Urinary Volatiles and Chemical Characterisation for the Non-invasive Detection of Prostate and Bladder Cancers

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Research Article

Keywords: Bladder cancer (BLC), Prostate cancer (PC), gas chromatography–ion mobility spectrometry (GC–IMS)

Posted Date: February 15th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-223926/v1

Abstract

Bladder cancer (BLC) and Prostate cancer (PC) are some of the most common cancers in the world, both having low five-year survival rates. In both BLC and PC, the diagnosis is often confirmed with an invasive technique that carries a risk to the patient. Consequently, a non-invasive diagnostic approach in these cancers would be medically desirable. The use of volatile organic compounds (VOCs) for disease diagnosis, including cancer, is a promising research area that could support the diagnosis process.

In this study, we investigated the urinary VOC profiles in BLC, PC patients and non-cancerous controls by using gas chromatography–ion mobility spectrometry (GC–IMS) and gas chromatography time-of-flight mass spectrometry (GC-TOF-MS) to analyse patient samples.

GC-IMS separated BLC from PC (AUC: 0.97 (0.93-1.00)), BLC Vs Non-cancerous (AUC: 0.95 (0.90-0.99).) and PC Vs Non-cancerous (AUC: 0.89 (0.83-0.94)) whereas GC-TOF-MS differentiated BLC from PC (AUC: 0.84 (0.73-0.93) BLC Vs Non-cancerous (AUC: 0.81 (0.70-0.90)) and PC vs Non-cancerous (AUC: 0.94 (0.90-0.97)). According to our study, a total of 34 biomarkers were found using GC-TOF-MS data, of which 13 VOCs were associated with BLC, 7 were associated with PC and 14 VOCs were found in the comparison of BLC and PC.

1. Introduction

Early detection and diagnosis of cancer remains a key goal to improve the prognosis and life expectancy of patients [1–4]. Globally, cancer results in some of the highest mortality rates for any disease. In 2020 alone there were more than 19 million new cancer diagnoses and almost 10 million deaths [5]. The UK is a major contributor to this, with some of the highest cancer rates in the world. It is amongst the top 10% of the countries with the highest number of new cases of cancer [6]. These figures emphasize the importance of using screening methods to improve disease diagnosis and to reduce cancer morbidity [7].

Bladder cancer (BLC) is the ninth most common cancer worldwide and is also one of the most difficult cancers to diagnose and clinically manage [8,9]. Prostate cancer (PC) is particularly prominent in men and is the sixth most common cancer worldwide [10–12]. Cystoscopy with biopsy and histological assessments are considered to be the ‘Gold Standard’ for the diagnosis of BLC currently [13]. However, Cystoscopy is invasive in nature, can cause pain, urinary infections, and blood loss in some cases [14,15]. To aid in the diagnosis of BLC, a range of urine tests have been developed including bladder tumour antigen (BTA) test, nuclear matrix protein 22 (NMP22), urinary bladder cancer antigen (UBC), and fibrin degradation products (FDP). Unfortunately, none of these tests have demonstrated sufficient specificity or sensitivity as a screening test [16].

For prostate cancer, PSA (Prostate-Specific Antigen) is a commonly used blood test. However, elevated PSA is not used as an indicator of PC in population screening, but rather as a method of monitoring PC progression in symptomatic patients [17]. The downsides of PSA as a diagnostic test for PC patients are

mainly related to the high false-positive rate. PSA can be raised in urinary and prostate infections or other conditions such as benign prostatic hyperplasia (BHP) [18]. Therefore, a raised PSA level can lead to unnecessary biopsies, which may end up causing fever, pain, bleeding, and infection to the patient [19–21].

One area receiving significant interest is in the use of volatile organic compounds (VOCs) as a way to diagnose and monitor cancer. VOCs are chemical compounds that are either produced *in vitro* or are introduced externally and can indicate the presence, or absence, of disease in the body. The concept first emerged after reports indicated that dogs could recognise cancer by sniffing biological samples [22]. Since this discovery, researchers have reported that VOCs could be used to detect a broad range of cancers including lung, colorectal and pancreatic cancer [23–25].

Urine is a common biological source of VOCs, as the components present are either the intermediate products or end products of metabolic activities occurring inside the human body [26]. A study published in 2016 provided significant evidence for the use of urinary VOCs for distinguishing BLC from a total of 72 urine samples and showed results with an accuracy of 89%, 90% sensitivity, and 88% specificity using PLS-DA (Partial least Squares Discriminant Analysis) on GC-MS data [27]. The gold standard for the analysis of VOCs remains GC-MS (Gas Chromatography-Mass Spectrometry) but it is expensive, requiring specialised equipment and trained staff, making it difficult to implement in a point of care scenario. However, more recently a range of other techniques have been reported that have the potential to be used at the point of care. GC-IMS (Gas Chromatography-Ion Mobility Spectrometry) is one such technique, it provides high sensitivity and selectivity, and the GC-IMS can be created in a portable form factor and can use nitrogen or air as the carrier gas. Our group has reported the use of this method with a range of different diseases [28,29].

