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VOLATOLOMICS COMBINED TERAHERTZ TIME - DOMAIN SPECTRAL ANALYSES OF COLON CANCER *IN VITRO*

Ahmad Zulhilmi Arshad[a], Yusof Munajat[a*], Sib Krishna Ghoshal[a], Rahman Jamal[b], Nor Adzimah Johdi[b], Raja Kamarulzaman Raja Ibrahim[a], Hanif Zulkhairi[b]

[a]Department of Physics, Faculty of Science, Universiti Teknologi Malaysia, 81310 Johor Bahru, Johor, Malaysia
[b]UKM Medical Molecular Biology Institute, Jalan Yaacob Latif, Bandar Tun Razak, 56000 Batu 9 Cheras, Wilayah Persekutuan Kuala Lumpur, Malaysia

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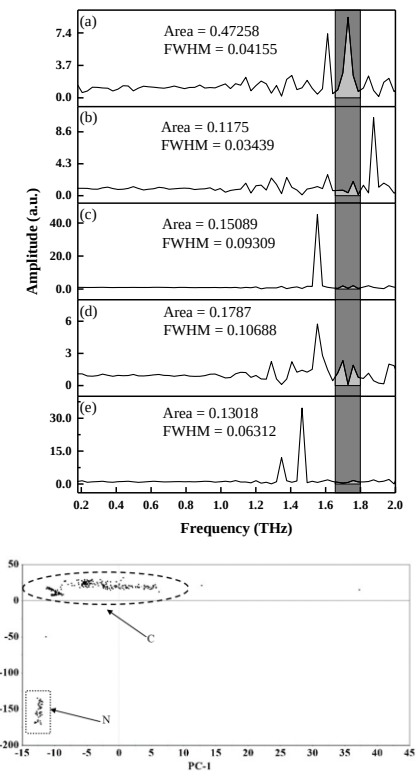
*Corresponding author
yusofm@utm.my

Graphical abstract



Abstract

Terahertz time-domain spectroscopy (THz-TDS) offers a great advantage for the analysis of biological samples. In this study, we combined THz-TDS with volatolomics analysis and analyzed 2 colon cell lines via *in vitro* settings. The release of volatile organic compounds (VOCs) was measured from the normal colon (CCD112CoN) and cancer colon (COLO320DM) cell lines which were grown in sealed flasks. Data validation were carried out with principal component analysis (PCA) and partial least square (PLS) scores while the chemometric analyses were performed using Camo Unscrambler X software. In-depth THz-TDS spectral analysis of the cancer colon (COLO320DM) cell line shows significant traces of benzamide gas when validated using gas chromatography-mass selective detection (GC-MSD) system. This preliminary data shows the potential use of identification and quantification of benzamide compound in the cancer colon cells and this could provide useful insight towards cancer drug design and therapy.

Keywords: Volatolomics analysis, colon cancer, Chromatography, Terahertz spectra, gas analysis

Abstrak

Spektroskopi domain masa Terahertz (THz-TDS) sangat berfaedah untuk menganalisis sampel biologi. Kami mengabungkan THz-TDS dengan analisis volatolomik dan menjalankan ujikaji menggunakan 2 jujukan sel kolon melalui teknik *in vitro*. Teknik ini digunakan untuk mengesan perlepasan sebatian organik yang meruap (VOCs) daripada jujukan sel normal kolon (CCD112CoN) dan juga jujukan sel kanser kolon (COLO320DM) yang dibiakkan di dalam kelalang yang tertutup. Pengesahan data dijalankan melalui skor-skor analisis komponen utama (PCA) dan separa kurang persegi (PLS) manakala analisis kemometrik dijalankan dengan menggunakan perisian Camo Unscrambler X. Analisis mendalam spektra THz-TDS bagi jujukan sel kolon kanser mendapati gas benzamide dikesan dan disahkan dengan menggunakan sistem kromatografi gas – pengesanan selektif jisim (GC-MSD). Berdasarkan hasil yang mengalakan ini, identifikasi dan kuatifikasi sebatian benzamide di dalam jujukan sel kolon kanser mempunyai potensi untuk diekplorasi dalam memberikan maklumat yang berguna ke arah rekabentuk ubat dan penyembuhan kanser kolon pada masa depan.

