ANALYSIS OF ALKALOIDS FROM PLANT CELL TISSUE CULTURES AND OTHER SOURCES

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DOCTOR OF PHILOSOPHY

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ABSTRACT

Specific and sensitive new analytical procedures for the separation and identification of *Cinchona* and opium alkaloids have been developed using on-line high performance liquid chromatography/mass spectrometry (HPLC/MS) with a thermospray (TSP) interface connected to a quadrupole instrument.

The *Cinchona* alkaloid separation was based on the extraction of the alkaloids from *Cinchona ledgeriana* cells using acid base extraction followed by isocratic reversed-phase HPLC. The eluent consisted of ammonium acetate buffer, methanol, and acetic acid, and a C_{18} stationary phase was used. The assays will allow the selection of high yielding plant strains and optimisation of the culture process for the production of these and other valuable compounds.

The method developed for opium alkaloids allowed simultaneous separation of codeine, thebaine, papaverine, noscapine, morphine and its mono and diacetyl conjugates, using an ammonium acetate buffer and acetonitrile, an application that has not previously been reported. Morphine was selectively acetylated to 3-monoacetylmorphine and

the other metabolites were obtained commercially. This assay will be of value to both clinicians and basic scientists.

The TSP spectra of these alkaloids showed only the protonated molecular ions and single ion monitoring provided sensitive and selective detection of the separated compounds. Tandem mass spectrometry experiments were carried out on the protonated molecular ions to allow multiple ion monitoring. Alternative ionisation methods including fast atom bombardment were also investigated.

The mass spectra and tandem mass spectra showed significant differences in the stability of the stereoisomers. These differences were used to identify the structural features responsible for their fragmentation in the ion source and they were also related to the biological activity, the epimers being modelled by molecular graphics. It was confirmed that H-bonding is possible in the stable isomers, and the differences in their activities agree with small differences in the Cheng's distances calculated.

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This thesis is dedicated to my parents, Ramiza and Mohamed Tahir Abdulrahman, and to my family, for their support and encouragement throughout this work.

1. INTRODUCTION

Alkaloids are one of the most important groups of pharmacologically active principles found in plants, and about 30 of them are used medicinally for a wide range of pharmacological effects (Tab. 1.1).

Table 1.1 Some pharmacologically important alkaloids

Pharmacological effect	Alkaloid
Analgesic	Morphine, codeine
Antiarrhythmic	Quinidine
Antimalarial	Quinine
Act on autonomic nervous system	Hyoscyamine, scopolamine
Muscle relaxant	Papaverine

Pharmacists continue to use purified natural products for medicinal purpose, some 25% of prescription drugs being derived from plants.[1] Plant tissue cultures have the ability to produce many secondary metabolites of pharmaceutical interest including a range of different alkaloids.[2] Biotechnology offers the promise of transferring the production of these chemicals to the more controlled conditions of the laboratory

or factory environment through the use of isolated cell tissue culture techniques which can improve yields, lower unit costs and reduce the risks associated with dependence on climatic and political factors.

The examples studied in this thesis are; (a) *Cinchona ledgeriana* root organ cultures which produces quinine and quinidine.[3,4] (b) *Papaver somniferum* plants, potential sources of morphinan alkaloids.[5] (c) *Datura candida* plants which produce tropane alkaloids.[6]

Analytical chemistry has an important role to play in the development and optimisation of these cultures. A rapid, reliable and sensitive method for the assay of the culture medium or extracts of the cultured material is highly desirable.

High performance liquid chromatography/mass spectrometry (HPLC/MS) with thermospray (TSP) ionisation offers several advantages for the rapid development of such assays. The development of derivatisation procedures, generally a requirement for the determination of polar drugs by gas chromatography (GC) is time-consuming; it is, however, unnecessary for HPLC analysis. In addition, the selection of the mass spectrometer

as a detector rather than UV, fluorescence or electrochemical detectors gives the advantage of mass specificity. This can reduce the time for development of a suitable extraction procedure. The TSP technique[7,8] provides a reliable and robust interface which is capable of handling reversed-phase chromatographic eluents with a high aqueous content and a flow-rate of 1-2 ml/min, that are commonly used for the quantitative analysis of polar drugs in such matrices. Furthermore the technique can be automated by combination with the advanced automated sample processor (AASP)[9] or autoinjector.[10]

1.1 FUNDAMENTALS OF PRESENT-DAY MASS SPECTROMETRY

Mass spectrometry occupies an increasingly important place in the natural products and clinical sciences because of its rather unique capabilities, to provide both qualitative and quantitative data that may not be readily available by other techniques. In particular, it can provide molecular weight, empirical formula (through precise mass measurement), detection of functional groups and other substituents, elucidation of structure, including in some cases stereochemical features and detection of compounds at the level of picogrammes or femtomoles. Since stable-isotope-labelled analogues behave like the unlabelled compounds but have spectral features that are shifted because of the label, such compounds can be easily used as internal standards for quantitation. Mixture analysis can be facilitated by the use of GC or HPLC inlet systems, or multistage mass spectral separation.

Various aspects of mass spectral analysis and fundamental processes involved in the technique have been the subject of several review articles and texts.[11-17]

1.1.1 MASS SPECTROMETER SYSTEMS

The mass spectrometer, no matter what type, has several essential components, indicated schematically in Fig. 1.1 : an inlet system, an ion source, a mass analyser, a detection/recording system, and a vacuum system.

1.1.2 THE INLET SYSTEM

Samples that are already gases may be introduced directly into the ion source, with only some device necessary to control the pressure or flow rate. Most samples of interest, however, are not gases and require some more elaborate arrangement.

Pure compounds or simple mixtures that have a measurable vapour pressure at 10^{-6} torr and thermal stability may be introduced via a direct insertion probe. The sample is introduced as a dilute solution in a small capillary tube, from which the solvent evaporates leaving a thin film of sample, the rate of evaporation of which is controlled by a temperature programable heater. For each of the "soft" ionisation modes that are suitable for nonvolatile or thermally unstable

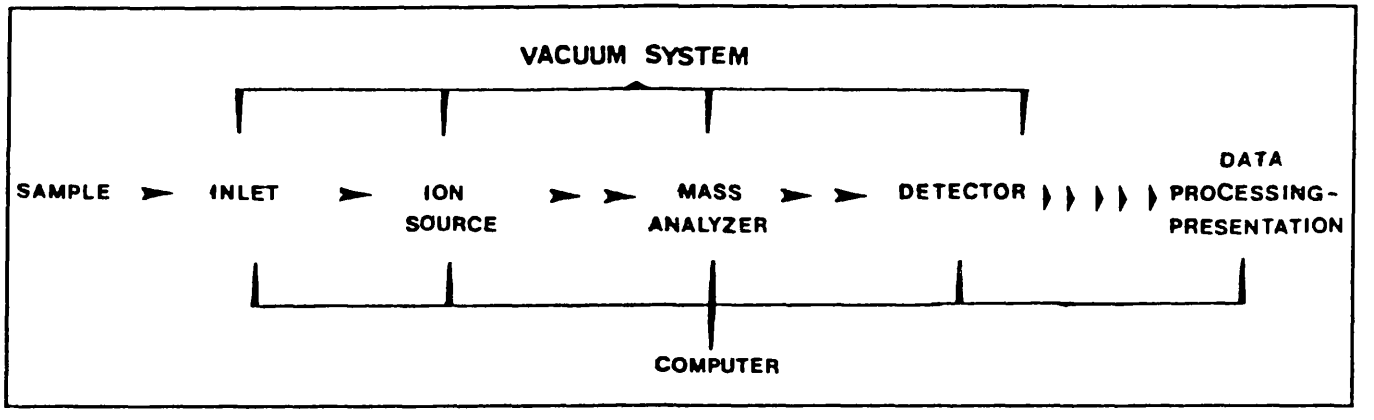


Fig. 1.1 Components of mass spectrometer/data system

samples, a specially constructed probe is required.

