

**THE DEVELOPMENT OF A SENSITIVE METHOD TO STUDY
VOLATILE ORGANIC COMPOUNDS IN GASEOUS EMISSIONS
OF LUNG CANCER CELL LINES**

A Thesis

by

ANUPAM MAROLY

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

May 2005

Major Subject: Biomedical Engineering

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ABSTRACT

The Development of a Sensitive Method to Study Volatile Organic Compounds
in Gaseous Emissions of Lung Cancer Cell Lines. (May 2005)

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The ultimate objective of this research was to develop a low cost, reliable system that would lead to early detection of lung cancer. Tests involved the quantitation of gaseous metabolic emissions from immortalized lung cancer cell lines in order to correlate the chemical markers to be of cancerous origin. The specific aims of the project were the study of gas emissions in selected cancer cell lines and identification of volatile organic compounds (VOCs) in them.

Disadvantages of earlier studies were that the measurements were not real time or state specific so that molecular identification was often inconclusive. Furthermore the methods of study used in the past were not quantitative, which limited their practicality for medical applications. We felt the need to prove or disprove these earlier results using a new technique.

The method we proposed is different and unique when compared to previous methods because cell lines have not been studied extensively for cancer markers. We have studied cancer cell lines which are adherent, immortalized cultures originating from primary tumors obtained from patients with no prior treatment for lung cancer.

We have used an alternative method for the spectrometric analysis and quantitation of the selected chemical markers. The pre-concentration method involved a Purge and Trap unit with a thermal desorber where the vapor concentration was enhanced. The concentrated head space gases were analyzed using a Gas Chromatograph – Mass Spectrometer setup. This setup eliminated the bulky apparatus used in earlier studies. It is

simpler in design and more comprehensive so that external factors such as patient's diet, habitat and lifestyle do not contribute to our study of recognition of cancer markers. Based on the results obtained in the above experiments, a more comprehensive, inexpensive study of lung cancer related markers could be made.

The first section, after giving an introduction to lung cancer, goes on to explain the background work done by other researchers on cancer. The third section gives a detailed explanation of the experimental setup. This is followed by all the tests conducted with corresponding results. The final section deals with the conclusions drawn from all experiments.

ACKNOWLEDGEMENTS

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INTRODUCTION

In the United States, more deaths are attributed to lung cancer than breast, prostate, and colon cancers combined. It is estimated that 169,400 new cases were diagnosed in 2002 and 154,900 deaths were attributed to lung cancer [1] [2]. According to studies by World Health Organization lung cancer is the most common cancer and the rate is increasing 0.5 % every year. More than 35 million Americans are living with chronic lung disease. It is the largest single cause of cancer deaths in the US. 28% of cancer deaths are due to lung cancer.

Structure of the lung

The lungs are in the chest, on either side of the heart. The right lung has 3 compartments or lobes and the left lung has 2 lobes. Air is inhaled through the nose and throat and flows past the voice box (larynx) into the windpipe (trachea). The windpipe divides into 2 tubes, the left and right bronchi, which supply air to each lung. Within the lung, the tubes get smaller and smaller (bronchioles) until they reach air sacs (alveoli). The alveoli's job is to add oxygen to the blood and to take waste gases out. The waste gas is removed from the body as we exhale. A slender barrier separates air in the alveoli of the lung from the blood in the capillaries. The volatile organic compounds (VOCs) in expired breath may give us information about general metabolic conditions in particular of the lung.

Lung cancer

Lung cancer is one of the more common forms of cancer in which the majority of these cancers arise in the bronchial epithelial cells. These cells can be cultured in vitro for a certain period before replication ceases.

This thesis follows the style of the Journal of Chromatography A.

Cancer occurs when normal cells undergo a transformation that causes them to grow and multiply without normal control. They form a mass or tumor that differs from the surrounding tissues from which it arises. Tumors are dangerous because they take oxygen, nutrients, and space from healthy cells and completely destroy them before spreading on to new tissue.

Types of lung cancer

Lung cancers are usually divided into 2 groups that account for about 95% of all cases. The division is based on the type of cells that make up the cancer. These two types are **small-cell lung cancer (SCLC)** and **non-small-cell lung cancer (NSCLC)**, which includes several types of tumors. NSCLC consists of **squamous cell carcinoma, adenocarcinoma and large cell carcinoma** (Fig 1).

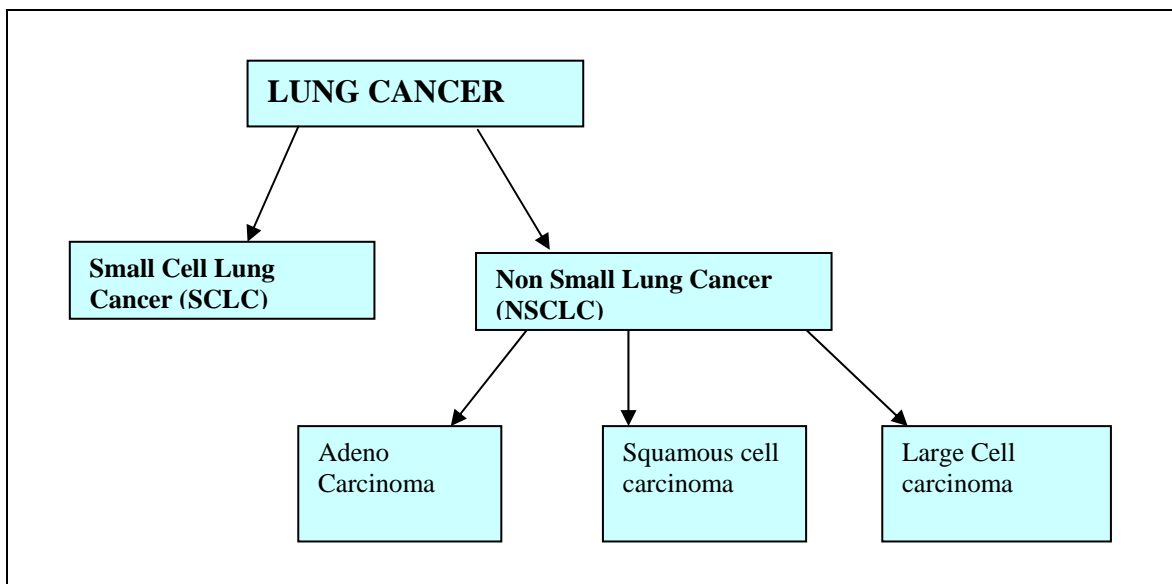


Fig. 1. Types of lung cancer

Adenocarcinoma of the lung is the most common histologic type of lung cancer [3, 4] and makes up 30-35%. Squamous cell carcinoma is the second most common type, making up about 30% of all lung cancers. SCLCs are less common, but they grow more quickly than NSCLCs and are more likely to metastasize. Often, they have already spread to other parts of the body when the disease is diagnosed. Large-cell cancer makes up 10% of all cases. SCLC makes up 20% of all cases.

Risk factors

These include active smoking (90%), radon (10%), exposure to carcinogens (9-15%), pollution (1-2%). Lung cancer causes 31% of deaths due to cigarette smoking [5]. Other causes of lung cancer include passive smoking, air pollution from motor vehicles, asbestos exposure. Among cancers that are associated with occupational exposures, cancer of the lung is the common [6]. Workers exposed to tar and soot (which contains benzo[a]pyrene), such as coke oven workers, [7, 8] in concentrations exceeding those present in urban air are at increased risk of lung cancer. Occupational exposures to a number of metals, including arsenic [9, 10] chromium, [11] and nickel [12] are carcinogenic [13].

BACKGROUND

Breath analysis

Extensive research of breath analysis has been carried out [14] in lung cancer studies since it requires minimum medical intervention and control and samples are easier to obtain than serum or urine [15, 16].

Normal human breath was analyzed using gas chromatography (GC), whereby several hundred VOCs in exhaled air were found [17]. Exhaled air from patients with lung cancer and 28 breath biomarkers including alkanes and benzene derivatives were found [18, 19]. A correlation between VOCs and lung cancer was made [17, 20]. VOCs from human breath have been studied as markers of oxidative stress with the investigation of alkanes and methylated alkanes since alkanes are known to be oxidized to alcohols by the enzyme which has shown to be active in case of lung cancer [18, 21]. Breath vapor from normal subjects was studied in order to understand its constitution using Gas-liquid partition chromatography [22] and Gas chromatography-Mass spectroscopy (GC-MS) [23] to provide a baseline for future studies.

GC-MS combined with some sample collection and concentration techniques such as cold trapping and adsorptive binding was developed for analysis of breath gas [17, 19, 20, 24, 25, 26]. A portable breath-collecting apparatus was developed and applied to determination of volatile markers [21, 27]. To determine whether the VOC is produced by external factors such as environment, food habits or internally in the body, researchers introduced the concept of alveolar gradient (AG). A positive AG shows higher concentration in the breath [26, 28].

Other studies

Non volatile markers such as proteins from breath of individuals with respiratory tract disorders have been studied which enabled the demonstration of their origin [29]. A

genetic alteration is seen in bronchial mucosa and plasma DNA of patients with the risk of lung cancer [30]. Most polar and non-volatile compounds are excreted and therefore all compounds in breath were concluded to be non polar [31].

Blood analysis

Blood was used to investigate VOCs studied including styrene, benzene, acetone, toluene [32]. VOCs have also been studied in blood samples spiked with analytes using headspace gas and capillary GC and ion trap detection [33, 34]. Since blood was an invasive technique, it has been considered an unsuitable approach by some researchers as there was a possibility of exposure of the analyst to infectious agents through accident spills [35].

Both head space analysis and purge and trap (P&T) concentration method have been used in blood analysis, but P&T was found capable of analyzing larger amounts of low concentration VOCs for analysis [36]. Blood analysis has been used to study styrene in urine and blood [37].

Techniques used

Many different detection and concentration techniques apart from P&T or GC-MS have been used in lung cancer studies. Each has their own advantages and disadvantages. Some of the more frequently used have been cited.

SIFT-MS - Single ion flow tube mass spectroscopy

It involves introduction of ions of trace gases in to the sample before detection [38]. This method was initially developed for the non-invasive real time breath analysis of workers for toxic solvents [39, 40]. It was also used to study acetaldehyde; acetone and ethanol in headspace of lung cancer cells [41] and to analyze trace gases in the breath for diabetes [42].

SPME – Single phase micro extraction- concentration technique

The simple and solvent-free technique of SPME has demonstrated a great deal of potential in the study of breath volatiles [43, 44]. Breath was studied to identify aliphatic and aromatic hydrocarbons as markers in non small cell lung cancer using SPME [45]. An array of non selective gas sensors made of quartz microbalance (QMB) was used to detect alkanes and aromatics in breath [46]. SPME was used to concentrate VOCs in blood alkanes, ketones, halogens and thioethers in order to study their origin [47]. Volatile biomarkers in lung cancer blood were studied using SPME and capillary GC-MS [48].

Collection of samples

Breath has most commonly been analyzed by collection in teflon bags, concentrated using traps packed with carbon molecular sieves [17, 19, 21, 49] and detected using GC-MS. Canisters have been used for breath collection instead of bags and trapping using cryogenic beads have been carried out [50, 51]. Breath has been simulated and studied in the nanomolar range with the use of SPME and carbon coated macrofibres [52].

Elutants studied in the past

1-pent-3-yne and 2, 5 di-ethyl furan occurred in high frequency in the breath of smokers but were absent from non smokers breaths [53]. Ethanol was studied extensively in breath using GC [25]. This was traced to antibiotics in food or acetaldehyde in metabolic pathways. Oxidative stress increases with age and so also does alkane production in the breath [54]. Isoprene was found to be the most abundant VOC in breath by some researchers [55]. Acetaldehyde was studied in breath by freezing VOC in liquid nitrogen [56]. Iso-propyl alcohol was studied by on column concentration which eliminated the need for pre concentration [57]. O-toluidine was detected in the breath of lung cancer patients [24].

Styrene seen in exhaled breath might have originated from smoking, so also benzene [58]. Proton transfer mass spectrometry enabled online detection of breath at parts per trillion levels [59], the study of propanol [60] and isoprene with respect to blood cholesterol levels [61], and the origin of isoprene and o-toluidine [62] in breath.

Having studied all the research that has been done for so far, we have designed a method to prove or disprove some of the results that have been reported. We have done this by designing a unique system to collect VOCs and analyze them. This method of collection has never been attempted on lung cancer cell lines before.

EXPERIMENTAL SETUP

Cell line study

Researchers found an excellent concordance between lung cancer tumor cell line and tissue morphology (100%) [63]. It was found that NSCLS cell lines of large majority of instances retain the property of their parental tumors for lengthy culture periods. These cell lines appear have shown to be representative of the lung cancer tumor from which they have been derived and thus provide suitable model systems for biomedical studies for this important neoplasm.

The cells are grown in a liquid media; their concentration is kept between low (too few cells for them to divide comfortably) and a high (too many cells, using up the media nutrients and beginning to die). The growth medium consists of tissue culture media, however a few additives are necessary since some components cannot be added by the company. Among these components are the fetal bovine serum (FBS), L-glutamine and gentamycin(antibiotic) (All supplied by Invitrogen Corporation, CA).

The standard media varies for different cell lines but all essentially contain many of the necessary nutrients such as inorganic salts, vitamins, amino acids and a pH indicator. The cells are grown for a period of 4-5 days and once dense growth has taken place; they have to be sub-cultured or analyzed. This procedure is essentially same for the cell lines used.

Cell culture

Cells are frozen in liquid nitrogen at -60°C when sold. The vial is thawed in a water bath at 37°C for two minutes. It is then decontaminated with 70% ethanol solution. The vial contents are transferred to 25 cm^2 tissue culture flasks and diluted with recommended media. The flasks are placed in an incubator (VWR Scientific, PA) (model 2310) for growth. The cells grow for a period of five days by adhering to the base of the culture

flask (Nunclon delta polystyrene) flasks. During the process, they come in contact with the neighboring cells and multiply in number. At all times the cells have to be contact with the media for growth.

During cell growth, due to oxidation and the formation of new cells there is a build up of acids leading to a change in pH. This is seen in the change of the color of the media from bright red to pale yellow. The phenyl red present in the media enables this color change with the change in pH. A distinct color change is sometimes seen in the media even with out the cells. According to distributes for cell cultures and their media, ATCC, when the temperature of the medium that is incubated increases, the equilibrium of the buffers shifts. This causes a slight decrease in the pH of the medium. This temperature change may cause media component to breakdown since the buffers included in the medium formulation are temperature sensitive.

This can be further explained with the CO_2 – bicarbonate system. In the headspace of a closed system initial CO_2 is used by the cells and gradually there is a build up of this in the flask over 5 days. Since there is no escape for the CO_2 , there is an increase in weakly dissociated NaHCO_3 producing excess H^+ ions in the medium and therefore a fall in pH. This is seen as a color change.

When the confluence has reached 90% the cells are ready to be sub-cultured (Fig 2).

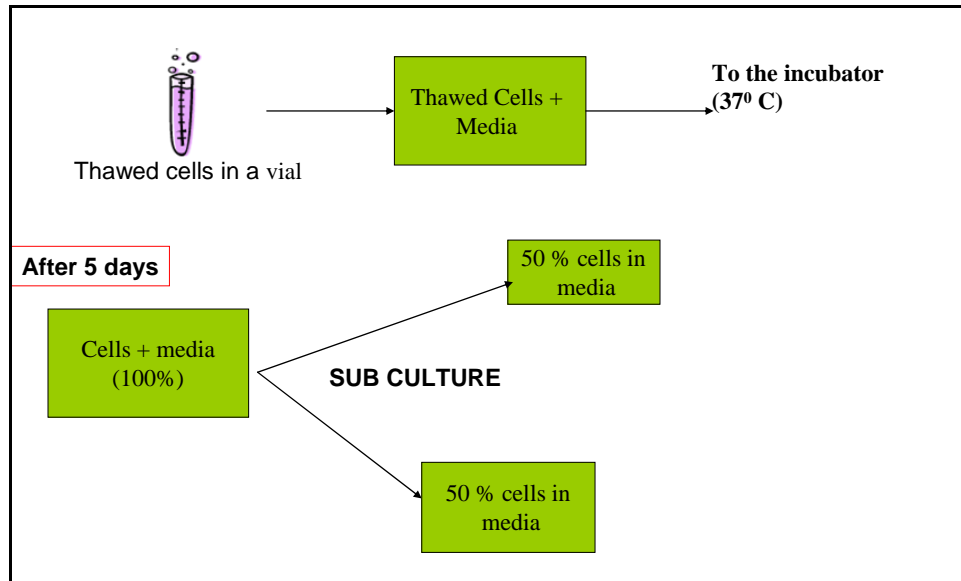


Fig. 2. Block diagram of cell culture process

Cell sub culture

Cell media solution is drained using a vacuum system to remove any dead suspended cells since the living cells adhere to the base. The flask is washed with 5 mL Dulbeccos phosphate buffer saline (DPBS) solution twice. 1 mL of Trypsin EDTA is added in order to free the cells from the base. The process takes 10 - 15 minutes. Once the cells can be seen freely floating in the media as observed on a microscope, 10mL of the media is added and then this is split into two batches of 5mL into two new clean sterile T-25 flasks (Fig 3).

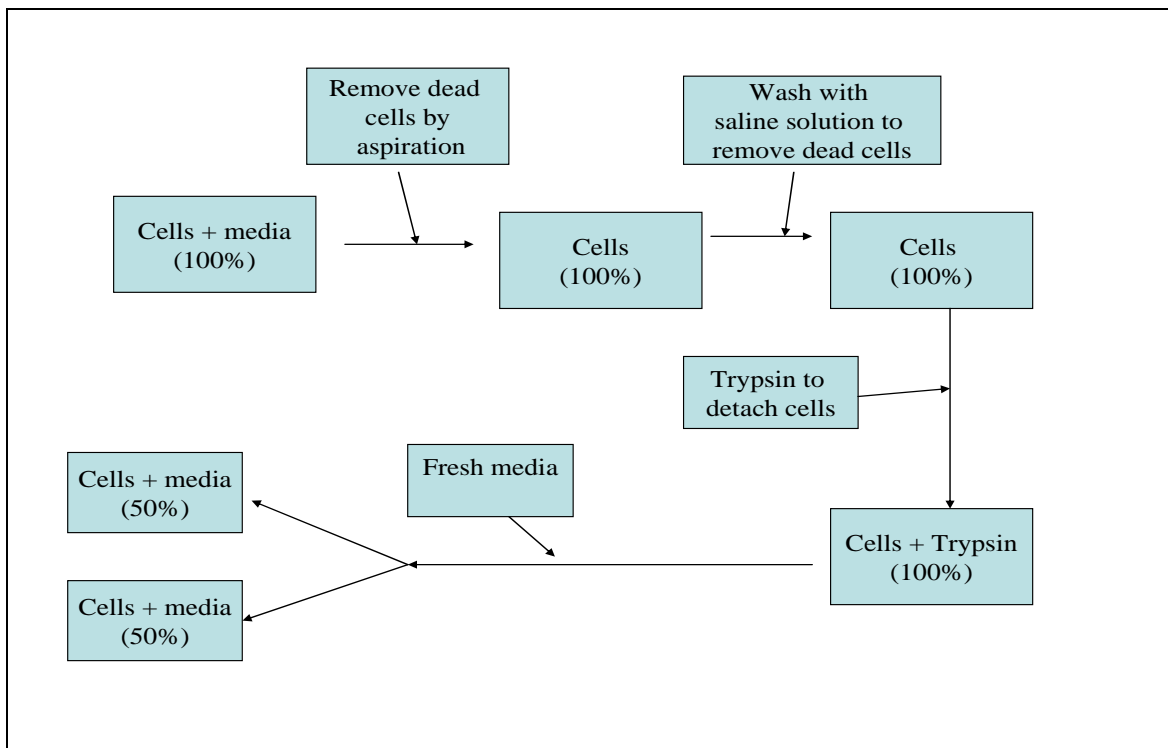


Fig. 3. Block diagram of the cell sub culture process

Purge and trap

Purge and trap gas chromatography (P&T–GC) first described by Swinnerton and Linnenbom[64] and developed by Bellar and Lichtenberg [57], has become a valuable and widely accepted method for the analysis of VOCs in aqueous samples [65, 66].

