CHAPTER II

METHODS AND TECHNIQUES
In the early stages, study of essential oils was restricted mainly to the isolation of the oils from the plant material and determination of their physico-chemical properties and their evaluation as perfumery materials. With the improvements in the techniques of separation of the constituents by fractional distillation (like spinning band column) and chromatography, it has now been possible to ascertain with greater confidence the structures of these fragrant principles. In addition to the normal chemical methods of reductive and oxidative degradations and specific colour reactions, an extensive use of spectroscopy such as infra-red (IR), ultra-violet (UV), and nuclear magnetic resonance (NMR), along with mass spectrometry, has greatly helped in establishing the structure and stereochemistry of these constituents.

**CHROMATOGRAPHY**

The age old technique of fractionation even with the improved techniques of separating the constituents boiling with a close range has the inherent danger of rearrangement change and polymerisation of the constituents. Chromatography has, therefore, largely replaced the methods of fractionation as separation procedure. This technique and its many variations which has been developed by Kuhn and Lederer\(^1\) (adsorption chromatography), Martin and Synge\(^2\) (partition chromatography), Adams and Holmes\(^3\) (ion-exchange chromatography), Consden,
Gorden and Martin⁴ (paper chromatography), Kirchner, Miller and Keller⁵ (thin layer chromatography), James and Martin⁶,⁷ (gas chromatography), and reviewed in standard books by Williams⁸, Fordon and Estoc⁹ etc., has proved useful even for the separation of the isomers of the same compounds.

The various forms of the chromatography may be executed by applying the three processes, elution development, frontal analysis and displacement development. In essential oil analysis column or adsorption chromatography, thin layer chromatography and gas liquid chromatography are of much use. Richter and Muscholl¹⁰ and Möhrhammer et al.¹¹ have described in detail new paper chromatographic methods and have given the chromatograms of several essential oils and mixtures of aromatic compounds.

Adsorption chromatography:

The method of choice which is in current practice in the adsorption chromatography of essential oils is that of fractional elution introduced by Reichstein, Ruzicka and their co-workers. The adsorbed materials are washed successively with a series of solvents of constantly increasing elutive power and thereby not only separated on column but also eluted one after the other. The eluate is collected into a series of fractions more or less arbitrarily on the
basis of preliminary experiments and then examined by any one of the variety of modern methods and techniques. Spectrophotometry, GLC, TLC, polarographic analysis, colour reactions, conductance measurements and potentiometric determination have been used. Column chromatographic separation of the saponifiable fats has not yet been widely applied although it is certain to become more prominent, since it offers the usual advantages of chromatography over fractionation or solvent separation method. Silver nitrate impregnated silica gel for column chromatography for the separation of methyl esters of fatty acids has also been used.

**Thin layer chromatography (TLC)**:

TLC is a very valuable supplement to column and paper chromatography and has become of great importance for analytical separation of natural products. It not only combines the advantages of paper and column chromatography but in certain respects, it is superior to either of them.

Kirchner, Miller and Keller\(^{12}\) have published numerous papers on the separation of terpenes and essential oils on chromatostrips, or on sheets for two dimensional separations, and described an apparatus for preparing chromatograms. Reitsema\(^{13}\) extended the technique by
preparing larger plates (5\" x 7\") and used them to separate essential oils. The wider applications of TLC were not, however, visualised until 1956, when Stahl\textsuperscript{14} described an apparatus for preparing chromatoplates and how they might be used to separate compounds other than terpenes. Several papers notably by De Mole\textsuperscript{15}, Stahl\textsuperscript{16}, and Wollish et al.\textsuperscript{17}, and recent books by Randerath\textsuperscript{18}, Truter\textsuperscript{19}, Bobbitt\textsuperscript{20}, Stahl\textsuperscript{21}, Marini\textsuperscript{22}, and Calvin\textsuperscript{23} have reviewed TLC in theory and practice.

Stahl\textsuperscript{24} has recently described an apparatus for the coating of glass plates with a thin layer of continuously changing composition. Chromatography on such layers permits the resolution of mixtures of compounds differing widely in polarity. This innovation in technique holds great promise as a means of separating mixtures that cannot be completely resolved on uniform layer.