Mixtures of volatile and thermally stable samples may be separated into individual components by GC[18], and the effluent from the GC column may be passed into the ion source either directly (if the gas flow is low) or through a separator that selectively removes the carrier gas from the effluent, leaving a sample-enriched flow to the ion source. GC-MS provides the analyst with a uniquely powerful combination, allowing rapid, efficient separation of complex mixtures with accurate and sensitive characterisation of the individual components. The method has proved so successful that GC-MS has had a major impact on mass spectrometer design.

Coupling of an HPLC is also possible and is a necessary choice for analysis of mixtures whose components are nonvolatile or thermally unstable. HPLC-MS interfaces[19] obviously present greater technical difficulties than do GC-MS interfaces. Because the liquid is more difficult to remove than is a gas, HPLC-MS interfaces are much less routine than are GC-MS interfaces, but their availability and role are growing steadily.

1.1.3 THE ION SOURCE

In the ion source sample molecules are converted to charged particles that can then be mass analysed. The oldest (and still the most widely used) ionisation method for organic analysis is electron impact (EI). Other softer ionisation methods now available include chemical ionisation (CI), desorption (direct) chemical ionisation (DCI), and fast atom bombardment (FAB), a category of secondary ion mass spectrometry (SIMS). Each of these may be utilised in either the positive or negative ion mode, other methods for ionisation include ^{252}Cf desorption and laser desorption.

i. ELECTRON IMPACT (EI)

A typical EI spectrum is obtained by transferring a range of energies from the electron beam to the sample molecules at pressures of 10^{-5}–10^{-6} torr so as to produce molecular radical cations $M^{+\cdot}$ with a range of internal energies. These radical cations can decompose to give structurally significant fragment ions (Eq. 1).

$$AB + e^- \longrightarrow {}^*AB^{+\cdot} + 2e^- \qquad \text{Eq. 1}$$

$$^*AB^{+\cdot} \longrightarrow A^+ + B\cdot \qquad A\cdot + B^+$$

This ionisation method provides a mixture of molecular ions, giving molecular weight information, and fragment ions, giving structural information.

There are two types of behaviour which reduce the utility of electron impact mass spectrometry (EIMS) for structure determination, both of which derive from the fact that EIMS is essentially a high energy process.

For some classes of compounds, $M^{+\cdot}$ is intrinsically unstable, so that almost all $M^{+\cdot}$ ions fragment within the ion source and the intensity of the peak given by these ions in the mass spectrum is negligible.

In other cases, isomeric samples yield identical EI mass spectra, indicating that structural information is lost before fragmentation occurs, owing to the fact that the molecular ions formed from the different isomers rearrange to a common structure or mixture of structures on a time scale that is short compared with that required for fragmentation.

ii. CHEMICAL IONISATION (CI)[20-22]

A quite different approach is used in chemical ionisation mass spectrometry (CIMS), in which ions characteristic of the sample molecules are formed in ion-molecule reactions between reagent ions and the sample. A reagent gas or mixture of gases at a pressure of ≈1 torr is bombarded with a beam of high energy electrons to produce a high yield of a reagent ions which may be positively or negatively charged.

The reagent ion may be a molecular ion, fragment ion, or product of an ion-molecule reaction between a primary ion and a reagent gas molecule (e.g., $N_2^{+\cdot}$ from N_2, $O^{-\cdot}$ from N_2O or CH_5^+ from CH_4) which is stable with respect to further reaction with the reagent gas. If a low concentration (0.1%) of sample is introduced into the ion source, very few ions will be formed from it through direct ionisation by electron impact. However, reagent ions may undergo ion-molecule reactions with the sample, the product ions of which constitute the CI mass spectrum of the compound.

It is clear that, for a given sample, the CI mass spectrum that is observed depends very much on the

type of reagent ion that is used to produce the spectrum, and an important feature of CIMS is that by making an appropriate choice of reagent ion, different types of ion-molecule reactions can be used to produce a variety of CI mass spectra. It is therefore possible to "tailor" a CI mass spectrum to suit a particular application under investigation. For example, a gas phase Bronsted acid XH^+ will donate a proton to a sample molecule M, providing that the proton affinity (PA) of M is greater than that of X:

$$XH^+ + M \text{ ------> } MH^+ + X \quad (\Delta H_1) \qquad \text{Eq. 2}$$

where ΔH_1 = PA(X) - PA(M). The extent of fragmentation of MH^+ depends upon the internal energy with which it is formed, which, in turn, is dependent upon ΔH_1. The magnitude of ΔH_1 can be controlled by suitable choice of X and the use of a strong gas phase acid such H_3^+ leads to considerable fragmentation of MH^+, whereas the much weaker acid $C_4H_9^+$ frequently gives MH^+ as the most abundant ion.

An example of ionisation of sample M with a reagent gas CH_4 is shown below:

1. Ionisation of reagent gas:

$CH_4 + e \longrightarrow CH_4^{+\cdot} + 2e$ (EI)

$CH_4^{+\cdot} \longrightarrow CH_3^+ + \cdot H$ (Fragmentation)

$CH_4^{+\cdot} + CH_4 \longrightarrow CH_5^+ + \cdot CH_3$ (Ion-molecule)

$CH_3^+ + CH_4 \longrightarrow C_2H_5^+ + H_2$ (Ion-molecule)

CH_5^+ and $C_2H_5^+$ are the main protonating agents

2A. Proton transfer:

$CH_5^+ + M \longrightarrow MH^+ + CH_4$

$C_2H_5^+ + M \longrightarrow MH^+ + C_2H_4$

Even-electron pseudo- or quasi- molecular ions MH^+ are more stable than the odd-electron molecular ion $M^{+\cdot}$

2B. Charge exchange:

$X^{+\cdot} + M \longrightarrow M^{+\cdot} + X$

Reagent gases: He, Ar, N_2, CO, C_6H_6. Stability and fragmentation of this $M^{+\cdot}$ depends on the internal energy transferred by the impact and on differences in the electron affinities and ionisation energies.

2C. Electrophilic addition:

$C_2H_5^+ + M \longrightarrow (M.C_2H_5)^+$

Reagent gases: CH_4, $i\text{-}C_4H_{10}$, NH_3.

2D. Anion abstraction:

$CH_5^+ + M \longrightarrow (M\text{-}H)^+ + CH_4 + H_2$

This is most evident when the sample is not a very good proton acceptor. This process usually accompanies some of the previous reactions 2a-2c.

iii. DESORPTION (DIRECT) CHEMICAL IONISATION (DCI)[23,99]

DCI makes use of a sample placed on an inert probe tip placed close to the electron beam in a conventional source. Under EI conditions, electron bombardment of the sample surface leads to what is essentially simultaneous evaporation and ionisation, minimising pyrolysis of the sample and yielding MH^+ ions presumably by "self" chemical ionisation. Under CI conditions, the probe tip is placed directly into the CI reagent ion plasma so that the surface is bombarded by reagent ions leading to rapid evaporation and ionisation of the sample. Very rapid heating of the probe under DCI conditions can produce quite satisfactory spectra from very labile molecules. Probe tips made from several metals have been used together with indirectly heated probe tips of polymers such as "Vespel".

Sample loading is usually effected using an electrospray method and CI reagent ions of low

energy (e.g., NH_4^+) are preferred so as to minimise fragmentation of the ion from which relative molecular mass information is derived. This is also affected by the position of the probe tip relative to the axis of the electron beam and by the probe temperature so that optimum conditions will usually be found only by repetitive scanning while conditions are changed slowly.

iv. FAST ATOM BOMBARDMENT (FAB)[24-26]

FAB is the preferred analytical technique for involatile and high mass molecules both on account of its ease of use and its capabilities of producing good quality spectra from a wide range of analytes. The sample is applied to a small quantity of relatively involatile liquid matrix (e.g. glycerol) which is coating a metal surface on the end of a probe (Fig. 1.2). The liquid surface is bombarded in the source with a beam of heavy atoms such as Ar or Xe, but a mixture of atoms and ions will serve as well and the use of Cs^+ is becoming more popular.

The analyte molecules are usually ionised to give protonated pseudo-molecular ions although both

positive and negative species are formed. If salts are present, adduct molecular ions are frequently seen. The ion beams are long lasting and stable, the lifetime of the experiment being limited only by the evaporation of the liquid matrix.

