In Vitro Cancer Cell Line Classification using Pattern Recognition Approach based on Metabolite Profiling

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***Abstract*—This study aims to evaluate the feasibility of metabolite profiling for the characterisation and discrimination volatile compounds using the pattern recognition from in vitro cancer cell lines, which are lung, breast and colon cancer together with the blank medium as a control group. This study implemented the A549 (lung), MCF7 (breast) and HCT116 (colon). Cells were harvested and maintained until they grow as monolayer adherent and reach confluence 70-90% before sampling. The volatiles profile from the targeted cell line was established using headspace solid phase microextraction coupled to gas chromatography-mass spectrometry (HS-SPME/GCMS). Multivariate data analysis employed principal component analysis (PCA) to better visualise the subtle similarities and the differences among these data sets. A total of 116 volatile organic compounds were detected focused on a limited range of retention time from 3rd until 17th minutes, and 33 compounds were recognized as targeted compounds (peak area>1%). According to both results, the score and the loading plot explained 83% of the total variance. The volatiles compound has shown to be significantly distinguished among cancerous and control group based on metabolite profiling using pattern recognition approach.**

***Index Terms*—GCMS; Headspace SPME; In Vitro Cell Line; Metabolite Profiling; Pattern Recognition.**

I. INTRODUCTION

According to the world statistic, the most prevalent cause of cancer-related death among men and women are lung, breast and colorectal [1][2]. Cancer itself derived from the dysfunction of the immune system [3]. More specifically, it started when cells begin to grow irregularly and become an abnormal population growth when cells multiply considerably [4]. Metabolomics can be defined as a field of research that aims the study of the metabolites from chemical process [5] presents within the cell, tissue or organism. The methods for metabolomics application are mostly focused on the analytical techniques which utilise Mass Spectrometry (MS) and Nuclear Magnetic Resonance (NMR) spectroscopy [6]. For the diagnostic purpose, metabolites obtained from the metabolomics technique have been used for the compounds determination. Volatile compounds from cancer cell lines as being investigated in this study indirectly provide the predictive signature for cancer biomarker based on the metabolites emitted [7]. Metabolomics study defined three major steps which emphasised targeted analysis, metabolite profiling, and metabolomics fingerprinting [8]. To date, metabolite profiling has become an emerging technique for cancer diagnosis because of non-invasive and potentially inexpensive. The application for volatiles compound production was executed using headspace solid phase microextraction coupled to gas chromatography-mass spectrometry (HS-SPME/GCMS) [9]. Additionally, volatile organic compounds can be described as an organic chemical which carbon-based with high vapour pressure and at ordinary room temperature [10]. Currently, this technique revealed the study of differences between cancer cell lines based on volatile compounds to be associated with the early diagnostic which instigated the analytical study [11]. As believed, the particular cell lines will discover the unique pattern of the metabolite profiling promoting the signature for cancer biomarker [12]. As a consequence, a preliminary study for classification of volatile compounds among the cancer cell lines needs further investigation before subjected to the biomarker discovery subsequently.

Hence, a multivariate statistical analysis which employed the principal component analysis has been a promising tool to differentiate samples from chromatographic methods [13]. It was reported that volatiles profile had been studied to discover the pattern from the different type of cancer and present the similarity utilising pattern recognition method [14]. Furthermore, in a study of characterisation of breast cancer [15], they have shown the potential to distinguish among cancerous and non-cancerous by employing the Principal Component Analysis (PCA) and Partial Least Square Discriminant Analysis (PLS-DA) techniques which presented high accuracy and specificity. There are few reports published based on volatile compounds in *Eugenia Uniflora* leaves sample which discriminated their biotypes orange, red and purple groups using HS-SPME by the methods of hierarchical clustering analysis (HCA) and PCA [16]. Presently, in a publication of apple classification [17], chemometric analysis has been a successful technique in differentiating samples related with SPME-GCMS. Besides, previous studies have shown the identification of biomarkers implemented the lung, colon, breast and ovarian from the metabolomics and significantly classified using the multivariate analysis [18]. The purpose of this work was thus first to employ the metabolite profiles for the characterisation of volatile compounds from three different cancer cell lines and blank medium as a control using pattern recognition approach.

