CHAPTER-4

EXPERIMENTAL

Chemicals

The chemicals used in analysis of air pollutants and toxicological examination were of highest purity available. Nitric acid, sulphuric acid, hydrolic acid, phosphoric acid, perchloric acid, iodine, mercuric chloride, potassium, dichromate, potassium iodide, sodium hydroxide, sodium, metabisulphite, sodium nitrite, sodium thiosulphate, acetone, formaldehyde and silica gel were procured from Merck/Qualigens/Spectrochem. p-Rosaniline hydrochloride of S.D. Fine Chemicals. Chromotropic acid, sulfanilamide, and 1-naphthyl ethylene diamine dihydrochloride (NEDA) and \( \delta \)-amino levulinic acid (\( \delta \)-ALA) of CDH Chemicals, HPCLgrade solvent (cyclohexane, pentane, methylene chloride, acetonitrile and water) of Spectrochem were used. Filter paper-GF/\( \AA \) and EPM 2000 of Whatman(India), photography film of Kodak 200 ASA, haemoglobin test combination kit, cat No. 124729 of Boehringer Mannheim India, were used. The PAHs standards of highest purity of laboratory of government Chemist, Teddington, U.K. were used.
**Experimental**

**Instrument**

Spectronic-2000 (Bausch and Lomb), atomic absorption spectrophotometer (Perkin Elmer model 5000), high volume sampler APM 415-411 (Envirotech Instrument India), HPLC model LC 10D (Shimadzu) equipped with UV/Visible detector and electronic digital balance (AFCO SET). Camera – Canon make model EOS 500 were used.

**Analytical Procedure**

High volume sampler is a basic instrument used primarily for measuring concentration of SPM in atmospheric air. Gaseous sampling attachment uses wet chemical methods for the absorption and detection of gaseous pollutants. Atmospheric air was passed through absorbers containing suitable reagents.

**Sampler Sequence**

1. The system was suitably located.
2. A fresh, pre-weighed glass fibre/filter paper (GF/A) was installed in filter holder.
3. Fluid in the orifice meter was adjusted at zero level.
4. In case both particulate and gaseous pollutants were to be sampled, impugners were filled with suitable reagents and placed in ice tray. Impugner outlet was connected to individual inlets of the gas manifold.
5. Initial flow rate of gases was measured.

Prior to completion of the sampling as per schedule, the following were noted.
(i) Flow rate indicated by orifice meter and rotometer was recorded.
(ii) Final sampling time indicated by the time totalize was recorded.
(iii) Final flow rate of gas was measured.
(iv) Filter paper was removed, folded along its length and stored in a clean envelope.

Air Sampling Schedule

<table>
<thead>
<tr>
<th></th>
<th>Sampling equipment</th>
<th>Flow rate</th>
<th>Duration of Sampling</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>High volume sampler</td>
<td>1.2-1.4 lit/min</td>
<td>24 hours</td>
<td>24 hours</td>
</tr>
<tr>
<td>2</td>
<td>High volume sampler</td>
<td>0.50.1 lit/min</td>
<td>24 hours</td>
<td>24 hours</td>
</tr>
<tr>
<td>3</td>
<td>High volume sampler</td>
<td>0.50.1 lit/min</td>
<td>24 hours</td>
<td>24 hours</td>
</tr>
<tr>
<td>4</td>
<td>High volume sampler</td>
<td>1.00.1 lit/min</td>
<td>24 hours</td>
<td>1 hours</td>
</tr>
</tbody>
</table>
Experimental

Estimation Of Air Pollutants

Gases and suspended particulate matter were collected as per our work plan 24 hours at each location twice in a month.