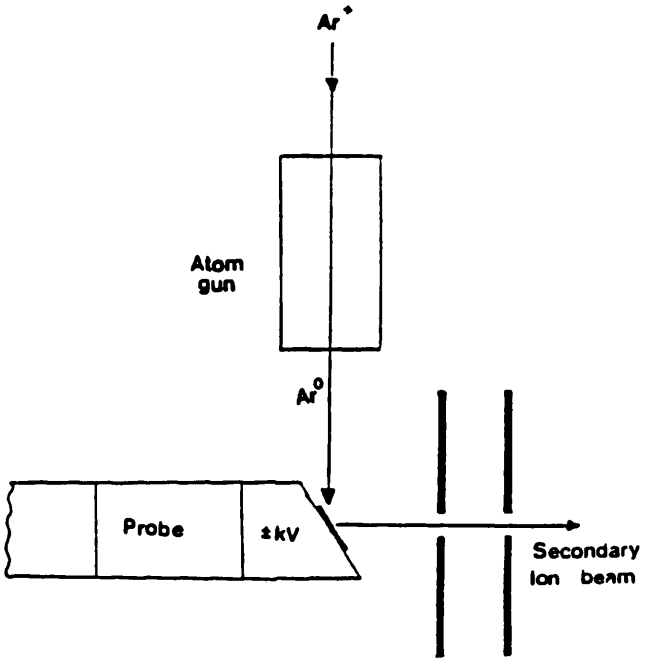


Fig. 1.2 Schematic diagram of FAB source

SIMS is very similar technique but one where no liquid matrix is used. This leads to very short lived though intense ion beams and is of less interest currently to organic mass spectroscopists.

1.1.4 MASS ANALYSERS

The ions produced in the ion source are accelerated by a small (positive) potential on the repeller plate into the mass analyser, where they are separated on the basis of their mass to charge ratio (*m/z*). Most of the ions have a single charge, and the *m/z* value is thus equivalent to the weight in atomic mass units (amu) or daltons (Da).

The most common types of mass analysers use (1) magnetic (B) or a combination of electrostatic (E) and magnetic fields or (2) quadrupoles (Q). Other instruments are available that separate ions on the basis of their time-of-flight (TOF) from ion source to detector or ion cyclotron resonance (ICR). The latter is acquired as a decay signal after an exciting pulse and analysed using a Fourier transform-hence the term Fourier transform mass spectrometry (FTMS). Only magnetic and quadrupole analysers are currently widely used.

i. SINGLE FOCUSING MAGNETIC SECTOR INSTRUMENTS

In a magnetic mass spectrometer the ions traversing the flight tube constitute the electric current and each ion experiences a deflecting force. An ion of mass (m) and charge (ze) accelerated out of the ion source by a voltage (V) will acquire velocity (v), and under the influence of a magnetic field (B) it will follow a circular path of radius (r). The following equations apply:

Ion kinetic energy $mv^2/2 = zeV$ Eq. 3

Deflecting force = centrifugal force

$Bzev = mv^2/r$ Eq. 4

Combining these: $m/z = B^2r^2e/2V$ Eq. 5

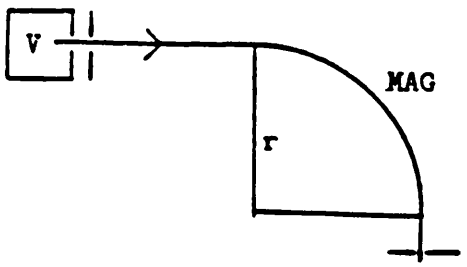


Fig. 1.3 The variables for separation of ions by a magnetic analyser

From (Eq. 5), it can be seen that by varying B or V (Fig. 1.3) it is possible to separate ions of different mass, though in practice it is generally the magnet that is scanned because scanning of the voltage leads to defocusing of the ion beam and loss in sensitivity. The radius r can also be treated as a variable, as in a photoplate detector, or more recently in a multichannel array detector.

Dividing (Eq. 4) by v shows that a magnetic sector is not a true mass analyser as the radius of the ion path actually depends upon the product of mass and velocity *m*v, i.e. the momentum. Single focusing magnetic sector instruments have a limited resolving power due to the spread of translational energies of the ions expelled from the ion source. This can be overcome by the use of an electric sector.

ii. DOUBLE FOCUSING MASS SPECTROMETERS

In a double focusing instrument the ions traverse both an electric and a magnetic field.[27] Ion deflection by an electric field is dependent on the kinetic energy of the ions rather than the momentum i.e. independent of mass. This is

described by (Eq. 6) for an electric field strength (E).

Deflecting force = centrifugal force

$zeE = mv^2/r$ Eq. 6

This is advantageous as the electric field can be used as a focusing element but it does not need to be scanned to allow through the full range of masses. The dimensions and field strengths of the two sectors are selected such that the ions are focused in both energy and in direction. The so called normal geometry instrument is shown in Fig. 1.4.

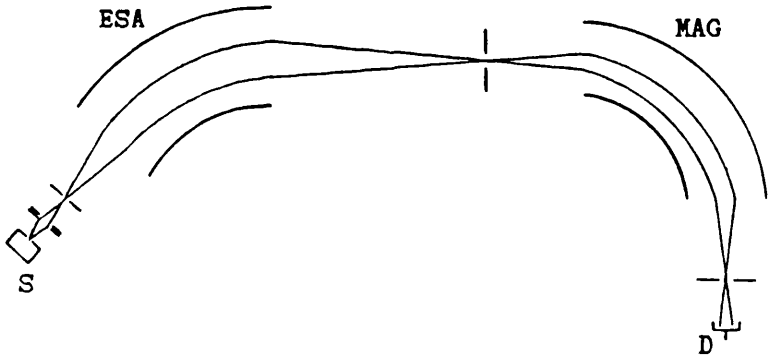


Fig. 1.4 Schematic diagram of a normal geometry double focusing mass spectrometer

It is possible to construct the mass spectrometer with the magnet preceding the electric field, the reverse geometry mass spectrometer[28] (Fig. 1.5).

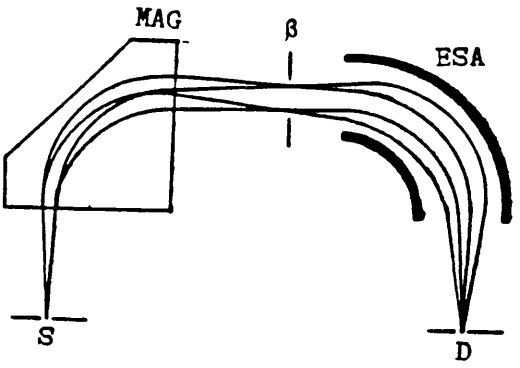


Fig. 1.5 Schematic diagram of a reverse geometry double focusing mass spectrometer

Here analysis, as the name suggests, is the reverse of a normal geometry instrument. Fig. 1.5 demonstrates how an angularly and energetically divergent beam of ions is focussed at the collector slit by the combination of magnetic and electric analysers.

This feature allows high mass resolution to be attained. The resolving power required to separate a peak due to ions of mass M from a peak of mass M+ΔM is defined as M/ΔM. Two peaks of equal height are usually defined as being resolved when the valley between them does not exceed 10%

of the peak height (10% valley definition) (Fig. 1.6).

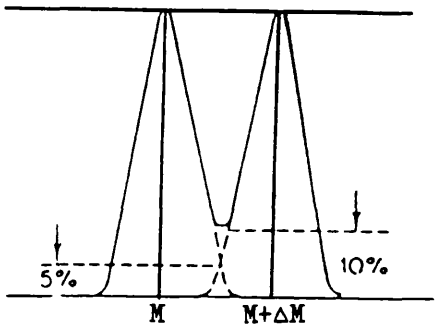


Fig. 1.6 Definitions of resolving power

iii. QUADRUPOLE ANALYSERS[29,30]

Quadrupole analysers are a more recent development than sectors. Quadrupole mass spectrometers are generally less expensive, lower performance instruments, not capable of high resolution, but they offer the advantages of flexibility, simplicity, ease of interfacing to LC and GC (as they do not use high potentials in the ion source), ease of computer interfacing and mass calibration (as they have a linear scan law), and speed of scanning and stepping between peaks for multiple ion monitoring.