II. Materials and Methods

A. Cell Lines and Culture Conditions

Three cancer cell lines employed for metabolic profiling analysis were obtained from Cell and Tissue Culture Engineering Lab, Department of Biotechnology Engineering, IIUM. Namely the A549 (ATCC CCL-185) human lung epithelial cell line, the HCT116 (ATCC CCL-247) human colorectal carcinoma cell line and the MCF7 (ATCC HTB-22) human breast adenocarcinoma cell line. A control group was provided with the blank medium containing no cells for each. Morphology condition which is epithelial from the cell lines designation was highlighted as the primary selection for this study. The cells were revived and cultured in growth medium included 90% DMEM (Dulbecco's Modified Eagles Medium) supplemented with 10% (v/v) FBS (Fetal Bovine Serum) as growth supplements. The cells were cultured and maintained in T-25cm^2 flasks in 5ml culture medium, under standard conditions in an incubator at 37°C/, 5% CO_2. The cells were grown and incubated until 70-90% confluence before the next subculture process. A549 cell line took two days to reach the confluence, while for HCT116 and MCF7 cell lines were incubated for three days respectively. Hereafter, once reach the confluence, all cell lines were undergone to the next passage with seeding of an initial cell density of 5×10^5 cells/ml in T-25cm^2 flasks separately in 5ml new medium. They were cultured and incubated until cells reached 70-90% confluence for almost two to four days. The culture was inspected daily for any signs of contamination.

B. Headspace Analysis by SPME GCMS

A divinylbenzene-carboxy-polydimethylsiloxane which is known as (DVB/CAR/PDMS) was used in the SPME extractions. The matrix has been shown to be an effective fibre coating [19] based on its high percentage of abstraction (achieving more than 100 volatile compounds) as well as its relatively good affinity between the compounds and the fibre [20][21]. The complex mixtures were evaluated using a GC-MS system (Agilent Technologies) under the following conditions: DB-WAX column (30m × 250µm × 0.25µm); helium gas flow: 1.18 mL/min; Oven program: 50°C for 0.5 min, then 10°C/min to 250°C for 5 min; temperature program 250°C; injection volume 1µL and mode split less [22]. Same parameter settings were for all runs. Upon reaching cell confluence of 70-90%, the SPME fibre was introduced into the headspace culture flask for 15 minutes in the incubator. Before sampling, the fibre was preconditioned in a GC injection port at 250°C for 8 minutes to clear localised background effects before any sampling. Then, the needle was injected into the GC injector at 250°C within 2 minutes splitless time. After 5 minutes, the injector was removed to avoid fibre damage. However, the samples were left to run in the GCMS system for about 24 minutes to complete the extraction time for metabolites production.

C. Statistical Analysis

Multivariate data analysis was applied to discover the potential difference of unique pattern from in vitro cancer cell lines. A total of 116 volatile compounds were tentatively recognised and evaluated. A data matrix consisted of the peak areas of all samples (3rd to 17th minutes of retention time) were subjected to the Principal Component Analysis (PCA) which utilised STATISTICA ver. 10.0 and The Unscramble X ver. 10.5. PCA was considered as an unsupervised method to explain the variance information of data and to establish the relationship between samples (score plot) and the peak area of volatile compounds (loading plot) [23][24][25]. This technique was instigated to perform a classification of cell lines based on their volatiles profile.