Calculation

The volume of air at STP (25 °C and 760 mm Hg) was calculated as follows in the case of SPM, lead and PAHs contents.

\[
V_s = \frac{(760 - P_m)}{760} \cdot \frac{298.2}{(t + 273.2)}
\]

Where:
- \( V \) = volume of air recorded at the sampling period
- \( P_m \) = Atmospheric pressure at the sampling period
- \( T \) = temperature recorded at the sampling period.
- \( V_s \) = Volume of air on STP in letter (at 25 °C and 760 mm Hg)

\[
V = Q \cdot T
\]

Where:
- \( Q \) = Average sampling rate (cu m/min)
- \( T \) = sampling time

\[
Q = \frac{Q_1 + Q_2}{2}
\]

Where:
- \( Q_1 \) = Initial sampling rate indicated by orifice meter.
- \( Q_2 \) = Final sampling rate indicated by orifice meter.
SUSPENDED PARTICULATE MATTER (SPM) [IS: 5182 Part IV 1987]

Suspended particulate matter was collected over the pre-weighted filter paper GF/A Whatman for 24 hours as per sampling schedule locations. The volume of air was measured in cubic meters.

Calculation

Weight in gms of suspended particulate (W) = $W_2 - W_1$

where $W_2$ = weight in gms of filter paper after sampling.

$W_1$ = weight in gms of filter paper before sampling.

$$W \times 10^6$$

Concentration of SPM ($\mu g/m^3$) = $$\frac{W \times 10^6}{Vs}$$

Where $Vs$ = Volume of air on STP in letter (at 25°C and 760 mm Hg)

Lead (Pb)

Glass and polythenewares: The corning glassware and high quality polythenewares used were initially washed with tap water and immersed in a bath containing 10% nitric acid for 48 hours. After soaking in nitric acid these wares were rinsed several times with double distilled water and finally with deionized water and air dried.

Reagents

The working lead standard solution was prepared on the day of analysis by suitable dilution of stock solution of lead (1000 ppm of Sigma Chemicals, USA) with deionized water.

Collection Of Sample: Suspended particulate matter (SPM) samples were collected from pre identified locations using high volume samplers operated at a rate of 1.5 m$^3$/min. The SPM concentrations
were determined by collecting the particulate matter for 24 hours on pre-weighted glass fiber filter of 20X25 cm size. Glass fiber filters were reweighed after sampling in order to determine the mass concentration of lead particles collected (Janssens & Dams, 1973). The concentration of in ambient air was computed in μg/m³ by dividing the net mass content with the volume of air.

**Sample Preparation:** Filter paper was digested three times with HNO₃ in a conical flask. The solution was filtered into another conical flask through 0.45-micron Whatman filter paper and insoluble residue on the filter paper was rinsed with 10ml of 10% nitric acid. A blank was prepared in similar fashion. The filtered digested solutions were evaporated to almost dryness. The residue were dissolved in 1%HNO₃ and made up in 10ml vol. Flask with 1%HNO₃. Finally, the digested samples were analysed by atomic absorption spectrophotometer. Five replicates were taken and the average values were used for calculating the results.

**Calculation**

\[
\text{Conc. of Pb in collected sample(μg)} = \frac{\text{Conc. of Pb(μg/ml)} \times \text{Vol. Of digested sample (ml)}}{\text{Wt. of sample used for digestion (gm)}}
\]

\[
\text{Conc. of Pb in ambient air(μg)} = \frac{\text{Conc. of Pb in sample collected(μg)}}{\text{Vol. of air(m³)}}
\]
Polycyclic Aromatic Hydrocarbons (PAHs)

Collection of sample: Suspended particulate matter (SPM) samples were collected from pre-identified locations using high volume samplers operated at a rate of 1.5 m$^3$/min. The SPM concentrations were determined by collecting the particulate matter for 24 hours on pre-weighted glass fiber filter of 20X25 cm size and reweighed after sampling in order to determine the mass concentration of particles collected. The concentration of particulate matter in ambient air were then computed on net mass content divide by volume of sample.

SPM was collected and brought to laboratory in black polythene to avoid photo degradation of PAHs.

Extraction: Filter paper was carefully placed into soxhlet apparatus, already wrapped with black carbon paper to avoid photodegradation during extraction procedure (EPA, 1996). PAHs were extracted with 300ml of dichloromethane for 16 hours. The extract was dried under reduced pressure. The dried was dissolved in the 5 ml of cyclohexane.