As explained above, the magnetic sector is actually a momentum analyser and the electric sector is a kinetic energy analyser, although both can give mass analysis under certain conditions. However, the quadrupole analyser is a true mass analyser as it is unaffected by changes in ion velocity, i.e. in momentum or kinetic energy.

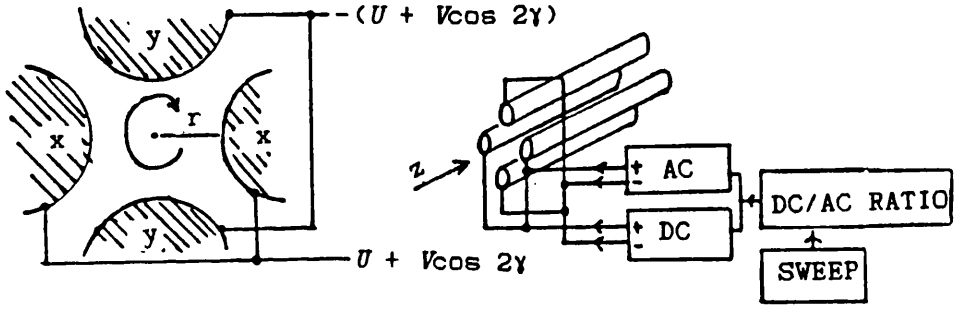


Fig. 1.7 Quadrupole analyser

Fig. 1.7 shows the quadrupole analyser, which consists of four parallel rods. The rods are electrically connected in diagonally opposite pairs and mass separation is achieved by applying a DC potential $+U$ to one pair (x) and $-U$ to the other (y), and superimposing a radio-frequency AC potential ($V\cos 2\gamma$), which differs in phase by 180° between the pairs of rods. The peak value of the AC voltage is greater than the DC voltage, so

the "positive" pair are sometimes negative, and *vice versa*, as shown in Fig. 1.8.

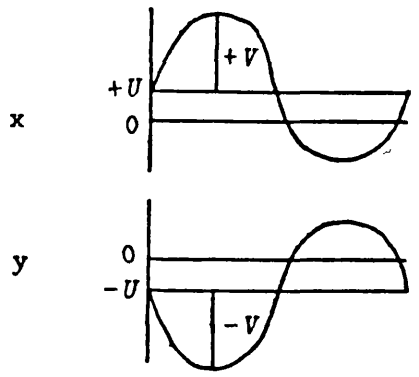


Fig. 1.8 AC and DC voltage

Low kinetic energy ions (≃ 20ev) are introduced and under the influence of the oscillating field they follow a spiral path through the analyser between the rods (along the z axis). Their motion on the x and y axes is defined by the Mathieu equations, which are of the form shown below for x (Eq. 7), with an identical equation for y:

$$d^2x/d\zeta^2 + (a + 2q\cos 2\zeta)x = 0 \qquad \text{Eq. 7}$$

Where x = displacement

ζ = time function = $(\omega t/2)$

The terms *a* and *q* are related to the geometry of the quadrupole system and to the applied potentials by:

$a = 8eU/mr^2\omega^2$ Eq. 8

$q = 4eV/mr^2\omega^2$ Eq. 9

where r = distance from rod surface to centre line

thus $a/q = 2U/V$ Eq. 10

It can be seen that if the AC frequency ω is kept constant, m is linearly proportional to U and V. In scanning the instrument the ratio a/q is kept constant by maintaining a fixed ratio between U and V (Eq. 10), which are scanned simultaneously to give a spectrum which is linear in mass.

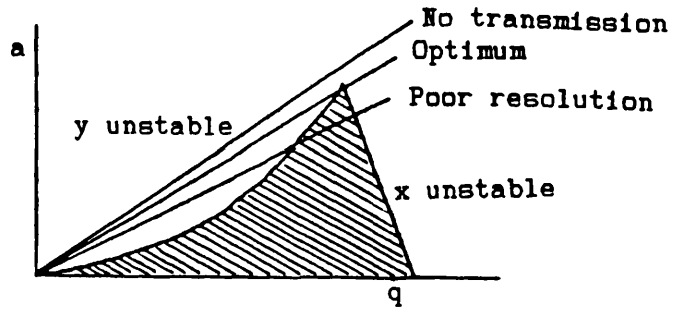


Fig. 1.9 Stability diagram for quadrupole analyser

The behaviour of the ions is defined by the stability diagram shown in Fig. 1.9. Within the shaded triangle the ions follow stable orbits and they are transmitted through the analyser, whereas outside this triangle the orbits become infinitely large and the ions are lost. The gradient of the

scan line determines the proportion of ions transmitted. If $U = 0$, i.e. no DC voltage, all ions will pass through and there will be no mass separation.

As the gradient of the scan line is increased, i.e. as U is increased relative to V, the proportion of ions following stable orbits decreases and the mass resolution increases. If the scan line is too steep it will not intersect with the triangle at all and no ions will be transmitted. The gradient a/q is therefore selected to cut the shaded triangle near its apex and give the optimum combination of transmission and mass resolution.

The resolving power of the quadrupole can not be defined by the $M/\Delta M$ relationship which applies to sectors, for which the actual peak width increases linearly with mass. With a quadrupole the peak width is constant throughout the mass range, and if the instrument is set up to resolve adjacent mass numbers it is described as having unit resolution.

iv. TANDEM MASS SPECTROMETRY

Tandem mass spectrometry can be carried out by mass selecting a specific ion[31] and following its unimolecular (metastable) or collision induced decomposition (CID)[32,33] between the two sectors (FFR2) by scanning the electric field voltage. The resultant mass analysed ion kinetic energy spectrum (MIKES) can be used for elucidation of ion structure, and energy release data can be obtained from the peak profiles. The CID spectrum of an ion is obtained by raising the pressure in a field free region of the mass spectrometer by the introduction of gas e.g. helium. The ions in the beam collide with the gas molecules which leads to some of the translational energy of the ions being converted into internal energy. This increase in internal energy of the ions causes subsequent decomposition of the ion, the resultant spectrum recorded being the CID spectrum for the chosen ion which is characteristic of the ion structure.

Similar information may be obtained from the forward geometry instrument by tuning the magnet to transmit a selected ion and then scanning accelerating voltage, i.e. high voltage (HV)[34] scans. Here all the parent ions for a given daughter ion may be recorded for processes

occurring in FFR1. This is limited by the range of HV that provides effective ion focusing.

A further technique available is that of linked scanning. B/E linked scanning is the most useful and widely used of the several linked scanning techniques.[35,36] Here the ratio of magnetic field and electric field is kept constant whilst scanning both fields. The resulting spectrum reveals all daughter ions from preselected parent, and gives higher resolution, but gives no information on the kinetic energies of these ions, unlike MIKES. Linked scanning is applicable to either geometry instrument.

The several types of mass analysers including quadrupoles can be combined to form even more powerful analytical instruments (EBE, BEB, BEQQ, QQQ, EBEB, BEEB, etc) that provide more than one dimension of mass separation, with the possibility for CAD of ions separated in the first stage before the second mass analysis. This feature allows structural studies of the relatively stable molecular ions produced by the "softer" ionisation modes.

1.1.5 DETECTORS

Ions exiting the mass analyser are usually detected with a secondary electron multiplier (SEM), which converts the ion current to an amplified electrical current, achieving a gain of 10^6 or more. This type of detector has fast response over a very wide dynamic range and low background noise. Other possibilities include photoplate detection, ion-counting, and continuous monitoring of ion currents with a faraday cup.

1.1.6 DATA ACQUISITION AND CONTROL SYSTEMS

The need for precise control of many operating parameters and the very high data production rate of the modern mass spectrometer make computer systems an essential requirement, particularly with GC/MS and LC/MS, for which spectra may be scanned at a repetition rate of perhaps one scan per second over long periods of time.

