6,7,8-hexahydro-6,6-dimethyl-	1.018
cis-p-mentha-1(7),8-dien-2-ol	1.016
2-Methyl-3-buten-2-ol	1.004
9,19-Cycloergost-24(28)-en-3-ol, 4,14-dimethyl-, acetate, (3.beta.,4.alpha.,5.alpha.)-	1.001
3-Butyn-2-amine, 2-methyl-	1.001
Benzene, (2-methyl-1-propenyl)-	1.000

Table S4. The variable importance for the projection (VIP) list of the optimal polar dataset for pancreatic adenocarcinoma (AD) (*n* = 28) and controls (C) (*n* = 33) processed by ChromSpace/Gavin 3.

Compound Name	VIP Value
2(3H)-Furanone, 5-heptyldihydro-	2.929
5-(2-Thienoyl)butyric acid	2.597
1,3-Benzenediamine, 4-methoxy-	2.385
2-Pentanone	2.328
Benzoic acid, 4-ethoxy-, ethyl ester	2.320
1,1,4,5,6-Pentamethyl-2,3-dihydro-1H-indene	2.315
1-Hydroxy-3-phenylacetone	2.283
1,1,1,3,5,5,5-Heptamethyltrisiloxane	2.134
Phthalic acid, methyl neopentyl ester	2.104
4-tert-Butyl-2,6-dimethylacetophenone	2.100
7-Octen-2-ol, 2,6-dimethyl-	2.086
2-Buten-1-amine, N-butyl-, (E)-	2.074
Thiophene-2-carboxylic acid, [2-(4-nitrophenyl)-2-oxo]ethyl ester	2.034
R(-)3,7-Dimethyl-1,6-octadiene	2.020
p-Cresol	2.006
Ethanone, 1-(2-furanyl)-	1.988
(2-Methoxyphenyl)carbamic acid, naphthalen-2-yl ester	1.960
Phthalic acid, methyl neopentyl ester	1.849
1-Butanol, 3-methyl-	1.846
2-Buten-1-one, 1-(2,6,6-trimethyl-1,3-cyclohexadien-1-yl)-	1.822
Methylenedioxyamphetamine, N-heptafluorobutyryl deriv.	1.797
Benzoyl chloride, 4-pentyl-	1.793
Acetic acid, [(2,4,6-triethylbenzoyl)thio]-	1.719
5,9,9-Trimethyl-spiro[3.5]non-5-en-1-one	1.697
Benzaldehyde, 4-propyl-	1.693
Levodopa	1.686
Hydroxyflutamide	1.663
3-Hexanone	1.637
D-Mannopentadecane-1,2,3,4,5-pentaol	1.636
2-Heptanol	1.625
3-Pentenoic acid, 4-methyl-	1.624
1-Butanol, 3-methoxy-	1.612
1,9-Nonanediol	1.599
Phenol	1.588
Silane, diethyldifluoro-	1.585
1,5,7-Octatrien-3-ol, 3,7-dimethyl-	1.578
Cyclohexanol, 5-methyl-2-(1-methylethyl)-	1.540
Carveol	1.514
4-Methyl-4-vinylbutyrolactone	1.505
1,3,5,7-Cyclooctatetraene	1.491
1,3-Dioxolane, 2-methyl-2-pentyl-	1.484
Benzene, 1,2,4,5-tetramethyl-	1.479
Naphthalene, 1,2,3,4-tetrahydro-1,1,6-trimethyl-	1.459
1,3,8-p-Menthatriene	1.446
Hexanal	1.444
1,2-Benzenediamine, N-methyl-	1.414
a-PVT	1.404
Furan, 2-(methoxymethyl)-	1.401
Cyclohexanol, 5-methyl-2-(1-methylethyl)-	1.388
Hydroxylamine, methyl-(1-phenylethyl)-	1.372
Benzoyl chloride, 4-hexyl-	1.360
1,3,8-p-Menthatriene	1.349
Cyclohexanemethanol, α,α,4-trimethyl-	1.343
1-Butyne, 3-(2-methoxypropoxy)-	1.338
Ethanol, 2-(1-methylethoxy)-	1.307
Mequinol	1.281
aR-Turmerone	1.276
1,3-Dithiolane, 2-benzyl-2-methyl-	1.247
Benzene, 1-methyl-3,5-bis(1-methylethyl)-	1.238
Naphthalene, 1,2-dihydro-1,5,8-trimethyl-	1.236
Sebacic acid, 3,5-dimethylphenyl isobutyl ester	1.221
Edulan II	1.211
Toluene, 4-(1,1-dimethyl-2-propynyloxy)-	1.193
Cyclohexane, ethylidene-	1.178

