CHAPTER 1

SECTION 1

NOVEL PHYSICO-CHEMICAL METHODS
FOR STRUCTURE DETERMINATIONS
Unlike the terrestrial organisms, studies on marine flora and fauna are beset with two critical problems: inadequate supply of organisms and low probability of their repeat collection for subsequent studies. These limitations effectively blocked any meaningful research on marine natural products for a long time. The classical way of structure elucidation included elaborate chemical degradations/derivatizations which required gram-quantities of materials. However, with the advancement in modern spectroscopic techniques, especially NMR and MS, it is now possible to determine not only the structure, but also the complete three-dimensional picture or configuration of compounds available in mg levels. In this chapter, some novel mass spectral techniques developed in order to determine the chemical structures of compounds available only in submilligram levels will be described.

CHEMICAL IONISATION MASS SPECTROMETRY

CHEMICAL IONISATION MASS SPECTROMETRY (CIMS), which uses relatively milder ionisation energies as compared to ELECTRON IMPACT MASS SPECTROMETRY (EIMS) is very useful for determining the molecular weights of thermally labile and nonvolatile compounds. Earlier Bose et.al., had used this technique quite extensively in natural products research,
especially in studying polar, underivatised compounds of biological interest\(^1\). They have observed that negative ion CIMS is much more sensitive than positive ion CIMS for many compounds. Subsequently, these workers improved this method and obtained the chloride / bromide adduct ions of various molecules by adding traces of NH\(_4\)Cl or NH\(_4\)Br to the samples before introducing them into the mass spectrometer. These pseudomolecular ions could be easily identified due to the presence of the corresponding isotope peaks. Thus, chloride adduct ion will show two molecular ions differing by 2 units corresponding to \([M+^{35}\text{Cl}]^-\) and \([M+^{37}\text{Cl}]^-\) in the ratio 3:1. Similarly, use of NH\(_4\)Br would generate twin peaks due to \([M+79]^-\) and \([M+81]^-\) respectively of equal intensity. When neither of these compounds are used, strong \([M-H]^-\) ions are observed. The CIMS may be carried out using several reagent gases; viz., ammonia, methane, argon, etc. It has been observed that if ammonia is used in the positive CIMS it gives rise to strong \([M+\text{NH}_4]^+\) pseudomolecular ions whereas CH\(_3\) or Ar generate strong \([M+H]^+\) ions. Another interesting observation was that use of Ar as reagent gas while little NH\(_4\)Cl is added to the sample gives rise to fragmentation, akin to EIMS\(^2\). This method becomes particularly valuable for observing McLafferty type fragmentations using CIMS instruments. Any uncertainty in the assignment of \([M+\text{NH}_4]^+\) peak could be checked by recording the spectrum using \(^{15}\text{NH}_4\)Cl, thereby exchanging the \([M+18]^-\) ion with \([M+19]^-\) ion.
Mass spectrometer is a powerful tool in the research on marine natural products. It gives accurate information about the molecular weights of organic compounds present as little as a few μg. Different methods such as electron impact mass spectra (EIMS), fast atom bombardment mass spectra (FABMS), field desorption mass spectra (FDMS), and chemical ionisation mass spectra (CIMS) are employed for ionising a given molecule depending on its nature and the type of information required. Among these, EIMS uses electron beams of about 70 ev for ionising molecules while all other techniques use much lower energy and are termed 'soft ionisation methods'. Consequently, EIMS yields relatively smaller molecular ions and more of fragment ions and are more suited for studying relatively less polar compounds. On the other hand, the other methods are more useful in studying polar compounds such as sugars, glycosides, proteins and peptides. Normally samples, as little as a few μg or less are introduced in small vials into the mass spectrometer. However, for meaningful results, it is imperative that these samples be very pure. A further instrumental development was in coupling a gas chromatograph with a mass spectrometer, in what is popularly known as GCMS. This enabled organic chemists to analyse impure mixtures such as fatty acid esters, sterols, etc. The major drawback of this method is that it involves purification using GC, which,
due to the high temperatures involved in separation, limits its use to relatively low polar, heat-stable compounds. With the development of new LCMS techniques, these drawbacks were mostly overcome. This method enables initial LC purification of impure samples and recording the mass spectra of purified compounds immediately. But modern LCMS instruments are very costly, which, at times prevent its widespread use.

As part of our studies on marine natural products, we happened to deal with both pure and impure compounds on mg and sometimes even sub mg levels. GCMS could not be adapted in many such situations due to sample volatility problems. This led us to develop a new instrumental technique for the routine analyses of these samples. The method described below involves introducing samples from TLC spots directly into a mass spectrometer and recording their mass spectra. Thus, this method can be considered as complimentary to GCMS and as a poor man's substitute to the LCMS.