P&T is the method of choice for extracting and concentrating VOCs from almost any matrix. It is particularly useful for concentrating VOCs that are insoluble or poorly soluble in water, have boiling points below 200°C, and having a higher octanol / water partition coefficient. The purge-and-trap system was a Model 4560 (O.I. Analytical, College Station).

Theory

The traps used in P&T (Fig 1) are generally packed with multiple beds of various sorbent materials so that a broad range of high and low molecular weight compounds, polar and nonpolar, can be trapped in a single tube. We selected a trap made up of Tenax (O.I. Analytical, College Station) (Fig 4), because of its capacity for reduced adsorption of water.

The procedure involves bubbling an inert gas such as helium, through an aqueous or gaseous sample at ambient temperature so that volatiles are transferred from the matrix to the vapor phase. The volatiles are then swept through a sorbent column where they are trapped. The column is heated and backflushed with helium to desorb the compounds which are transferred to the GC.



Fig. 4. Purge and trap unit

The working of the concentrator involves three phases- **Purge, Desorption and Bake** cycles.

Purge

During the purge phase of sampling, helium gas is pumped through the sample so that VOCs from the sample are released. The lower molecular weight compounds pass through the initial adsorbent beds, but are trapped by succeeding beds. Each bed protects the next, by preventing compounds from being held too strongly so that can be desorbed quickly without decomposition.

Desorption

The purge volatiles are now adsorbed on to the trap of the P&T with the help of the carrier gas. The adsorbent trap is rapidly heated to the desorb temperature and the valve is switched to align the carrier gas flow in-line with the trap. The trap is then held at the desorb temperature for an optimal time to thermally desorb the analytes into the carrier gas. The carrier gas passes through the trap in the reverse direction of purge flow, so that higher molecular weight compounds never come in contact with the stronger (innermost) sorbents.

Bake

The adsorbed VOCs are now baked off the trap at high temperatures and transferred to the GC. An adsorbent material that traps and then releases a group of compounds efficiently will help provide high recoveries, sharp peaks, and good resolution, allowing accurate quantification of the analytes. The absence of interferences from contaminants or water vapor is also essential for accurate quantification. Similar to extraction efficiency, desorption performance is dependent on the rate at which the trap is heated; the final trap temperature, and the total desorption flow (i.e., the amount of gas that is passed through the trap). The different temperatures and times of each cycle are tabulated in Table 1.

Table 1
Temperature and time settings for each cycle in P&T

Cycle	Temperature (in °C)	Time (in min)
Purge	20	11
Desorb	180	0.5
Bake	180	10

The flow-rate of carrier gas, helium is 40 mL/min and the system pressure is between 6 and 11 psi.

Water management

In the process of purging the volatile target analytes from the water matrix (in our case cell culture media), some of the water will inevitably be carried along with the inert gas and onto the trap. This is minimized by heating the transfer tubing between P&T and GC to 100°C.

Head space analysis

This involves the study of the VOCs collected above the sample over a fixed period of time. The advantage of the dynamic headspace method is its capability to isolate volatiles in their natural form. Head space analysis is done by two methods.

1) Sparge vessel method - Here the sample has to be manually injected in to the sparge vessel of the P&T using a gastight syringe. The disadvantages are limited volume, more chance for human error.

2) Air tube method - Here an air tube is used to trap VOCs off the head space of the sample flask. The trapped VOCs are then transferred to the second trap in the P&T using an air tube desorber. The principle of working is as described below.

Air tube

There are number of adsorbents that can be used as packing material in air tubes (Fig 2). Tenax (2,6 diphenylene oxide) air tubes (OI Analytical, College Station) were chosen since it can retain a specific or a group of analytes for a specified sample volume. It has been designed for trapping volatiles and semi volatiles from liquid and solid matrices. It is a low bleeding material with low level of impurities. Due to its low affinity for water it is useful for purging of volatiles with high moisture. It is used to detect C5- C26 compounds, and compounds with low affinity for water. Compounds which are to be detected by this method are non polar organics having boiling points in the range 80-200°C.



Fig. 5. Air tube placement in the P&T unit

Once fixed on the air desorber unit (Fig 5) the sample concentrator pre-purges the air-tube at ambient temperature to remove oxygen and moisture accumulated during sample

collection. The analytes migrate through the adsorbent bed where at some point in time some of the analytes breakthrough whereas some are retained by the adsorbent.

The tube is heated causing adsorbed materials to release from the trapping material. Purge flow sweeps the tube transferring desorbed compounds to the sample concentrator's trap. Thermal desorption of the P&T trap releases the analytes on the GC/MS system for detection. The sample transfers as a discrete plug to the analytical column for separation and analysis.

The advantages of this method are

- 1) The errors in extracting the sample such as loss of gases to the atmosphere while handling the syringe are minimized.
- 2) Back pressure created by the needle of the syringe is avoided.
- 3) Transfer of water vapor from the sample is minimized due to the properties of Tenax.

Gas chromatography

Gas chromatography (GC) is an analytical technique for separating compounds based primarily on their volatilities. It provides both qualitative and quantitative information for individual compounds present in a sample. In this technique, a sample is converted to the vapor state and a flowing stream of carrier gas (helium) sweeps the sample into a column.

The compounds partition between a **stationary phase**, which can be either solid or liquid, and a **mobile phase** (gas). The differential partitioning into the stationary phase allows the compounds to be separated in time and space. The carrier gas serves as the mobile phase that moves the sample through the column.

The injector is the point where the sample is introduced into the column from the P&T unit. It contains a heated chamber containing a glass liner into which the sample is injected through the septum. The sample vaporizes to form a mixture of carrier gas, vaporized solvent and vaporized solutes. When the sample is introduced into the GC unit from the P&T, it takes the form of a narrow band. The rate at which the sample passes through the column depends on the size of the particle and how well it is adsorbed onto the column (attraction of the particle for the column material).

The GC column is the heart of the system. It is coated with a stationary phase which greatly influences the separation of the compounds. The structure of the stationary phase affects the amount of time the compounds take to move through the column. Typical stationary phases are large molecular weight polysiloxane, polyethylene glycol, or polyester polymers of 0.1 to 2.5 μm film thickness.

The column is placed in an oven where the temperature can be controlled very accurately over a wide range of temperatures. Typically, GC oven temperatures range from room temperature to 250°C. As the sample mixture moves through the column, sample components that interact strongly with the stationary phase spend more time in the stationary phase vs. the moving gas phase and thus require more time to move through the column.



Fig. 6. GC-MS unit

The gas chromatograph was a Hewlett-Packard 5890 (Palo Alto, CA), equipped with an electron ionization detector (EID) for quantification (Fig 6).

Temperature program for GC

A DB-Wax capillary column (50 m×0.32 mm I.D., 0.52 m film thickness) (Agilent Technology, CA) was used for GC analysis. The carrier gas was helium (99.999%) at flow-rates of 1.20 mL/min. The oven temperature was held at 30°C for 5 min, and then increased at 20°C/min to 200°C, where it was held for 3 min; the injection temperature was 250°C and the detector temperature was 280°C. The mass spectra were obtained at an ionization voltage of 70 eV and were recorded in the total ion scan mode from 20 to 250 amu.

Once the molecules leave the column, they are monitored by a detector. The compound and detector interact to generate a signal. The size of the signal corresponds to the amount the compound present in the sample. The type of detector used depends on the compounds to be analyzed. These detectors can measure from 10^{-15} to 10^{-6} grams of a single component.

Chromatograms

Analytes enter the detector and generate an electronic signal called response. This response is displayed as a graph where the x axis is the retention time and the y axis is a measure of the intensity of the response. In chromatography, this graph is called a **chromatogram**. When the run begins, there are no analytes in the detector; the response line produced on the chromatogram is called the **baseline**. The size of the peak is proportional to the concentration of the analyte. The concentration of the analyte is measured by calculation the area of the peak.

While polarity is usually the major factor governing separation, the boiling points of components of the sample also play a role in determining retention time. The retention

time, is qualitatively indicative of the type of compound. Components with higher volatility (lower boiling points) tend to spend less time in the moving gas phase and therefore tend to have shorter retention times.

Mass spectrometry

Mass spectrometry is an instrumental approach that allows for the mass measurement of molecules.

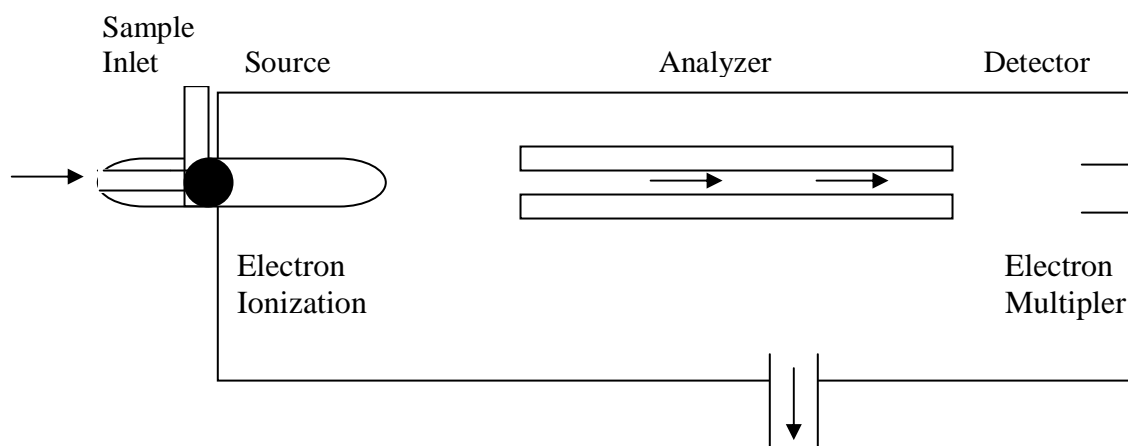


Fig. 7. Structure of mass spectrometer

The five basic parts of any mass spectrometer are: a vacuum system; a sample introduction device; an ionization source; a mass analyzer; and an ion detector (Fig 7). Combining these parts a mass spectrometer determines the molecular weight of chemical compounds by ionizing, separating, and measuring molecular ions according to their mass-to-charge ratio (m/z). The ions are generated in the ionization source by inducing either the loss or the gain of a charge (e.g. electron ejection, protonation, or deprotonation).

Once the ions are formed in the gas phase they can be electrostatically directed into a mass analyzer and then on to the ion detector. The detector allows a mass spectrometer to generate a signal current from incident ions by generating secondary electrons, which are further amplified, separated according to mass and finally detected. The result of ionization, ion separation, and detection is a mass spectrum that can provide molecular weight and structural information of the samples being analyzed (Fig 8).

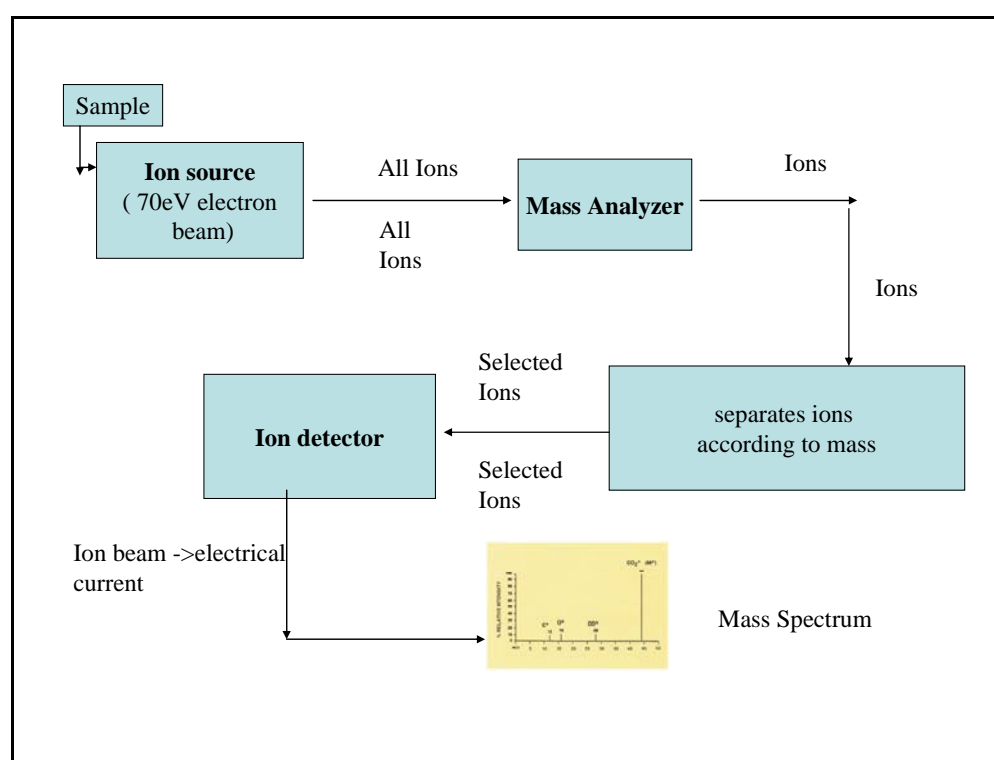


Fig. 8. Block diagram of the working of a mass spectrometer

Electron ionization plays an important role in the routine analysis of small molecules. Databases combined with current computer storage capacity and searching algorithms, allow for rapid comparison with known mass spectra, thus facilitating the structural determination of small molecules.

The lab setup is as shown in Fig 9.

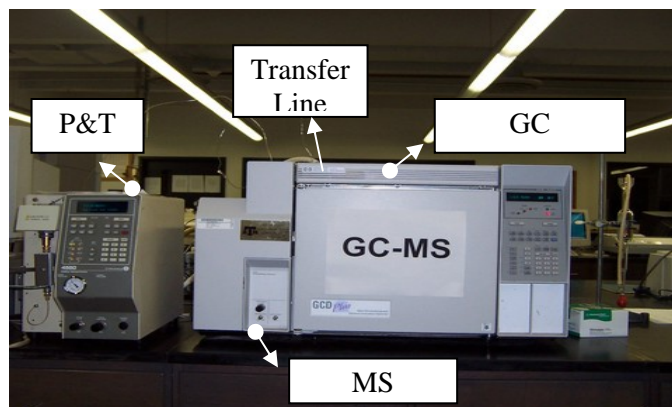


Fig. 9. Equipment setup

EXPERIMENTS AND RESULTS

Various VOCs have been studied and reported as lung cancer markers viz. toluene, styrene, dodecane, benzene, decane, cyclohexane, ethyl benzene, acetaldehyde [17, 19, 24, 41, 52]. Our project was to study some of these 'markers' and check for the validity of these reports.

Initial studies

Three compounds were short listed to be studied initially. They were analine, tetrahydro furan (THF) and methyl ethyl ketone (MEK) since they had good detection efficiency on GC-MS equipment. The detection efficiency of these compounds on P&T was analyzed by running standard solutions 100, 200, 500 and 1000 ppb for each of the selected compounds (Figs 10, 11, 12) on a P&T-GC-MS unit. These experiments were carried out in an off campus lab at O.I. Analytical Instruments, College Station.

In all the spectra generated from the P&T equipment the initial peak seen is CO₂ and according to the suppliers of the equipment, O.I. Analytical, this is generated from the equipment and not the sample. This peak is seen in all spectra reported in this report.