Methyl-2-O-methylαd-glucopyranoside	1.171
p-Cymene	1.167
2-Methyl-2-nonanol	1.166
1,2,5-Oxadiazole-3-carboxamide, 4-amino-N-[2-[[(4-fluorophenyl)methyl]amino]ethyl]-	1.152
(±)-Dihydro-ar-turmerone	1.127
(2-Hydroxy-3-methoxy-phenyl)-(3,5,5-trimethyl-4,5-dihydro-pyrazol-1-yl)-acetonitrile	1.127
Furan, 2,3,5-trimethyl-	1.126
Benzene, (2-methyl-1-propenyl)-	1.122
Thiophene, 2-methoxy-5-methyl-	1.122
1,1,4,5,6-Pentamethyl-2,3-dihydro-1H-indene	1.110
(-)-Carvone	1.110
o-Isopropenyltoluene	1.106
Cyanamide, dimethyl-	1.106
4-Ethoxystyrene	1.101
Methyl Isobutyl Ketone	1.097
2-Furancarboxylic acid, hydrazide	1.096
Methyl Isobutyl Ketone	1.094
α-Bromomesitylene	1.076
3-Isopropylbenzaldehyde	1.075
(R)-(+)-3-Methylcyclopentanone	1.069
Nonane, 2,2,4,4,6,8,8-heptamethyl-	1.065
Terbutaline, tetrakis-trifluoroacetyl-	1.035
N-Chloroacetyl-dl-erythro-O-methylthreonine	1.033
Benzene, undecyl-	1.025
α-Phellandrene	1.020
2,4-Dimethylfuran	1.012
Bicyclo[2.2.1]hept-2-ene, 1,7,7-trimethyl-	1.001

Table S5. The variable importance for the projection (VIP) list of the optimal polar dataset for pancreatic adenocarcinoma (AD) (*n* = 28) and controls (C) (*n* = 33) processed by MSHub/GNPS.