III. Results and Discussions

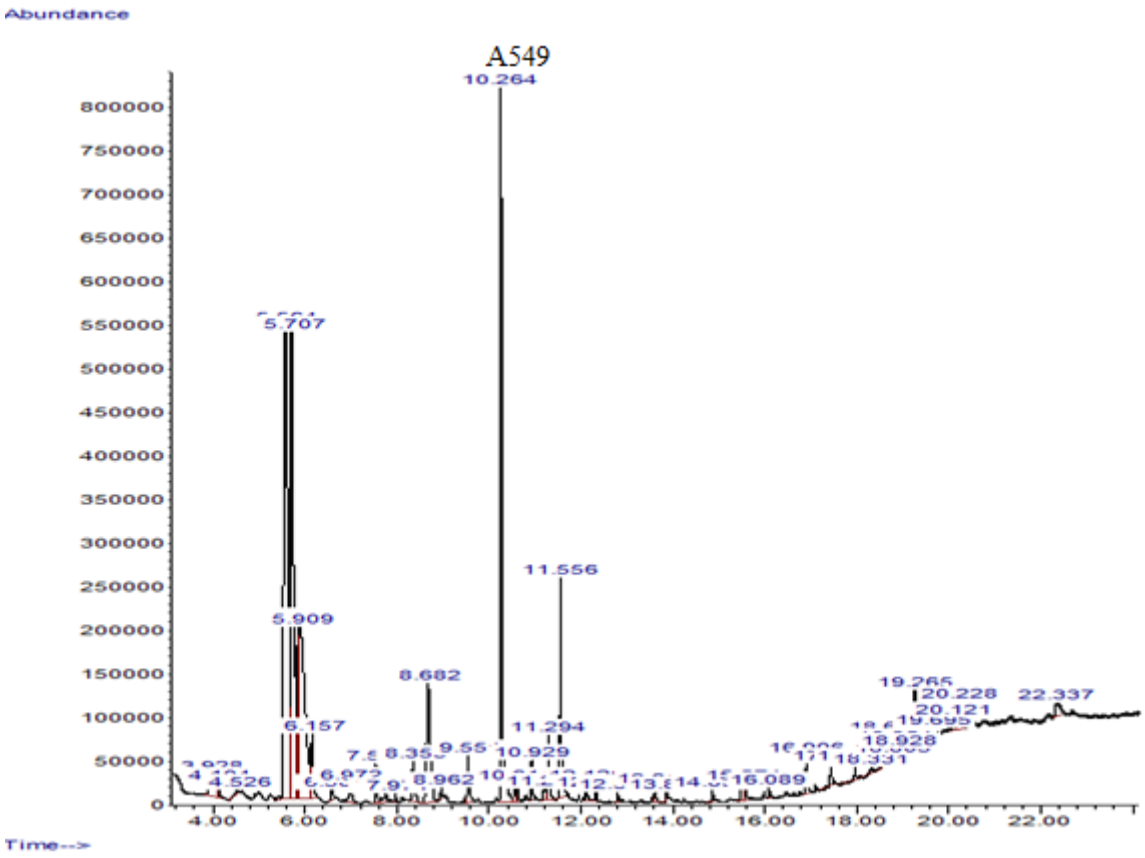
A. Characterization of Volatile Compounds for Cell Lines

A total of 116 volatile compounds were discovered, containing the total area of the peaks revealed from A549 (34%), MCF7 (21%), HCT116 (55%) and blank medium (6%) respectively. Out of 116 volatiles, 33 VOCs were recognised as targeted compounds which the peak area is greater than 1% and employing spectral library matching (higher than 80% match) as presented in Table 1. Figure 1 shows the chromatograms of the volatile characteristic profile of all samples. Volatiles were identified through the National Institute of Standards and Technology (NIST 2009) mass spectral database and retention time libraries. The chromatographic region between 3 and 17 minutes was modelled and selected. It was reported that compounds recognised after 17th minutes were considered as noise which the patterns can be seen from chromatograms in Figure 1. It can be valued that clear variances exist in pattern profiles between cancer cell line and blank medium. The differences in volatile profiles may be attributed to the dissimilarity of the relative concentration from these complexes in the vapour phase. Besides, there a few factors that result in variation composition which are extraction time, extraction temperature, incubation time and the background influence during the running sample process [26].