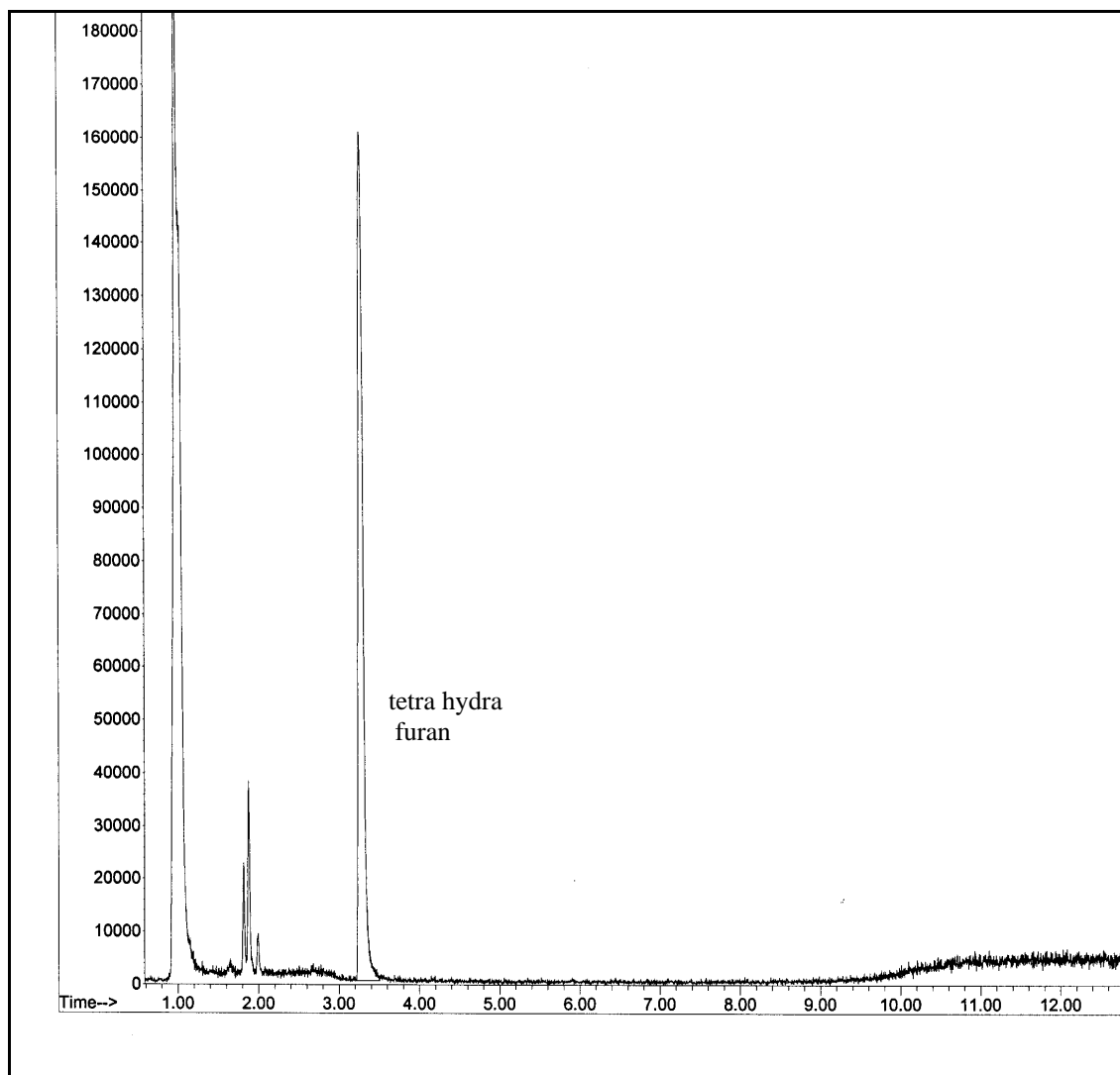


Fig. 10. Spectrum of THF at 200 ppb

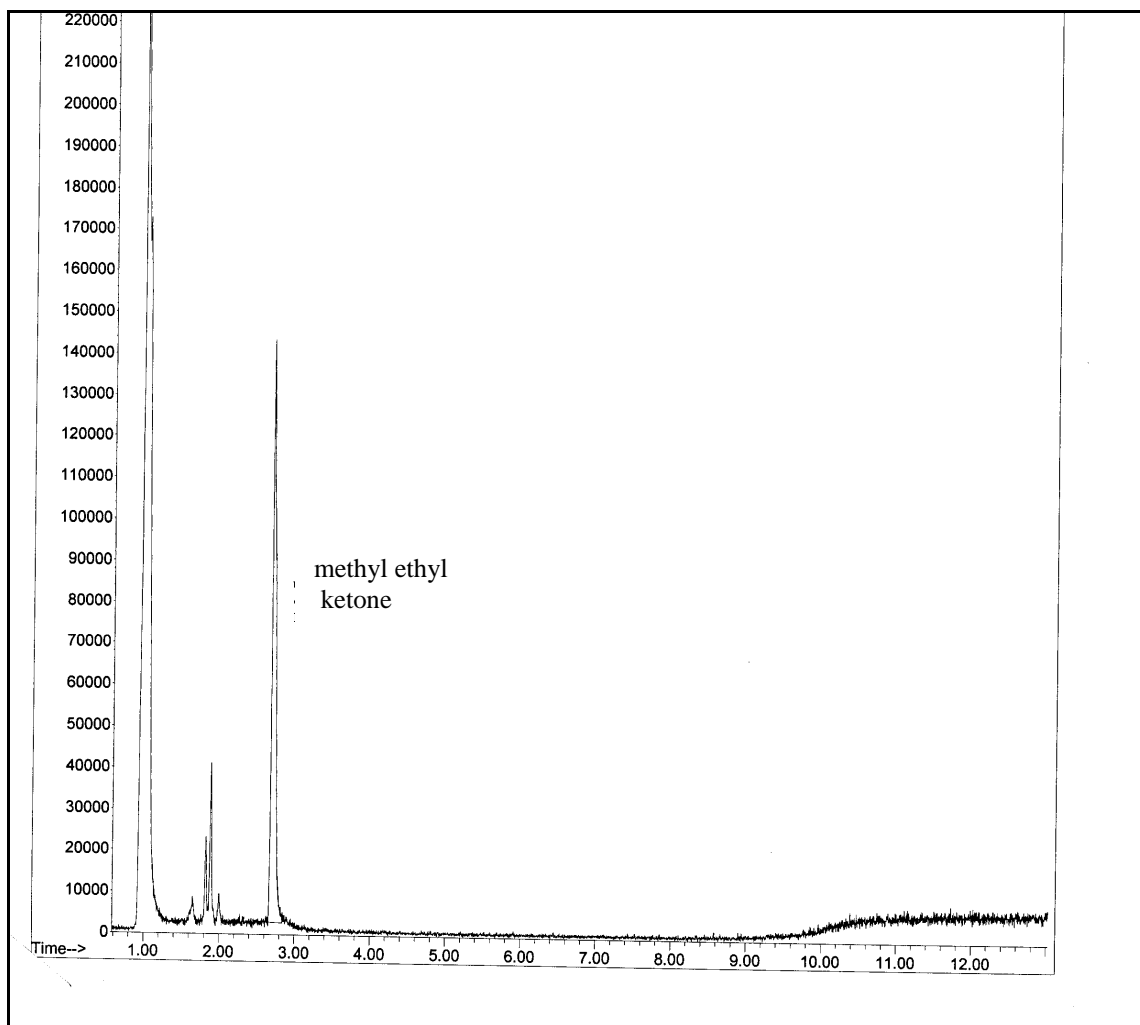


Fig. 11. Spectrum of MEK at 200 ppb

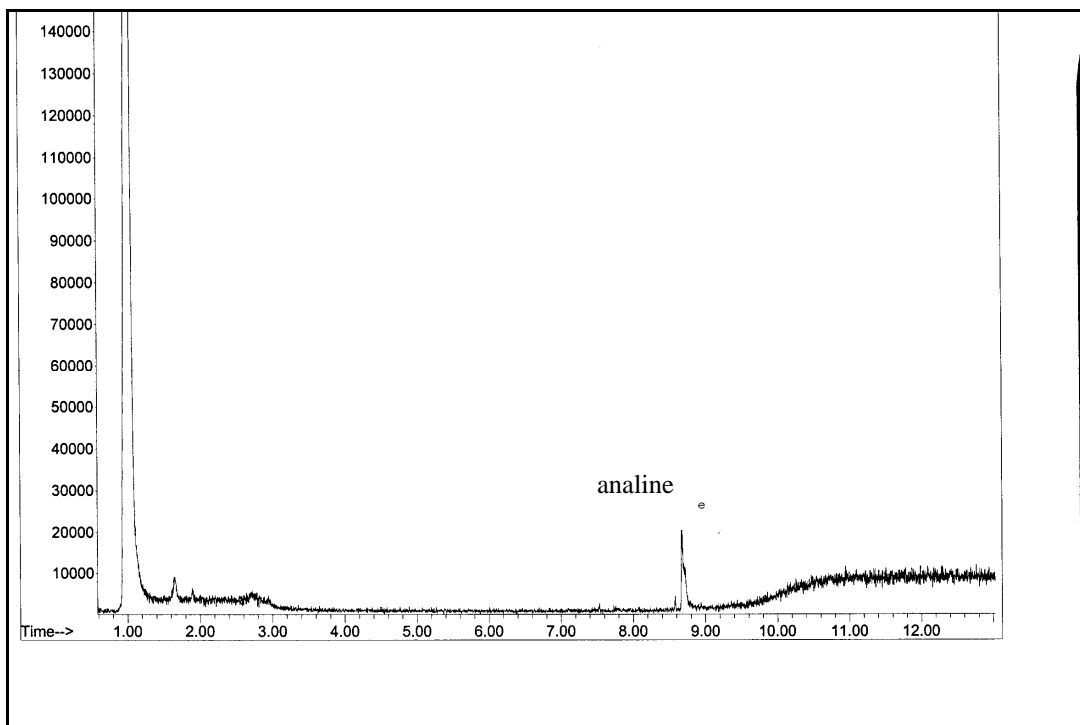


Fig. 12. Spectrum of aniline at 200 ppb

The limits of detection were determined by running standards of known concentration and the results were as tabulated (Table 2).

Table 2

Observations of early VOCs studied

VOC	Retention time	Observations
Aniline	8.67 min	not detected at 50ppb or 100 ppb, poor repeatability at 500 ppb
MEK	2.7 min	Excellent sensitivity and repeatability at 500 and 1000ppb
THF	3.27 min	excellent sensitivity and repeatability at 500 ppb and 1000ppb

Cell line studies

Initial tests were followed by working with actual cell lines. First the cell lines to be studied were short listed and then ordered from American Tissue Culture Company (ATCC). We used Dr. Lori Bernstein's laboratory at the department of Pathology, TAMU for the growth and study of the cells. Table 3 lists the different cell lines and their corresponding media.

Table 3**Type of cell lines and their propagation media**

Cell line	Type	Media and additives
HTB 59	Squamous cell carcinoma	Leibovitz L-15 medium (Invitrogen, CA) with 2mM L-glutamine- 90% FBS- 10%
HTB 58	Squamous cell carcinoma	Eagle's essential medium with Earle's BSS (Invitrogen, CA) and 2mM L-glutamine - 90%. FBS- 10%
CRL 5810	Adenocarcinoma	ATCC medium: RPMI 1640 medium (Invitrogen, CA) - 90%, FBS-10%

The media and additives used to grow the cells is a complex mixture of inorganic salts, amino acids and hormones (Table 4).

Table 4
Components of media and additives

HTB-59	Leibovitz L- 15 medium	Inorganic Salts CaCl ₂ , MgCl ₂ , MgSO ₄ , KCl, KH ₂ PO ₄ , NaCl , Na ₂ HPO ₄ Amino Acids L-Alanine, L-Arginine, L-Asparagine, L-Cysteine, L-Glutamine, Glycine, L-Histidine, L-Isoleucine, L-Leucine , L-Lysine, L-Methionine, L-Phenylalanine, L-Serine , L-Threonine, L-Tryptophan, L-Tyrosine, L-Valine Vitamins Choline Chloride, Riboflavin, Folic Acid, myo-Inositol, Nicotinamide, D-Pantothenic Acid, Pyridoxine, Thiamine Other D-Galactose , Phenol Red, Sodium Salt , Sodium Pyruvate
Additives for all media used	Fetal bovine Serum	Albumin, Glucose, Alkaline Phosphatase, Iron, Magnesium, bicarbonate, Bilirubin, Phosphorous, Potassium, Blood urea nitrogen, Protein, Calcium, Chloride, sodium, Cholesterol, Triglycerides, Creatinine, Globulin, uric acid. Hormones Insulin, Progesterone, Thyroxine, Estradiol, testosterone.
	DPBS	Potassium chloride, Potassium phosphate, sodium chloride, sodium phosphate
	Trypsin	Inorganic salts EDTA, potassium chloride, potassium phosphate, sodium bicarbonate, sodium chloride, sodium phosphate Other Dextrose anhydrous, phenol red, trypsin porcine
HTB- 58	Eagle's essential medium with Earle's BSS	L-glutamine, sodium bicarbonate, non-essential amino acids, sodium pyruvate
CRL-5810	RPMI 1640	L-glutamine, sodium bicarbonate, glucose, HEPES, sodium pyruvate

On introducing cell media (without the cells) in to the P&T-GC unit there was excessive foaming of the media liquid which posed a threat to the functioning of the equipment [67].

Ideally VOCs of these lung cancer cell lines should have been compared to similar tissue from a healthy samples, therefore two cell lines were considered viz. CCL- 95.1 and CRL 2078. However due to the very complex nature of their growth media the cell lines from a normal epithelial tissue were not used. For e.g. CRL -2078 required keratinocyte serum free media, cholera toxin, bovine pituitary extract, and recombinant human epidermal growth factor which our lab was not certified to handle. Therefore, as a control, it was decided to study the media without any cells.

Analysis of VOCs

Before the setup of our lab on campus, initial experiments on cell medium were conducted in O.I. Analytical. 1cc of the medium was introduced in to the P&T-GC-MS system using 10 cc syringes (SGE). This was done for the media of cell lines HTB-59 and CRL-5810, both with and without the cells for comparison (Figs 13, 14, 15, 16, 17, 18).

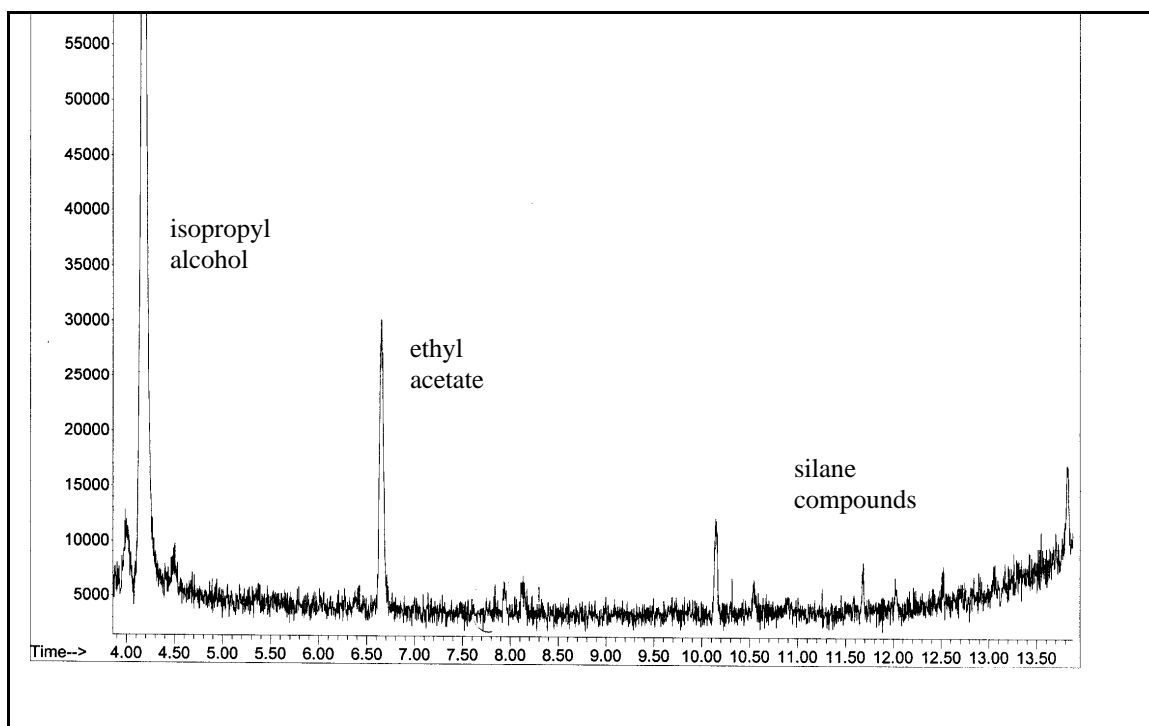


Fig. 13. Spectrum of media without cells (CRL-5810)

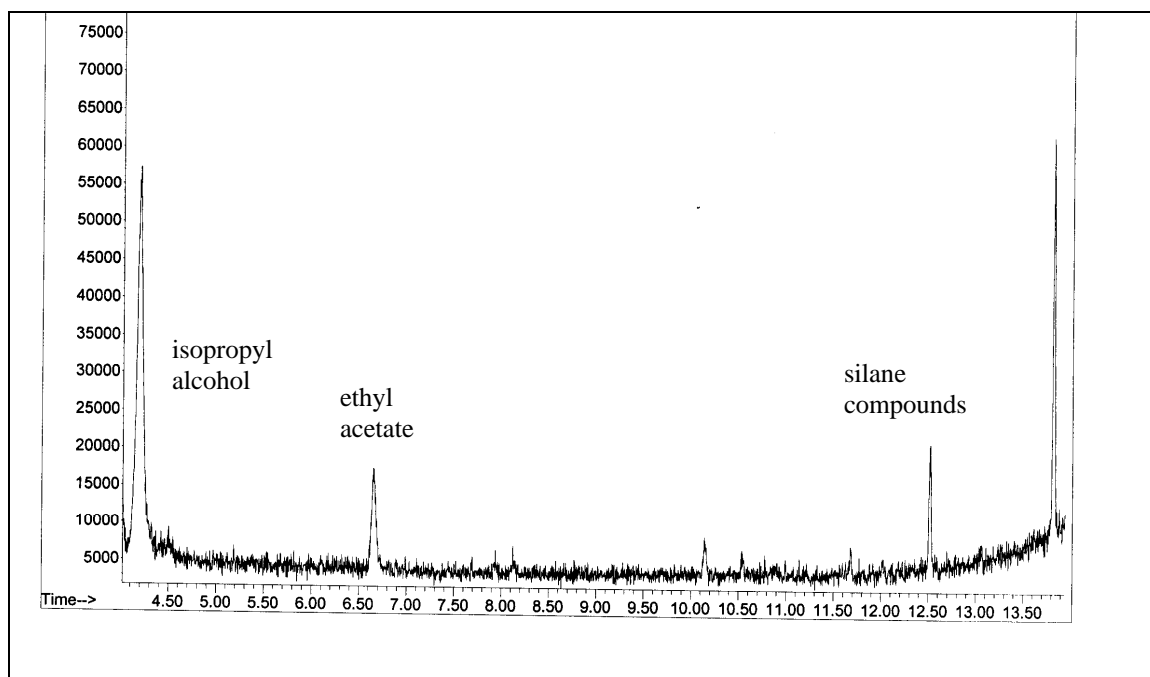


Fig. 14. Spectrum of media with cells (CRL-5810)

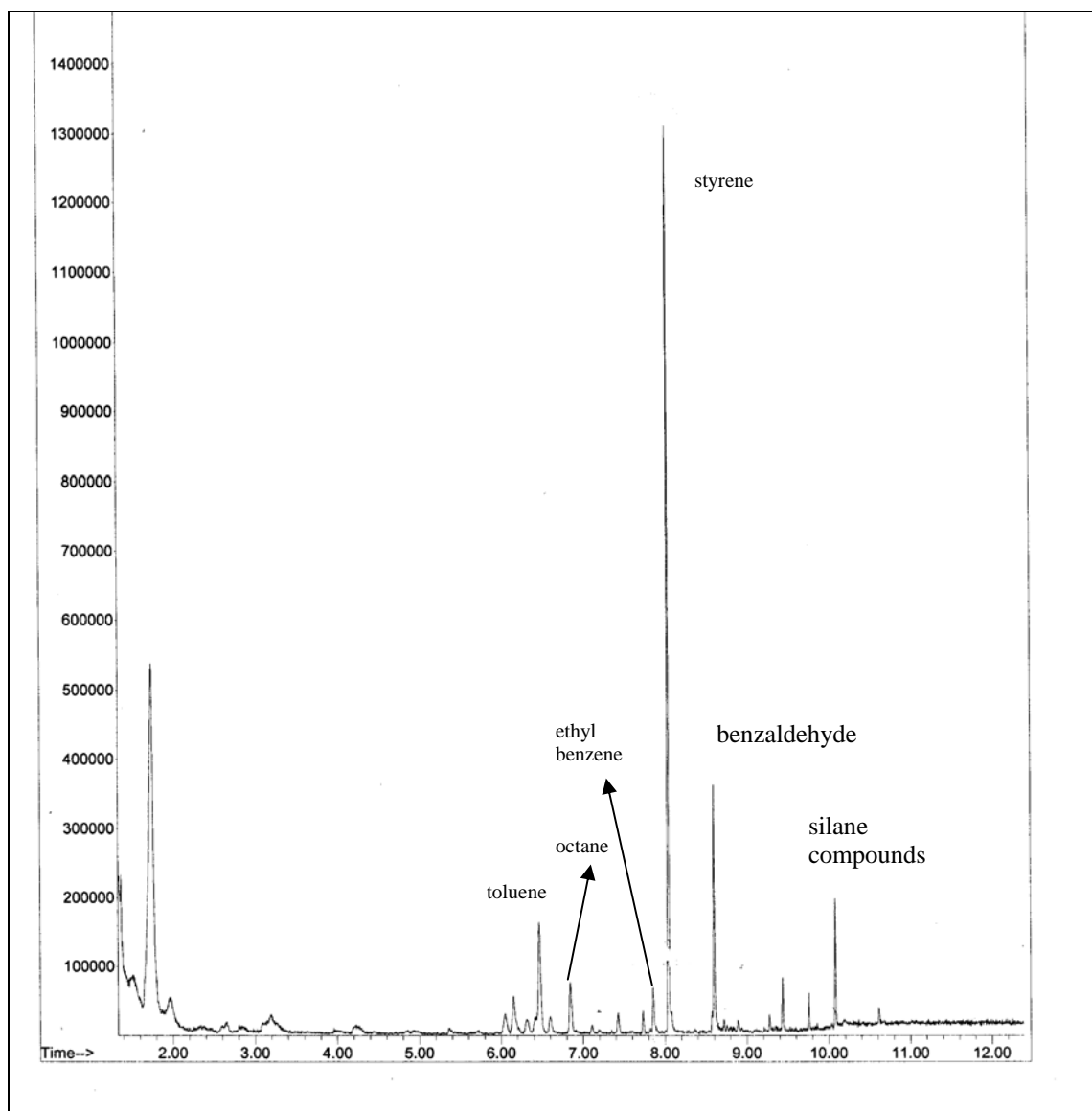


Fig. 15. Spectrum of media without cells (HTB-59)