The data system is usually capable of three basic operations: (1) control of the scanning of the mass spectrometer and perhaps various focusing parameters through digital/analogue converters, (2) data acquisition from the mass spectrometer

through a complex interface that incorporates analogue/digital conversion with various thresholding and peak detection devices, and (3) data processing. Ideally it should perform all three functions essentially simultaneously. Modern computers with micro processor controlled interfaces are capable of acquiring data at 250 kHz with a dynamic range of $1:10^6$ for peak detection. As well as controlling the mass spectrometer and acquiring the data they can also display a "real-time" chromatogram for total ion current or for several selected ions, or they can display selected mass spectra. Subsequent data processing allows a variety of manipulations including background subtraction, smoothing, spectrum averaging and the display and out-putting of the data in a variety of formats.

1.1.7 VACUUM SYSTEMS

In order to permit the use of high voltages, to avoid unwanted collisions of ions and molecules, and to prevent contamination of the MS, an effective vacuum system is required. For high vacuum requirements, there is a choice of diffusion pumps, turbomolecular pumps, cryopumps, and ion-getter pumps. Selection will depend on

factors such as expected pumping load, ultimate vacuum desired, initial cost, maintenance cost, and reliability. Chromatographic interface and ionisation methods requiring high gas flows such as CI, thermospray, electrospray and FAB put extra loads on ion-source pumping. Techniques such as thermospray which involve direct introduction of liquids at high flow rates generally demand the use of high-capacity mechanical pumps to remove the majority of the vapour.

2. ANALYTICAL TECHNIQUES FOR MONITORING OF DRUGS AND THEIR METABOLITES

The variety of sensitive and specific methods currently available to the analyst is quite diverse and covers a wide linear dynamic range for quantitation[37] (Fig. 2.1). These include chromatographic techniques with a variety of selective detectors to ensure specificity, e.g., gas chromatography (GC) with ionisation detectors such as electron-capture (ECD), nitrogen-phosphorus specific detector (NPD), mass spectrometric-chemical ionisation detectors (CI-MS), HPLC with UV, fluorescence and electrochemical detectors (oxidative and reductive-polarographic), high-performance thin-layer chromatography (HPTLC) with in situ spectrophotometry/fluorodensitometry. There are also non-chromatographic techniques such as spectrophotometry (UV-Vis), luminescence methods (fluorescence and phosphorescence), differential pulse polarography (DPP) and radioimmunoassay (RIA). All of these methods are capable of quantitation over a wide linear dynamic concentration range.

Among the whole range of instrumentation used, chromatography has long played a vital role. The

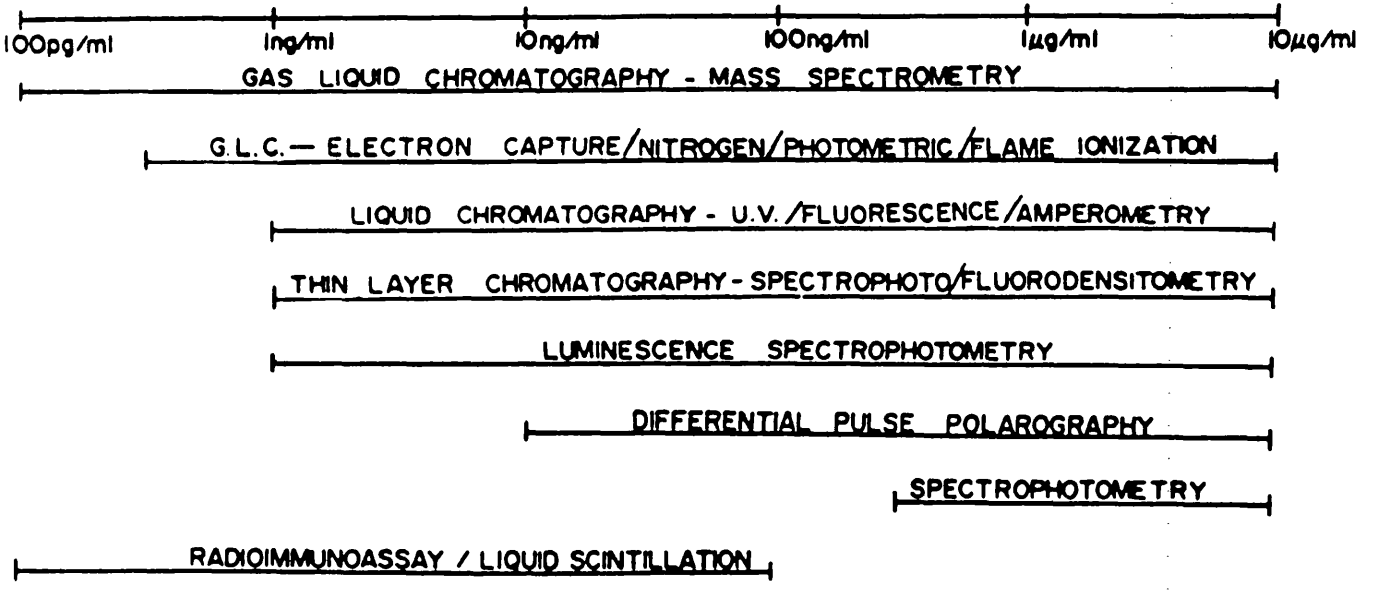


Fig. 2.1 Practical range of usefulness of analytical techniques.[37]

separation and identification of trace amounts of drug alkaloids in complex mixtures can be carried out by TLC, GC, or HPLC, with choice of the analytical method being governed either by the intrinsic chemical and physical properties of the molecule or its amenability to chemical derivatisation to render it compatible with quantitation by a specific method.[38,39] Factors responsible for compound losses during sample preparation (adsorption, stability) are critical at low concentrations and may adversely affect the reliability of an assay. Consequently, maximizing the overall recovery of the assay is essential not only for sensitivity but also for good precision and accuracy. The type of assay selected will also govern the amount of sample preparation and clean-up required.[40]

All of the three chromatographic techniques referred to above are well established[41] and possess a high degree of selectivity with good sensitivity.[42] However, when used in combination with conventional detection systems they are not in themselves sufficiently specific, and often provide only minimal structural information for unknown compounds. Mass spectrometry is an analytical technique which combines high sensitivity with structural specificity, and by

coupling this technique with chromatographic separation, a very powerful analytical tool is created.

GC has been used extensively in the analysis of alkaloids. Many compounds which are not directly amenable to gas chromatographic study because of their low volatility and/or thermal lability can be chemically converted into compounds which can then be handled by this technique. Typical derivatisation reactions include the protection of alcohol, amine and acid moieties by alkylation, trialkylsilylation, esterification, acetylation, etc. However, derivatisation is not always an effective approach, particularly if one does not know the nature of the compounds being studied. Further complications can arise from chemical reactions taking unexpected courses and incomplete derivatisation. There are also many compounds which are not readily amenable to conversion to volatile derivatives.

HPLC has many advantages over some of the earlier separation techniques, including high selectivity, application to non-volatile samples, speed, and ease of quantitation. Generally, liquid chromatographic procedures require fewer extraction steps as compared to those of GC and

they are well suited to the analysis of polar materials. Precision and accuracy are thus enhanced. For such reasons HPLC is finding increasing use in alkaloid analysis.

2.1 HPLC OF ALKALOIDS

The analysis of alkaloids by high-performance liquid chromatography (HPLC) has always been hampered by the basic properties of the alkaloids and the weakly acidic properties of the silanol groups present in silica gel. Even in chemically bonded stationary phases, uncovered silanol groups may interact with the bases, resulting in chemisorption, usually recognized by tailing of peaks.[43-45] The addition of basic modifiers such as ammonia or amines to the mobile phase[46,47] often improves the column performance and the peak shapes of basic compounds. Dimethylformamide has also been reported as a useful additive for improving the peak shapes of basic compounds in reversed-phase HPLC.[48] As chemically bonded phases and silica gel deteriorate rapidly under basic conditions owing to dissolution of the silica, either guard columns can be used to protect them, or the mobile phase can be saturated

with silica[49] to prevent dissolution of the stationary phase.