Micro TLC technique\textsuperscript{25}, using microscopic slides instead of glass plates, has also recently been largely used. It is possible to make circular chromatograms over runs\textsuperscript{26,27} and two-dimensional chromatograms, and it is also possible to use the method for quantitative or preparative use.\textsuperscript{28-31}

\textbf{Detection of the spots}:

Various methods have been used for the detection of the spots obtained from various compounds in the different
type of TLC. The sprays of the following reagents are generally used in the analysis of essential oils \(^{21}\):

- Antimony tri and pentachloride
- Diphenyl carbazoine
- Potassium iodide - starch reagent
- Dimethyl amino benzaldehyde - glacial acetic acid
- Phosphomolybdic acid
- Anisaldehyde - sulphuric acid
- 2:4 diphenyl hydrazine agents etc.

In addition, some of the other methods adopted for qualitative analysis and quantitative estimations are:

1. Planimetric analysis\(^{32-34}\) (size of the spots are measured and then compared with a calibrated curve drawn from known solutions),
2. Densitometric\(^{35}\),
3. Spectrometric\(^{36,37}\),
4. Colorimetric\(^{38,39}\),
5. Polarographic\(^{40}\),
6. Fluorescence measurements\(^{41,42}\), and
7. Measurement of radio isotopes\(^{43,44}\).

Paris and Goden\(^{45}\), who examined 20 essential oils have demonstrated that how TLC may be used to differentiate between closely related products such as anise oil and staranise oil.

Bhalla, Nayak and Dev\(^{46}\) have described 'Inverted dry column chromatography', a method which has been found to reproduce faithfully the TLC on the column. The method is effective, quick, easy to manipulate and useful both for synthetic and natural mixtures.
GAS LIQUID CHROMATOGRAPHY:

Seldom in the history of science and technology has a new research tool become as universally and rapidly adopted as gas chromatography. The technique of gas liquid chromatography, was introduced for the first time by James and Martin (1952). It has rendered possible, the separation and estimation of very small amount of volatile substances, with a wide range of boiling points and has thus given a great impetus to many fields of research. It is useful, both for separation as well as identification.

The gas liquid chromatography process may be briefly described as follows. A typical, very high boiling and essentially non-volatile liquid (stationary phase) is impregnated on a suitable granular and inert material. In the analysis of essential oils, the stationary phases generally used are apiezon grease, polyethylene glycol, reoplex and silicon oil, didecyl phthalate and dicyclohexyl phthalate etc. The impregnated granular material in turn, is filled in a U-shaped column (long with a narrow tubing). A stream of carrier gas, such as hydrogen, nitrogen or helium etc., whose flow rate is carefully regulated, is passed through the inlet of the column. Samples of about 0.02 ml. are introduced at the top of the column by means of a syringe.
The components of the sample travel through the column at varying rates with elution times of the order of a few minutes to an hour. The separation of the components takes place mainly by partition by the repeated equilibria between the moving gas phase and the high boiling organic compound, which is called a 'stationary phase'. The exit stream of gas is passed through a detector which responds to the presence of materials other than the carrier gas. Because of its negligible vapour pressure at the temperature of the column, the stationary phase is retained as such, while the volatile mixture passes out of the column. Thus, one and the same column filling can be used repeatedly. Thermal conductivity cells are the most generally used detectors, although others, such as gas density balances, discharge tubes, ionisation detectors, flame detectors and automatic titrators have also been employed.\textsuperscript{35} The detector signal is usually plotted against time on a chart by an automatic recorder. The chart is called a chromatogram. From such a chromatogram, qualitative information from retention values the number of components present from the peak heights or peak area measurements, can be known. Ideally, a separate peak is produced for each component in the sample mixture, and there is negligible overlap of adjacent components.
Retention behaviour are quite dependent on temperature of the column. Drew and co-workers have also pointed out that two solutes whose peaks overlap, can be more completely resolved by reducing the column temperature. Investigations of Grant and Vaughan have made it possible to calculate the temperature required to obtain a given degree of separation between two homologous solutes. Commercially available instruments now have 'linear programmed' temperature control, i.e., temperature increasing with time and they have proved to be very successful in separating complex mixtures having compounds of wide range boiling points.