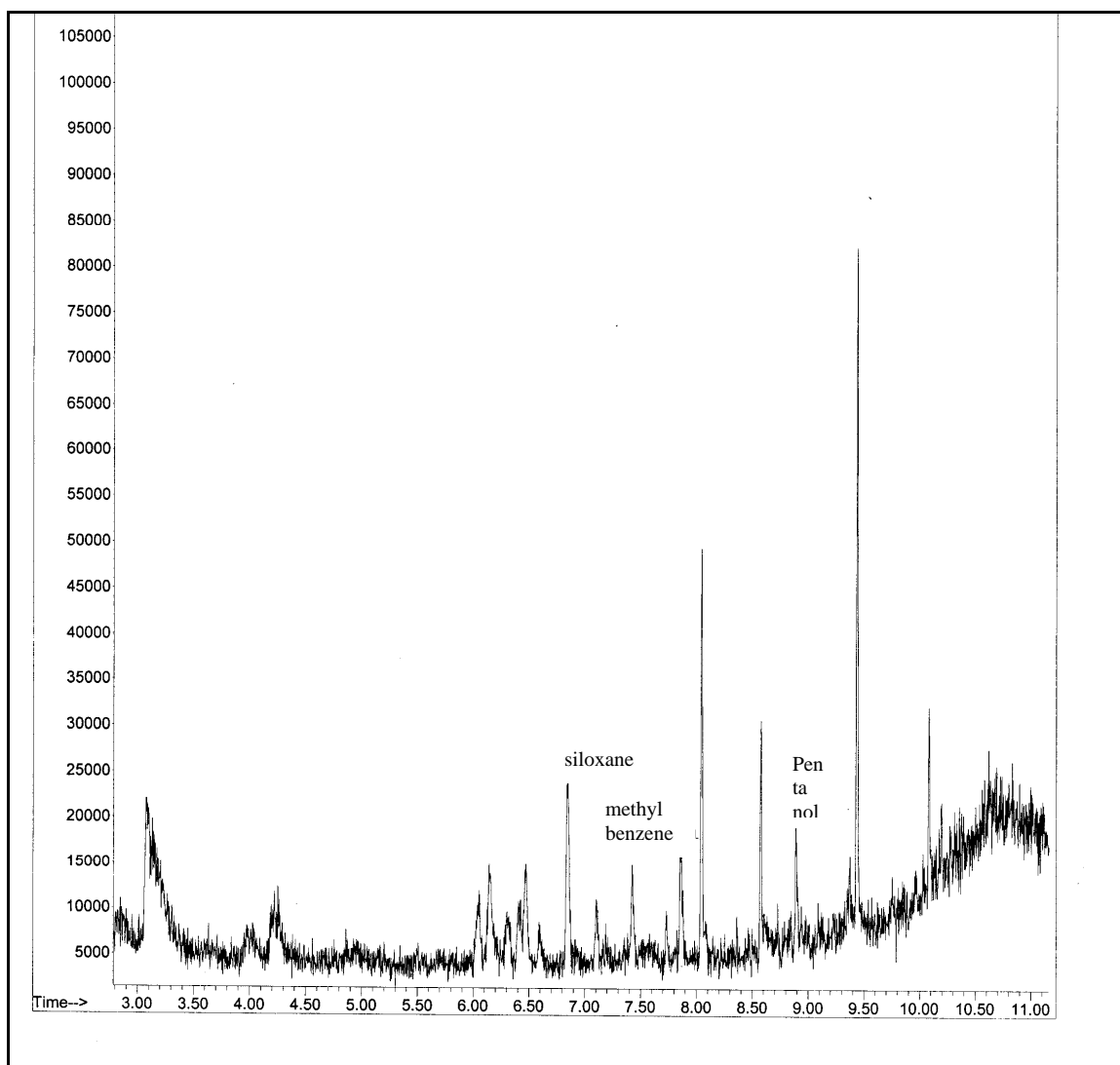


Fig. 16. Spectrum of media with cells (HTB-59)

As seen from Figs 13, 14, 15, 16 for both cell lines CRL 5810 and HTB-59 the spectra did not have much reproducibility and there was not much difference seen between the media with and without cells.

Using an antifoam

The media of HTB-59 and CRL-5810 cell lines caused intense foaming in the sparge vessel of the P&T equipment. This proved extremely harmful for the equipment and damaged certain internal circuits. Therefore 0.5cc of anti foam (O/I Analytical, College Station) was added to the medium and analyzed in subsequent tests. Antifoam consists of a silicone emulsion, hydro-carbons, alcohols and polymers designed to control foaming. These agents inhibit the build up of foam by causing the bubbles to burst, thus releasing the air.

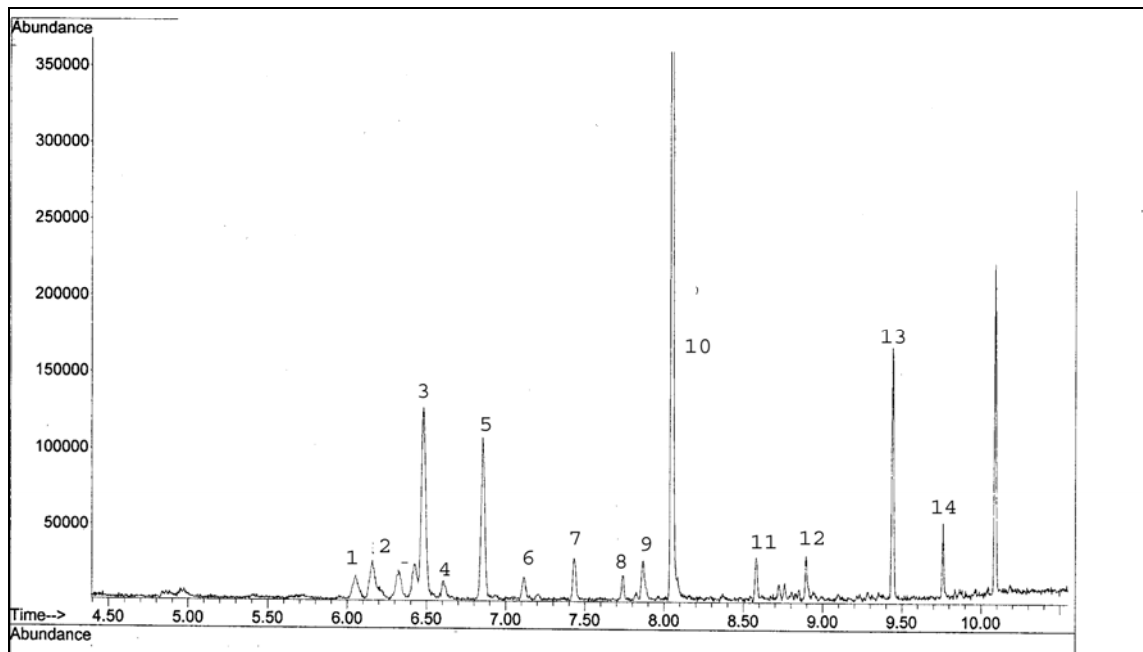


Fig. 17. Spectrum of media with cells (without antifoam)

The peaks seen in the spectra are as numbered

- | | | |
|-----------------------|-------------------------|---------------------|
| 1- Tri methyl pentane | 2- Dimethyl hexane | 3- Toluene |
| 4- Dimethyl hexane | 5- Dimethyl heptane | 6- Tri siloxane |
| 7- Dimethyl heptene | 8- Ethyl benzene | 9- Dimethyl benzene |
| 10- Styrene | 11- Tri siloxane | 12- Octane |
| 13- Ethyl hexanol | 14- Decyl hydroxylamine | |

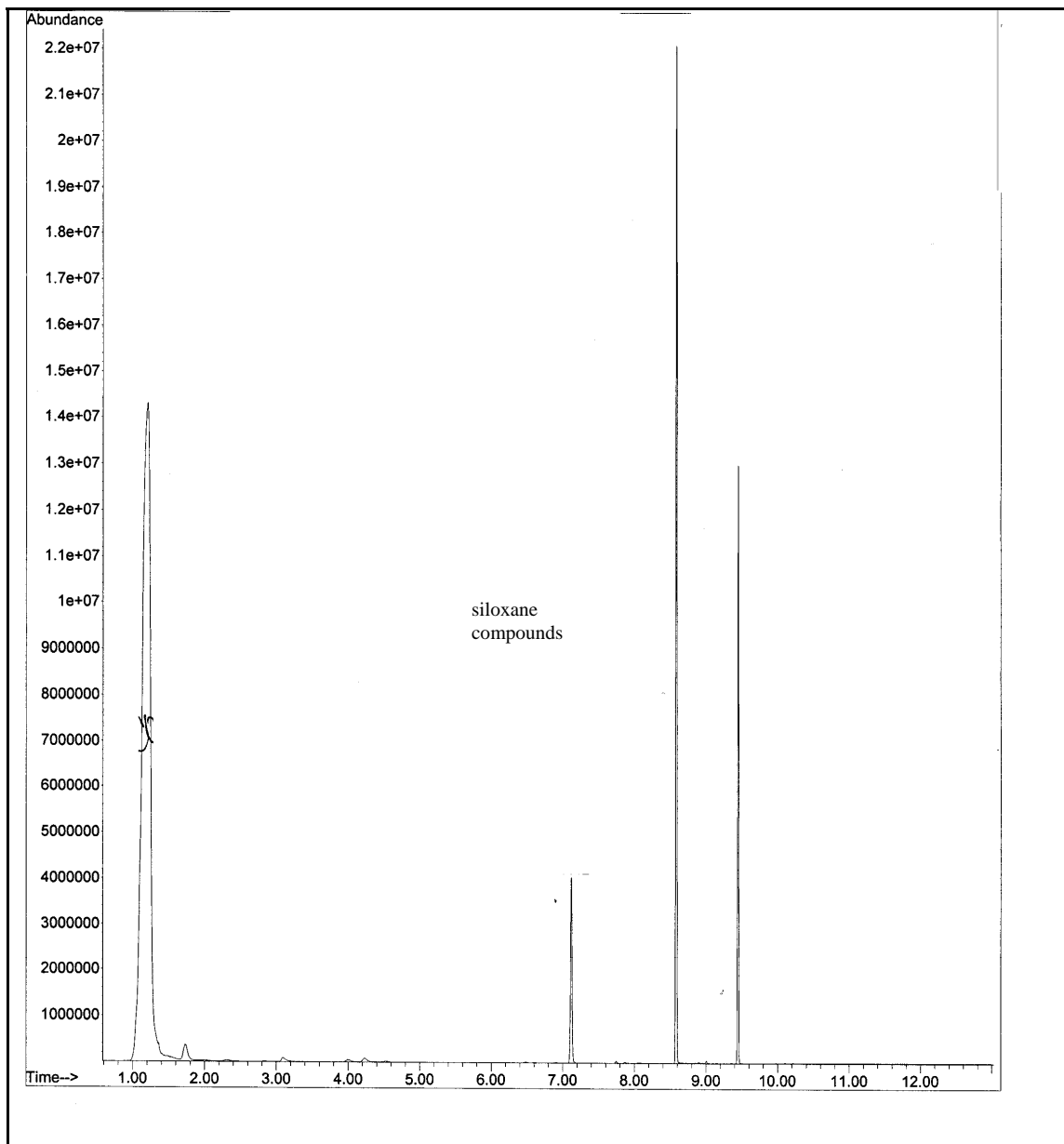


Fig. 18. Spectrum of media with cells (with antifoam)

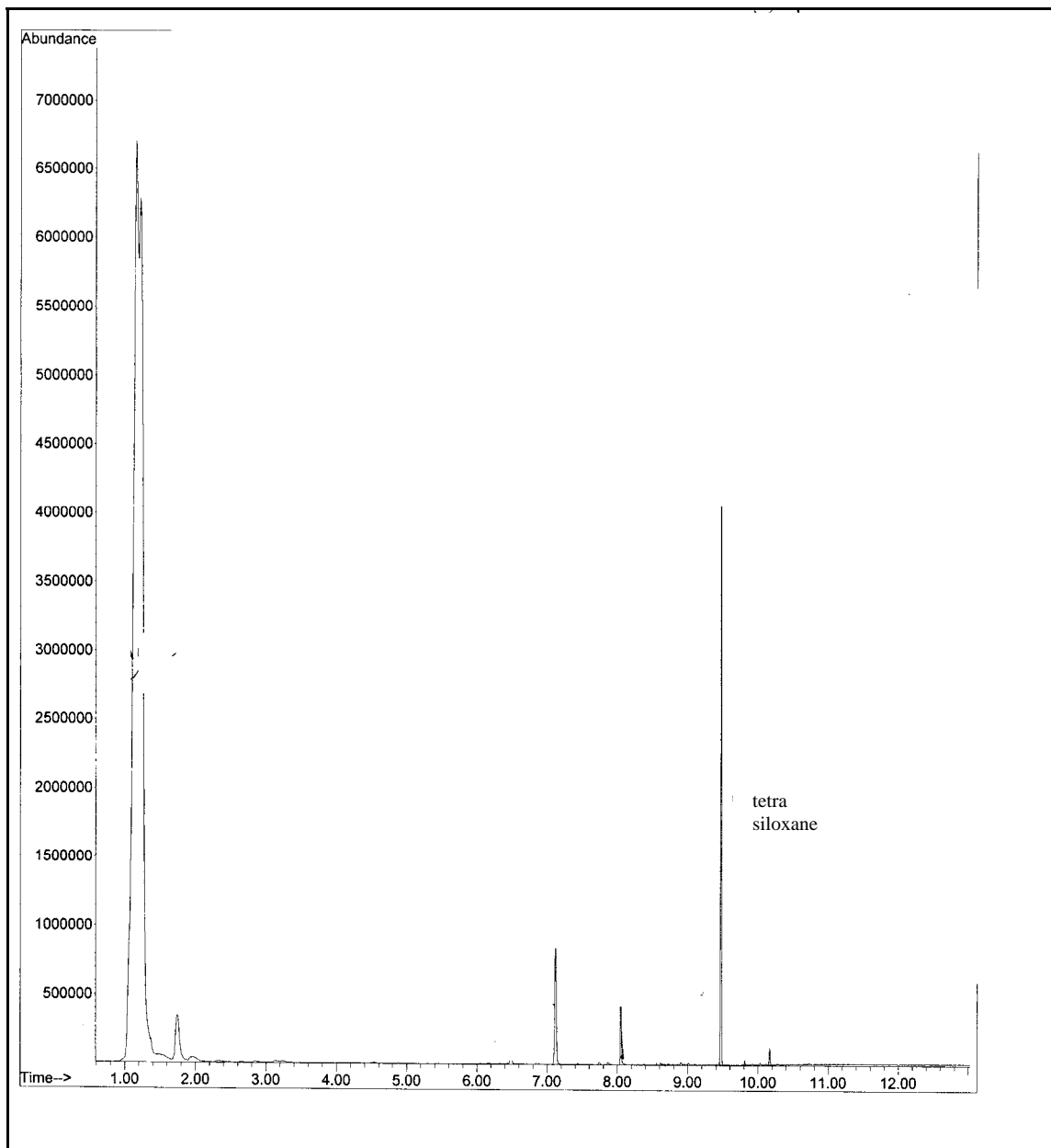


Fig. 19. Spectrum of media without cells (with antifoam)

The use of antifoam reduces the foam (Figs 18, 19) but also wipes out most of the VOC spectra seen in Fig 17. All the peaks seen in tests using antifoam originate from the

components of the antifoam and not from the sample. Therefore the use of antifoam was discontinued in our tests.

Once our lab was setup at Dr. Marian Hyman's lab at analytical chemistry all further experiments were carried out there. We continued tests with cell media by injecting 1cc of the media with and without cells in to the sparge vessel of the P&T equipment.

The media which is known to consist of amino acids and minerals offered complicated spectra of peaks which were not reproducible. Though some of the previously studied markers such as Aniline [17, 18, 19] were seen, the spectra were not reproducible.