The problems of column degradation mentioned can be circumvented if the analyses are performed under acidic conditions. Under such conditions the alkaloids are protonated and require a more polar eluent in normal-phase HPLC than do non-protonated alkaloids, whereas the opposite holds true for reversed-phase HPLC.

The HPLC of some alkaloids has been reported using ion-exchange,[50-52] ion-pair,[53,54] straight-phase[55] and reversed-phase.[56,57] The application of ion-pair HPLC to the analysis of alkaloids has proved to be a very useful technique, but ion-pairing reagents are not suitable for TSP LC/MS analysis. Simple ammonium acetate systems can often replace complicated buffers, without ion-pairing agents, with improved column selectivity and efficiency. Ammonium acetate also offers the best sensitivity in methanol-water and acetonitrile-water solutions in thermospray analysis,[58] and NH_4^+ decreases polar interactions by masking free silanol groups on the bonded stationary phase.[59]

2.1.1 ADVANTAGES OF AMMONIUM ACETATE

Ammonium acetate has many properties which are particularly attractive as a general purpose buffer. It is: (1) chemically stable, non-toxic, inexpensive, and readily available; (2) fully ionised and almost neutral in water; (3) highly soluble in methanol and acetonitrile; (4) an excellent masking agent for residual silanol groups on chromatographic media leading to greatly improved separation; and (5) relatively volatile and easily removed after preparative separation or when used in conjunction with a mass spectrometer.

2.1.2 DISADVANTAGES OF AMMONIUM ACETATE

One of the most obvious disadvantages of ammonium acetate, as of all carboxylic acid buffers, is its relatively high UV cut-off value (220 nm). However, since the great majority of medicinally important alkaloids have absorptions above 220 nm, this disadvantage is generally minimal. However, it is a serious disadvantage in case of *Datura* alkaloids, as the optimum absorption of these alkaloids occurs at 210-215 nm.

2.1.3 RETENTION MECHANISMS WITH AMMONIUM ACETATE BUFFERS

The most important retention mechanism with ammonium acetate buffer on reversed-phase columns is undoubtedly hydrophobic interaction between the solutes and the non-polar stationary phase surface. The hydrophobic interaction is, however, not the only basis for separation. It is known that highly polar or strongly basic molecules can interact with the free silanol functions at the surface of the stationary phase through hydrogen bonding and/or ion-pairing mechanisms.[60,61] Furthermore, basic ions extracted by the stationary phase may act as ion-exchangers. Ammonium acetate is also able to form ion-pairs with solutes, while the association of the NH_4^+ ions with the stationary phase may lead to ion-exchange properties.

3. LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY (LC/MS)

The coupling of chromatographic separating techniques with the high sensitivity and structural specificity of mass spectrometry has been utilised over the last twenty-six years with GC-MS[62,63] and more recently and to a lesser extent with TLC-MS[64].

GC-MS has had a major impact in the analysis of organic compounds. Unfortunately, GC has certain limitations in that it is not well suited to handle highly polar compounds which may be non-volatile or thermally labile. This covers a large number of biologically active compounds, such as drugs, peptides, and antibiotics. Consequently HPLC has become the method of choice for the separation of complex mixtures.[65]

One of the problems with HPLC alone is that there is no universal detection system available. UV detectors are the most commonly used, and usually selected wavelengths are monitored. This means that compounds which do not absorb at the wavelength being monitored are overlooked, and that for compounds which do not have suitable chromophores for UV detection, other forms of

detection must be used. Thus LC/MS can assist in the characterisation of compounds not otherwise observed with UV detection systems. Alternative methods such as fluorimetry are also extremely compound-specific.

The other problem with LC alone is that the efficiency of conventional LC columns is poor compared with capillary GC columns. Thus, HPLC chromatographic peaks may contain unresolved compounds that nonspecific detectors cannot differentiate. The mass spectrometer enables multicomponent peaks to be identified and components to be resolved, and stable isotopically labelled compounds can be similarly resolved when used as internal standards for quantitation. This, coupled with need for a more specific, selective and sensitive detector for HPLC, has generated considerable interest in the development of routine LC/MS.

In the sixteen years since reports of on-line HPLC/MS first appeared (Baldwin *et. al.*[66]), many different approaches to this inherently-difficult interfacing problem have been tried,[67-69] each showing early promise but few really maturing into a routine analytical tool.

Coupling of HPLC to MS is a difficult task, as the MS works at high vacuum, e.g., 10^{-7} torr, whereas conventional LC conditions use 1-2 ml/min of liquid eluent. In order to couple these two techniques the excess solvent must be removed either prior to introduction into the MS, i.e., indirect coupling, or during introduction of the sample into the MS, i.e. direct coupling.

3.1 INDIRECT COUPLING

3.1.1 MOVING BELT

The original moving belt systems are based on Scott's[70] studies using a moving wire, onto which the LC eluent was passed. Solvent was removed in two vacuum locks and, electrical heating was used to vaporize the solute into the ion source of a quadrupole mass spectrometer, where EI or CI spectra were obtained. This approach suffered from poor transfer efficiencies, and considerable improvements were obtained by use of a continuously moving belt of stainless steel.[71] Kapton (polyimide) has proved to be a more suitable belt material causing less sample decomposition than stainless steel.

A number of methods for improving the performance of interfaces of the moving belt type have been described. Deposition of the LC eluent onto the belt system by use of a nebulizer[72,73] overcomes the problems encountered in handling high percentage aqueous mobile phases and eliminates the need to use a splitting device for handling lower percentage aqueous mobile phases, which results in improved on-column detection limits. Use of microbore LC produces similar results,[74-76] but less sample must be injected on-column if chromatographic performance is to be maintained and sample injection volumes are also limited. Most interfaces for LC/MS have problems in coping with non-volatile buffers and ion pair systems. This difficulty has been overcome with the moving belt by use of a continuous extraction interface.[77-80]

One of the major existing problems with moving belt interfaces is the decomposition of thermally labile compounds at low levels,[81] this will limit the utility of interfaces of this type for quantitative studies.

Recent reports by Games *et al.*, 1989[82] suggest that although the detection of simple mixtures of low polarity compounds is possible with moving

belt HPLC-MS, it is unsuitable for the analysis of complex mixtures including high-polarity and involatile compounds.

In order to extend the application range of the belt interfaces, surface ionisation methods will have to be used. The use of secondary ion mass spectrometry (SIMS) has been demonstrated with transport interfaces[83] and the potential for the use of laser desorption has also been shown.[84] A discontinuous system using ^{252}Cf plasma desorption has been described,[85] as has a semi-automated laser desorption system.[86]

The combination of the belt with the technique of FAB[87,88] allows the ionisation and determination of involatile high molecular weight compounds that are not amenable to other modes of analysis. Moving belt systems, within the range of compounds which they can presently handle, have proved to be reliable and relatively easy to use. The provision of both EI and CI data with a free choice of reactant gases[89] is particularly useful in qualitative studies when the types of compounds present are not known.

The technique of supercritical fluid chromatography/mass spectrometry (SFC/MS)[90]

appears uniquely suited to the moving belt LC/MS. The deposition of SFC effluent onto the moving belt is a convenient means of accomplishing packed-column SFC/MS while obtaining either EI or CI mass spectra. This technique is likely to be developed further, and holds great promise for future applications.

3.2 DIRECT COUPLING

3.2.1 ATMOSPHERIC PRESSURE IONISATION (API)

Atmospheric pressure ionisation has the advantage that all the eluent from the liquid chromatograph can be introduced into the ion source. Ionisation is effected by a discharge or radioactive source and the ionised solvent molecules then ionise the solute molecules, which are mass analysed in a quadrupole mass analyser. This was one of the earliest approaches to LC/MS,[91] however early systems suffered from difficulties in vaporizing even moderately volatile compounds. Other problems included the simple spectra produced which, although they provide molecular weight information gave little structural information, and the presence of cluster ions due to the

solvent which caused problems in obtaining total ion current traces. Although excellent sensitivity was obtained for compounds with high electron affinities in the negative mode, sensitivities for other classes of compound were poor. Finally highly purified solvents were necessary.