Kata kunci: Analisis gas, Analisis Volatolomik, Kanser Kolon, Kromatografi, Spektra Terahertz

1.0 INTRODUCTION

Breath analysis has emerged as one of the most popular approaches in examining biomarkers in diverse diseases including cancer [1]–[4]. It allows detection of various biochemical agents from the volatile organic compounds (VOCs) analysis. The measurement of VOCs can also be extended to VOCs released by the human body, odor, breath, stool and urine, cells, tissues, and organs. To accomplish this goal, volatolomics analysis is integrated with advanced terahertz time-domain spectroscopy (THz-TDS) for broad arrays biochemical studies related to human diseases.

Over the years, dedicated efforts have been made towards breath analysis for the disease diagnosis including cancer. In this regard, varieties of analytical techniques have been utilized such as gas chromatography-mass spectrometer (GC-MS), proton transfer reaction mass spectrometry (PTR-MS), selected ion flow tube mass spectrometry (SIFT-MS), ion mobility spectrometry (IMS), Fourier transform infrared spectroscopy (FTIR), etc. [5]–[8]. Each of these techniques has it owns advantages and disadvantages in terms of their efficiency, cost-effectiveness, databases, accuracy, rigor, speed, etc. For instance, GC-MS is suitable for the identification and validation of the targeted compounds. However, it is limited to unknown samples or compounds that require several columns to analyze precisely. In fact, the position and number of absorptions by each molecule depend on different forces within the particular species. The molecular structure plays a significant role in the energy levels of the rotational transitions [9].

Gas sensing using terahertz radiation is beneficial due to various advantages. Heavy gas molecules display rotational resonance at microwave (MW) frequencies. Conversely, the light gas molecules show rotational resonances in the mid-infrared (MIR) frequencies. Generally, the absorption of terahertz radiation occurs due to the rotational and rovibrational modes of few compounds group present in the sample. Though rotational transitions in the terahertz region are three to six times stronger than the one occurs in the MW region. Research using terahertz radiation is an emergent area, wherein researchers have been mostly focusing on the optimization of THz radiation generation and detection. Thus, the database information involving the functional groups of diverse chemical compounds in the gaseous form obtained using terahertz spectroscopy is still limited. On top, THz-TDS is a new spectroscopic technique similar to FTIR covering a wide range of frequencies from 0.3 - 4.00 THz. Table 1 shows the peak position of some specific VOCs obtained using terahertz spectral analyses.

Table 1 THz spectral peak for various chemical compounds

Chemical Composition	Compounds Name	Spectral Peak Position (THz)	Reference
CH_3CN	Acetonitrile	0.50	[10]
C_2H_3N	Acetonitrile vapor	0.60	[11]
C_2H_3CHO	Acrolein	25.1886 ($\times10^{-2}$)	[12]
Atmospheric	Mixed Compounds	0.56 to 1.92	[13]
C_3H_7CN	Butyronitrile	0.22	[10]
CO_2	Carbon dioxide	1.84 to 3.06	[14]
$CHCl_3$	Chloroform	25.1766 ($\times10^{-2}$)	[12]
CH_2Cl_2	Dichloromethane	25.1870 ($\times10^{-2}$)	[12]
C_2H_6O	Ethanol	0.25 to 9.08	[15]
C_2H_6O	Ethanol	0.21	[16]
H_2O	Humid air	0.45 to 2.27	[11]
HCl	Hydrogen chloride	0.62 to 1.88	[11]
CH_3OH	Methanol	25.1738 ($\times10^{-2}$) to 25.1884 ($\times10^{-2}$)	[12]
CH_3CN	Methyl cyanide (Acenonitrile)	0.22 to 0.57	[16]
CH_3NCO	Methyl isocyanate	25.1744 ($\times10^{-2}$) to 25.1830 ($\times10^{-2}$)	[12]
C_2H_5CN	Propanenitrile	0.33	[10]
$(CH_2)_5N$	Pyridine	25.1749 ($\times10^{-2}$)	[12]
$(CH_2)_3S$	Thietane	25.1881 ($\times10^{-2}$)	[12]
$(CH_2)_4S$	Thiophene	25.1770 ($\times10^{-2}$)	[12]
H_2O	Water vapor	0.56 to 1.92	[11]
H_2O	Water vapor	2.40 to 4.53	[14]
OCS	Carbonyl sulfide	0.164	[17]
N_2O	Nitrous oxide	0.163	[17]
C_7H_7NO	Benzamide	1.73	[18]