Compound Name	VIP Value
1,3,3-Trimethylbicyclo[2.2.1]heptan-2-one	2.265
3-Nonanone	2.104
(2E)-1-(2,6,6-Trimethyl-1,3-cyclohexadien-1-yl)-2-buten-1-one	2.020
Curcumenol	1.975
1,1,5,6-Tetramethyl-1,2,3,4-tetrahydronaphthalene	1.887
(5E)-3,7-dimethyl-3-octa-1,5,7-trienol	1.883
Cyclopentanone, 3,4-bis(2-oxopropyl)-, trans-	1.791
Cyclohexanone, 4R-acetamido-2,3-cis-epoxy-	1.780
Myrcenol-dihydro	1.758
2-Pentanone	1.740
2-Butanone, 3-methyl-	1.736
Isobutyl-2-heptenone	1.734
Dodecyl fluoroacetate	1.672
Thiourea, 1-(2,4,6-trimethylphenyl)-3-(2-propynyl)-	1.641
Cycloprop[e]indene-1a,2(1H)-dicarboxaldehyde, 3a,4,5,6,6a,6b-hexahydro-5,5,6b-trimethyl-, (1a.alpha.,3a.beta.,6a.beta.,6b.alpha.)-(+)-	1.597
10,13-dimethyl-2,3,4,7,8,9,11,12,14,15-decahydro-1H-cyclopenta[a]phenanthren-3-ol	1.552
.alpha.-Dehydro-ar-himachalene	1.543
2-Methyl-5-propylfuran	1.488
Cyclohexane, 1,1,3-trimethyl-2,3-epoxy-2-(3-methylcyclobuten-2-yl-1)-4-acetyloxy-	1.481
3-Methyl-2-[(2Z)-2-pentenyl]cyclopentanol	1.469
1-Isopropyl-2,3,4,5-tetramethylbenzene	1.447
3-Cyclopenten-1-one, 2,2,5,5-tetramethyl-	1.436
Nootkaton-11,12-epoxide	1.426
.delta.-Cadinene	1.422
Bicyclo[2.2.1]heptane, 2,2-dimethyl-3-methylene-	1.411
(-)-Spathulenol	1.405
3-Hepten-2-one	1.400
Neomenthol	1.382
1,3-Cyclohexadiene, 1-methyl-4-(1-methylethyl)-	1.353
Heptan-3-one	1.337
Alpha bisabolol	1.331
Linalol oxyde THE-E	1.328
2-Heptanone, 3-methyl-	1.318
2(3H)-Furanone, dihydro-4,4-dimethyl-	1.318
1,4,4,7a-Tetramethyl-2,4,5,6,7,7a-hexahydro-1H-indene-1,7-diol	1.314
Limonene	1.302
Phenol, 3,5-dimethyl-	1.297
Modhephene	1.297
2-Ethyl-5,6,7,8-tetrahydro-8,9,9(5,9,9)trimethyl-5,8-	1.293
2H-Pyran, 2-ethenyltetrahydro-2,6,6-trimethyl-	1.293
4,7,7-Trimethylbicyclo[3.2.0]hept-3-en-6-one	1.287
3-Nonanol, 1,2:6,7-diepoxy-3,7-dimethyl-, acetate	1.283
Naphthalene, 1,2-dihydro-1,5,8-trimethyl-	1.281
Hexan-3-one	1.253
1,2-Dihydroxyadamantane	1.253
Octanoic acid methyl ester	1.249
Beta thujone	1.244
Naphthalene, 1,2,4a,5,8,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-, [1S-(1.alpha.,4a.beta.,8a.alpha.)]-	1.243
3,3-Dimethyl-6-methylenecyclohexene	1.236
3-Buten-2-one, 4-(2,6,6-trimethyl-1-cyclohexen-1-yl)-	1.227
2,3,4-Trimethyl-2-pentanol	1.213
Cyclohexanone, 2,2,6-trimethyl-	1.210
(3E,5E)-2,6-Dimethylocta-3,5,7-trien-2-ol	1.209
1,2,4,5-Tetramethylbenzene	1.205
Bicyclo[2.2.1]hept-2-ene, 2,3-dimethyl-	1.195
Ethanone, 1-(4-methylphenyl)-	1.186
4-Isopropylcyclohexa-1,3-dienecarbaldehyde	1.185
Benzene, triisopropyl-	1.184
Alpha curcumene (Ar-curcumene)	1.183
2H-1-Benzopyran, 3,4,4a,5,6,8a-hexahydro-2,5,5,8a-tetramethyl-, (2.alpha.,4a.alpha.,8a.alpha.)-	1.182
Acetic acid, hexyl ester	1.181
2-Furanpentanal, 5-methyl-	1.179