Clean up: 10mm inner dia chromatography column was prepared by 10g of activated silica gel in the methylene chloride. The slurry was placed into column on the glass wool and allowed to settle.
down. A uniform bed was prepared. The anhydrous sodium sulphate was placed to avoid moisture from air. Column was preeluted with 40ml of pentane to remove any contamination in column materials and discarded the eluted solvent. The cyclohexane containing extract was poured into column. The column was run by 25ml of solvents mixture (dichloromethane and pentane 2:3). The elute was dried under reduced pressure and dissolve in cyclohexane and made up the volume to 10ml in volumetric flask with the same solvent.

**Analysis:** The cleaned extract was applied on HPCL under following analytical conditions to have a good reproducible chromatograms for analysis of naphthalein, anthracene, acenepthylene, fluorine, fluoranthene, benzo(ghi)pyrene, acenepthylene, pyrene, chrysene, benzo(k)flourene, benzo(a)pyrene and phenanthrene.

**Instrument:** HPCL Shimadzu make model LC 10D; Column: C\(^{18}\) (250x4mm) column length (E.merck made); Detector: UV/Visible (SPD-10) Mobile phase: Acetonitrile and water (HPCL grade) in the ratio of 70:30. Flow rate 1.75 ml/min.

**Calculation**

\[
\text{Concentration of PAHs (ppm)} = \frac{\text{Area of unknown} \times \text{Conc. of PAH (ng)} \times \text{Vol. of sample made up}}{\text{Area of known} \times \text{Vol. of injected sample (µl)} \times \text{Wt. of sample (gm)}}
\]

\[
\text{Actual amount of PAHs (ng/m}^3) = \frac{\text{Conc. of PAHs (ng/mg)} \times \text{Wt. of sample (mg)}}{\text{Vol. of air (m}^3\text{)}}
\]
SULPHUR DIOXIDE (S0₂) IN AIR [IS: 5182, Part-II, 1989(West and Gaeke)]

Reagents

(a) Absorbing solution for SO₂ (0.1M Sodium tetrachloromercurate)

27.2g of mercuric chloride was mixed with 11.7g of sodium chloride in 1000 ml of distilled water.

(b) Formaldehyde solution (0.2%).

1.25 ml of formaldehyde (assay 40%) was diluted to 250 ml of distilled water.

(c) p-Rosaniline hydrochloride dye (0.04%).

0.10 g of p-Rosaniline hydrochloride was dissolved in the 50 ml distilled water solution kept in dark and after 48 hour it was filtered. The solution was finally made up to 50 ml which remains stable for 3 months. It was stored in the dark and at refrigerated temperature. 10 ml of the solution was mixed with 3 ml of HCl and kept in the dark for 5 minutes. Then the volume was made up to 50 ml.
(d) Sodium thiosulphate solution (0.01M)

25.0 g of sodium thiosulphate was dissolved in 1000 ml of distilled water. The Stock solution was approximately 0.1 N solutions. The 100 ml of stock was diluted to 1000 ml with distilled water.

(e) Potassium dichromate solution

4.904 g of potassium dichromate was dissolved in the 1000 ml of distilled water.

(f) Iodine solution (0.1N)

25 g of potassium iodide was dissolved in the distilled water then the 12.7 g of iodine was added and diluted up to 1 liter.

(g) Iodine (0.01 N)

0.01 N iodine solution was obtained by diluting 10 times the solution of 0.1 N iodine and standardized with 0.01 N sodium thiosulphate solution.

(h) Starch

To the paste of 1 g soluble starch in 2.0 ml of distilled water 100 ml of boiling water is added cooled and then 5 ml of chloroform was added.
(i) Standard sodium metabisulphite solution

Sodium metabisulphite (assay 95 %) 441.3 mg was dissolved in 1000 ml of distilled water. The sodium contained approximately 0.40 mg /ml of SO₂. Stock solution of sodium metabisulphite was standardized by 0.01N of iodine and the normality of solution was adjusted to 0.0123 N. The solution contained 150 μ 1 SO₂ (25° C and 760 mm Hg).

Procedure

(a) Standard curve

2ml of standard stock sodium metabisulphite solution was diluted by 100 ml of distilled water. Solution contained 3.0 μl SO₂/ml. The standard curve was prepared by taking different concentration ranges 0.5 to 3.0 μl SO₂/ml. The standard solution were further processed as per procedure described below for unknown samples.