Table 2 shows the examples of VOCs found in colon cancer using breath analysis. So far, only a few investigations have been made on colon cancer via breath analysis [19],[20]. These studies employed the GC-MS technique for the detection of colon cancer where different types of column filters were

considered for compounds identification. The molecular composition and structure are highly sensitive to the THz and MW radiation due to the characteristic frequency of the molecular rotation. The moment of the inertia of the molecule associated with each transition is the main parameter that affects the spectral peak position [21].

Table 2 Identified compounds from colon cancer using breath analysis

Compounds Name	Reference
Acetone, isopropanol, hydrogen, methane, carbon dioxide, acetate, propionate, nutyrate, hydrogen cyanide, aldehydes, alkanes, ammonia, isovalerix, isobutyric acid, phenolics, hydrogen sulphide, pentane, ethane, ethylene	[19]
Benzaldehyde, Decan-1-ol, Methyl dodecanoate, Nonan-2-one, Undecan-2-ol, Pentadecan-2-one, 3-Methylbutan-1-ol, Heptan-1-ol, Octan-1-ol	[20]

Based on current data from The Global Cancer Observatory 2018 (GLOBOCAN 2018), colon cancer is the third leading cause of cancer in both males and females globally [22]. Colon cancer is difficult to diagnose and require thorough clinical examinations such as colonoscopy and biopsy for confirmation [23]. This study provides preliminary results for improving and assisting patients' diagnosis in the future.

2.0 METHODOLOGY

2.1 Preparation of Cell Culture

The colon cell lines COLO 320DM (cancer) and CCD-112CoN (normal) were grown under the manufacturer's recommendations (American Type Culture Collection). COLO 320DM cells were maintained with RPMI-1640 media while EMEM media was used for CCD-112CoN cells (Table 3). Both media were supplemented with 10% of fetal bovine serum (FBS). Cells were sub-cultured at confluency above 70%. The standard seeding density was (2×10^4 to 4×10^4) cells/cm^2 in a sterile tissue culture flask of area 75 cm^2. Incubation was carried out at 37 °C in the CO_2 humidified atmosphere of 5%. All analytical chemical reagents (high purity) were purchased from Gibco® Thermo Fisher Scientific (Malaysia) and other consumables were obtained from Thermo scientific (Malaysia). Cell culture was performed in the Biosafety Cabinet (BSC) class II under sterile conditions.

Cell viability test was carried out to ensure the cells are viable. This is done using the Trypan Blue exclusion method. It is based on the principle that the live cells contain intact cell membranes and exclude the trypan blue dye, whereas the dead cells are stained with trypan blue dye. This step is important to ensure that the VOCs results were from live cells and not from dead cells. The cells' viability was counted using ThermoFisher Scientific Countess II FL Automated Cell Counter due to its higher accuracy of counting than the conventional method (hemocytometer with manual counting).

Normally, the growth media for both colon cell lines were replaced three days interval to ensure the optimum growth of cells. As the colon cell lines grow, the cells produce a high volume of gases in the flask and these gases were collected and measured. The cell culture flask was constantly monitored and precautions were taken to ensure the gas did not escape from the flask.

Table 3 Descriptions of cell lines used in the study

Cell Line	Tissue Origin	Media
COLO 320DM	Colon (cancer Dukes' type C)	RPMI-1640 Medium
CCD-112CoN	Colon (Normal)	EMEM

2.2 Experimental Arrangements

The released VOCs from the cell lines were collected by inserting a sterile glass cylinder (length of 10 cm and diameter of 4 mm) through a hole existed at the flask's cap. The flask's cap and the glass cylinder were properly sealed to prevent external air contamination. This ensured the accumulation of VOCs released by the cells within the tissue culture flask. The VOCs were drawn using a syringe of volume 50 ml and collected into a special Tedlar sample bag gas collector (Sigma-Aldrich, Malaysia). Each of the cell lines was grown in five tissue culture flasks to allow the collection of VOCs at different time durations (24, 48, 72, 96 and 120 hours). Every measurement was conducted thrice.