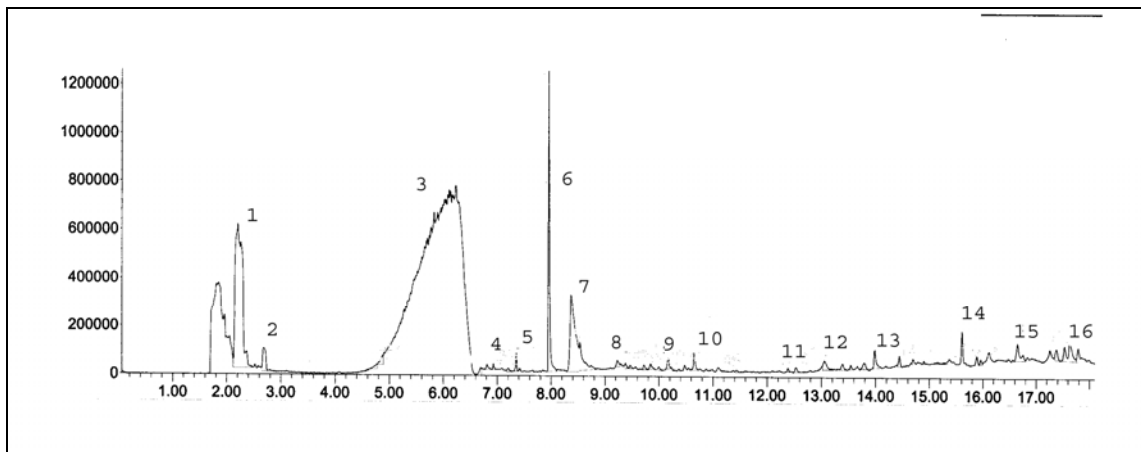


Fig. 20. Spectrum of media without cells

The peaks seen in the spectra are as numbered

- | | | |
|-------------------|--------------------|-----------------------|
| 1- Methyl butanal | 2- Toluene | 3- Dimethyl sulfonium |
| 4- Benzaldehyde | 5- Acetic acid | 6- Styrene |
| 7- Benzaldehyde | 8- Fluro acetamide | 9- Aniline |
| 10- Acetamide | 11- Tri siloxane | 12- Phenol |
| 13- Methyl phenol | 14- Penta siloxane | 15- Pyranone |
| 16- Siloxanes | | |

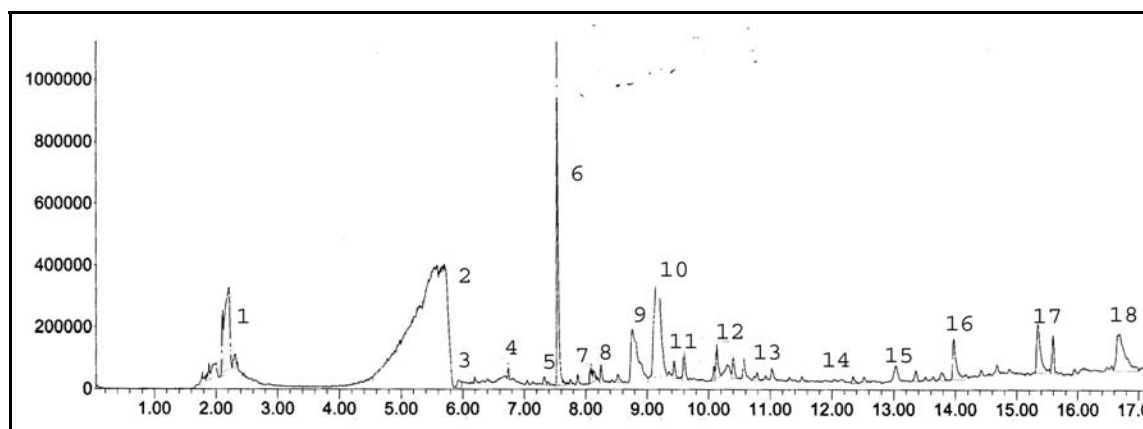


Fig. 21. Spectrum of media with cells

The peaks seen in the spectra are as numbered

- | | | |
|--------------------|-------------------------|-----------------|
| 1- methyl butanal | 2- methyl bezenediamine | 3- benzaldehyde |
| 4- acetic acid | 5- acetic acid | 6- styrene |
| 7-formic acid | 8- tri methyl furan | 9- benzaldehyde |
| 10-fluro acetamide | 11-methyl butanoic acid | 12-analine |
| 13-oxime | 14-bromo butane | 15- siloxanes |
| 16- phenol | 17- methyl phenol | 18- siloxanes |

Comparing the spectra between Figs 20 & 21 we see there most peaks are common. Aniline is seen both in the media with and without the cells.

Head space analysis

The switch was made to analysis of headspace (HS) above the cells. This involved using a 10mL gas tight syringe (SGE, Austin) to extract 30cc of head space gases above the cells and injecting the headspace gases directly in to the P&T sparge vessel. Initially HS was studied in plastic flasks but the flask itself gave off some of the VOCs of interest such styrene, toluene, decane (Figs 22, 23).

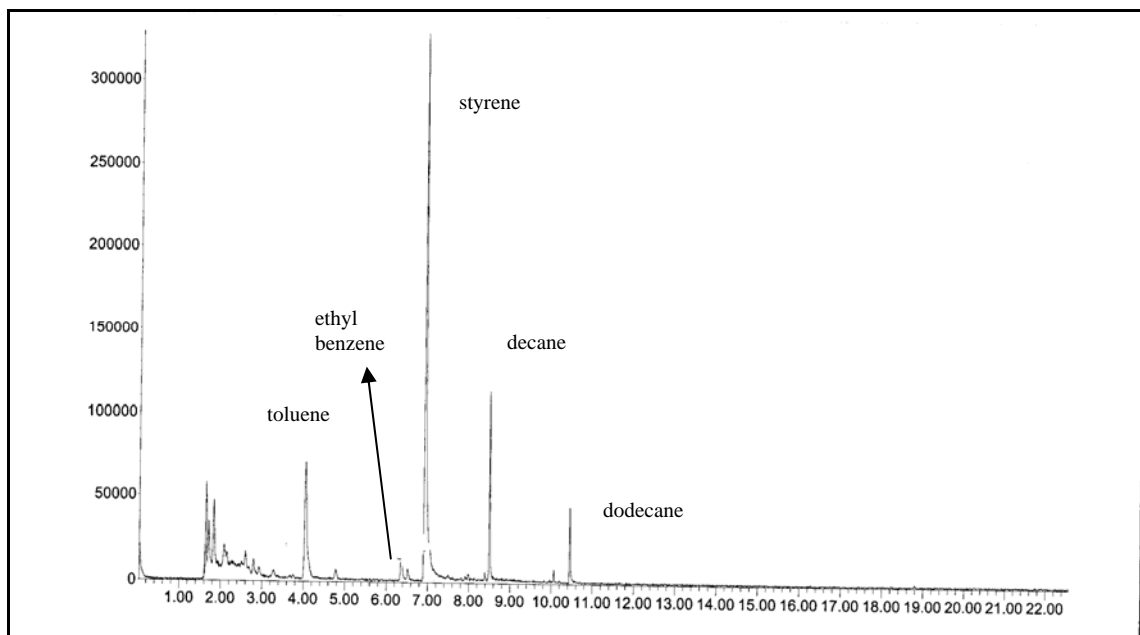


Fig. 22. Spectrum of HS of media with cells in plastic flask- trial 1

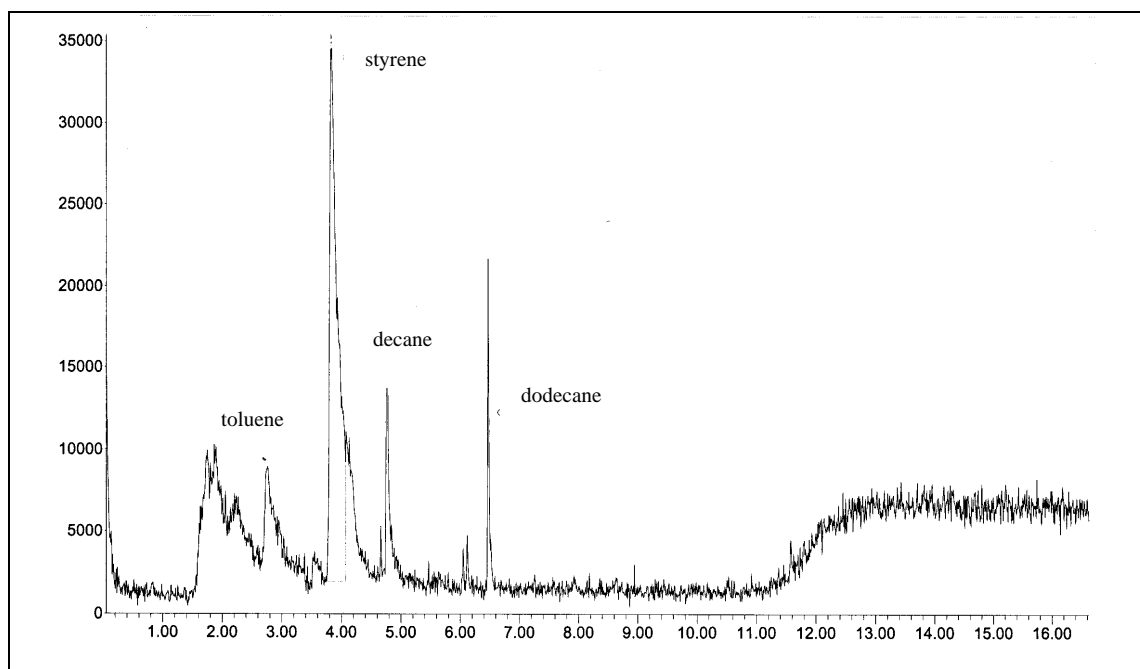


Fig. 23. Spectrum of HS of media with cells in plastic flask- trial 2

Next a comparison was made between HS of an empty plastic flask and HS of cells in plastic.

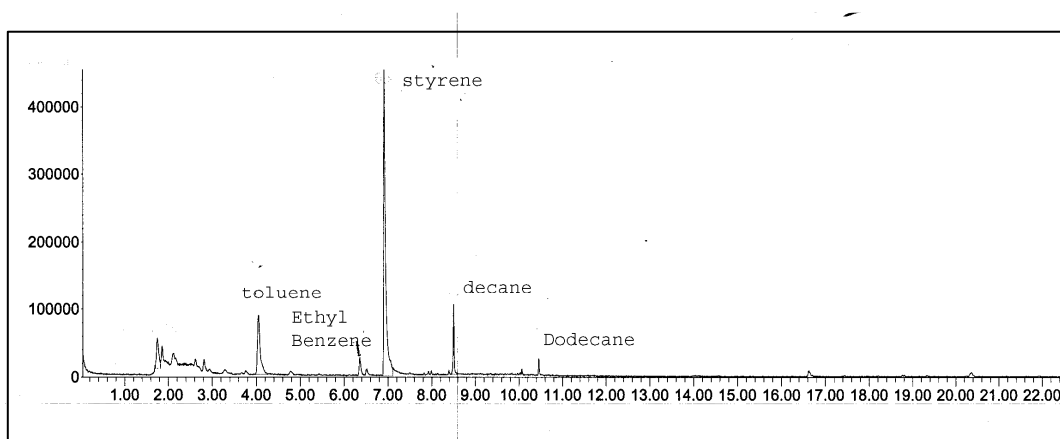


Fig. 24. Spectrum of HS of an empty plastic flask

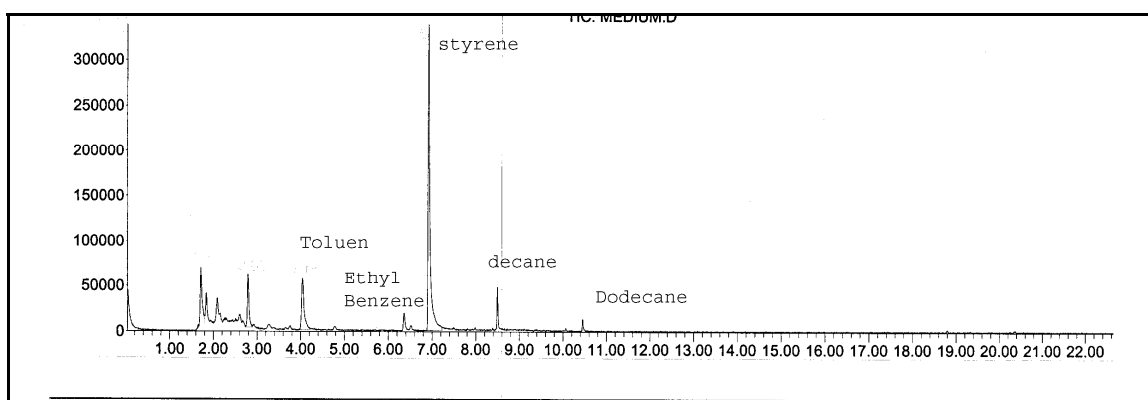


Fig. 25. Spectrum of HS of cells in media in a plastic flask

Comparing Figs 24 & 25 we see that all the peaks are being contributed by the plastic ware itself. Therefore it was decided to discontinue analysis in plastic and continue only with glass.

Glass flasks

40 mL glass vials (Fisher brand economical EPA) made of borosilicate glass were adapted to grow cells. Glass unlike plastic does not have a layer of protein coating that enables adherence of the cells to the base. This coating for the cells to adhere to had to therefore be made artificially. The sample bottles were coated with poly lysine (VWR Scientific) for 7 minutes and air dried overnight in a sterile environment. Glass since reusable had to be autoclaved before every use and before coating. There is a general exchange of O_2 and CO_2 between the cells and the air in the incubator. But it is found that in glass vials this exchange did not take place. The media was analyzed before and after sub-culturing to study the differences. The headspace of the cells in media was tested both before and after sub culturing of the cells (Figs 26, 27).

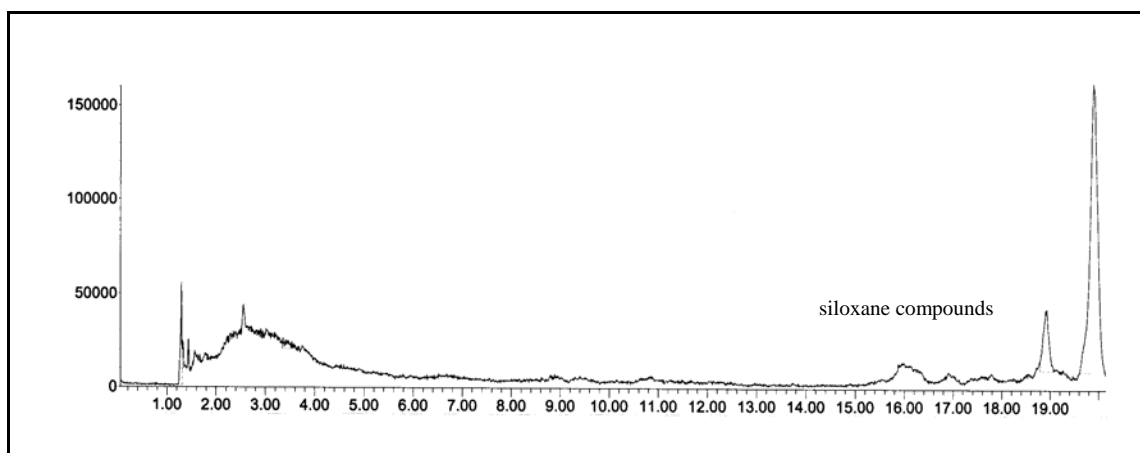


Fig. 26. Spectrum of HS of cells in media in a glass flask before subculture

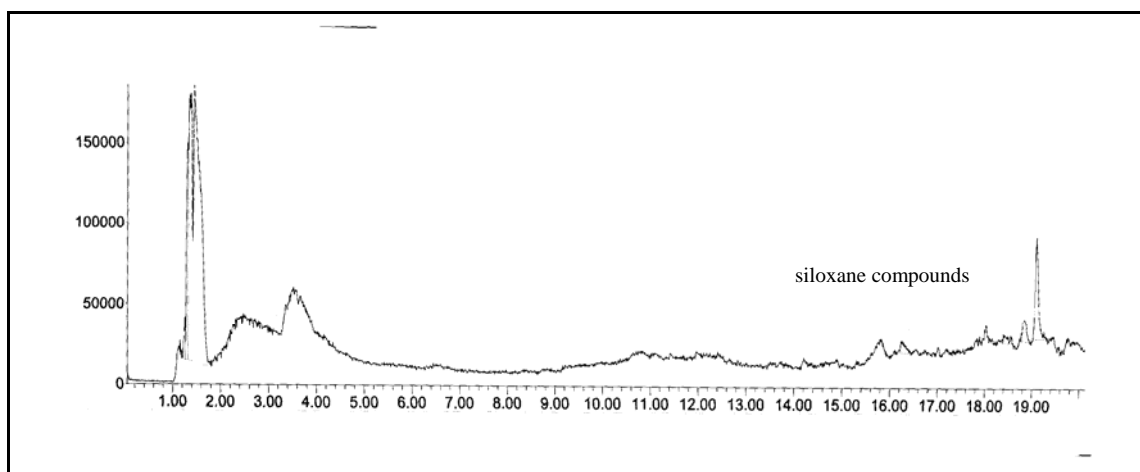


Fig. 27. Spectrum of HS of cells in media in a glass flask after subculture

It was seen there is much difference between the HS of media and HS of cells both before and after sub culturing. Moreover, since glass wasn't the natural medium for the cells to grow, the cell survival rate was very low. Due to the complex nature of the cell media there were no major peaks seen. Glass was disastrous for the cell line CRL 5810 since cells could not adhere to the base and therefore did not survive. Since it was decided to continue with glass as the material for growing the cells, this cell line was discontinued in our studies.

Styrene was seen in head space of glass vials when the samples of cells were heated in an incubator for 60°C for 30 min (Fig 28).

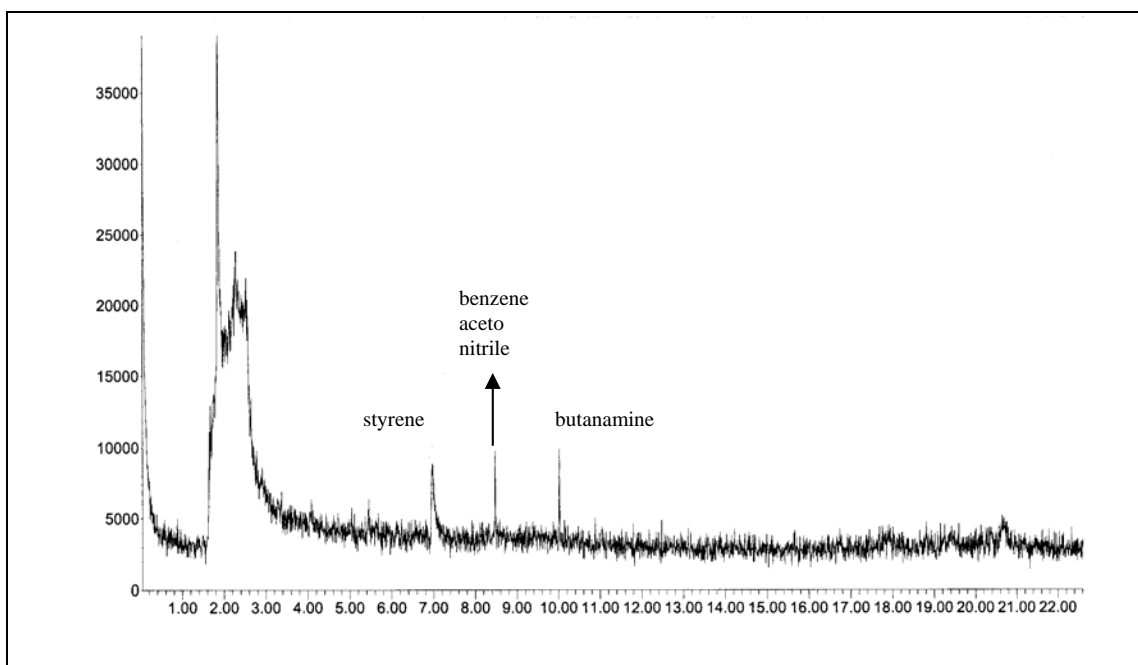


Fig. 28. Spectrum of HS of cells in media in a glass flask after heating

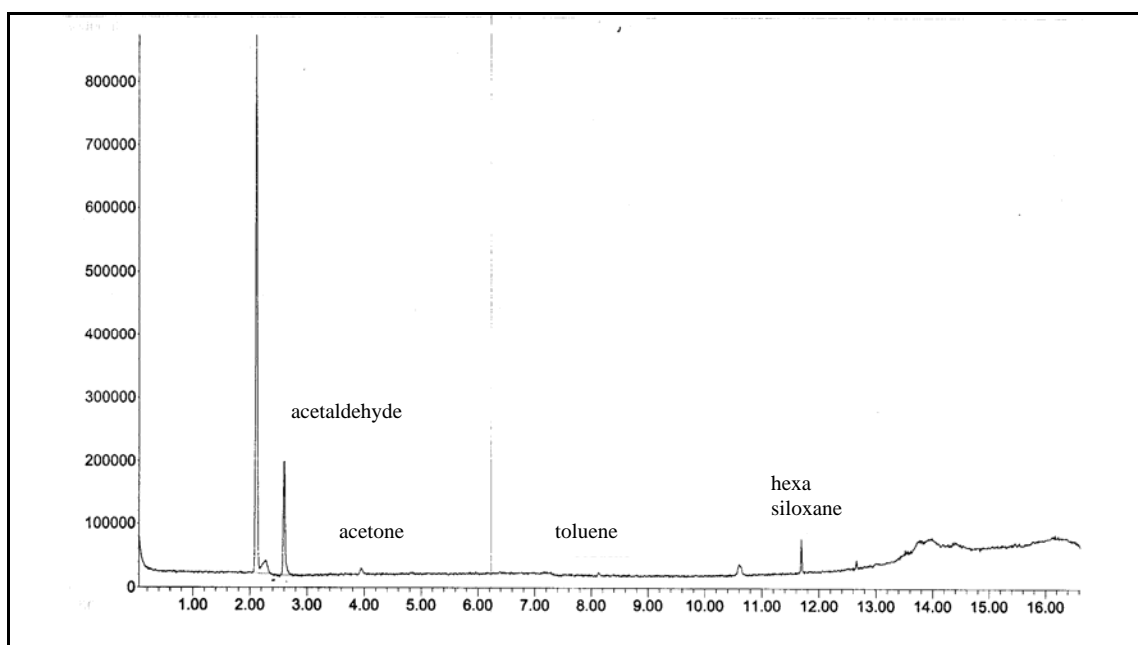


Fig. 29. Spectrum of HS of cells in media in a glass flask

Figs 26 & 29 clearly show the inconsistency in the results since both represent the HS of media with cells in the glass flask. Acetaldehyde and toluene were seen in some results but not in others. We further developed this method in to the next technique.