(b) Sample collection

The air was drawn through 10 ml of absorbing solution (0.1M sodium tetrachloromercurate) at the flow rate of 0.5 l/m for 8 hours. The sample was carried to laboratory for analysis.
(c) Analysis

1.0 ml of formaldehyde solution was added into 10 ml of sample. Then 1.0 ml of p-rosainline was added and mixed well. Absorbance was recorded at 560 nm on spectrophotometer after 20 minutes.

Calculation

\[ \text{Volume of air at } 25^\circ \text{C and 760 mm Hg (STP)} \times \frac{V(760-Pm)}{760} = X \]

\[ (t+273.2) \]

Where \( V \) = Volume of air in liters during sampling period

\( Pm = \) Atmospheric pressure at the sampling period

\( t = \) temperature recorded at the sampling period

\( Vs = \) Volume of air on STP in liter (at 25°C at 760 mm Hg)

\[ V = F \times T \]

Where \( F = \) Average flow rate of gas.

\( T = \) Time

\[ F_1 + F_2 \]

\[ F = \frac{F_1 + F_2}{2} \]

Where \( F_1 = \) initial flow rate of gas in lit/min

\( F_2 = \) final flow rate of gas in lit/min

\( (SO_2) \) ppm by Vol. = \[
\frac{\mu l \text{ of } SO_2}{Vs}\]

\( (SO_2) \) in \( \mu g/m^3 = \) ppm by volume \( (64 \times 10^{6}/24470) \)
Oxides of Nitrogen (Nox) IS: 5182 Part VI, 1992
(Jacob and Hochheiser)

Reagents

(a) Absorbing solution of NOx
It was prepared by 4.0 g of NaOH and 1 g of sodium arsenite in 1000 ml of distilled water.

(b) Sulphanilamide solution
10 g of sulphanilamide dissolved in 250ml of concentrated phosphoric acid (85%) diluted.

(c) Hydrogen Peroxide
0.2 % of $\text{H}_2\text{O}_2(30\%)$ diluted with 250 ml of distilled water.

(d) 1-naphthyl ethyl diamine hydrazide(NEDA)
0.05% g of NEDA was dissolved in the 50 ml of distilled water.
(all the reagents were stored in the amber bottle at refrigerated temperature for a week).
(e) Standard nitrite solution:

The amount of sodium nitrite was calculated as.

\[
\frac{1.5 \times 100}{A}
\]

Where \( G \) = amounting of sod. Nitrite
\( A \) = Assay percent of sodium nitrite used
1.5 = Gravimetric conversion factor.

Sodium nitrite (assay 97%) 1.5464 g was dissolved in the 1000 ml of distilled water, Stock solution contains 1000 \( \mu \)g NO\textsubscript{2}/ml.

**Procedure**

(a) Calibration curve

2.5 ml of stock sodium solution (1000\( \mu \)g /ml) was diluted to 100 ml with distilled water. The solution contained 25\( \mu \)g NO\textsubscript{2}/ml. The standard curve was prepared by taking different concentration of NO\textsubscript{X} ranges 0.5-5.0\( \mu \)g NO\textsubscript{2}/ml. The standard solution were further processed as per procedure described below for unknown sample.

(b) Sample collection

The air was drawn through 10 ml of absorbing solution (0.4% NaOH solution) at the flow rate of 0.51/m for 8 hours. The sample was carried to lab for analysis.
(c) Analysis

10 ml of sulphanilamide was added into 10.0 ml of sample followed by the addition of 1.0 ml of H₂O₂ and 1.4 ml of NEDA. Blank was prepared in the similar fashion. The test tube was inverted up and down several times. Absorbance was recorded at 540 nm on the spectrophotometer after 30 minutes.

Calculation

\[ V = F \times T \times 10^6 \]

Where \( F \) = Average flow rate of gas

\( T \) = Time

\[ \frac{V (760 - P_m)}{760} \times \frac{298.2}{(t + 273.2)} \]

Where \( V \) = volume of air in litre measured during sampling time

\( P_m \) = Barometric pressure in mm of Hg during sampling time.