The gas cell was vacuumed using a vacuum pump before the released VOCs were transferred from the cell culture flasks into the Tedlar bag. This guaranteed the precise detection of minute VOC samples emanated from the gas cell. The gas cell was attached to a THz-TDS spectrometer (Ekspla) to allow the accurate detection and analysis of the emitted VOCs from the cancerous colon cells. Present THz-TDS facility was available at Photonics Research Centre (PRC) of Universiti Malaya (Kuala Lumpur, Malaysia). The THz-TDS was attached to a Spectra-Physics Short Pulse Tsunami laser generator. The THz radiation was generated and detected via photoconductive antennas. Using this procedure, all the feasible key species of VOCs were identified and compared with the earlier reports. Table 4 provides the specifications of the Spectra-Physics short pulse Tsunami laser system used in this study.

Figure 1 shows the optical layout of the THz-TDS attached with a gas cell. Various windows (L) were used to transmit the laser from the pumping source to the target. Mirrors (M) were utilized to reflect the laser beam. The laser signal (mode-locked at 780 nm) was emitted by the THz emitter (E) and detected using a THz detector (D). The gas cell sampling tools (S) were located in between the emitter (E) and detector (D). The laser beam was split into bi-direction using a beam splitter (B). One of the beams was projected onto the emitter (E) through delay stage (H), while the other was directed towards the detector (D). The set of mirrors (M) were used to collect, collimate, and focus the terahertz beam. The half-wave crystal plate (W) was utilized to reduce the phase difference by half cycle between the two components of the traversing polarized terahertz laser beam. A narrow range of THz frequency (from 0.175 to 4.0 THz) was used. Only the narrow frequency range from 0.175 and 2.0 THz was focused. Nevertheless, the spectral response between 2 to 4 THz was highly noisy during calibration via the THz-TDS system. The poly-ethylene gas cell (length of 100 mm) was obtained from Pike Technologies Ltd. The gas cell containing the samples was placed between windows and the THz radiation was transmitted through the samples.

Table 4 Detail specifications of the used laser system

Parameter	Value	Range	Unit
Average power	400	Greater than	mW
Bandwidth	65	Greater than	nm
Pulse width	50	Less than	fs
Repetition rate	80	± 1	MHz
Wavelength	800	± 1	nm
Power stability	± 1	N/A	%

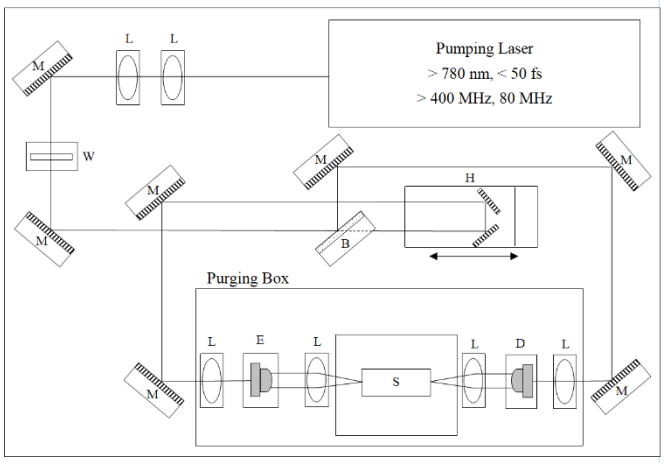


Figure 1 Optical transmission configuration of the Ekspla THz-TDS kit attached to the gas cell

3.0 RESULTS AND DISCUSSION

Tables 5 and 6 depict the mean viability of the grown cancerous colon cell lines and normal colon cell lines at different time periods. Normal and cancerous colon cell lines were selected respectively for 120 hours and 96 hours to analyze using THz-TDS (Table 7). Living cells having maximum abundance and the cell viability were selected as the optimum sample for further analyses using THz-TDS.