Lysing of cells

We wanted to investigate the possibility of splitting the cells to release the VOCs if any inside the cells. This was done using a French press. It essentially works like a pestle and mortar. The cells are transferred to a test tube which is immersed in a bucket of ice. The pestle consists of a long steel rod which is used to mechanically pound the cells in media 5 times. The results obtained were a number of peaks with poor repeatability as seen earlier (Figs 30, 31). We also heated the sample for 30 min at 60°C but there didn't see any improvement in results. Styrene which was seen earlier when glass was heated wasn't seen in this case possibly since the VOCs might have escaped while handling.

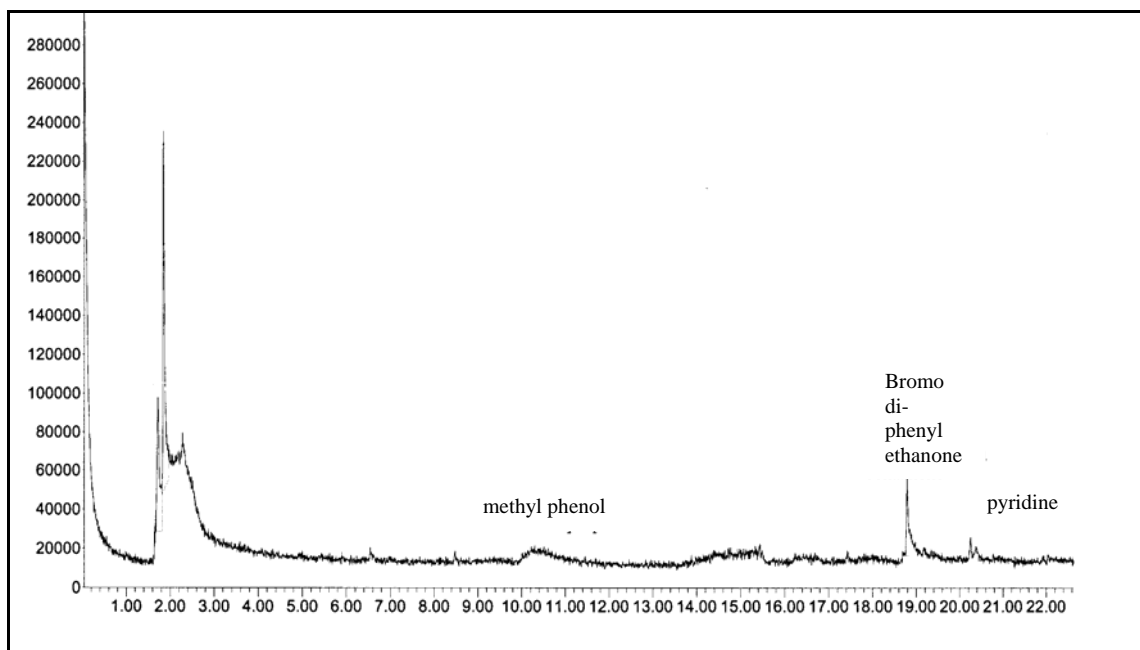


Fig. 30. Spectrum of HS of lysed cells in media in a glass flask

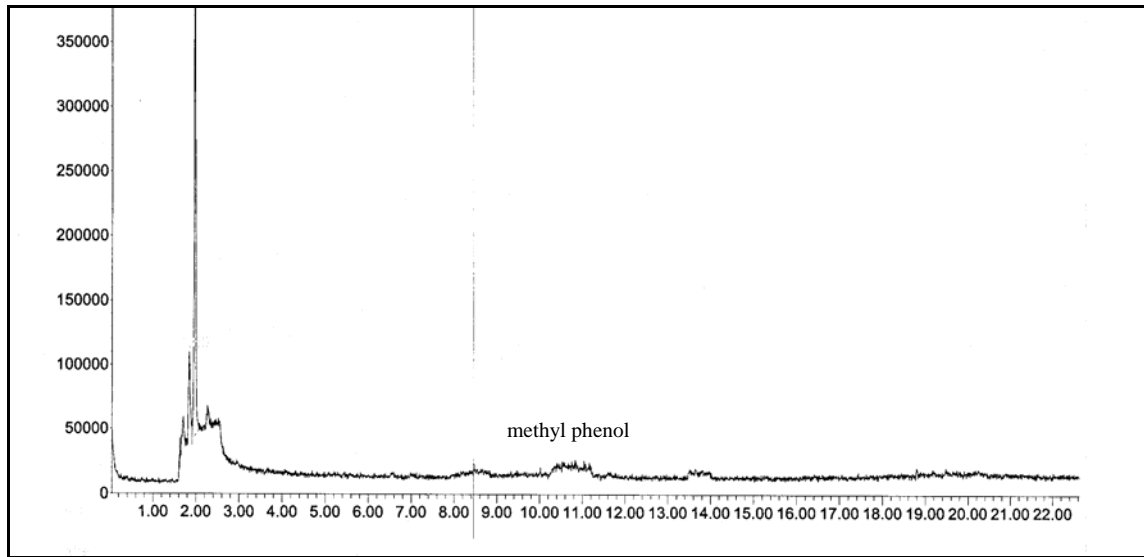


Fig. 31. Spectrum of HS of lysed cells in media in a glass flask after heating

Scale up process

We felt that we were not able to see the VOCs of interest due to insufficient number of cells in the sample and therefore decided to increase the number of cells using scale up methods.

Glass beads

As a part of scale up process glass beads were used for scale up by suspending media with cells in a plastic and glass containers containing the coated beads. Glass beads from the Chemistry department stock room were used to increase the surface area in both plastic and glass cases. 100 beads were coated with poly lysine for 7 minutes and dried overnight.

The cells however did not survive, which was due to the effect of gravity which pulled the cells to the bottom of the flask and prevented them from adhering to the curved

surface of the glass beads. This was confirmed by the fact that there was no color change seen in the media. This method proves that cells adhered to a flat surface only. No growth was seen either in plastic or glass.

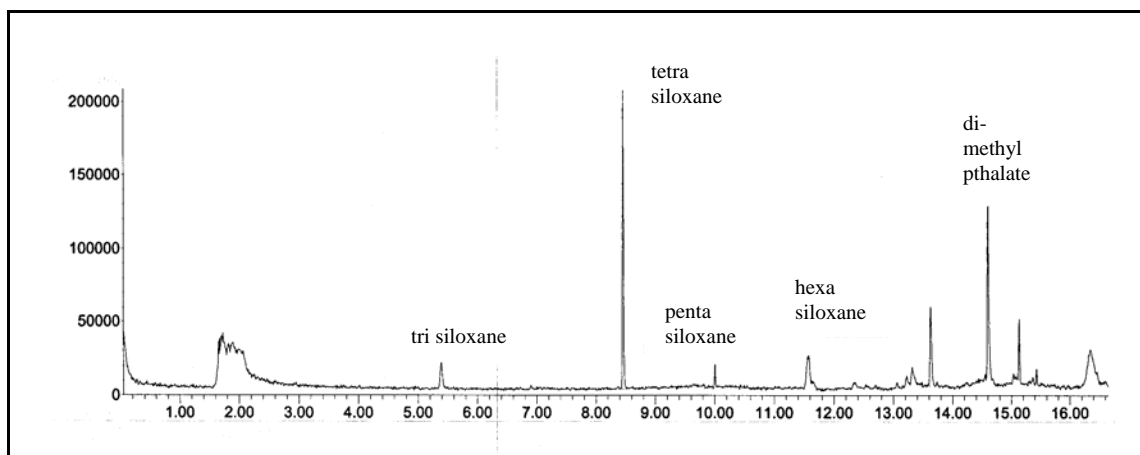


Fig. 32. Spectrum of HS of media in a glass flask with glass beads

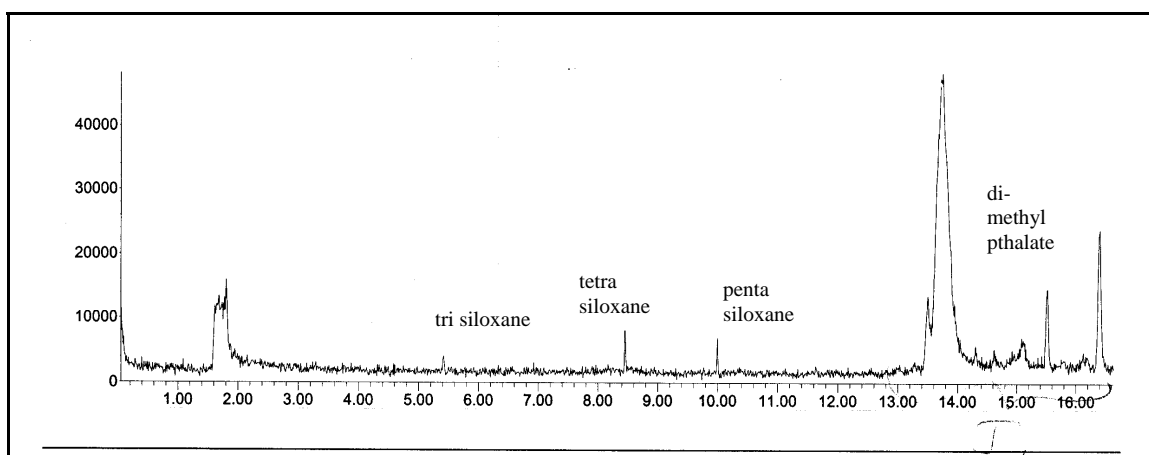


Fig. 33. Spectrum of HS of cells in media in a glass flask with glass beads

Figs 32, 33 shows there is no difference between the media with and media without the cells since most the cells died. Therefore this technique of scale up was abandoned.

Triple Flasks

Another known method for scale up was using triple flasks (Nunclon delta -175 cm²) to scale up the number of cells. Cells were grown for 7 days in these flasks and transferred to an 800mL glass bottle (VWR, PA) before being analyzed. There was an 11 times scale up in plastic and 9 times scale up in glass as seen in Table 5.

Table 5
Statistics of scale up experiment

Type of culture flask	Total volume	Media volume	Area of cell growth	Number of cells
Plastic-T 25	25mL	5mL	38.7 sq cm	2 million
Plastic-T 175	600mL	100mL	435.48 sq cm	40 million
Glass -small	40mL	10mL	38.7 sq cm	1.5 million
Glass -big	800mL	100mL	173 sq cm	15 million

Counting of the cells was done using a cell cytometer designed by Dr.Bernstein's group. A drop of media is observed on a cover slip slide under the microscope. Cells in a 1 X 1 sq. cm area are counted and the number is multiplied by 10⁴/mL.

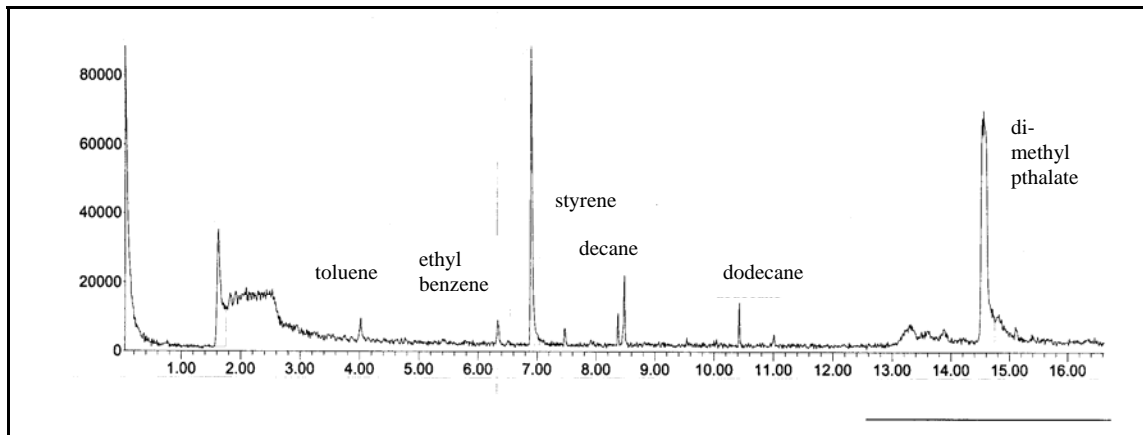


Fig. 34. Spectrum of HS of media with cells in a large plastic flask

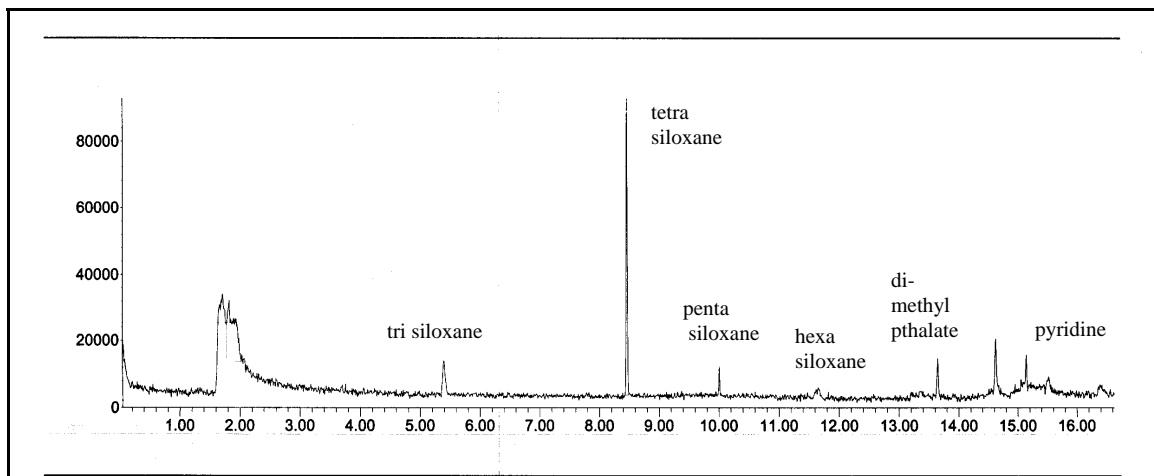


Fig. 35. Spectrum of HS of media in a large glass flask

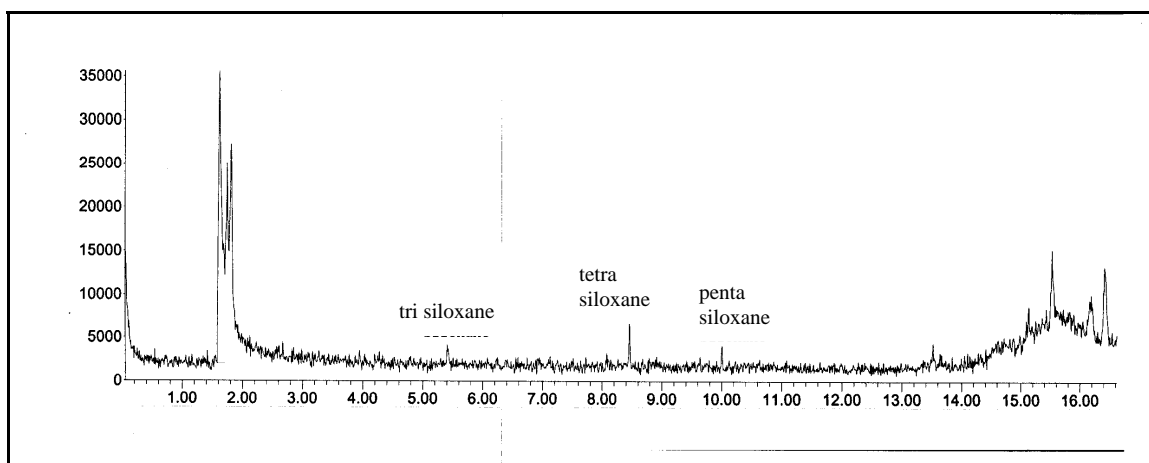


Fig. 36. Spectrum of HS of media with cells in a large glass flask – trial 1

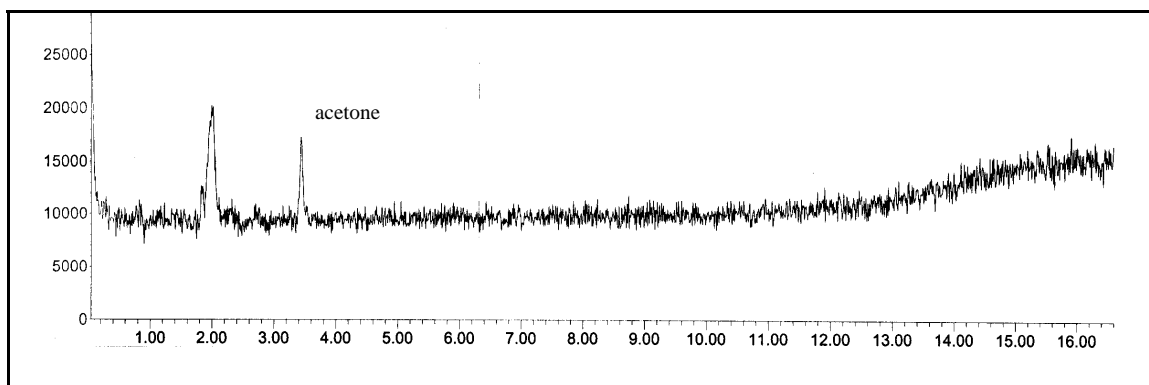


Fig. 37. Spectrum of HS of media with cells in a large glass flask – trial 2

On analyzing the HS the components of the plastic were seen as expected (Fig 34). In HS of glass both without cells (Fig 35) and with cells (Figs 36, 37) not many VOCs were seen apart from silicanes and components of plastic. There was inconsistency when the HS of glass was tested in consecutive trials. Whether HS of the small or big bottle was done, acetone, toluene and sometimes ethanol was seen. For the next trials the media with cells was heated for 60 minutes and tested. On comparison of this spectrum to

media that wasn't heated the only visible VOC seen was styrene (Figs 35, 38). This was due to plastic cap of the glass flask and rubber lining.