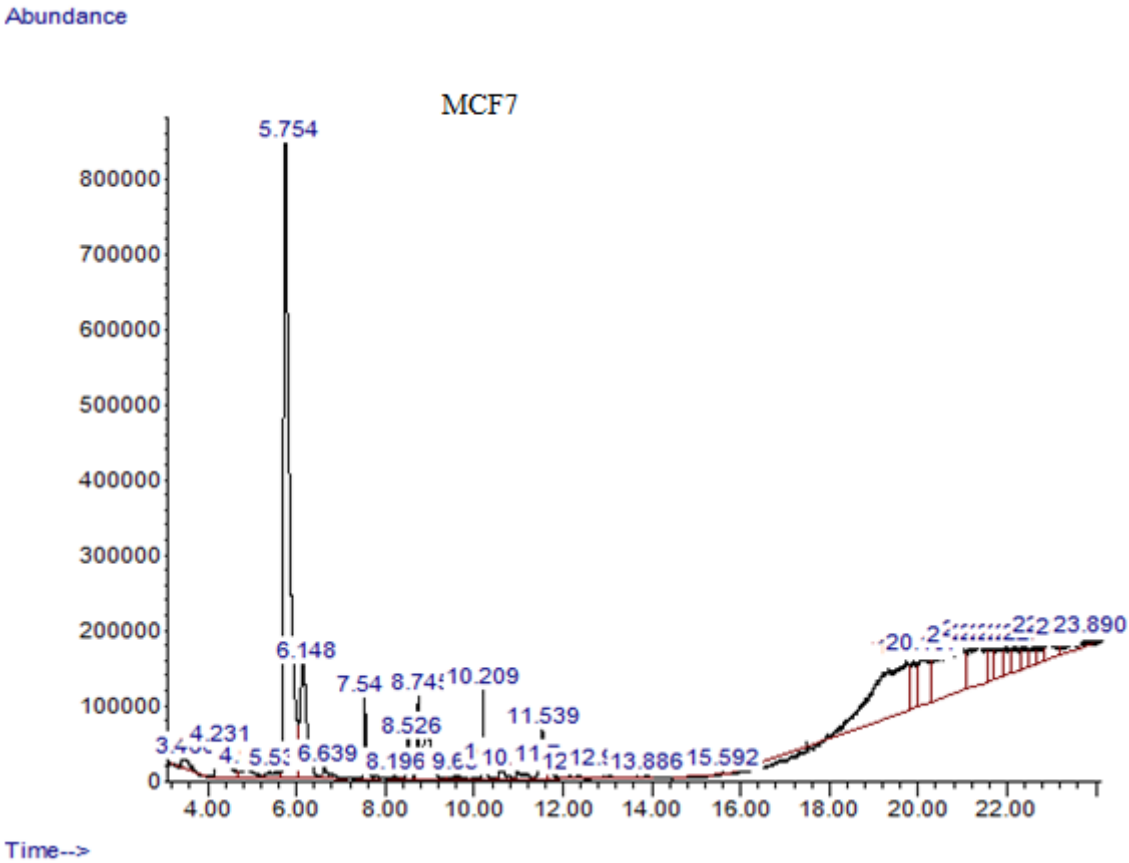
Different cell lines emit different volatile compounds that could be used as biomarkers for diseases. The similar peak may be detected due to the same compound emitted during the extraction time. Table 1 shows that styrene has been found in all samples at the specific retention time respectively; A549 (5.5636, 5.7082, 5.8548, and 5.9093 minutes), HCT116 (5.7751 minutes), MCF7 (5.7540 minutes) and Blank medium (5.7396 minutes). This finding suggests that styrene could be a degradation product of the polystyrene flask. A similar finding has been reported in a previous study [27]. Meanwhile, Ethylbenzene was only found in A549, HCT116, and MCF7 cell line between 3.92 and 4.32 minutes, whereas Benzaldehyde present only in the blank medium at 8.9957 minutes. This compound of Benzaldehyde may probably be metabolised by the cells as reported in the previous study of human skin cell line[28] and also found from volatile products of human colon cell line [29].

Table 1
List of VOCs Obtained from the A549, HCT116, MCF7 Cell Lines and Blank Medium, (Peak Area >1%)