The study aimed to identify and test the potential of urinary biomarkers to distinguish between two different cancers and healthy controls using both GC-TOF-MS and GC-IMS. We believe this is the first time that GC-IMS has been used with these cancers in combination with GC-TOF-MS.

2. Materials And Methods

2.1. Urine samples:

A total of 106 patients were recruited after providing informed consent at University Hospital Coventry & Warwickshire NHS Trust, UK, between July 2013 and November 2019. This study was approved by Coventry and Warwickshire and North-East Yorkshire NHS Ethics Committees (Ref 18717 and Ref 260179). Urine samples were collected in standard universal sterile specimen containers and frozen within 2 hours at -80°C for subsequent batch analysis and according to standard operating procedures compliant with tissue bank requirements under Human Tissue Act 2004. Prior to analysis the samples were transferred to the University of Warwick and briefly stored at -20°C. The samples were defrosted in a laboratory fridge at 4°C and aliquoted into 20mL glass sample vials with a crimp cap. 5mL of each urine

sample was used for the analysis using GC-IMS and GC-TOF-MS. Of the 106 urine samples collected, 15 patients had confirmed BLC, 55 were confirmed PC, and 36 non-cancerous controls. The mean age of the BLC patients was 70 years and the mean age of the PC patients was 72 years. The demographic data of the subjects are illustrated in Table 1.

Table 1
Demographic data for subject groups

Group	Bladder cancer	PC	Non-Cancerous
Number of samples	15	55	36
Mean Age (years)	70.0	71.9	62.5
Sex: Male/Female	12:3	All Male	24:12
Avg. BMI	24.4	27.5	30.9
Current Smoker (number and % of patients)	1 (6.7%)	6 (10.9%)	3 (8.3)%

2.2. Analytical devices:

2.2.1. G.A.S. FlavourSpec GC-IMS

The G.A.S FlavourSpec (Germany) uses a GC-IMS measurement technique to analyse VOCs. GC-IMS is a method used in various applications, such as detection of explosives and chemicals [30–32], air quality [33], health and disease detection [28,34–36] and food [37–39]. The method is formed of two stages. The first stage is a GC component that pre-separates chemicals based on their interaction with a retentive coating on the inside of a GC column. Thus, chemicals elude from the GC at different times [40]. These chemicals are further analysed using a drift-tube IMS method. Here, the chemicals are ionised (tritium in our case) and pass along a drift-tube, propelled by a high electric field. Against the flow of ions, a buffer gas (nitrogen in this case) is passed. The buffer gas and the ions collide resulting in a loss of momentum of the ions. Thus, transit time along the tube is a function of the interaction of the ion with the electric field and the number of collisions with the buffer gas. This provides two-dimensional separation of the chemical components [38,41].

For analysis, glass vials containing samples were transferred to an autosampler fitted to the GC-IMS. The sample tray is chilled to 4°C to reduce sample degradation during sample analysis. Each sample is heated to 40°C and agitated to 10 minutes before sampling. The autosampler then takes 0.5 mL of sample headspace and directly injects it into the GC-IMS. The machine settings for analysis were as follows: E1: 150 mL/min (for the drift tube IMS), E2: 20 mL/min (for the GC column), and the pump set to 25%. The total run time per sample was 10 minutes. The temperatures were set to T1 (IMS): 45°C, T2 (column): 80°C, and T3 (injector): 70°C.

2.2.2. Markes GC-TOF-MS

GC-TOF-MS operates by analysing the time of flight of ions and analyse them according to their mass-to-charge ratio. The GC-TOF-MS system used is a combination of TRACE 1300 GC (Thermo Fisher Scientific, Loughborough, UK) and BenchTOF-HD TOF-MS (Markes Intl., Llantrisant, UK). This system also includes a high-throughput autosampler and a thermal desorption unit, ULTRA-xr and UNITY-xr, respectively (both from Markes Intl.). The GC separates the chemicals in the same way as explained previously. The separated chemicals are detected by TOF MS once they enter the TOF 'flight box'. TOF-MS separates fragment ions instead of molecular ions as in an IMS. The ions are detected depending upon the mass-to-charge ratio of the ions after passing through the drift tube [42,43].

For analysis, a thermal desorption (TD) sorbent tube (C2-AXXX-5149, Markes Intl., Llantrisant, UK) was inserted through the septum and into the headspace above the sample and then heated at 40°C for 20 minutes. A pump was then attached to the TD tube, and whilst still heated to 40°C, the headspace VOCs were then pulled onto the tubes at 20mL/minute for a further 20 minutes. The sorbent tubes were then placed in an autosampler for analysis. The analysis started with ULTRA-xr with a stand-by split set to 150°C. The GC run time for samples was 25 min with a programmed temperature ramp from 40°C to 280°C at 20°C/min. Each sample was pre-purged for 1 min and then desorbed at 250°C for 10 min, with the trap purge time set to 1 min. These traps were then cooled at -30°C and the trap was then purged for 3 min at a temperature of 300°C. The temperature for both transfer line and ion source was heated to 250°C. The data from GC-TOF-MS analysis were identified using the national institute of standards and technology (NIST) list (2011).