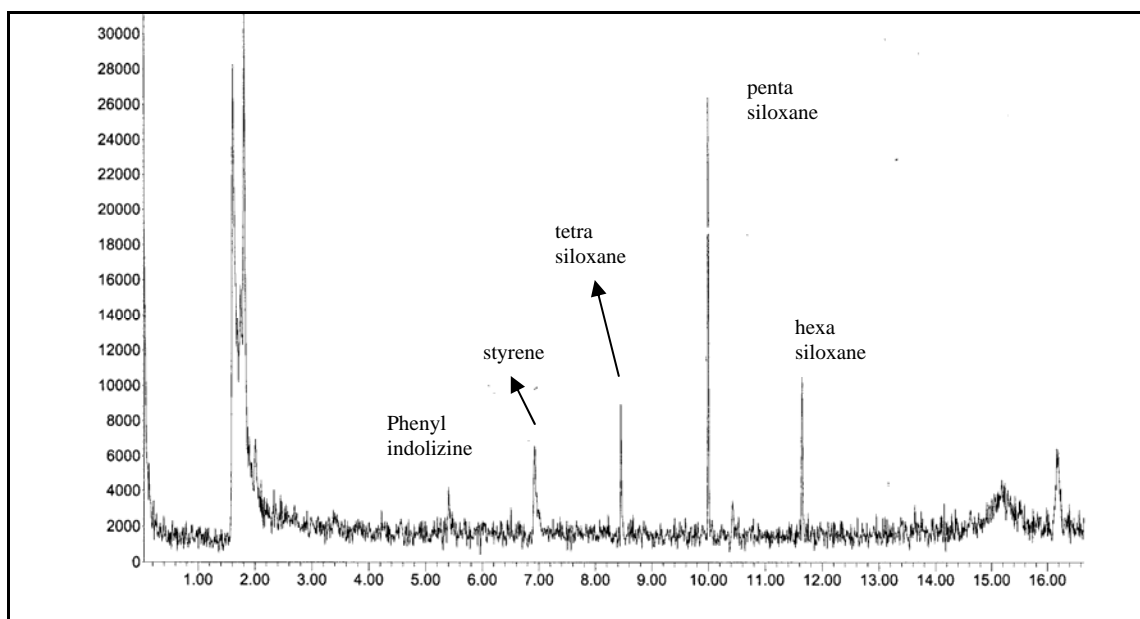


Fig. 38. Spectrum of HS of media with cells in a large glass flask after heating

Calibration of results

Calibration of VOCs was carried out by running of known standards in the P&T- GC-MS and calculation of the area under the spectrum. The plot of the area vs. the concentration yields a **calibration curve**. This enables the detection of the concentration of any unknown if the area of the spectrum is known. It also gives a measure of the sensitivity of the method as the lowest detectable concentration can be determined. Using the calibration curves for acetone and toluene (Figs 39, 40) with the sparge vessel method the concentration of acetone was found to be between 200 -300 ppb and in the concentration of toluene was found to be 50 – 200 ppb in the sparge vessel method.

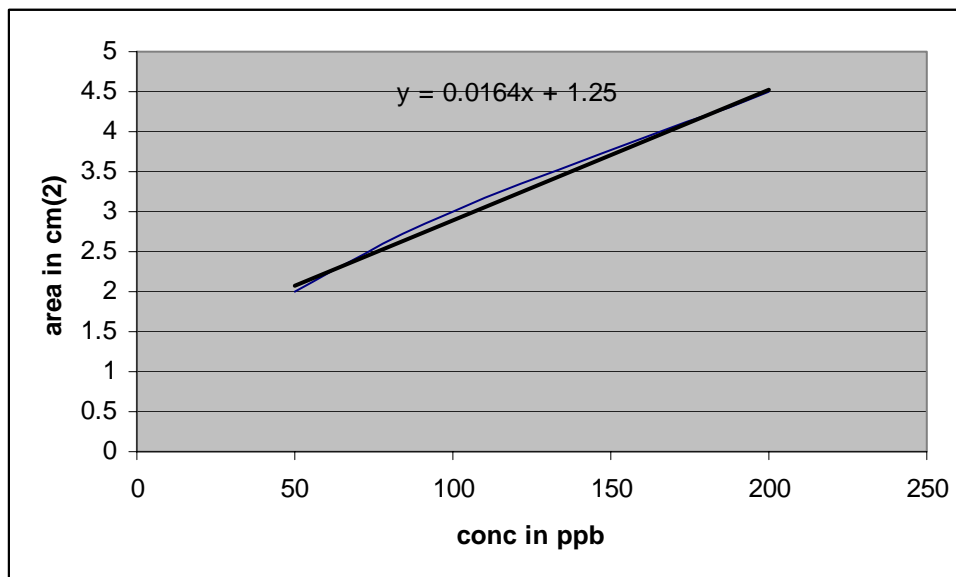


Fig. 39. Calibration curve for acetone using sparge vessel method

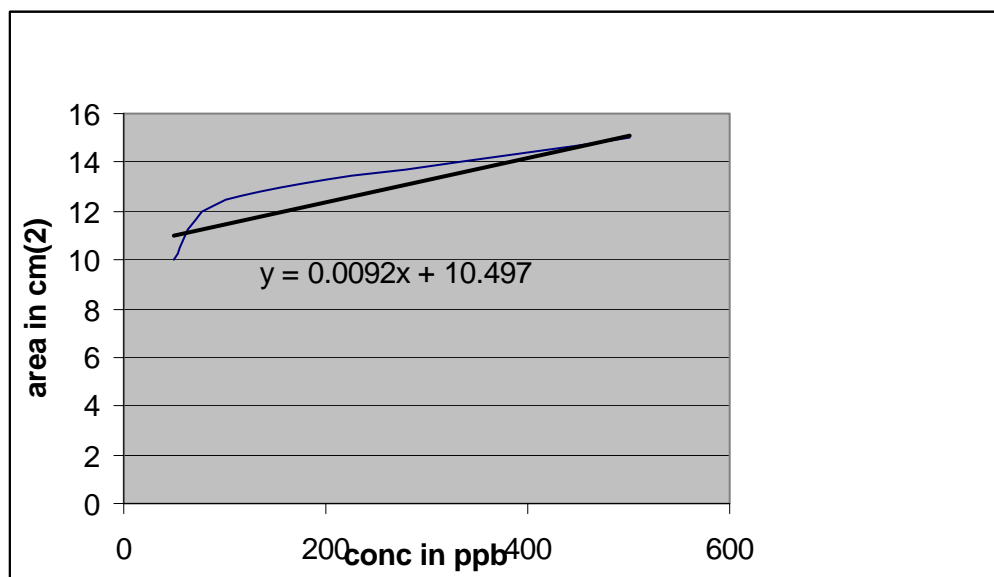


Fig. 40. Calibration curve for toluene using sparge vessel method

The sparge vessel injection method also had its disadvantages. It involved manually retrieving samples from the culture flasks using gas tight syringes which would some times lead to dead space volume, pressure differences and inability to monitor flow rate. A large amount of water vapor from a sparger carried by the purge gas condensed onto the cold surface could create chromatographic interference. Key issues to address when setting up HS analysis systems include minimizing system dead volume, maintaining inert sample flow path and achieving efficient sample transfer.

It was decided to change the cell line to HTB- 58 (ATCC, VA), a cell line studied in the past [42]. It is an epithelial squamous cell carcinoma type cell line. Initial tests consisted of checking the HS in both the small 40mL bottle and 800mL bottle (Fig 41, 42).

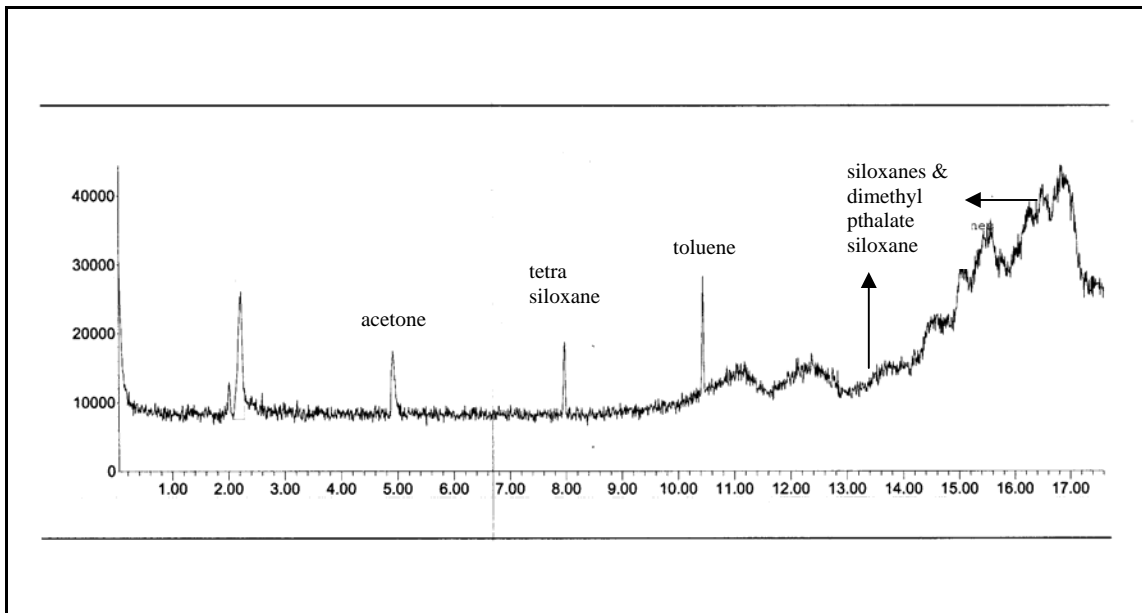


Fig. 41. Spectrum of HS of media with cells in small glass flask (HTB- 58)

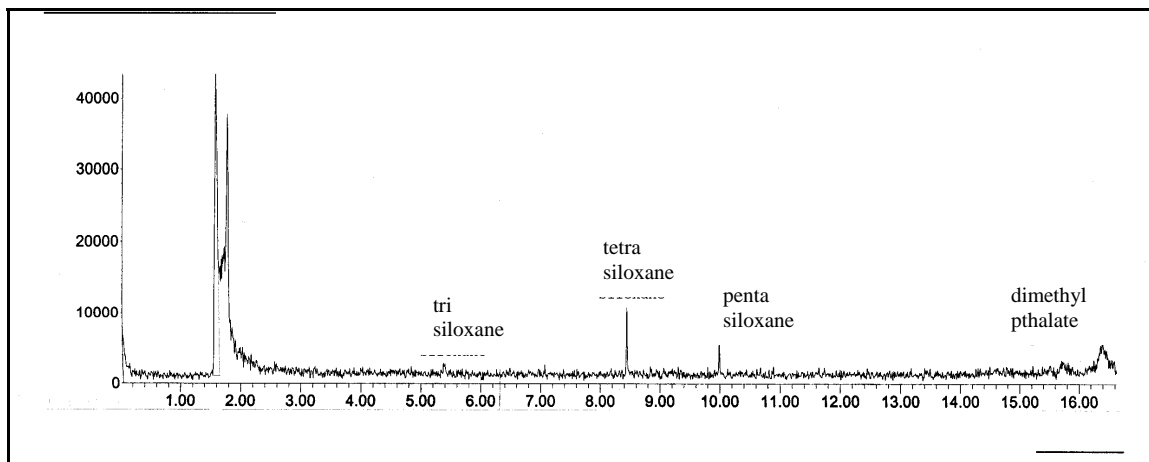


Fig. 42. Spectrum of HS of media with cells in large glass flask (HTB- 58)

The results of HTB-58 cell line were consistent with earlier results for other cell lines and did not give us any new results. Next, we designed a new method that has never been attempted viz. adsorption of VOCs using airtubes.

Air tube desorption

We realized we needed a more sensitive method to overcome all the above mentioned disadvantages of earlier methods used for the collection of the VOCs. For this we used the **Air tube method** of detection. This method has never been attempted before in the study of lung cancer. We basically were using the principle of the P&T equipment but were pre concentrating the VOCs on an air tube before even introducing the sample in to the concentrator. Essentially concentration of the VOCs was taking place twice.

This is the second method of HS analysis apart from the manual sparge vessel method described earlier. It involved using nitrogen gas at a flow rate of 20mL/min and pressure of 10 psi to drive the head space gases from the glass bottle on to an air tube packed with Tenax (Fig 43).

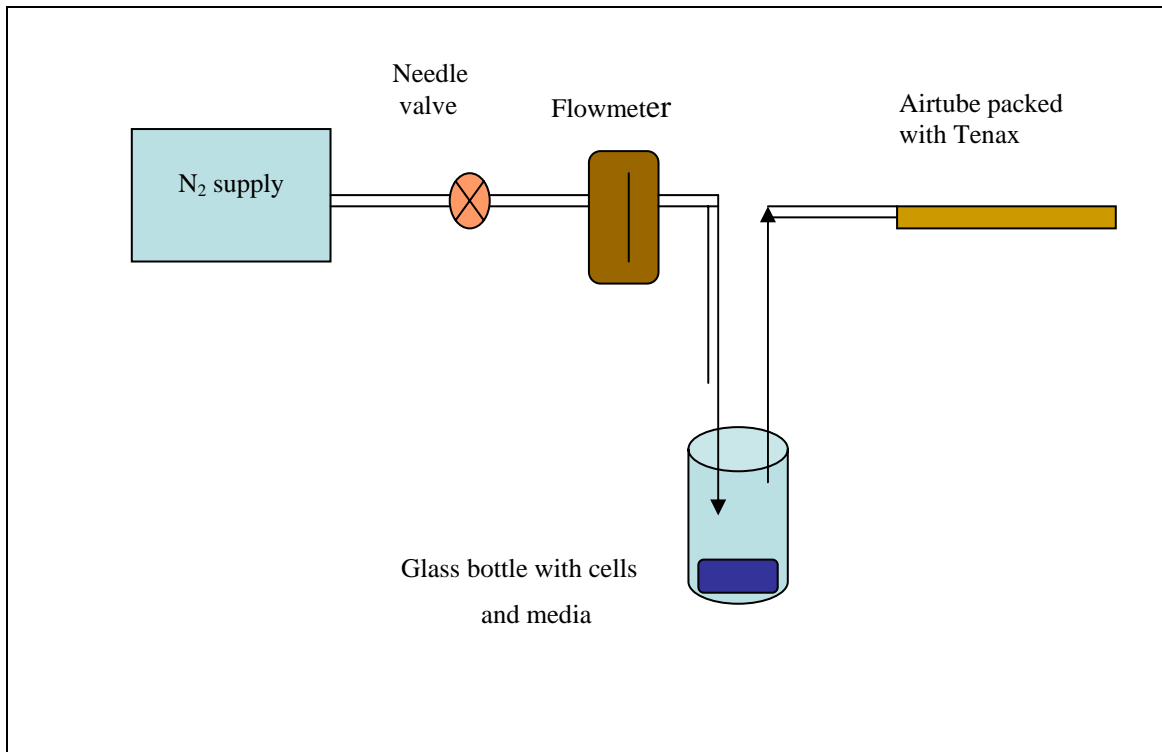


Fig. 43. Block diagram for the design for the airtube experiment

Nitrogen is used as the carrier gas since it is purer than compressed air. If compressed air was used, the adsorbents would have concentrated the slightest contaminants. Also moisture is present in air. The carrier gas drives the HS gases from the glass to an air tube packed with Tenax. The flow rate was monitored using a 65mm flow meter (Cole Parmer, IL). The experimental setup with the air tube both outside and inside the incubator is as illustrated in Figs 44 & 45 respectively.

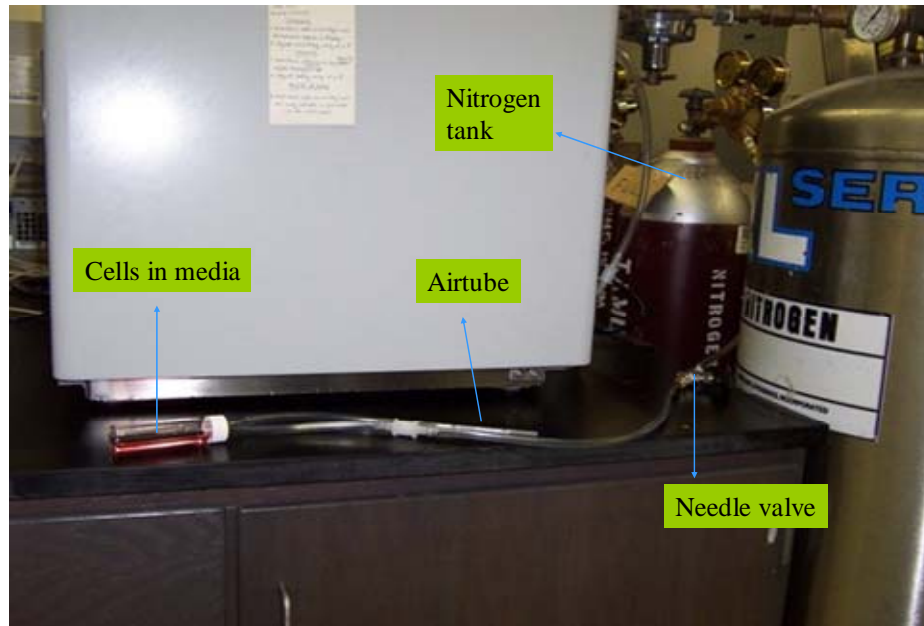


Fig. 44. Setup of air tube experiment in our lab (outside incubator)

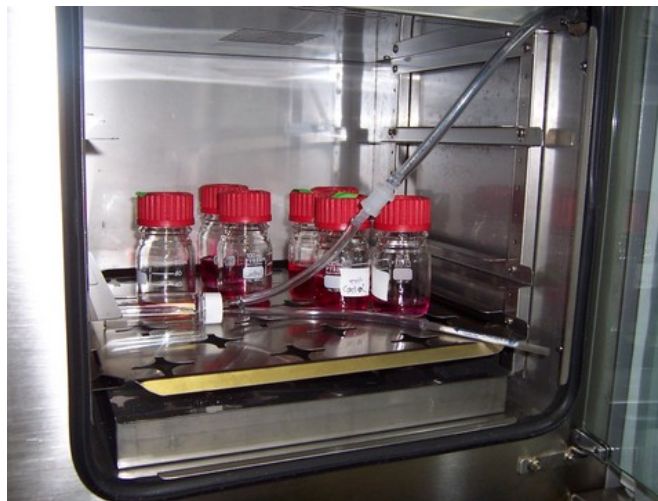


Fig. 45. Setup with air tube (inside the incubator)

All compounds have a breakthrough volume after which they are no longer retained by adsorbent. According to Supelco company, the manufacturers of Tenax for an airtube packed with 143 mg of Tenax, at 10 psi the breakthrough volumes of acetone was 1.4L and toluene 63L. This showed the excellent adsorption of these VOCs with Tenax.