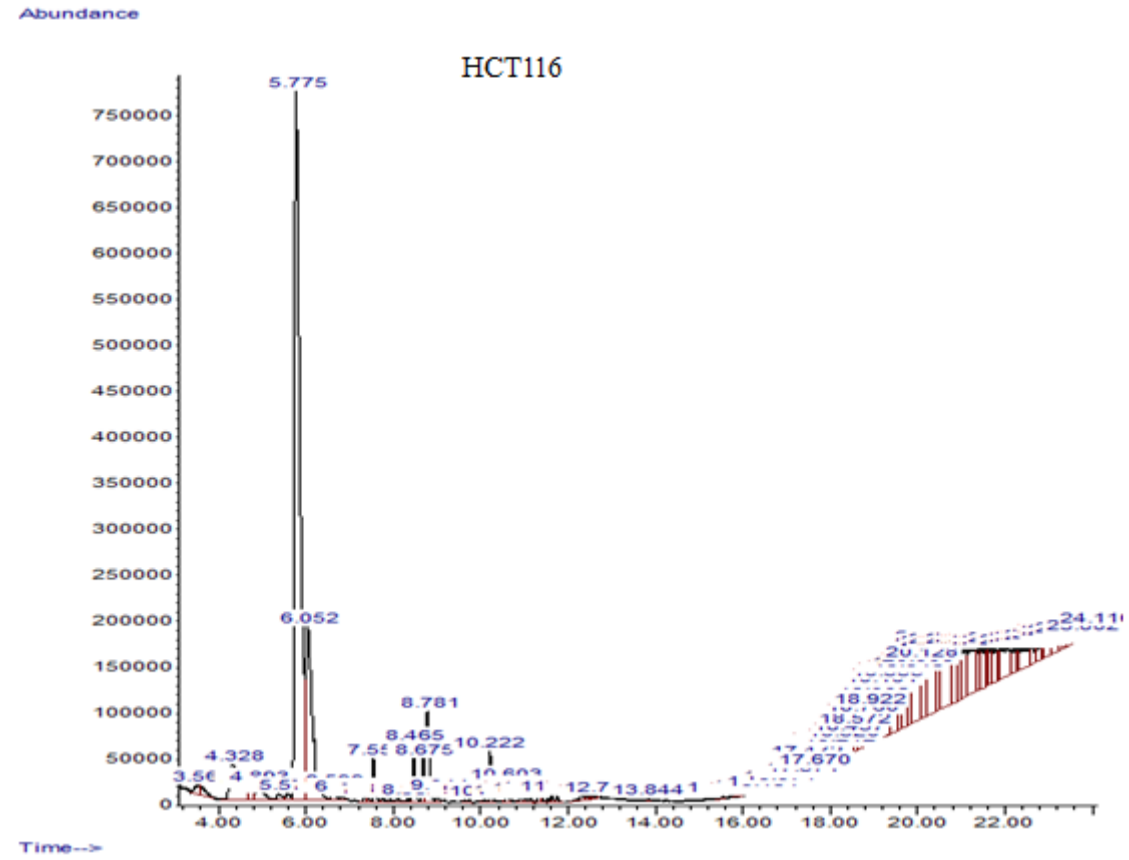
Retention Time (min)	A549	HCT116	MCF7	BLANK MEDIUM
3.9272	Ethylbenzene			
4.2307			Ethylbenzene	
4.3273		Ethylbenzene		
5.5636	Styrene			
5.7082	Styrene			
5.7396				Styrene
5.7540			Styrene	
5.7751		Styrene		
5.8548	Styrene			
5.9093	Styrene			
6.0517		Dodecane		
6.1479			Dodecane	
6.1566	2-Propanone, 1-hydroxy-			
7.5413			Cyclohexanol	
8.3524	Benzene,1,3 – bis(1,1-dimethylethyl)			
8.4655		Benzene, 1,3-bis(1,1-dimethylethyl)		
8.5261			Benzene, 1,4-bis(1,1-dimethylethyl)	
8.6793				Tetradecane
8.6814	Tetradecane			
8.7461			1-Hexanol, 2-ethyl-	
8.7819		Tetradecane		
8.9957				Benzaldehyde
9.5510	Tetradecane			
10.2087			Silanediol, dimethyl-	
10.2213		Silanediol, dimethyl-		
10.2570				Silanediol, dimethyl-
10.2634	Silanediol, dimethyl-			
10.9233				2-Pyrrolidinone, 1-methyl
11.2942	2-Amino-5-methylbenzoic acid			
11.5392			Oxime-, methoxy-phenyl-	
11.5561				Oxime-, methoxy-phenyl-
11.5562	Oxime-, methoxy-phenyl-			
16.9055	Oxazolidine, 2-ethyl-2,3-dimethyl-			