E-11-Tetradecen-1-ol trifluoroacetate	1.173
1,2,3,4,5-Pentamethyl-1,3-dicyclopenta-dienylcarb onsaeuremethylester	1.168
1,3-Cyclopentadiene, 1,2,3,4,5-pentamethyl-	1.168
Naphthalene, 1,6,7-trimethyl-	1.163
Pulegone	1.157
Myrtenal	1.154
Vitispirane	1.152
N'-(2-Cyanoacetyl)acetohydrazide	1.145
Benzene, 2-(1-decylundecyl)-1,4-dimethyl-	1.144
(1R,3aR,5aR,9aS)-1,4,4,7-Tetramethyl-1,2,3,3a,4,5a,8,9-octahydrocyclopenta[c]benzofuran	1.144
Styrene	1.139
Pentandioic acid, (p-t-butylphenyl) ester	1.105
trifluoro(fluorosulfonyloxy)methane	1.092
Dicyclohexylmethanol	1.075
Hexanal	1.074
p-Mentha-1,5,8-triene	1.054
Butyl carbamate	1.049
Alpha-cedrene	1.042
.alpha.-Selinene	1.028
2-Butyl-1-methyl-1,2,3,4-tetrahydronaphthalen-1-ol	1.026
3,9-Phenanthrenedione,1,2,3,4,4a,9,10,10a-octahydro-5,8-dimethyl	1.019
14-Hydroxy-9-epi-E-caryophyllene	1.017
Dillether	1.016
2,3,3,4,7-Pentamethyl-2,3-dihydro-1-benzofuran	1.015
3-Hydroxy-2-methyl-7-[(1E)-1-propenyl]-2,3-dihydro-4H,5H-pyrano[4,3-b]pyran-4,5-dione	1.010

APPENDIX 4 Investigation for confounding factors

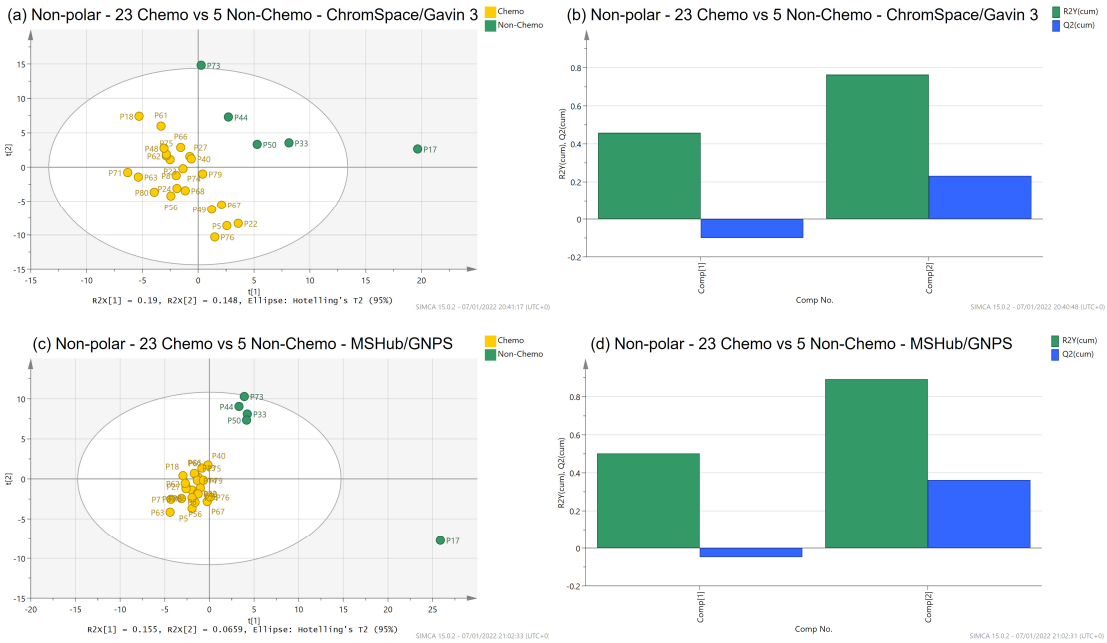


Figure S1. PLS-DA for the effect of chemotherapy (non-polar).

PLS-DA score plot of the optimal non-polar dataset for post chemotherapy pancreatic adenocarcinoma (Chemo) (*n* = 23) and chemotherapy naïve (Non-Chemo) (*n* = 5) processed by (a) ChromSpace/Gavin 3 and (c) MSHub/GNPS. Internal validation for both models ((b) ChromSpace/Gavin 3: Component 1, R^2Y = 0.458, Q^2 = -0.1; Component 2, R^2Y = 0.763, Q^2 = 0.232) ((d) MSHub/GNPS: Component 1, R^2Y = 0.502, Q^2 = -0.048; Component 2, R^2Y = 0.892, Q^2 = 0.362) indicated model overfitting and poor predictive capability.