In this method, TLC of a mixture of compounds (synthetic or natural products) is carried out in the usual manner. The chromatogram is later visualized in an iodine chamber or under ultra violet light. About 2-5 mg of carbowax 20 (polyethylene glycol with very high molecular weight), is placed at the tip of a microvial which is in turn attached to the end of a heated solid probe of the mass spectrometer. The small flake of the carbowax is then warmed to 60°C in order
to make it soft and sticky. Touching the TLC spot of interest earlier visualized using iodine vapour or under UV light with this sticky wax enables efficient transfer of the compound of interest from the TLC plate to the microvial which is then introduced into the mass spectrometer with or without NH₄Cl, NH₄Br, etc. Using this technique intense CI mass spectra of several compounds have been obtained. Contamination with background peaks due to carbowax can be avoided by keeping the ion source temperature below 220°C. Some of the mass spectra obtained in this way will be described in the following chapters.

**TLC-O₂-CIMS**

Next, we decided to study the determination of the position of double bonds in unsaturated compounds, especially in long chain compounds such as fatty acids and alcohols, their esters, waxes, etc. This was always very difficult and laborious. The classical way of doing this involves ozonolysis, decomposition of the ozonides into the respective aldehydes, ketones or acids and their purification prior to identification. Evidently, this method cannot be adopted while working with limited quantities or in routine analyses.
With this view, we extended the TLC-CIMS technique for obtaining information about the position of unsaturations in the following manner. Initially the molecular weight of the compound under a TLC spot is determined as described above. Later another spot of the same compound is exposed to ozonised air for 1-2 min. The unsaturated compounds on the TLC plate get ozonised in this way. Subsequently the TLC-CIMS of the spot is recorded in the usual manner. Inside the Mass Spectrometer the ozonides decompose into the respective carboxylic acids or ketones depending on the substitution pattern on the vinyl carbons. Significantly this reaction, which normally is catalysed by reagents such as dimethyl sulphide in the laboratory seems to be catalysed within the Spectrometer by the traces of water present in the silica gel. The carboxylic acids, generated in this manner, produce intense pseudo molecular ions in the negative CI mass spectra. We have found this method to be highly sensitive and very easy to perform. Satisfactory results could be obtained for samples even as low as 200 μg. Oleic acid methyl ester upon this treatment yielded azelaic acid and zuberic acid ester. Petroselinic acid ester, upon similar treatment yielded the corresponding C₁₀ monocarboxylic acid and the C₁₂ dicarboxylic acid monoester. Cholesterol upon TLC-ozonolysis followed by the negative CIMS in presence of traces of NH₄Cl provided intense peaks at m/z 451 and 453 in the ratio 3:1. This corresponded to the combined weight \([\text{Cholesterol}+O_2+\text{Cl}-\text{H}_2\text{O}]^+\), which is expected from trisubstituted double bond...
Fig. 1  TLC - O₃-CIMS of Docosahexaenoic acid.
forming part of a ring system. We have carried out this reaction further, to locate the positions of double bonds in polyenoic fatty acid esters. The NCIMS of docosahexaenoic acid methyl ester is illustrated in FIG 1.

Having successfully adapted this technique to individual compounds, we next attempted to study mixtures of fatty acids, and sterols without prior purification. All organisms contain such mixtures and it was construed as worthwhile to develop simple analytical methods for their analysis. The immediate problem as far as the fatty acids were concerned was the development of a proper technique to separate them into different spots on a TLC plate. These compounds could subsequently be analysed using the TLC-CIMS technique described earlier. Normally these fatty acids or their methyl esters are inseparable on ordinary TLC plates. They can, however, be separated on a TLC plate doped with AgNO₃. This argentation TLC is in fact a very powerful technique in separating close homologues such as mixtures of fatty acids, sterols etc. However, we encountered problems in visualizing the separated spots on these TLC plates. After chromatography we noticed that the entire plate turned black in colour making it impossible to visualize the spots either by iodine vapours or under UV lamp. To overcome this problem we decided to dope only half of the plate with AgNO₃. The other half was left intact. On the former side was spotted a mixture of unsaturated fatty acid esters, viz., methyl esters of
Fig. 2  Separation of a mixture of fatty acid methyl esters (C\textsubscript{16:0}, C\textsubscript{18:0}, C\textsubscript{18:1}, C\textsubscript{18:2} and C\textsubscript{18:3}) on semi-impregnated TLC plate.
Palmitic, Stearic, Oleic, Linoleic and Linolenic acids (16:0, 18:0, 18:1, 18:2 and 18:3 respectively) and chromatographed using 15% EtOAc in pet.ether. Subsequently the plate was turned by 90° and redeveloped in a more polar solvent system such as 40-50% EtOAc-pet.ether in order to transfer these spots into the non-doped silica gel portion. They were then visualized in an iodine chamber and analysed by TLC-CIMS and TLC-O₂-NCIMS techniques. As evident from FIG 2, four separate TLC spots could be identified on the TLC plate. From the TLC-PCIMS it was clear that the least polar (uppermost) spot contained both Palmitic and Stearic acid esters while the most polar (lowest) spot contained linolenic acid ester. The other two spots of intermediate polarity were due to oleic and linoleic acid esters in the order of polarity. Subsequent TLC-O₂-NCIMS revealed the position of the double bonds in oleic acid at C-9 position. However the results with the polyenoic acids were not very satisfactory. This could be probably due to the spreading of these acids on the TLC plates, as a result of the double development or due to the masking of some of the double bonds by iodine vapours, which might have hindered the formation of the ozonides.