Abundance
A549
10.264
5.707
11.556
5.909
8.682
6.157
11.294
10.929
4.526
16.089
18.928
18.331
19.265
20.228
22.337
20.121
Time-->

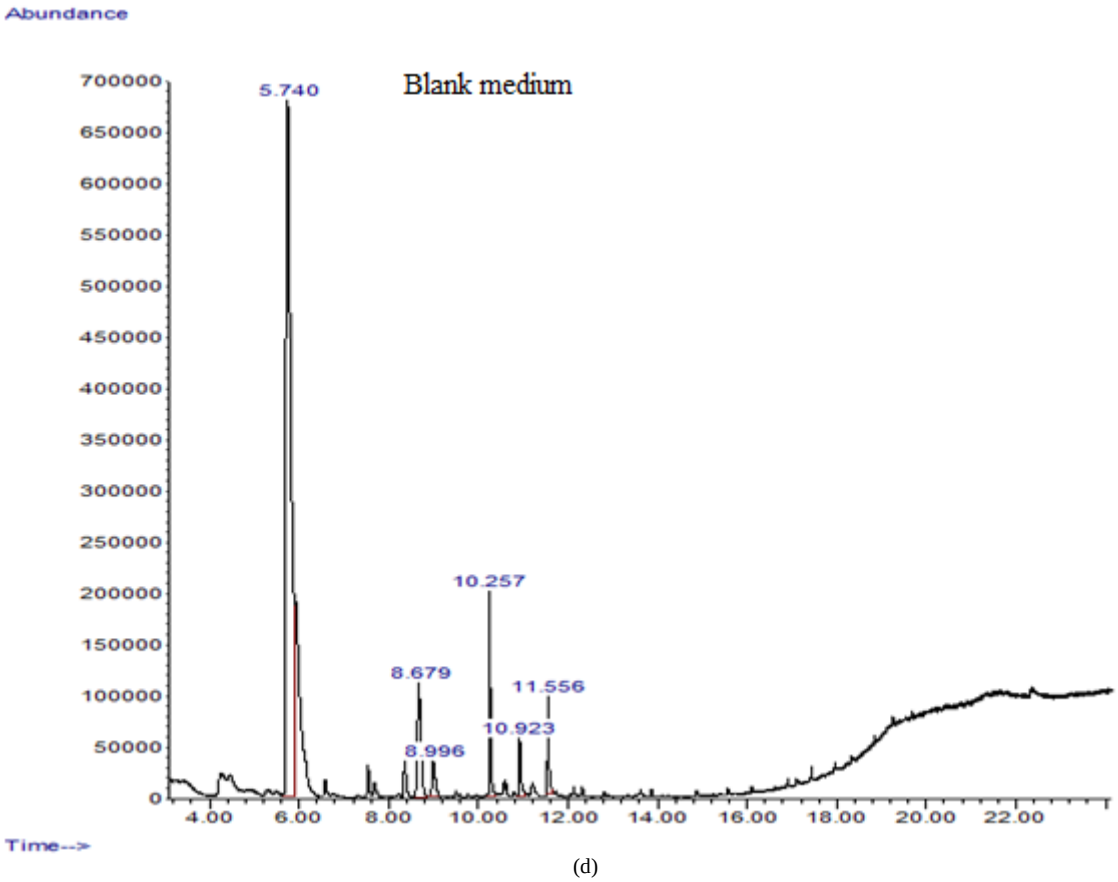
(a)


Abundance
MCF7
5.754
6.148
7.54
10.209
8.526
11.539
4.231
6.639
13.886
15.592
23.890
Time-->

(b)



(c)



(d)

Figure 1: VOC chromatograms (Abundance versus Time (minutes)) obtained using the HS-SPME/GCMS method.
(a) A549 (lung cancer), (b) MCF7 (breast cancer),
(c) HCT116 (colon cancer), and (d) Blank medium