Beveritt, and Cheshire have discussed conditions which give optimum performance in the gas chromatographic analysis of essential oils. Bayer and co-workers have used the technique for geranium oil. A new technique for reactions, in gas chromatography has been demonstrated by Hoff and Feit where the chemical reaction is made to occur in the injection syringe. Gas chromatography, together with TLC and IR spectroscopy, has been employed for better identification and characterisation of the components of essential oils. Recently the interpretation and value of GLC data for essential oils has been reviewed by Kingston. The characterisation
of essential oils containing linalool and linalyl acetate and that of isomeric citral $^4_5\alpha$ and $\beta$, has been successfully accomplished by GLC. GLC has also been successfully employed for the separation of fatty acids or their methyl esters.$^4_6$

GENERAL METHODS OF STRUCTURE DETERMINATION:

(1) **Analytical methods**:

Determination of C, H and O content by combustion and molecular weight determination of purified terpenes, are of fundamental importance in structural investigations. The estimation of C-methyl groups can be effected by the method of Kuhn and Roth$^4_7$, which depends on the estimation of acetic acid obtained from the chromic acid oxidation of C-methyl groups. Gem-dimethyl groups (\(\begin{array}{c} \text{C} \\ \text{CH}_3 \end{array}\)) however, give unreliable results by this method. The isopropylidene group (\(\text{CH}_3 - \text{C} = \text{CH}_3\)) is estimated by ozonolysis, by its conversion to acetone. Active hydrogen in terpenoid compounds may be estimated by Zerewitinoff's method, or by modified lithium aluminium hydride method. The degree of unsaturation may be determined by quantitative catalytic hydrogenation using palladium or platinum oxide as
catalysts. Ethylenic linkages may be estimated by titration of the terpene against an organic per-acid e.g., mono-perphthalic acid.

(2) **Chemical methods**

Functional groups of the compounds present are detected by the usual familiar reactions. Formation of addition compounds with bromine, hydrogen chloride and nitrosyl chloride (Tilden's reagent) demonstrates the unsaturation. The addition of the unsymmetrical reagents is governed by Markownikov's rule, e.g., dipentene forms the dihydrochloride as shown below:

\[
\text{Dipentene} + 2\text{HCl} \rightarrow \text{Dipentene dihydrochloride}
\]

Nitrosyl chloride (NOCl) reacts readily with the double bond of the terpenes to form additive compounds. These addition products are of great significance in the terpene chemistry mainly for two reasons. Firstly, they are
usually crystalline compounds with sharp melting points and hence can be used for the separation and identification of the terpenes and secondly, they can be made to undergo an important series of reactions terminating in the ketonic derivatives, which are extremely reactive and capable of undergoing numerous transformation reactions and thus serve for the elucidation of the structure of the terpene molecule. A conjugated diene may be detected by the Diels-Alder or diene addition reaction, in which compounds containing a 1:3 diene system reaction with dienophilic molecules like maleic anhydride to give a crystalline adduct.

**Dehydrogenation:** Dehydrogenation with sulphur or selenium also gives a clue to the structure. Dehydrogenation by means of selenium is recommended by Vesterberg. The reaction takes place at a high temperature (200-300°C), often in an inert solvent. Under these conditions cyclic terpenes are usually converted smoothly into aromatic compounds, which are easy to identify. Ruzicka has used this technique extensively in the sesquiterpene group. An interesting example is the case of bicyclic sesquiterpene alcohol eudesmol which on dehydrogenation gives eudalene(XXXIV) containing one carbon atom less. The elimination of a carbon atom during the dehydrogenation indicates that eudesmol has an angular methyl group, which is lost as
methane when the reduced naphthalene system is aromatised.

\[
\text{OH}
\]

\[
\text{OH}
\]

(XXXIII) Eudesmol (XXXIV) Eudalene

**Oxidative degradation**: Various methods of oxidative degradation have been used extensively in terpene chemistry. The degradation is usually carried out by ozonolysis or oxidation with hypo-iodate, potassium permanganate, nitric acid or chromic acid. Permanganate oxidations are sometimes conveniently conducted in acetone solution, since the insolubility of salts of intermediate acidic oxidation products may protect them from further oxidation.