2.3. Statistical Methods:

For GC-IMS data analysis, the data was extracted using the G.A.S VOCal (v0.1.3, G.A.S., Dortmund, Germany) software. This was followed by pre-processing steps to reduce the data's dimensionality. Among all the data points, the central section contains all of the computationally significant chemical information and thus all the other data was removed through a cropping process. This was followed by applying a small threshold to remove the background information, which is a value just above the background noise level. The same data cropping and threshold values were used on all the data and it was undertaken using an automated program. The data was then analysed using a 10-fold cross-validation, undertaken using a bespoke R program (version 3.6.2). Within each fold training set, feature selection was undertaken using a Wilcoxon rank-sum test between the different cancer groups and non-cancerous group. That resulted in the identification of the 20 most discriminatory features between the two groups and the features trained by three models, XGBoost, logistic regression, and random forest. The model was then applied to the test set to create class probabilities. Once all the samples have been within a test set, statistical results were generated from the probabilities, including a Receiver Operator Characteristic (ROC) curve, p-values, sensitivity, selectivity, specificity, positive predictive value (PPV), and negative predictive value (NPV).

An analogous approach was used for GC-TOF-MS data analysis. For GC-TOF-MS, the chemicals and the abundance of the chemicals were identified. Using the TOF-DS software, a background correction was

applied, and the chromatogram was integrated, and the peaks were identified using the NIST list which was exported. The data obtained from GC-TOF-MS was converted into text files of chemical lists and abundances. The data was then processed using an 'R' program that was similar to that used for GC-IMS, where chemical components of discriminative power were identified. Figure 1 provides a flow diagram of the data analysis steps.

3. Results

Figure 2 shows a typical output from the GC-IMS method from a urine sample in which the x-axis represents the drift time of the IMS and the y-axis represents the retention time of the GC. In the figure, the 'dots' are the chemicals detected by the IMS and the intensity of the peak represents the number of ions. Those 'dots' in red are the most intense. The red line in the figure is the default output of the instrument where no chemicals are present. G.A.S VOCal (v0.1.3, G.A.S., Dortmund, Germany) was used to view the GC-IMS data. Figure 3 provides an example output from the GC-TOF-MS method. Here, the x-axis refers to the retention time, and the y-axis, the total ion count.

The results of the statistical analysis of the GC-IMS gathered results between different cancer groups and the non-cancerous group are given in Table 2. The results demonstrate high sensitivity and specificity, indicating that there are significant differences between the VOC profiles of the different groups. Importantly good separation between the two different cancers - BLC vs PC, was also achieved.

Table 2
GC-IMS diagnostic group results

Comparisons	Classifiers	AUC	Sensitivity	Specificity	PPV	NPV
BLC vs PC	Logistic Regression with Elastic Net Regularisation	0.97 (0.93-1.00)	0.60 (0.38–0.80)	0.98 (0.95-1.00)	0.90	0.90
BLC vs Non-Cancerous	Logistic Regression with Elastic Net Regularisation	0.95 (0.90–0.99)	0.87 (0.70-1.00)	0.92 (0.84–0.98)	0.81	0.95
PC vs Non-Cancerous	Extreme Gradient Boosting	0.89 (0.83–0.94)	0.76 (0.64–0.88)	0.88 (0.80–0.95)	0.81	0.85

The ROC curves obtained from GC-IMS data comparing BLC and the non-cancerous group, BLC and PC groups, and PC and non-cancerous groups are shown in Fig. 4. The results indicate that among BLC patients and PC patients, AUC (area under the curve) was 0.97 (0.93-1.00) with sensitivity and specificity of 0.60 (0.38–0.80) and 0.98 (0.95-1.00) respectively. However, the separation between BLC and non-cancerous samples was even higher with a sensitivity 0.87 (0.70-1.00), specificity 0.92 (0.84–0.98) and

AUC 0.95 (0.90–0.99). Similarly, for PC Vs non-cancerous samples using GC-IMS, the separation was significant with sensitivity 0.76 (0.64–0.88), specificity 0.88 (0.80–0.95) and AUC 0.89 (0.83–0.94).

The results of the statistical analysis between different cancer groups for GC-TOF-IMS are given in Table 3. The results demonstrate high sensitivity and specificity, indicating that there are significant differences between the VOC profiles of different cancer groups, which was also shown in the GC-IMS data.