Table 5 Mean viability for cancerous colon cell lines

Time Duration (Hours)	Living Cells (10^6 cells/mL)	Dead Cells (10^6 cells/mL)	Total Number of Cells (10^6 cells/mL)	Viability (%)
24	0.76	0.05	0.81	94
48	1.30	0.10	1.40	93
72	1.60	0.10	1.70	91
96	1.60	0.09	1.69	95
120	3.00	0.11	3.11	97

Table 6 Mean viability for normal colon cell lines

Time Duration (Hours)	Living Cells (10^6 cells/mL)	Dead Cells (10^6 cells/mL)	Total Number of Cells (10^6 cells/mL)	Viability (%)
24	0.93	0.08	1.01	92
48	1.60	0.08	1.68	95
72	5.90	0.01	5.91	100
96	7.40	0.18	7.58	98
120	3.30	0.90	4.2	79

Table 7 Selected types of cell culture based on their most abundant living cells in flasks

Cells Types	Time Duration (Hours)
Colon Cancer	120
Normal Colon	96

Figure 2 illustrates the time duration dependent spectra in the frequency range of 0.2 – 2.0 THz for the studied colon cell lines. Table 8 enlists the results obtained from the THz-TDS spectral analysis of the investigated cell lines compared to various control samples as indicated. Cancerous colon cells revealed a Benzamide peak at 1.73 THz (Figure 2(a)), which was consistent with the literature value when compared [18]. Most of the observed significant peaks (Table 8) could not be compared and allocated to the appropriate chemical compounds due to the lack of available database in the literature. Figure 2(c) shows the THz spectra of the empty flask, in which the occurrence of a sharp peak at 1.54 THz was assigned to the atmospheric compounds. It is worth noting that the THz-TDS measurement system is an open system and can be influenced by environmental conditions. In short, the benzamide compound was selected as one of the key species of colon cancer because it exhibited an

intense peak in the spectra which is complete absent in normal colon cells. It was affirmed that detection and quantification of benzamide compound in the cancerous colon cells may provide valuable information towards cancer drug development and treatment.

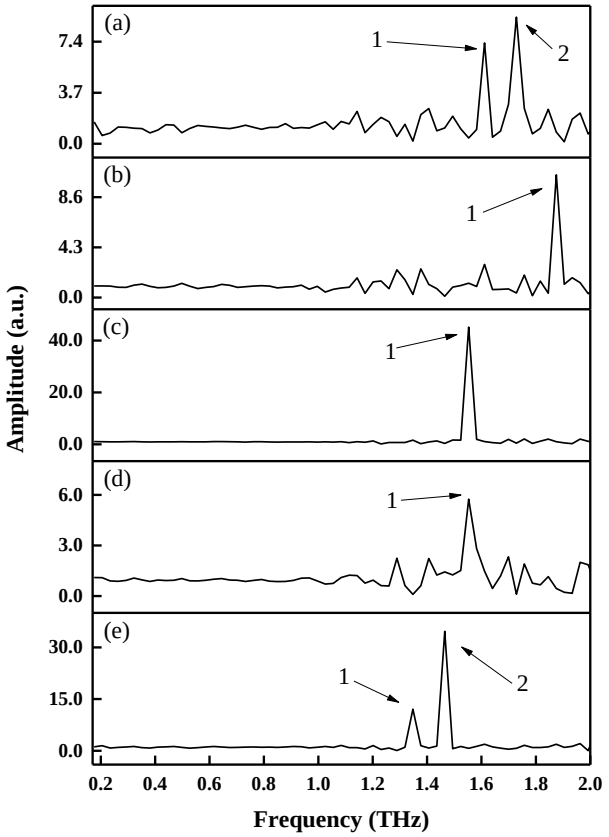


Figure 2 Time duration dependent THz-TD spectra revealing the peaks (arrow) of (a) cancerous colon cell lines at 120 hours, (b) normal colon cell lines at 96 hours, (c) control 1 – empty flask, (d) control 2 – with RPMI media and without cells, and (e) control 3 –with EMEM media and without cells