B. Multivariate Data Analysis

The principal component analysis was applied to explore the proficiency of the HS-SPME/GCMS in distinguishing the cell lines and blank medium based on their volatile compounds profile. Total variance of 83% explained the distribution of the data. Figure 2 shows scatter plot of normal probability; which is important in identifying the fundamental departures from normality [13]. It was found that all samples were fit to the straight line suggesting that the data fit the distribution. Meanwhile, Figure 3 presents matrix plots graph which visualising a dispersion of data associated with retention time separately. The number of metabolites considerably increased as the incubation time increased. In another factor, VOCs were undergoing pre-conditioning phase to enrich the metabolites detection of interest. The conception of scattered data highlighted the HCT116 (colon cancer) contributes the most spreading data among other cells. The last influence was from the blank medium which can be concluded that compounds without cells not much consumed during the adsorption time [30]. The second most dispersion of the data was A549 (lung cancer), followed by the MCF7 (breast cancer). Even though the cancer cells emitted quite similar volatiles, but to be specific, it is of vital importance to explore the particular compounds for cancer biomarkers study.

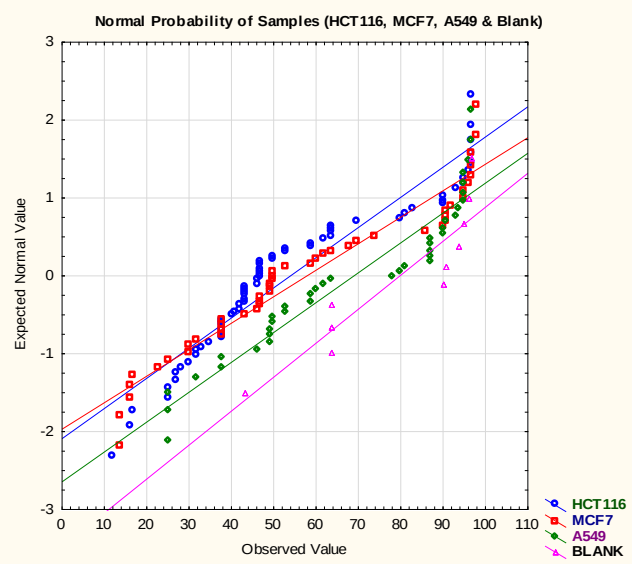


Figure 2: Normal probability of samples

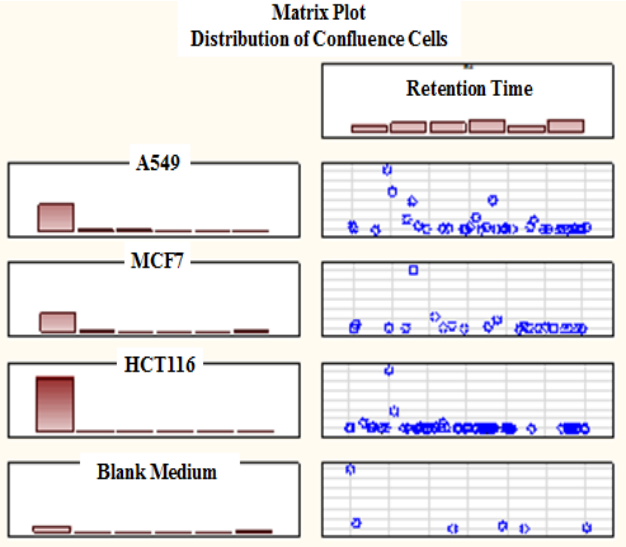


Figure 3: Matrix plot of the distribution of confluence cells

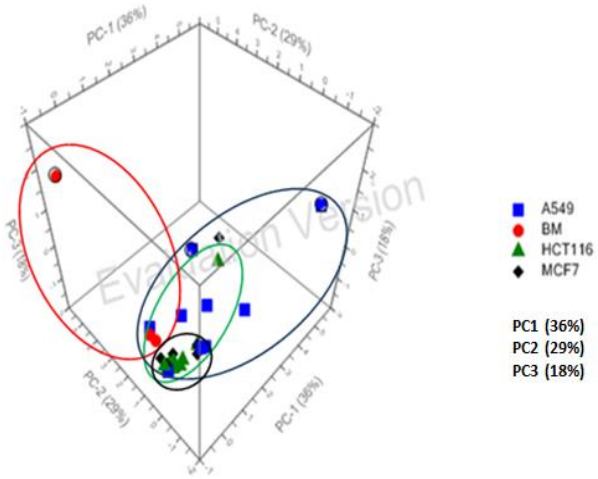


Figure 4: PCA model for score plot of VOCs profiles

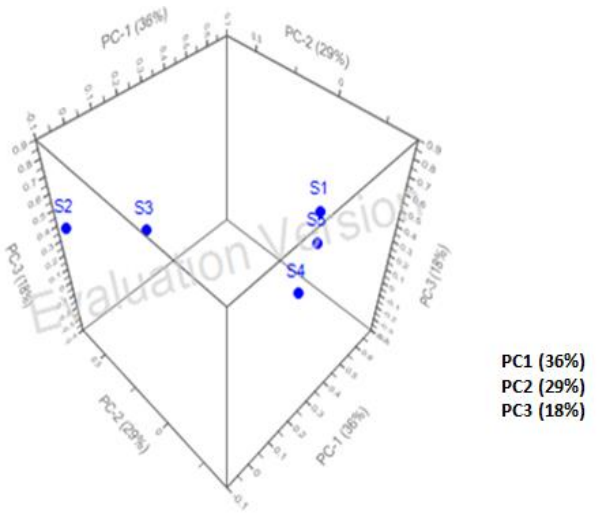


Figure 5: PCA model for loading plot of VOCs profiles

Figure 4 shows PCA score plot model results of the first three principal components. The variance of PC1, PC2, and PC3 were 36%, 29%, and 18% correspondingly, representing 83% of the total VOCs of variability data. PCA offers a

picturing of three main components definite and relatively grouping models concerning different colours designated from all samples. Figure 5 shows PCA loading plot which is related to the correlation of variables. Each variable constituted of retention time respectively and associated with the recognised compounds for all samples. S1 until S5 label designated from the range of retention time; S1 (3 to 5.9 minutes), S2 (6 to 8.9 minutes), S3 (9 to 11.9 minutes), S4 (12 to 14.9 minutes) and S5 (15 to 17 minutes). The