Table 3
GC-TOF-MS diagnostic group results

Comparisons	Classifiers	AUC	Sensitivity	Specificity	PPV	NPV
BLC-PC	Logistic Regression with Elastic Net Regularisation	0.84 (0.73–0.93)	0.53 (0.33–0.75)	0.90 (0.83–0.96)	0.62	0.87
BLC - Non - Cancerous	Random Forest	0.81 (0.70–0.90)	0.27 (0.09–0.46)	0.94 (0.88-1.00)	0.33	0.71
PC - Non - Cancerous	Random Forest	0.94 (0.90–0.97)	0.78 (0.66–0.89)	0.88 (0.80–0.95)	0.82	0.85

The ROC curves obtained from GC-TOF-MS data comparing BLC and non-cancerous groups, BLC and PC groups, and PC and non-cancerous groups are illustrated in Fig. 5. The results indicate that GC-TOF-MS was able to differentiate BLC and PC with AUC 0.84 (0.73–0.93), sensitivity and specificity of 0.53 (0.33–0.75) and 0.90 (0.83–0.96). The separation between BLC and non-cancerous samples was very poor with sensitivity only 0.27 (0.9 – 0.46), specificity 0.94 (0.88-1.00) and AUC 0.82 (0.72–0.90). However, the separation was more significant with sensitivity 0.78 (0.66–0.89), specificity 0.88 (0.80–0.95) and AUC 0.94 (0.90–0.97) for PC and non-cancerous groups.

In our results, we analysed different VOCs linked to BLC and PC for the screening and diagnosis of these cancers. A total of 34 biomarkers were found using TOF-DS software. These VOCs were verified using PubChem, NIST (National Institute of Standards and Technology), and previously published papers. Out of 34, 13 VOCs were found in the comparison of BLC and non-cancerous groups specific to BLC, 7 in PC and non-cancerous groups specific to PC, 14 VOCs were found in the comparison of BLC and PC group out of which 3 VOCs do not overlap either with BLC or PC Specific VOCs, which may indicate that they are new markers.

4. Discussion

In this study, we developed urinary VOC profiles linked with BLC and PC. Table 4 consists of the chemicals that have been identified in our study and have been cross verified using PubChem, NIST and previously published research, which may have particular relevance to BLC diagnosis. Out of 13 VOCs found noteworthy to BLC, Biphenyl, Heptanal, and 2, 6, 10, 14-tetramethyl- Pentadecane were the three distinct biomarkers found in our study that did not overlap with other studies. Biphenyl has been identified as the most significant biomarker in our study. Biphenyl has been linked to various diseases, including carcinoma. It has been proven that Biphenyl is a promoter of BLC in rats [44]. Nonanal, Tetradecane, Dodecane, Hexadecane, Naphthalene, and Methyl Isobutyl Ketone have been suggested by Rodrigues *et al.* [45] in their study using GC-MS on BLC cell lines. Whereas 2-pentanone and 4-Heptanone overlap with the findings of Cauchi *et al.* [27]. Benzoic acid was another chemical found in our study that overlapped in both Rodrigues *et al.* [45] and Cauchi *et al.* [27] studies.

Table 4
A list of possible biomarkers from the analysis of urine samples by GC-TOF-MS identified using PubChem, NIST and publications significant to Bladder Cancer.

	Chemicals	p-values
1	Biphenyl	< 0.01
2	Nonanal	< 0.01
3	Tetradecane	< 0.01
4	Pentadecane, 2,6,10,14-tetramethyl-	0.012
5	2-Pentanone	0.012
6	Undecane	0.014
7	4-Heptanone	0.018
8	Dodecane	0.025
9	Hexadecane	0.026
10	Heptanal	0.026
11	Methyl Isobutyl Ketone	0.045
12	Naphthalene	0.046
13	Benzoic acid	0.049

From the analysis of PC urine samples, a total of 7 distinct VOCs have been identified and are summarised in Table 5. In our study, we found toluene as the most significant chemical for PC. Toluene has been published previously as a significant biomarker for PC [46]. Also, there are significant results published that toluene has been reported in testicular diseases [47,48]. Pyrrole has been reported by Smith

et al. in their study with 24 controls and 13 patients with PC. They tested the urine samples to assess VOC profiles and found Pyrrole as one of the significant markers for PC [49]. 2-ethyl-1-Hexanol, phenol and dimethyl disulphide [50], Acetic acid [51], 2-methyl Cyclopentanone [52] were also found in our study, which overlaps with previous studies.

Table 5
List of possible biomarkers from the analysis of urine samples by GC-TOF-MS identified using PubChem, NIST and publications significant to PC.

	Chemicals	p-values
1	Toluene	< 0.01
2	Phenol	< 0.01
3	Acetic acid	< 0.01
4	1-Hexanol, 2-ethyl-	0.011
5	Disulfide, dimethyl	0.012
6	Cyclopentanone, 2-methyl-	0.017
7	Pyrrole	0.033

Table 6 represents all the chemicals found in the analysis of urine samples for prostate and BLC. Most of the chemicals present in this list are the chemicals present in the lists for BLC and non-cancerous group and PC and non-cancerous group. 2-Hexanone, p-Xylene, and 3-methyl Nonane are the only significant chemicals out of 14 chemicals in this list that are important for both bladder and PC. 2-Hexanone and p-Xylene have previously been reported as a significant marker for the PC [50,52]. There is no significant evidence for both 2-Hexanone and p-Xylene as a potential biomarker for BLC. However, 3-methyl-Nonane has not yet been reported as a biomarker for either bladder or PC, although it has been reported as a biomarker for lung cancer in different studies [53,54]. This may signify the importance of 3-methyl-Nonane as a potentially significant marker. The results reported in this paper support the findings of other groups for the validation of these chemicals as potential biomarkers in both PC and BLC. It has been noted that the chemicals found in all the cancer groups were different and there was almost no overlapping of the VOCs fingerprints for BLC and PC. This adds further support to the unique VOC fingerprint in cancers of different cell origins.