Table 8 THz-TDS spectral analysis of the investigated cell lines compared to various control samples

Samples Tested	Marker on the Figure	Peak Position (THz)	Identified Compounds
Cancerous colon cells	(a) 1	1.62	NA
	(a) 2	1.73	Benzamide
Normal colon cells	(b) 1	1.89	NA
Colon control 1 – Empty flask	(c) 1	1.54	Atmospheric
Colon control 2 – Media I (RPMI)	(d) 1	1.55	NA
Colon control 3 – Media II (EMEM)	(e) 1	1.35	NA
	(e) 2	1.47	NA

**NA = data not available yet*

Figure 3 displays the area under the benzamide peak (A) and the corresponding full width at half maximum (FWHM) in the 1.64 – 1.79 THz spectral region for cancerous colon cells compared to normal colon cell lines as well as various controls. The FWHM was used to signify the broadening of the spectral peak. The peak positions indicated the occurrences of various elements and chemical compounds. The FWHM acted as indicators of chemical state changes and physical influences of the cell structures. Meanwhile, the broadening of a peak signified an alteration in the number of chemical bonds contributing to the peak shape and variation in the sample condition and/or differential types of the samples [24].

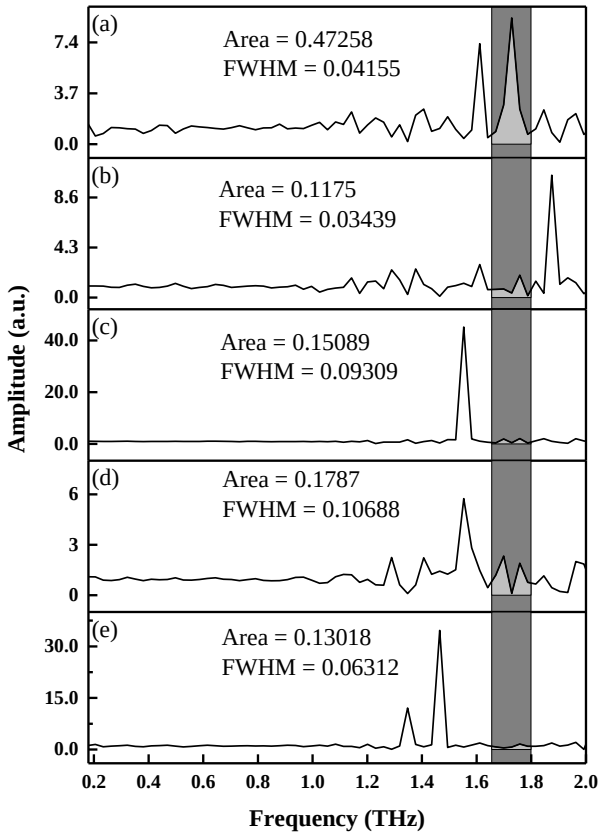


Figure 3 Area under the benzamide peak (shadowed region) and the corresponding full width at half maximum (FWHM) in the 1.64 – 1.79 THz spectral region for (a) cancerous colon cell lines at 5 days, (b) normal colon cell lines at 4 days, (c) control 1 – empty flask, (d) control 2 – with RPMI media, and (e) control 3 –with EMEM media

Table 9 summarizes the results obtained from THz-TD spectral analysis such as the area under the benzamide peak, FWHM and various ratios. The areal ratio of colon cancer to normal, control 1, control 2 and control 3 was found to be 44:12:14:17:13. Quantification of the selected VOC (benzamide) was possible because it could be identified in the standard database.