\( t \) = temperature recorded in degree celcious (°C) during sampling time

\[ \text{NO}_x(\mu g/m^3) = \frac{(\mu g \text{ NO}_x / ml) \times (10 \text{ ml of sample})}{0.45 \times V_s} \]

Formaldehyde (HCHO) [NIOSH, 1997]

Reagents

(a) Absorbing solution

Double distilled water.

(b) Chromotropic acid
Chromotropic acid 0.1g was dissolved in 10.0 ml of distilled water.

(c) Iodine (0.1 N approximately)

Potassium iodide 25.0 gm was dissolved in 25 ml of distilled water and 12.7 g of iodine was added to this finally diluted to 1000 ml with distilled water.

(d) Iodine (0.01 N)

100 ml of stock iodine solution was diluted to 1000 ml with distilled water.

(e) Standardization of iodine solution

0.01N iodine solution was standardized by 0.01 N sodium thiosulphate solution.

(f) Starch

The solution was prepared as described earlier.

(g) Sodium carbonate buffer solution

80 g of anhydrous sodium carbonate was dissolved in the 500 ml of distilled water. Then 20 ml of glacial acetic acid was added slowly and made up to 1000 ml with distilled water.

(h) Sodium bisulphate (1%)

1 g of sodium bisulphate solution was dissolved in 100 ml of distilled water.
(i) **Formaldehyde solution A (1 mg/ml)**

Formaline Solution (37%) 2.7 ml was diluted to 1000 ml with distilled water. The solution was standardized as given below:

(j) **Formaldehyde standard solution B (10 μg/ml)** 1.0 ml of standard A was diluted to 100 ml with distilled water.

(k) **Standardization of formaldehyde solution**

10.0 ml of (1%) of sodium bisulfite and 1 ml of starch solution was added in the iodine flask containing 1 ml of formaldehyde solution and in blank solution having 1.0 mg distilled water. Then the solution was titrated by 0.1 N of iodine to a dark blue colour. The excess of iodine was destroyed by 0.05 N of sodium thiosulphate. Then solution was titrated by 0.01 N iodine solution. At the end of titration faint blue colour appeared. The excess of inorganic bisulfite was completely oxidized to sulfate and the solution was ready for the assay of formaldehyde bisulfite addition product.

The 25.0 ml of chilled sodium carbonate buffer was added in chilled flask containing the product which was finally titrated with 0.01 N iodine solution.

0.01 N Iodine = 0.15 mg formaldehyde
Procedure

(a) Standard curve
Standard curve was prepared by taking different concentration of formaldehyde solution B (10μg/ml) ranging from 0.5 to 5.0 μg/ml and diluted by 4.0 ml of distilled water. Standard solution was further processed as per procedure described below for unknown samples.

(b) Sampling collection
Two impingers were consented in the series containing each 20.0 ml of distilled water. The air was drawn for 1 hour at flow rate of 1 l per minute sample was carried to the lab for analysis.

(c) Analysis
The 4.0 ml of aliquot was taken from each sampling solutions into separate test tube and a blank containing 4.0 ml of distilled water. 1.0 ml of chromotropic acid solution was added. The 6.0 ml of conc. H₂SO₄ was cautiously added very slowly and allowed to cool at room temperature. The absorbance was recorded on the spectrophotometer at 580 nm.

Calculation

\[ V = F \times T \]

Where \( F \) = Average flow rate of gas
\( T \) = Time

\[ V_s = \frac{V \times P}{298} \times \frac{760}{(T+273)} \]
Experimental

where $V$ = Volume of air in liter during sampling period

$P$ = Atmospheric pressure in liter during sample period

$T$ = Temperature recorded in degree Celsius ($^\circ$C) during sampling period

$$C_T = C_A \times F_A + F_B \times C_B$$

Where $C_T$ = Total $\mu$g of formaldehyde in the sample

$C_A$ & $C_B$ = Formaldehyde concentration in $\mu$g of sample aliquot taken impinger A. and B form calibration curve.