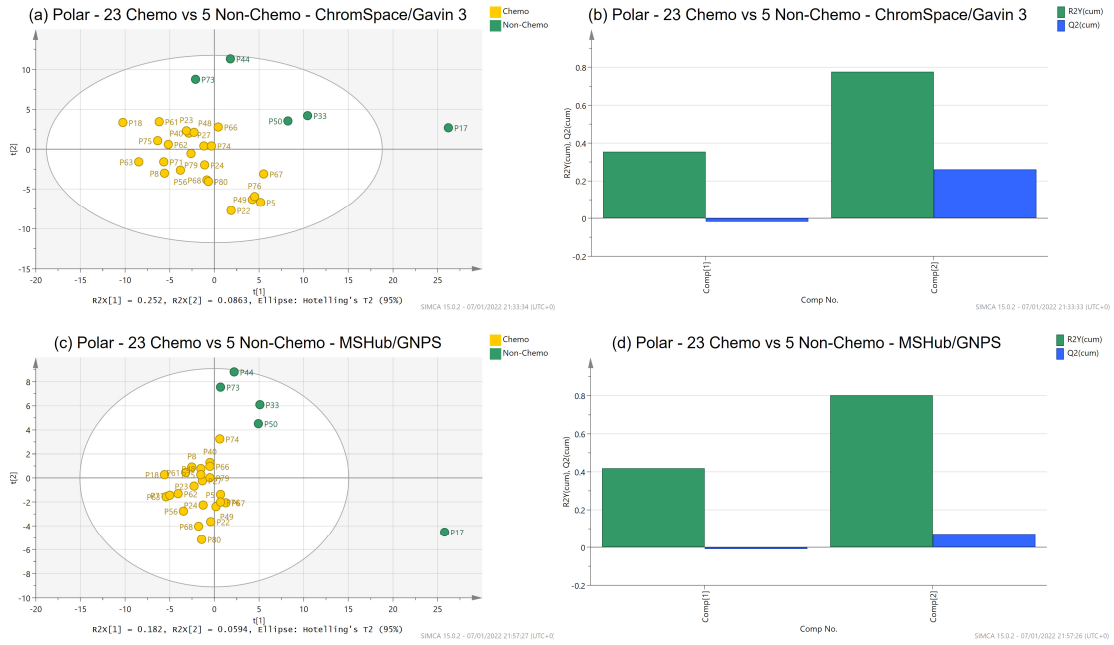


Figure S2. PLS-DA for the effect of chemotherapy (polar).

PLS-DA score plot of the optimal polar dataset for post chemotherapy pancreatic adenocarcinoma (Chemo) (*n* = 23) and chemotherapy naïve (Non-Chemo) (*n* = 5) processed by (a) ChromSpace/Gavin 3 and (c) MSHub/GNPS. Internal validation for both models ((b) ChromSpace/Gavin 3: Component 1, R^2Y = 0.355, Q^2 = -0.019; Component 2, R^2Y = 0.777, Q^2 = 0.261) ((d) MSHub/GNPS: Component 1, R^2Y = 0.419, Q^2 = -0.009; Component 2, R^2Y = 0.802, Q^2 = 0.072) indicated model overfitting and poor predictive capability.