scores in PC1, which describes 36% of the total variance, present the clustering with the A549 (blue), HCT116 (green), and MCF7 (black) is outlined in PC1 positive and associated closely. Meanwhile, the group respected to the blank medium (red), is nominated with a separation in PC1. The loading plot model shows that volatiles emitted attributed to the retention time of S1, S4, and S5 are responsible for the discrimination of A549, HCT116, and MCF7which volatile compounds produced from S2 and S3 of retention time are attributed to the blank medium. This finding suggests the compounds released are mostly consumed by cells [31] after minutes of 12^{th} and within 3^{rd} to 5^{th} minutes subsequently.

In conjunction with PC2, the total variance explained 29% shows that the grouping of the blank medium is visualised positively in PC2, while the cluster of A549, HCT116, and MCF7 are calculated in negative PC2. From the loading plot, it indicates that the compounds emitted from S2 and S3 of retention time comprised of the blank medium the most, hence, created a distance from other cell lines as projected in PC2. Based on the score plot, the compounds were overlapped with the A549, HCT116 and MCF7 samples resulted from the similar compounds produced [32] namely ethylbenzene, styrene, silanediol, dimethyl- and tetradecane. Thus, our results defined that those compounds were close to each other due to the same VOCs produced and may have a close association with the retention time respectively. Even though the differences in samples are well illustrated, the clustering tendencies by the metabolites emitted are not easily distinguishable for the predictive signature for each cancer cell line.

IV. Conclusion

A simple method was developed to establish the visualisation and discrimination from the volatile compounds profiles in A549, HCT116, MCF7 and blank medium based on the identified compounds utilising HS-SPME/GCMS method. The acceptable percentage which is about 83% of the total VOCs variability has been observed well from the PCA score plot and loading plot. The application of PCA as chemometric tools for the preliminary study provides a picturing of discrimination between the samples. A good separation of blank medium and other cells confirmed the pattern observed with and without cells. Nevertheless, further studies using different multivariate data analysis are necessary to validate VOCs pattern for future research work.

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