**Reduction**: For the characterisation of the number and type of unsaturated centres in a molecule, quite a good number of methods of reduction are available. In recent years complex metal hydrides of graded reactivity
have been used, because of their selectivity for carbonyl, carboxylic and ester groups. Catalytic hydrogenation has also been found to be very helpful in elucidation of the structure of cadinene, bisabolene and zingiberene. The principle that hydrogenation will occur preferentially on the less hindered side of a molecule has been widely applied to triterpenes\textsuperscript{51,52} as well as to the simpler substances such as eudesmol.

Stereoisomers, e.g., acyclic monoterpene alcohols, geraniol and nerol, are assigned a configuration based on chemical and physico-chemical evidence. An important chemical method for the determination of the configuration depends on the formation of ring compounds from the geometrical isomers.

(3) \textbf{Physical methods:}

The chemical methods used for the analysis of essential oils, require comparatively larger amount of the oil and entail the danger of polymerisation and structural changes. It is at this stage that the application of the physical methods\textsuperscript{54,55} comes to the rescue of the essential oil chemist. The use of physical methods\textsuperscript{56} such as optical rotatory dispersion, molecular refraction,
ultraviolet (UV), infra-red (IR), and nuclear magnetic resonance (NMR) spectroscopy and more recently mass spectrometry thus has greatly advanced, nay revolutionised the successful elucidation of the structure of organic compounds. Some of these important physical methods so used are summarised below:

**Molecular refractivity:** Molecular refraction is an additive property and depends upon the constitution of the molecule in general. The molecular refraction \( R_M \) of a liquid is given by the Lorentz-Lorenz equation as given below:

\[
R_M = \frac{n^2 - \lambda}{n^2 + 2} \cdot \frac{M}{d}
\]

where \( n \), \( d \) and \( M \) are refractive index, density and molecular weight of the compound respectively. In general, agreement is found between the observed and calculated values, but investigations on a large number of mono and sesquiterpenoids have shown an anomaly wherever a system of conjugated double bonds is present, the observed values of \( R_M \) are somewhat higher than the calculated one. This increase or exaltation in the molecular refraction has proved very useful in the detection of conjugated systems in terpenes, e.g., \( \beta \)-phellandrene shows an exaltation of 1.06 due to its conjugated system. The
application of molecular refractivity is, however, limited to pure components only. By making use of the graphical representation, Sutherland\textsuperscript{57} has extended the scope of this property.

\textbf{Optical rotation:} Since most natural products are optically active, Klyne\textsuperscript{58} and others found it very useful in the prediction of the configuration of a number of terpenoid compounds. The property shows clearly that the triterpenes are of the same enantiomeric type as the steroids. The rotation and the structure in case of terpenoids have been correlated by Barton and Jones.\textsuperscript{59}

\textbf{Absorption spectroscopy:}

(a) \textbf{Ultra violet:} The absorption of ultraviolet or visible radiations by a molecule involves a high order energy which results in a modification of the electron arrangement within the molecule. Hantzsch\textsuperscript{60} and Auwers\textsuperscript{61} first suggested the use of UV absorption spectra in the chemistry of terpenes, in a series of papers in 1910. The important features of UV absorption curve are the wavelength of a maximum absorption (\(\lambda_{\text{Max.}}\), expressed in \(\mu\) or \(\text{A}\)) and the corresponding molar extinction coefficient (\(\varepsilon_{\text{molar}}\)).
Various types of structure of terpene hydrocarbons are readily distinguished by their ultra-violet spectra and this may serve as a basis for preliminary characterisation. The position of the double bond in the side chain of eugenol and isoeugenol was ascertained by taking the help of their ultra-violet spectrum. The absorption spectra of a number of unsaturated ketones have been examined and the new data discussed as a test of Woodward's generalisation that the nature of the substitution of the chromophoric group in these compounds can be predicted from the location of main absorption bands. Woodward's rule combined with the exact positions and the intensities of the bands, has proved a successful tool for determining the degree of substitution in such a system. Woodward's scheme has been modified by Fieser. In a few favourable instances, it is possible to assign a substantial portion of the molecular structure of a natural product almost entirely on the basis of the ultraviolet absorption data.