Table 9 The area under the benzamide peak, FWHM and various ratios obtained from the THz-TD spectral analysis

Marker on the Figure	Area Under the Benzamide Peak (A)	FWHM (THz)	Ratio (A/Total Area)
(a)	0.47	0.0145	0.44
(b)	0.12	0.0343	0.12
(c)	0.15	0.0930	0.14
(d)	0.18	0.1079	0.17
(e)	0.14	0.1536	0.13
Total	1.07	0.4033	1.00

The experimental area (A_{exp}) under the detected spectral peak and the standard area (A_{std}) acquired from the available database was used to quantify the amount of benzamide (N_{ppm}) present in the studied cancerous cell lines (in part-per-million *ppm*) via the relation:

$$N_{ppm} = \frac{A_{exp} \times L_{std} \times N_{std}}{A_{std} \times L_{exp}} \quad (1)$$

where N_{std} is the concentration of the gaseous VOC listed in the standard database, L_{exp} and L_{std} are experimental and standard path length of the gas cell, respectively. The standard analysis is performed at room temperature (25 °C) with a gas cell of path length 1 meter and gas concentration of 100 ppm.

Figure 4 presents the of retention time-dependent variation in the abundance of various chemical compounds present in the colon cell lines analyzed via GC-MSD system (Agilent Technologies – 6890N Network GC system interfaced with 5975 Mass Selective Detector). Gas-chromatography (GC) was combined with mass spectrometry (MS) to identify different substances present in the test sample. Using GC-MSD technique, the presence of benzamide in the cancerous colon cell lines was detected, in which standard non-polar column was utilized. Table 10 depicts the validation result of benzamide compounds detected using GC-MSD system at a retention time of 4.054 min.

Benzamide (also known as benzoic acid amide or benzoylamide or phenylcarboxyamide) is a colorless compound which is generally used to make other chemicals such as benzoyl chloride and ammonia or ammonium carbonate. Benzamide is harmful to the human when inhaled and absorbed through the skin. Being a strong irritant to the eyes, nose and throat its inhalation often causes nausea, vomiting and abdominal pain. It is enlisted in the hazardous substance list by the US Department of Environmental Protection (DEP) [25] and the US Environmental Protection Agency (EPA) [26]. Thus, its detection and quantification are not only significant for cancerous cells but also for environmental protection as well as human safety.

Observation made from other studies found out that benzamide could be detected in emphysema disease [27].

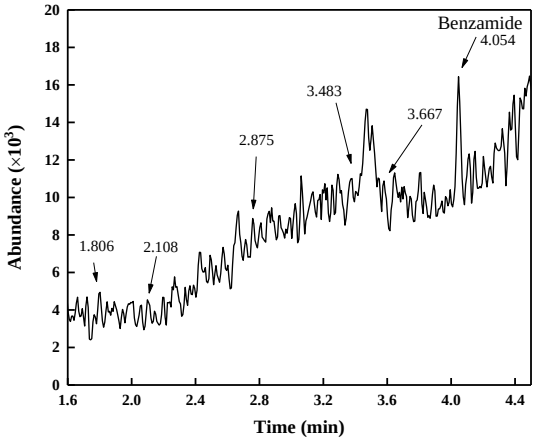


Figure 4 Spectra of retention time-dependent abundance for compounds analyzed via GC-MSD

Table 10 Compounds identified at different retention times using GC-MSD system

Retention Time (min)	Detected Compounds
1.810	Perhydro-htx-2-one
2.106	Titanium
2.876	2,5-Cyclohexadien-1-one
3.484	2H-1,4-Benzodiazepin-2-one
3.669	3-Indoleacetic acid
4.054	Benzamide

Figure 5 and 6 show the results (three-dimensional projections) from principal component analysis (PCA) and partial least square (PLS) scores of colon cancer, normal colon and control samples. These chemometric analyses were performed using Camo Unscrambler X software. The PCA revealed the variation and strong patterns in the dataset. The partial least square (PLS) clearly showed the fundamental relations between two variables under consideration. There is a significant separation between control and cancer-normal samples. This PCA and PLS prove the key species is not from normal and control samples. However, there is no difference between cancer and normal cells.