$F_A$ & $F_B$ = respective aliquot factor= sampling soln. Vol in ml/ml aliquot used

Concentration of formaldehyde at $25^\circ$C and 760 mm Hg ppm

$$\frac{C_T \times 24.47}{(Vol)} = \frac{(Vol)}{Vs \cdot M.W.}$$

Where $Vs$ = liter of air samples under standard conditions

$M.W. = $ Molecular weight of formaldehyde = 30.03

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**Biochemical Assay in Blood**

**Blood**

Blood was collected for the 5 ml plastic syringe (not lead stabilized) and immediately transferred to heparnized tube stored at $4^\circ$C in the laboratory. Blood ALAD and haemoglobin were analyzed on the same day and the platelet within a week from date of sample collection.
Haemoglobin

Blood haemoglobin was measured by the Cynamethanoglobin colorimetric method (Van Kampen & Zijlstra, 1961).

Reagents

Mixture solution

Reagents bottle NO. 1 and 2 supplied in the Kit were mixed and made up to 1000 ml with distilled water (mixture solution) solution was stored in brown bottle and stable for six month + 15 to 25 °C.

Procedure

To 5.0 ml of mixture solution was added in 0.02 ml of blood to avoid the clumping of erythrocytes. The samples were vigorously shaken. Then sample was incubated for 20-25 °C. After incubation absorbance (A) of sample against distilled water was recorded on the spectrophotometer Spectronic 2001 at the wavelength of 546 nm. The measurement was carried after 3 min. of the addition of blood into mixture solution.

Calculation

Concentration of hemoglobin in mg % obtained by talling absorbance obtained and the absorbance already mentioned in the Table (supplied with hemoglobin kit of boehringer Manheim).

\[ C = 36.77 \times A \text{ (g/100ml)} \]

Where \( A \) = absorbance of the sample.

\[ C = \text{concentration of hemoglobin in percent.} \]
Blood δ-ALAD (δ- Aminolevulinic acid dehydrates)

The activity of δ-ALAD was determined by the method of Berlin and Schauer (1974).

The assay system consisted of 0.2m heparinized blood and 1.3 ml of distilled water. Mixture was incubated at 37 °C for 10 min for complete hemolysis. After 10 min 1 ml of standard δ- ALA solution (0.083 g ALA in 50 ml of phosphate buffer, pH 7.4) was added to the experimental tubes while 1 ml TCA to blank tubes (10% TCA containing 1.35 g HgCl₂ in 100 ml) incubated for 60 min at 37 °C. The reaction was stopped after 1 hr by adding 1 ml of TCA in experimental tubes and 1 ml of ALA was added in blank. Mixture was centrifuged and 1 ml of aliquot were taken in a test tube and an equal volume of Ehrlich reagent (0.5 g dimethyliminobenzaldehyde in 12.5 ml acetic acid + 6 ml of perchloric acid + 1 ml of 2.5% HgCl₂ was added to it. After 5 min. absorbance was read at 55 nm. The enzyme activity was expressed as alpha-aminolevulinic acid μ mol/min/ml erythrocytes.

Calculation:

δ-ALAD (μδ-mol/min erythrocytes = absorbance x 100 x 2.35 (μg/l)/Hematocrit (%) x 60 x 0.062.

Blood Lead

'Five ml of blood sample (heparinized) was treated with 1 ml of 5% triton-X-100 solution and 1 ml of 2% ammonium tetramethylene dithiocarbamate (APDC) solution. After vigorous shaking, 5 ml of water
saturated methyl iso butyl ketone (MIBK) was added. After shaking for 5 minutes, the sample was centrifuged at 700g for 10 minutes. The solvent layer was separated and readings were recorded at 283.3 nm on AAS. The working standards of 1,2,3 and 4 ppm of lead and a blank (deionized water) were prepared using the same extraction procedure (Hesse, 1968).

**Calculation:**

\[
\mu g/ml = \frac{S-B \times \text{dilution factor}}{\text{vol. of sample}}
\]

\[
\text{Blood Pb concentration in } \mu g/dl = \text{blood Pb concentrationin } \mu g/ml \times 100
\]

where

- \(S\) = sample
- \(B\) = blank.