Table 6
List of possible biomarkers from the analysis of urine samples by GC-TOF-MS identified using PubChem, NIST and publications significant to PC and Bladder cancer.

	Chemicals	p-values
1	Toluene	< 0.01
2	Methyl Isobutyl Ketone	< 0.01
3	Dodecane	< 0.01
4	Phenol	< 0.01
5	Cyclopentanone, 2-methyl-	< 0.01
6	2-Hexanone	< 0.01
7	Heptanal	< 0.01
8	p-Xylene	< 0.01
9	Nonane, 3-methyl-	< 0.01
10	Tetradecane	< 0.01
11	Nonanal	< 0.01
12	Biphenyl	0.019
13	Acetic acid	0.025
14	2-Pentanone	0.032

The use of urinary VOC analysis is an attractive option due to the non-invasive nature. It also has the potential to be used in early cancer diagnosis with further validation studies. This approach may also prove to be efficient, whilst lowering the cost per patient, and increasing patient compliance due to its non-invasive nature. The results of using GC-IMS as an analysis tool are significant as the method is much simpler than using a high-end analytical method, such as GC-MS, without the need for a laboratory environment.

Our results were limited by not accounting for the contributory factors that can also lead to abnormal metabolism with subsequent excretion of differing concentrations of these chemicals in the urine. These factors include stress, alcohol, smoking, certain food products, medicines and different environmental factors. We aim to consider these further in the next study. We also did not undertake full chemical identification with calibration standards. However, many of the chemicals we found correlate with other studies and therefore, there is evidence that these are correct.

5. Conclusion

In this paper, GC-IMS and GC-TOF-MS methods were used to identify VOC fingerprints using urine headspace and establish an interdependence between BLC, PC and non-cancerous samples. It was found that both GC-IMS and GC-TOF-MS have potential to differentiate between different cancer groups with respective AUC for different diagnostic groups: For GC-IMS, BLC and PC [0.97 (0.93-1)], BLC and Non-Cancerous [0.95 (0.90–0.99)], PC and Non-Cancerous [0.89 (0.83–0.94)] and for GC-TOF-MS, BLC and PC [0.84 (0.73–0.93)], BLC and Non-Cancerous [0.81(0.70–0.90)], PC and Non-Cancerous [0.94 (0.90–0.97)]. A total of 35 VOCs were found to be relevant for identifying these cancer groups, with several VOCs distinct to each cancer. VOCs from this study were supported by findings from previous studies. This signifies that VOCs for both bladder and prostate cancer have different profiles, which may be helpful in future to distinguish them. In the future, these VOC profiles obtained from these analytical devices can be used as a reference for developing low-cost devices.

Declarations

Acknowledgments

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Author Contributions:

H.T. : Conceptualisation , Methodology, Formal analysis, Investigation, Data Curation, Writing - Original Draft, Software, Visualization.

E.D. : Conceptualisation, Methodology, Writing - Review & Editing

A.S.B. : Writing - Review & Editing, Data Curation

R.P.A.: Resources, Project administration, Writing - Review & Editing

J.A.C: Conceptualisation, Supervision, Resources, Visualization, Writing - Review & Editing, Project administration.

Competing interest statement:

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Figures

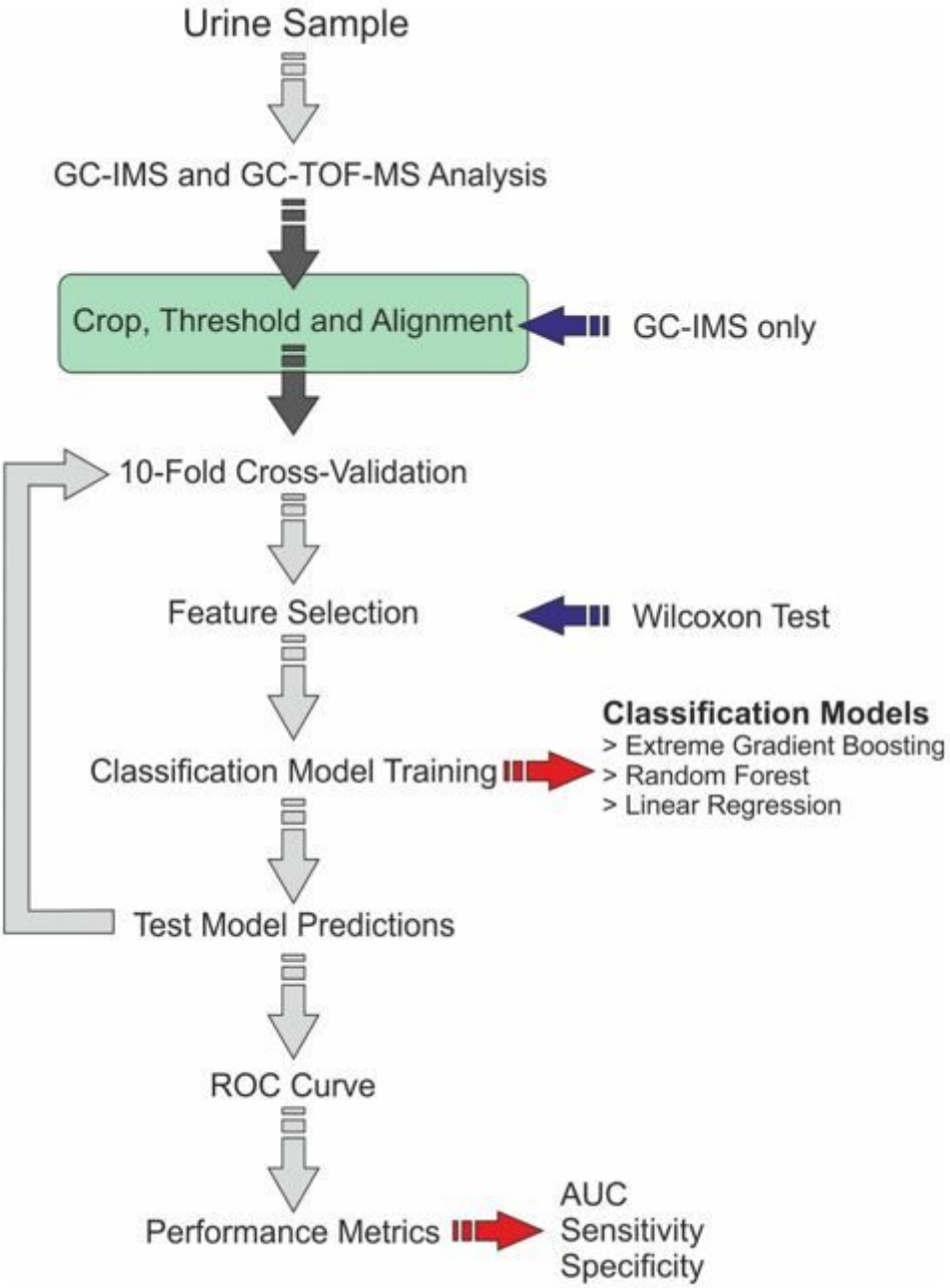

Figure 1

Data analysis pipeline

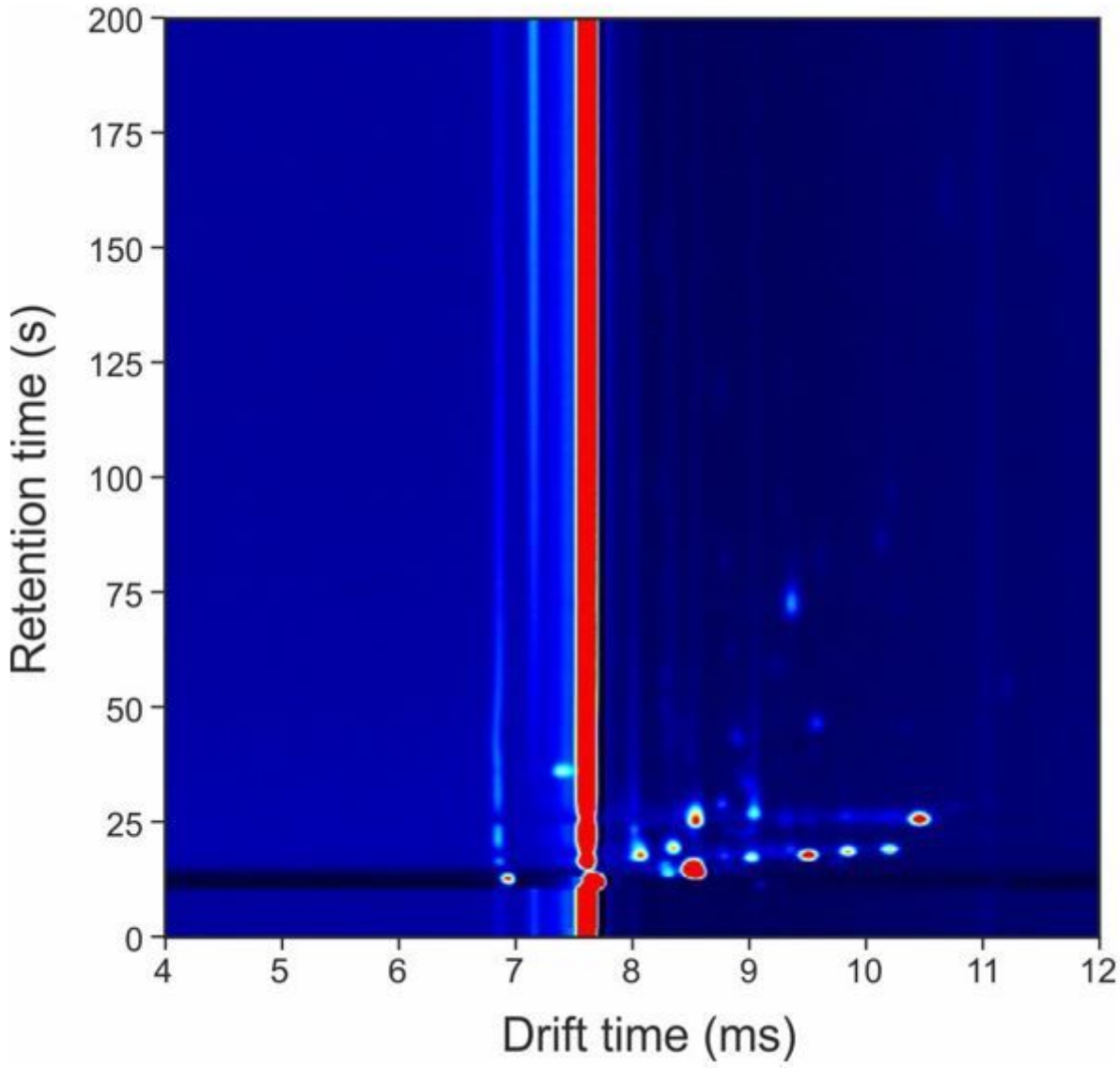

Figure 2

Typical output plot from the GC-IMS instrument