We no longer manually introduced samples as done in the previous method. Therefore the errors in extracting the sample such as loss of gases to the atmosphere while handling the syringe, back pressure created by the needle of the syringe, transfer of water vapor from the sample were avoided. Advantages of this method as compared to the sparge vessel method are minimum human error, less unknowns and much greater volume of sample.

In the air tube experiments HS of the cells in plastic and glass were analyzed. At the rate of 20mL/min for 60 minutes, 1.2 L of the HS gases was adsorbed on to the air tube. This air tube was then attached to the air desorber unit of the P&T unit. Pre concentration by heating of the air tube drives the VOCs in to the trap of the P&T for further concentration.

Fig 46 shows the VOCs given off by the glass flask. Acetone and ethyl alcohol were seen above the cell lines in the air tube method and an enhancement was seen in the media with cells as compared to the media without the cells (Figs 47, 48, 49, 50). The abundance of acetone is shown on the y-axis it is clearly increased in the media with cells; this enhancement was seen because the cells themselves were contributing some acetone.

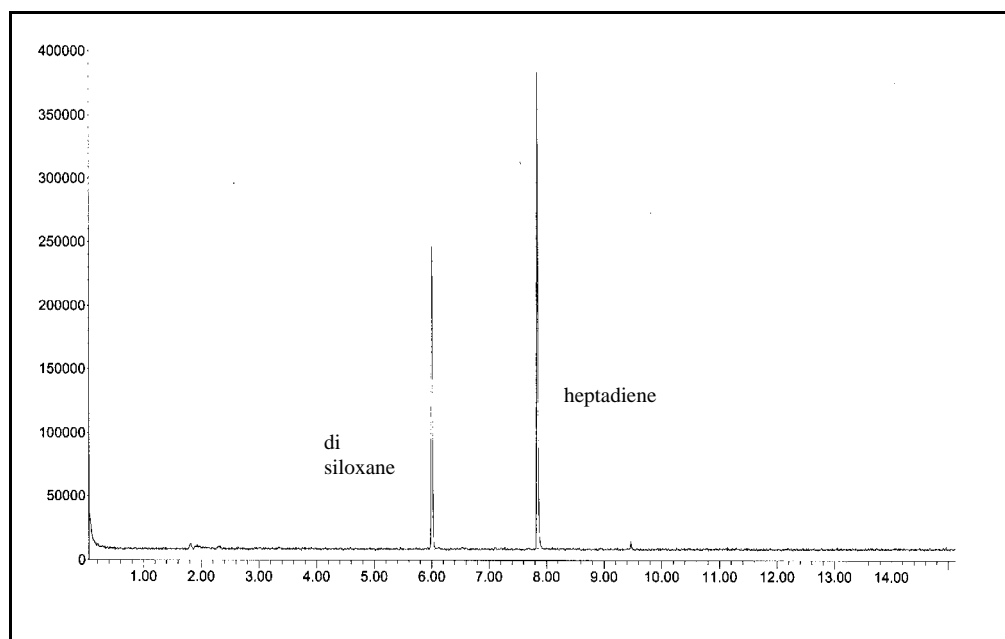


Fig. 46. Spectrum of HS of glass flask using air tube method

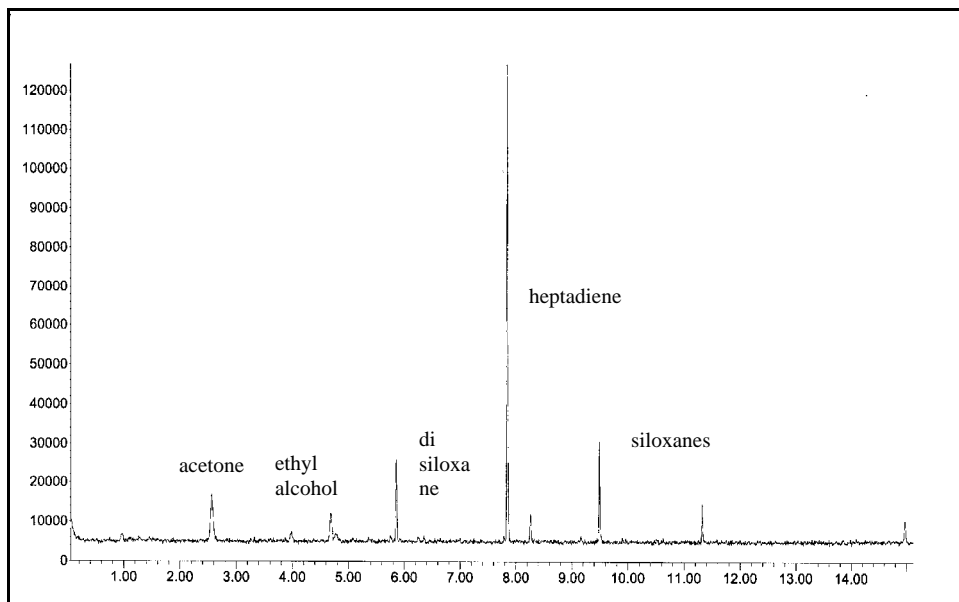


Fig. 47. Spectrum of HS of media using air tube method- trial 1

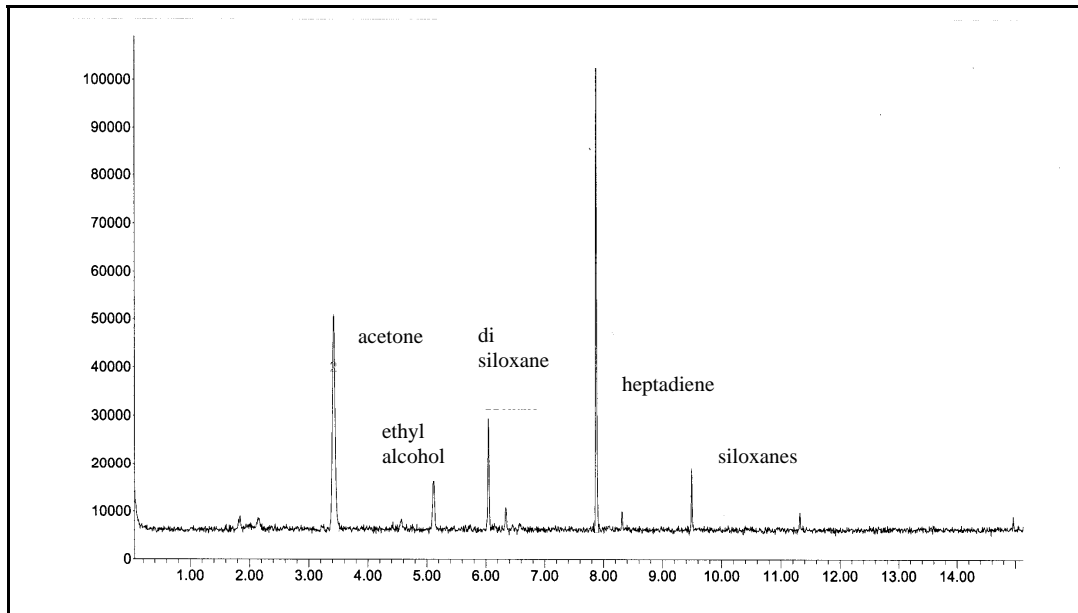


Fig. 48. Spectrum of HS of media with cells using air tube method- trial 1

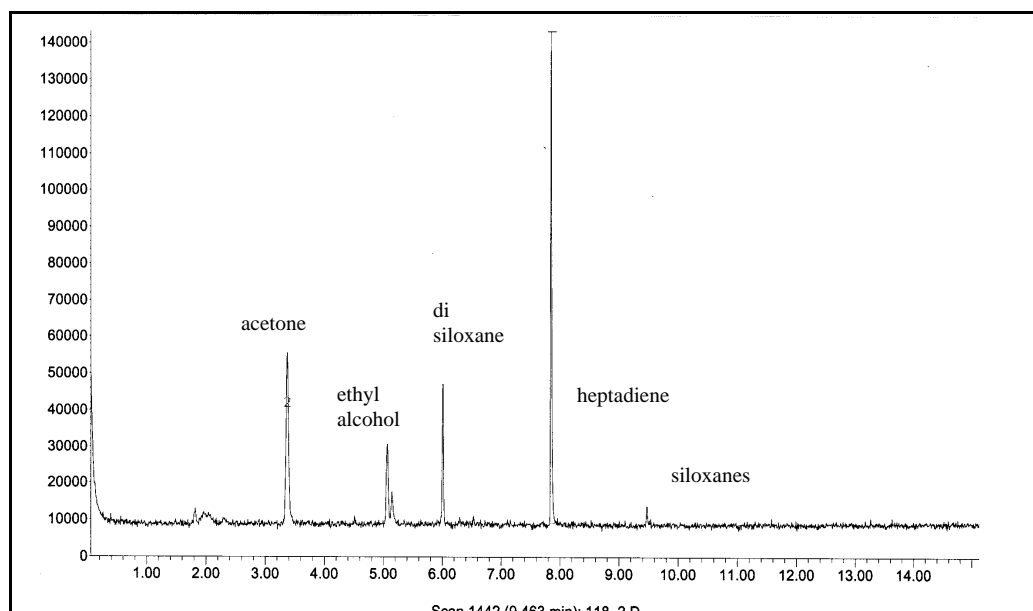


Fig. 49. Spectrum of HS of media using air tube method- trial 2

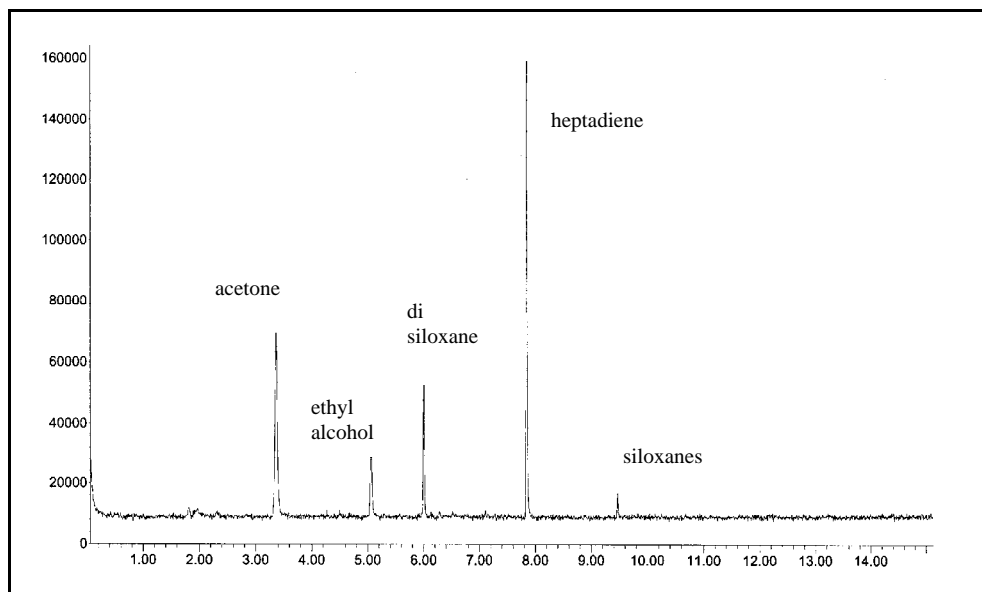


Fig. 50. Spectrum of HS of media with cells using air tube method- trial 2

Calibration curves in AT method

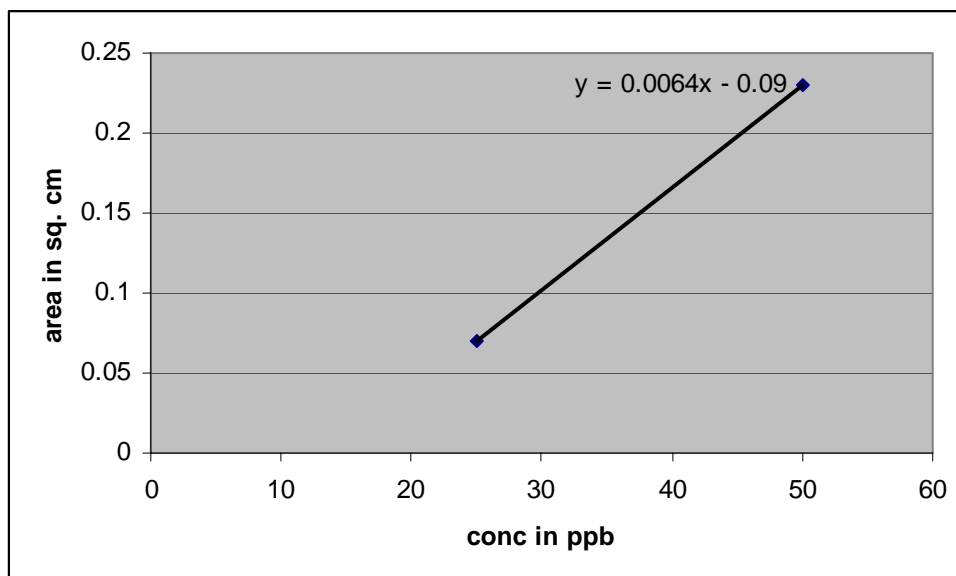


Fig. 51. Calibration curve for toluene using air tube method

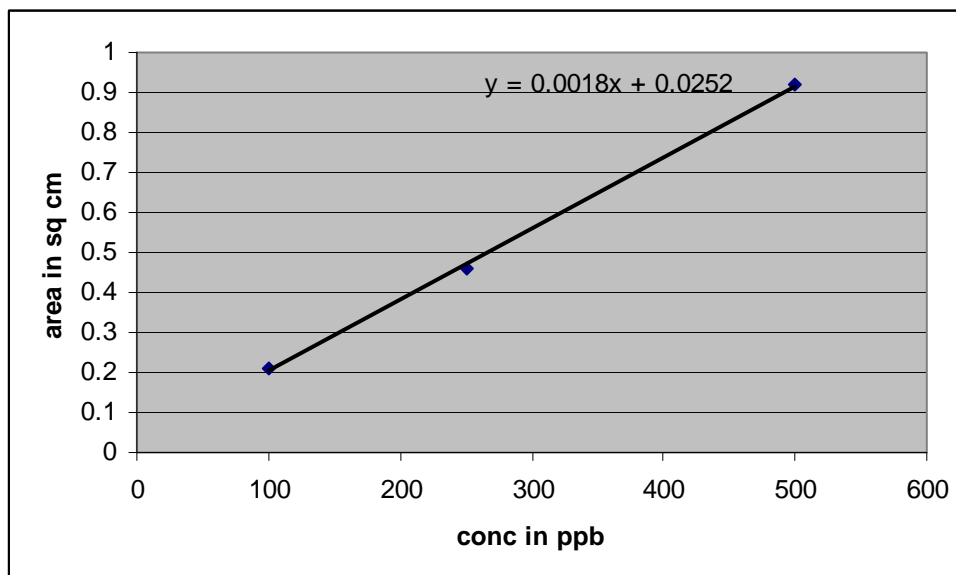


Fig. 52. Calibration curve for acetone using air tube method

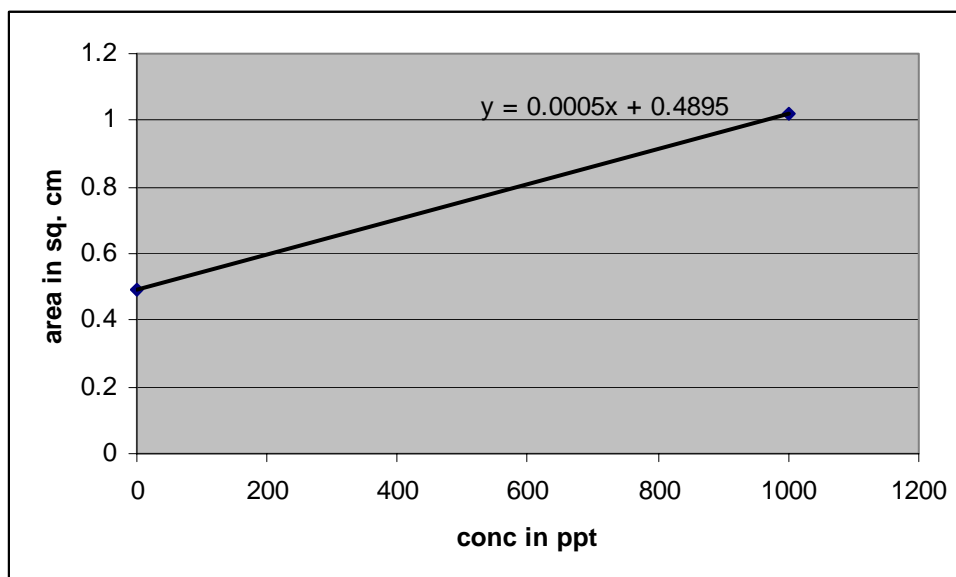


Fig. 53. Calibration curve for ethyl benzene using air tube method

The calibration curves were plotted for acetone, toluene and ethyl benzene (Figs 51, 52, 53). Ethyl benzene and toluene both of which have excellent detection efficiency with Tenax air tube were detectible at the ppb and parts per trillion (ppt) respectively proving the sensitivity of this method with non polar volatiles. The enhancement seen in acetone concentration in the media with and without the cells is tabulated in Table 6.

Table 6

Concentration enhancement seen in acetone by air tube method

Trial	Culture flask	Concentration in media (in ppb)	Concentration in cells + media (in ppb)
1	40mL bottle (10mL)	2.6	74
1	800mL bottle (100mL)	191	408
2	800mL bottle	141	219
3	800mL bottle	174	263

From the tabulated values it is seen for the smaller glass bottle there is the concentration of the acetone with the cells is increased 28 times whereas with the larger 800mL bottle there is average of 1.6 times increase. This could be due to the smaller HS area in the smaller bottle, this could lead to the fact there is a larger concentration of VOCs at the end of 5 days in the smaller area. The larger bottles have a larger HS and therefore there is a possible dilution and spreading out of VOCs.

The average concentration of acetone in the media is 168 ppm with an average %error of 16.32%. With the cells, the average concentration of acetone is 296 ppm and the % error is 24%.

CONCLUSIONS

Cell lines are extremely complicated and many factors influence their successful growth. Many factors influence the analysis of these cell lines, such as optimum temperature, pressure and a sterile environment. These reasons are probably why not many investigators have chosen it to study lung cancer. In order to develop a sensitive system for VOC analysis in lung cancer cell lines we have tried and tested numerous methods for a period of 3 years ranging from media to headspace analysis both on campus and off campus labs for various cell lines using various techniques like centrifugation, lysis, and heating of cells.

Acetaldehyde, which has reportedly been seen in the headspace of the same cell line [42], was seen only at very high concentrations of 500 ppb in the air tube method. This can be attributed to the fact that acetaldehyde is an extremely volatile compound, very polar, very soluble in water; therefore its purge efficiency is extremely low even when heated according to the manufacturers of Tenax -O/I Analytical. Tenax on the other hand is suitable for non polar compounds with high boiling point.

The air tube method of collection of VOCs was designed in our lab and has proved extremely sensitive system for non polar compounds in cell lines with minimum human error. The fact that it is able to detect up to 10 ppt of toluene questions earlier claims made that toluene listed as a cancer marker [17, 18, 19, 20] since we were never able to see any reproducibility in all methods used to study cancer cell line emissions. Hence it is safe to conclude that the air tube setup can be used further to build a prototypical instrument for cell line or breath analysis in the future.

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