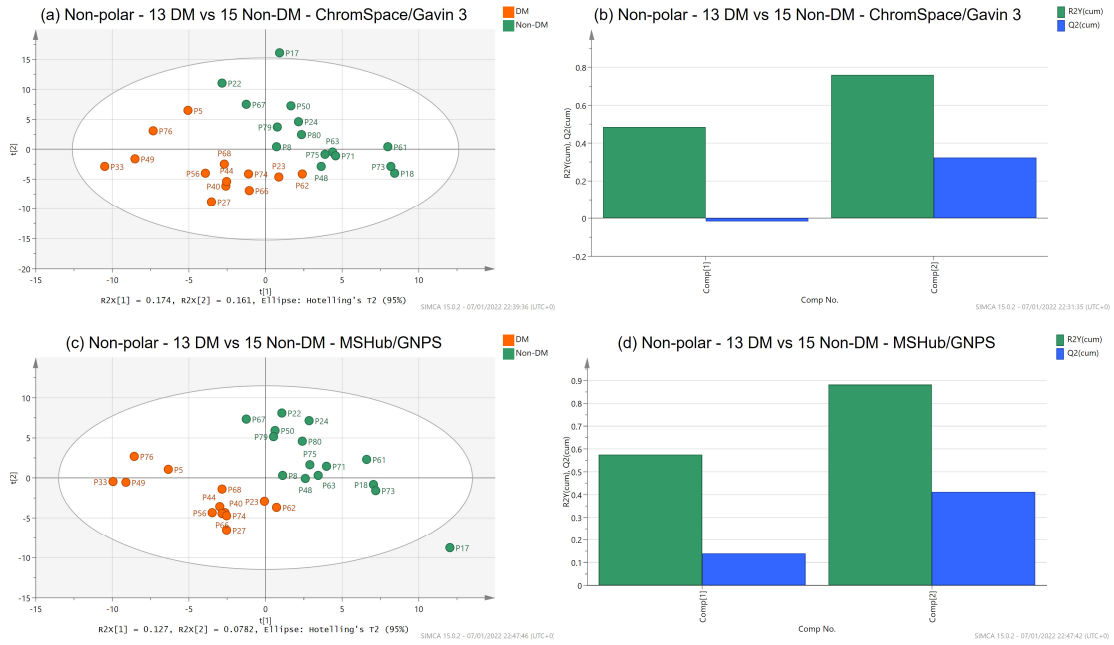


Figure S3. PLS-DA for the effect of diabetes (non-polar).

PLS-DA score plot of the optimal non-polar dataset for pancreatic adenocarcinoma patients with diabetes (DM) (*n* = 13) and without diabetes (Non-DM) (*n* = 15) processed by (a) ChromSpace/Gavin 3 and (c) MSHub/GNPS. Internal validation for both models ((b) ChromSpace/Gavin 3: Component 1, R^2Y = 0.485, Q^2 = -0.018; Component 2, R^2Y = 0.760, Q^2 = 0.324) ((d) MSHub/GNPS: Component 1, R^2Y = 0.575, Q^2 = 0.140; Component 2, R^2Y = 0.882, Q^2 = 0.412) indicated model overfitting and poor predictive capability.