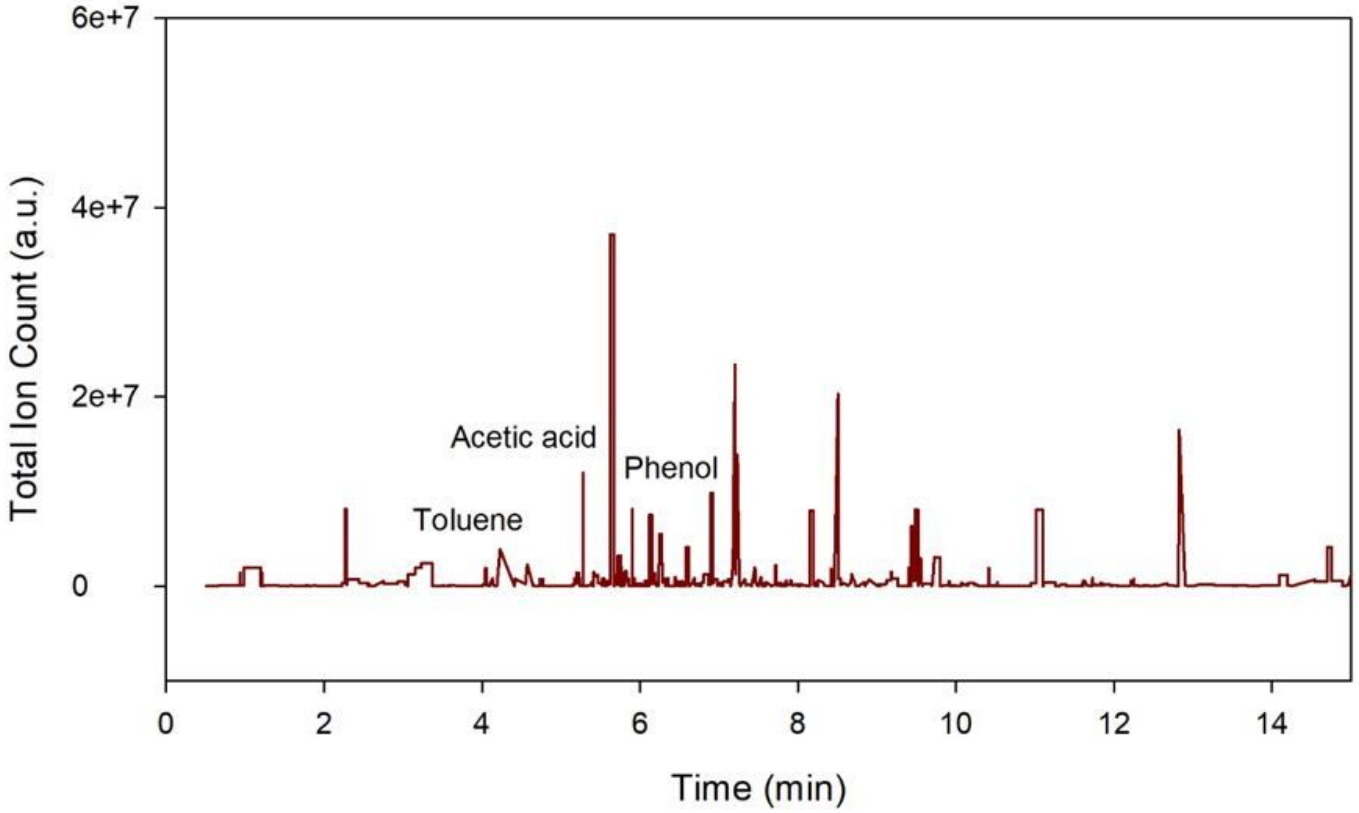

Figure 3

illustrates a typical output plot of GC-TOF-MS. The x-axis in the plot represents the retention time and y-axis lists the chemical according to their abundance in the sample.