Recent developments have overcome some of these problems. Using a nebulizing system to introduce the sample enables lower volatility compounds to be handled[92,93] and liquid ion evaporation techniques now provide an excellent method for handling ionic compounds.[94,95] More structural information can be obtained either by variation of the potential at the pinhole leading to the mass analyser[96,97] or by using collision induced decomposition in a triple quadrupole mass spectrometer.[98]

3.2.2 DIRECT LIQUID INTRODUCTION (DLI)

The basis of the direct liquid introduction (DLI) approach to LC/MS is:

1. The observation that introduction of a sample in solution into the CI source of a mass spectrometer has a beneficial effect in that

vaporization of the sample takes place at a lower temperature than if conventional sample introduction is used.[99]

2. 1-2% of the eluent from an analytical liquid chromatograph can be introduced into the CI source of a mass spectrometer and solvent induced CI spectra of the solute are obtained.[66]

Early systems used a simple capillary inlet and had problems in handling low volatility compounds because the inlet was easily blocked. A major advance was the development of a water cooled introduction system having a diaphragm with a small diameter hole, enabling nebulization of the eluent from the liquid chromatograph into the mass spectrometer ion source.[100,101] In addition, incorporation of a desolvation chamber which assisted in the desolvation and ionisation of solute molecules was found necessary to study low volatility compounds.[102-104]

A second problem with this approach to LC/MS is the on-column sensitivity that can be obtained if conventional LC is used, since only a small portion of the eluent from a conventional analytical liquid chromatograph is fed into the mass spectrometer ion source. Improvements in

mass spectral pumping, particularly the use of cryogenic pumps assist in the handling of higher flow rates of some solvent systems.[106] However the most dramatic improvements in detection limits are obtained by the use of microbore LC; here, 0.5-1 mm i.d. columns are used with flow rates of 20 µl/min and all of the eluent from the liquid chromatograph can be fed into the ion source of the mass spectrometer.[106] A splitting system will still be needed if these columns are going to be used at their optimal flow rates. An alternative approach has been the use of capillary columns,[107] with flow rates of 5 µl/min, although these do present considerably more technical problems than do microbore systems.

The major difficulties with these interfaces are:

1. Only solvent induced CI spectra are obtained, although it has been shown that conventional CI reagent gases can be introduced to improve spectral quality.[101] Such spectra are often very simple, consisting mainly of protonated molecular ions with few structurally useful fragment ions. One solution to this problem is to use collision induced decomposition to obtain more structural information.[108]
2. The solvent system which optimises spectral

quality may not be the optimum for LC separation. Also changes in solvent composition when gradient LC is used may affect the spectra obtained.

3. Care must be exercised in the quality of solvents used, and filtration systems have to be incorporated to ensure that blocking of the inlet does not occur.
4. Sensitivities measured in terms of sample injected on-column are poor if conventional LC is used.
5. The technique appears to be limited to the use of normal phase LC by the requirement for the solvent to be volatile. This could place severe restrictions on the quality of the LC for some types of study where reversed-phase columns are preferable.

However the technique has been shown to be very useful, particularly when known compounds must be identified. Also, because of the lack of decomposition with thermally labile compounds, it has advantages over interfaces of the moving belt type in quantitative studies on this class of compound.

3.2.3 CONTINUOUS FLOW FAB (CF-FAB)

FAB ionisation see (1.1.3.iv), which is carried out in the cold without any filaments and other hot surfaces, could provide a simple method for implementing the combination of mass spectrometry with other chromatographic techniquies.[109]

Basically, in CF-FAB, a direct insertion probe containing a fused-silica capillary transfer tube is introduced directly into the mass spectrometer ion source. The capillary terminates at the target of the probe, which is bombarded by a beam of heavy atoms. The sample is introduced into the flow of 95% water and 5% matrix (usually glycerol), via a micro HPLC injection valve. Glycerol is required to provide a nonvolatile hydrophilic coating on the target so that the sample solution flows smoothly over the surface and is either absorbed in a filter pad below the target or evaporated by gentle heating. Sample injection volumes range from ≈0.5 to 5 μl. Of course, the larger the injection volume becomes, the longer the time the sample will persist on the target.

Conventional FAB ionisation produces a spectrum with a signal at every *m/z* value over the entire

range of masses scanned. These signals are mostly derived from ions produced by the matrix, and this effect can lead to an unacceptably low signal-to-noise ratio for samples of low concentration. It was soon realised that the reduction in the concentration of glycerol in the CF-FAB device gave FAB spectra which were considerably improved in this regard.[110] Together with the ability to perform on-line HPLC/MS experiments this interface has shown considerable promise for analysing low levels of polar substances. There are, however, surprisingly few reports in the literature of the use of this device for routine quantitative studies and the major reason for this would appear to lie in the difficulty in maintaining a stable ion beam for a prolonged period of time.

The most common reasons for unstable flowing FAB ion beams lies in the formation of droplets of liquid from the carrier flow which evaporate suddenly into the ion source giving pressure surges and defocussing the ion beam as well as contaminating the source and slits. It is essentail to create conditions in which a stable thin film of liquid is formed and maintained on the FAB sample stage.[111]

Recently the use of on-line RP-HPLC FAB MS in structural studies of proteins, glycoproteins and synthetic peptides has been investigated by Roberts et al.[112] The application of the technique in determining environmental pollutants has been reported by Brumley.[113] Subnanogramme sensitivity of CF-FAB with MS/MS techniques has been reported by Marbury and Charles.[114]

Two other techniques are coming into increased use, especially in the biological field, in which analytes are present in flows of liquid at about 1-5 µl/min. These are separation by capillary zone electrophoresis and analysis through the use of microdialysis probes.[115] In the former an electrophoretic separation is carried out in a buffer solution contained in a fine fused silica capillary column. Ionisable species, typically peptides and proteins, separate according to their charge and flow through the capillary by electro-osmosis. The application of the on-line CF-FAB MS with microdialysis in vivo measurements of drug pharmacokinetics has been reported by Caprioli and Lin.[116]

3.2.4 ELECTROSPRAY

The basic principle of electrospray is the evaporation of ions from an electrolyte containing liquid by the application of a high electric field, 8-10 kV, to the surface of the liquid.[117] The first experiments on an electrospray ion source were carried out by Dole *et al.*,[118] They attempted to produce beams of charged macromolecules by electrospraying a solution of polystyrene molecules into a bath gas to form a dispersion of macroions that was expanded through an orifice as a supersonic free jet into vacuum.[119,120] These authors believed that as the droplets evaporated, the increasing coulombic repulsive forces came to exceed the surface tension so that the droplet underwent fission. Successive fissions ultimately gave rise to droplets containing a single solute molecule that retained the droplet charge as the remaining solvent evaporated.

Electrospray is now enjoying a renaissance with its coupling to an atmospheric ionisation source which produces highly multiply charged ions from practical LC mobile phases and hence allows high mass molecules to be mass analysed on low mass range instruments. Proteins of masses greater

than 100,000 Da have been studied successfully, and early results suggest that higher mass accuracies can be obtained with electrospray/quadrupole mass spectrometers than with laser desorption and plasma desorption time of flight mass spectrometers.[121]

The electrospray-type approach offers some attractive features for LC/MS. These include the absence of critical temperature control as in thermospray LC/MS see (3.2.5), GC/MS-like sensitivity and the absence of a small orifice, which can cause practical difficulties.