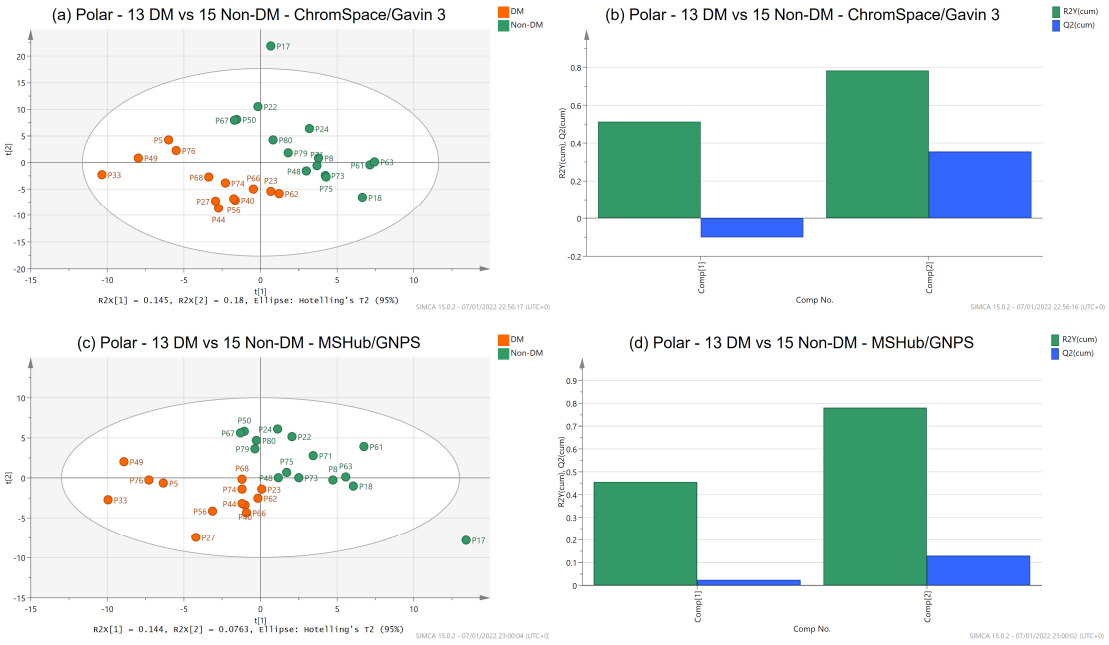


Figure S4. PLS-DA for the effect of diabetes (polar).

PLS-DA score plot of the optimal polar dataset for pancreatic adenocarcinoma patients with diabetes (DM) (*n* = 13) and without diabetes (Non-DM) (*n* = 15) processed by (a) ChromSpace/Gavin 3 and (c) MSHub/GNPS. Internal validation for both models ((b) ChromSpace/Gavin 3: Component 1, R^2Y = 0.513, Q^2 = -0.1; Component 2, R^2Y = 0.784, Q^2 = 0.356) ((d) MSHub/GNPS: Component 1, R^2Y = 0.456, Q^2 = 0.025; Component 2, R^2Y = 0.780, Q^2 = 0.129) indicated model overfitting and poor predictive capability.

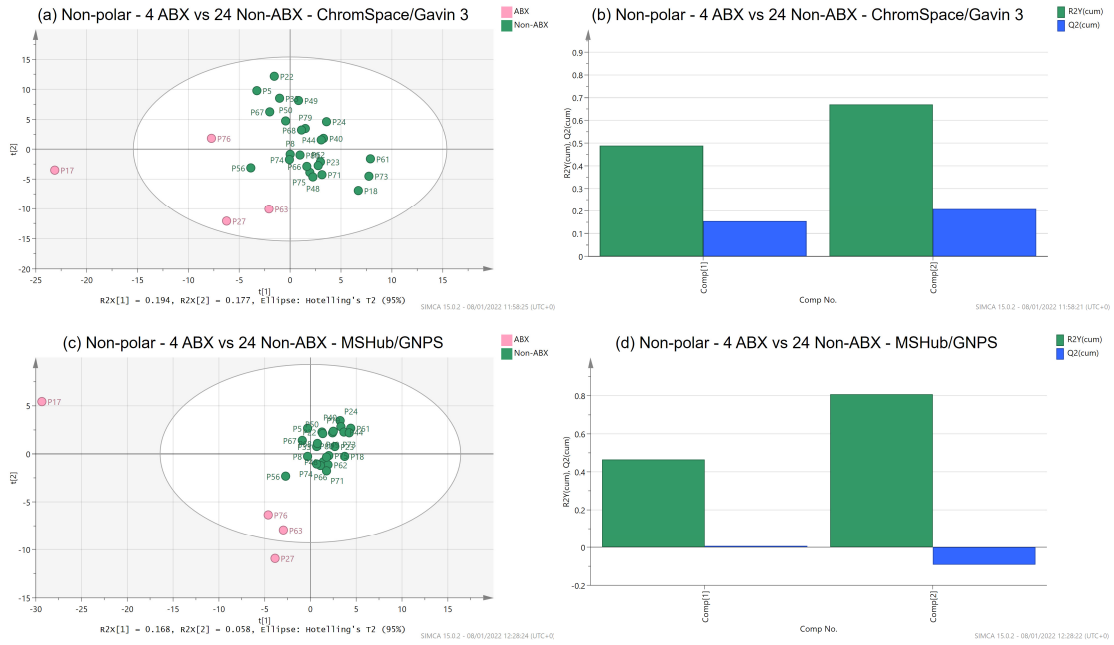


Figure S5. PLS-DA for the effect of antibiotics (non-polar).