(b) Infra-red: In recent years the IR absorption spectroscopy of terpenes has been employed to an ever increasing extent in structural problems. Its advantage in the qualitative and quantitative analysis of essential oils is considerable. No procedure offers so much information in so short a time. The operation of the instrument also requires very little skill. The interpretation of the
results, however, calls for a good knowledge of spectroscopy and organic chemistry, and a considerable acquaintance with the chemistry of essential oils. The transition from classical methods to IR offers no difficulty, as the compo-
ents tend to occur as groups, e.g., 'free alcohols', 'esters', 'aldehydes', etc. In addition, the type of alcohol or aldehyde can be identified and knowing this the individual components can be determined quantitatively. For instance, the citrals in lemon grass oil can be determined either as a group or separately as citral a and b and isocitral.

The absorption is measured in the 600 cm$^{-1}$
- 4000 cm$^{-1}$ region of the spectrum yielding a complex absorption curve containing many bands or peaks of varying intensities corresponding to the characteristic groups and their concentration. 68-70 A brief, review of the absorption bands characteristic of the various groups usually found in essential oils has been given by Carroll and Price. 71

First papers dealing with the uses of IR spectrophotometric examination in the essential oil field were published by Thompson and Whiffen, 72 Sörm et al. 73 and Lundin et al. 74 It is worthy of note that Sörm was the first to suggest that the sesquiterpene caryophyllene contained a nine membered ring by the IR spectrum studies. A number of naturally occuring lactones have been found to have the
and thus their detection can be carried out with the help of IR spectra. Gunthard and Plattner\textsuperscript{75} studied the IR spectra of azulenes. Mixtures of compounds can also be identified by comparison with reference spectra. Quantitative estimations of camphor\textsuperscript{76} in sassafras oil, menthofuran\textsuperscript{77} in mint oil, cienol(1:8) in lavender oil have been reported through the peak intensities. Carroll and Price\textsuperscript{79} have analysed the geranium and cinctronella oils, both qualitatively and quantitatively by the IR absorption spectra studies.

\textbf{Nuclear magnetic resonance spectrometry:} Though high-resolution nuclear magnetic resonance spectrometry\textsuperscript{80} of hydrogen containing compounds, has proved to be a versatile and major analytical tool for the physical and organic chemists, yet it does not replace any of the other methods but complements and extends the other techniques in a way which has proved valuable for the entire field of chemistry. Under appropriate conditions a sample can absorb electromagnetic radiations in radio frequency region, at frequencies governed by the
characteristics of the sample. A plot of the frequencies of the absorption peaks against peak intensities constitutes an NMR spectrum. The NMR spectra are exhibited only by atoms with nuclear magnetic moments, i.e., nuclei not possessing an even atomic and an even mass number. Thus, the atoms of $^1H$, $^{19}P$, $^{15}C$, $^{17}O$, $^{31}P$ and isotope $^{13}C$ yield nuclear magnetic resonance spectra but $^{12}C$ and $^{16}O$ do not.

Detection and identification of double bonds, the determination of the number of rings in the compound, and to some extent the analysis of mixture of terpenes, includes the application of NMR to essential oil studies. Dauben and Hance reported one of the first application of NMR in the chemistry of $\gamma$ santonin (XXXV). They showed that santonin contained no olefinic protons, whereas a hydrogenolysis product, 1-keto-7 hydroxy santonin acid (XXXVI) as one olefinic proton.

\[ \text{XXXV) } \gamma \text{ Santonin} \]

\[ \text{XXXVI) 1-keto-7 hydroxy santonin acid} \]
Dev proved the structures of Nerumbone and humulene by the help of NMR studies. Work has also been reported on the structures of thujopsene and hinkolic acid, valerenic acid, and an oxidation product of sabinol. A very interesting example of semiquantitative analysis of terpene mixture by NMR has been reported, whereby a mixture of several terpenes has been analysed from a knowledge of the assignments of the pure components. Apparently this application could prove complementary to VPC in the analysis of terpene mixtures.

X-ray diffraction: The X-ray diffraction method is the most direct of the physical methods of structure determination. Structures deduced from chemical and other physico-chemical evidence have been confirmed by the plot of electron density maps. A remarkable application of this method has been in the elucidation of the structure of the sesquiterpene longifolene (XXXVII) at a time when little was known about the chemistry of the compound. The structure assigned by X-ray diffraction studies, has been confirmed by chemical methods. The X-ray diffraction pattern of various essential oils have been obtained by Mohan and the interplanner distances utilized to identify the main constituent of the oils.

(XXXVII) Longifolene
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