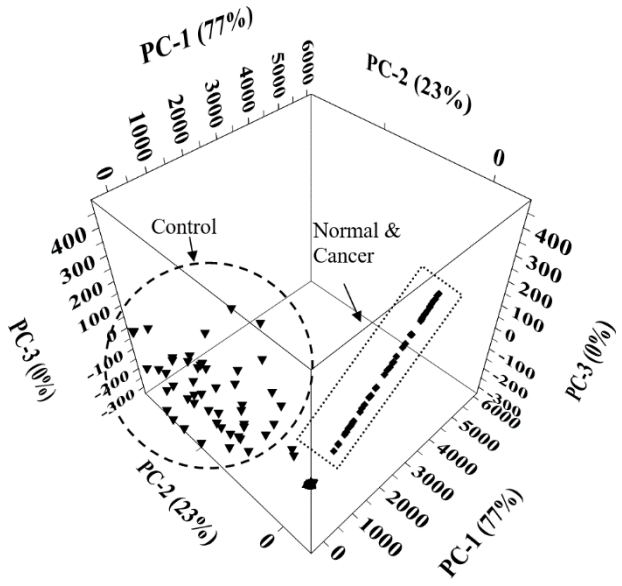


Figure 5 PCA outcome of normal and cancerous colon cells

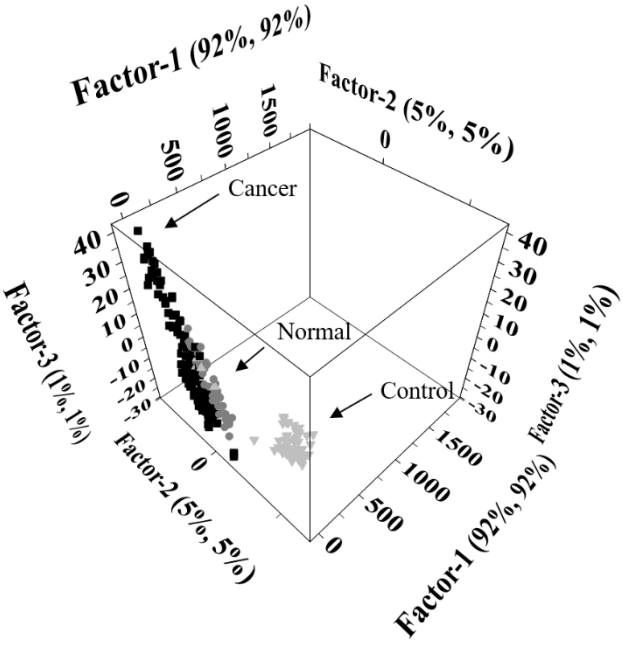


Figure 6 PLS scores of cancer, normal and control sample cells

Figure 7 displays the PCA score between PC-1 and PC-2 of cancerous colon versus normal colon cell lines. This score revealed no correlation between normal and cancer cell lines and the marker was exclusively not from normal cells. The PCA and PLS analysis provided an important platform for rapid interpretation of information-rich spectral datasets to draw comprehensive conclusions [28], [29] by differentiating the selected datasets (e.g. cancer, normal and control region of the samples).

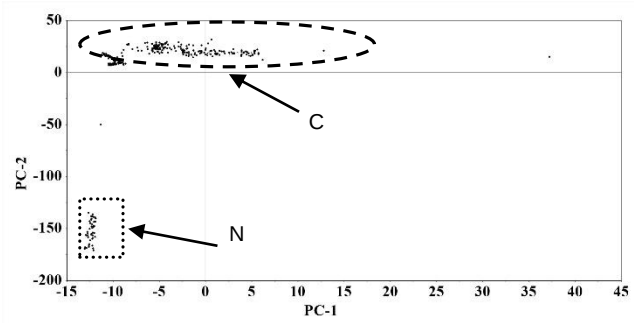


Figure 7 PCA scores normal (N) and cancerous colon (C) cells

4.0 CONCLUSION

We used THz-TDS together with volatolomics analysis to quantify the colon cancer cells (*in vitro*). Normal and cancerous colon cell lines were tested to determine the emission of VOCs from such cells. The authenticity of the spectral data was validated using PCA and PLS scores evaluation. This GC-MSD combined THz-TDS spectral analysis confirmed the release of benzamide gas from the studied cancerous colon cells. The PCA of the cancerous colon cells clearly distinguished them from normal cells and control samples. This systematic approach of detecting and quantifying the VOCs emission from cancerous cells is useful as biomarker identification in cancer patients.

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