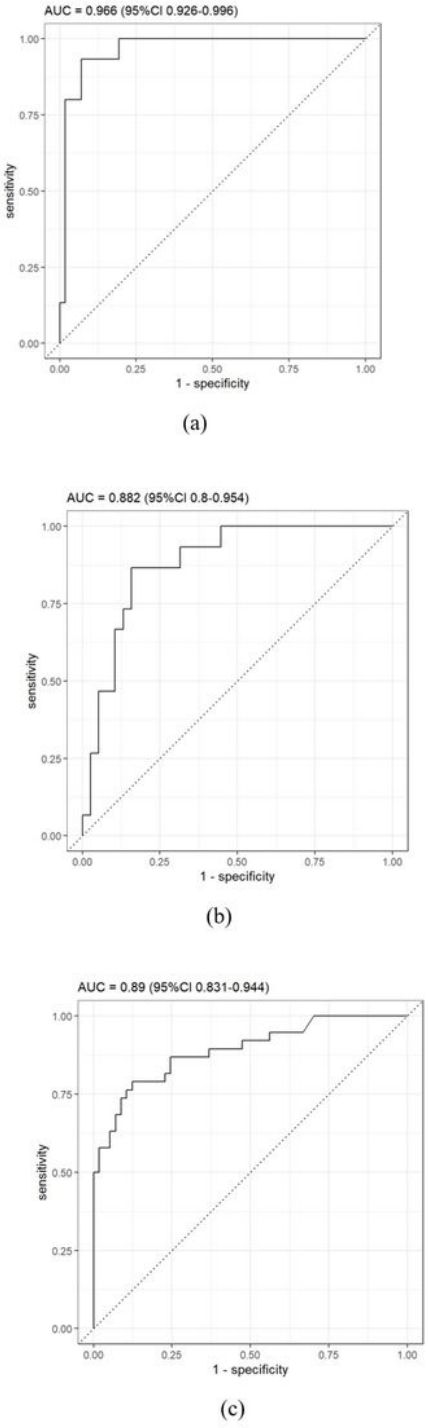

Figure 4

ROC for (a) Bladder Vs PC, (b) Bladder Vs Non-cancerous group and (c) Prostate Vs Non-cancerous group using GC-IMS.

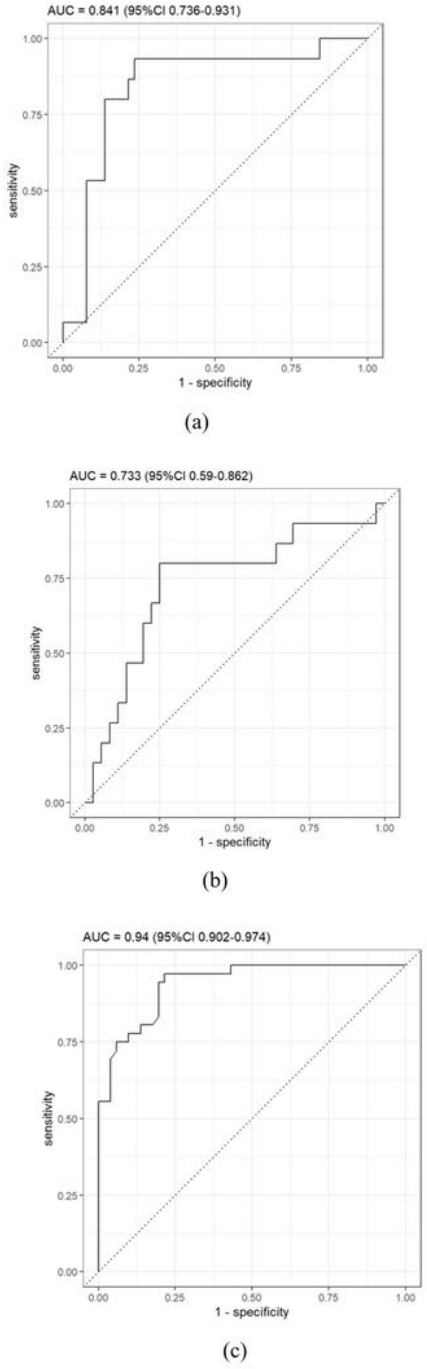

Figure 5

ROC for (a) Bladder Vs PC, (b) Bladder Vs Non-cancerous group and (c) Prostate Vs Non-cancerous group using GC-TOF-MS.