PLS-DA score plot of the optimal non-polar dataset for post antibiotics pancreatic adenocarcinoma (ABX) (*n* = 4) and antibiotics naïve (Non-ABX) (*n* = 24) processed by (a) ChromSpace/Gavin 3 and (c) MSHub/GNPS. Internal validation for both models ((b) ChromSpace/Gavin 3: Component 1, R^2Y = 0.489, Q^2 = 0.154; Component 2, R^2Y = 0.670, Q^2 = 0.208) ((d) MSHub/GNPS: Component 1, R^2Y = 0.464, Q^2 = 0.008; Component 2, R^2Y = 0.807, Q^2 = -0.092) indicated model overfitting and poor predictive capability.

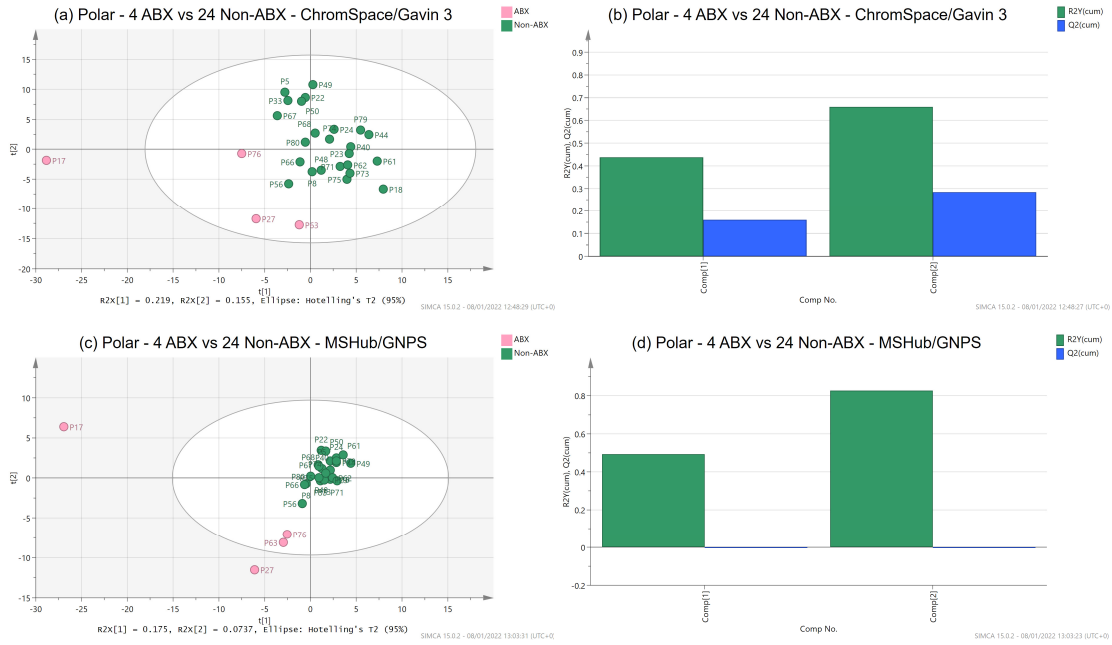


Figure S6. PLS-DA for the effect of antibiotics (polar).

PLS-DA score plot of the optimal polar dataset for post antibiotics pancreatic adenocarcinoma (ABX) (*n* = 4) and antibiotics naïve (Non-ABX) (*n* = 24) processed by (a) ChromSpace/Gavin 3 and (c) MSHub/GNPS. Internal validation ((b) ChromSpace/Gavin 3: Component 1, R^2Y = 0.438, Q^2 = 0.160; Component 2, R^2Y = 0.660, Q^2 = 0.284) ((d) MSHub/GNPS: Component 1, R^2Y = 0.493, Q^2 = -0.004; Component 2, R^2Y = 0.826, Q^2 = -0.004) indicated model overfitting and poor predictive capability.