3.2.5 THERMOSPRAY (TSP)

Since the commercial introduction of thermospray, approximately six years ago, this technique has gained tremendous popularity both as a new soft ionisation technique and as a versatile LC/MS interface. A great deal of excellent work has been reported from many laboratories throughout the world.[122]

i. PRINCIPLE

The TSP technique has emerged from efforts to develop an LC/MS interface suitable for efficiently analysing samples dissolved in aqueous mobile phases at typical analytical flow rates on the order of 1 ml/min. Early work employed a focused CO_2 laser to vaporize the liquid.[123] Later the laser was replaced by an array of oxy-hydrogen torches.[124,125] In the present version, an inexpensive electrical heater is employed. The original approach involved the production of a molecular beam of the vaporized effluent which could be directed into an EI or CI source. In the course of this work it was found that, under certain conditions, ions were produced even though the hot filament normally used to produce the primary ionising beam was turned off.[7]

Initial measurements of mass spectra produced from nonvolatile compounds such as peptides, showed that the spectra were quite different from those obtained by chemical ionisation and were, in fact, most similar to those obtained by field desorption.

ii. INSTRUMENTATION

A typical schematic diagram for TSP experiments is shown in Fig. 3.1. Solvent flow from the chromatographic pump is directed through an injector to a selector valve that permits the operator to select a direct sample loop or to perform a chromatographic separation. The direct sample loop is extremely valuable for samples containing only a small number of components and is also used for a preliminary survey of complex mixtures prior to LC separation. From the loop or column, the sample passes through a protection valve and into the TSP vaporizer. The vaporizer consists of a stainless-steel capillary with a

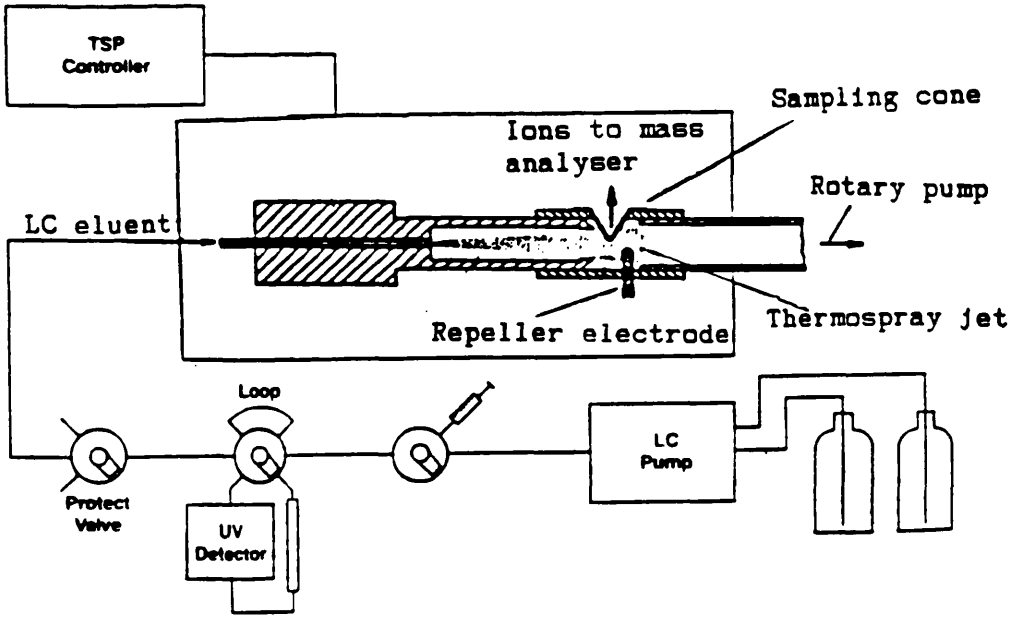


Fig. 3.1 Schematic diagram of thermospray LC-MS system

nominal I.D. of 0.15 mm. In most configurations, the liquid is carefully heated by passing a current directly through the stainless-steel tubing so that the liquid emerges from the tip of the vaporizer as a fine spray, directed through the jet chamber or source block.[126] Because the droplets formed in this process are small, effective evaporation occurs with minimal thermal contact with the walls of the jet chamber.

iii. IONISATION MODES[127]

Three processes are currently used for sample ionisation; buffer ionisation, filament ionisation and discharge ionisation.

The buffer mode requires that the chromatographic effluent contain a buffer salt, which is preferably volatile. Ammonium acetate has proven to be the most versatile buffer. It can be present during the chromatographic separations or, if desired, can be added post-column using an additional high-pressure pump and a zero-dead-volume tee.

Filament and discharge ionisation are complementary ionisation modes that are closely

related to the chemical ionisation process used with conventional ion sources. Filament ionisation requires a rugged emitter (thoriated iridium is commonly used) and at least 600-800 V acceleration to obtain effective penetration of electrons into the jet chamber.

Discharge ionisation can be obtained with a high voltage electrode which protrudes slightly into the jet chamber. Because of the relatively high pressure in the TSP jet chamber (5-15 torr), a stable discharge is obtained in a voltage range of 800-1400 V.

The reasons for using filament or discharge ionisation are: (1) low-polarity solvents cannot dissolve a buffer salt; (2) the presence of buffer may impair chromatographic separations; (3) some samples do not ionise efficiently by buffer ionisation; (4) for low-flow studies (i.e., below 0.4 cm^3/min), where buffer ionisation is ineffective; (5) some compounds that give only molecular adduct ions by buffer ionisation may give fragment ions in filament or discharge-ionisation mode. These auxilliary modes of operation clearly extend the use and versatility of the TSP system.

iv. BUFFER IONISATION MECHANISMS[128]

The overall mechanism is illustrated schematically in Fig. 3.2. The primary ions produced in the thermospray process are apparently identical to those present in solution. For example, an ammonium acetate solution produces NH_4^+, CH_3COO^-, and clusters of these ions with water, ammonia, and acetic acid. Approximately equal total intensities of positive and negative ions are observed. The ion current is strongly dependent on the liquid flow rate and vaporizer temperature.

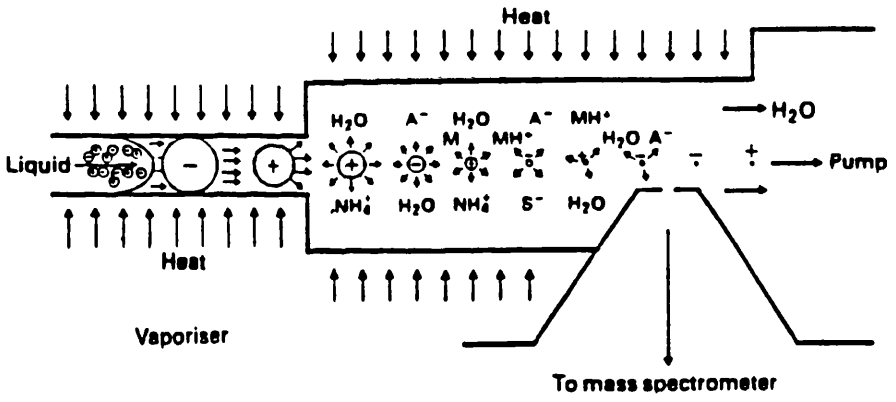


Fig. 3.2 Schematic illustration of the mechanism of ion production in the thermospray process

Intact molecular ions are observed for large nonvolatile molecules which are ionised in solution. Many molecules which are neutral in solution have been observed as either $(M+H)^+$ or

$(M-H)^-$ ions in the mass spectrum. Fragment ions are sometimes observed.

The mechanism which appears to be consistent with the experimental observations and known physical and chemical processes involves the following five steps:

1. Nearly complete vaporization of the liquid at the rate at which it is supplied to the vaporizer to produce a superheated mist carried in a supersonic jet of vapour. Non-volatile molecules are preferentially retained in the droplets of the mist.
2. Droplets of the mist are charged positively or negatively.
3. Molecular ions clustered with a few solvent molecules evaporate from the superheated droplets assisted by the high local electrical fields generated by the charge on the droplet.
4. Cluster ions rapidly equilibrate with the vapour in the ion source to produce the degree of solvation appropriate to the temperature and pressure of the vapour in the source. Ion-molecule reactions will occur if exothermic channels are available.
5. Ions diffusing to the sampling aperture are transmitted to the mass analyser.

v. FRAGMENTATION IN TSP

Four ways that ion fragmentation can be obtained or controlled in TSP experiments are: (1) control of the vaporizer temperature; (2) selection of the ionisation mode; (filament or discharge ionisation); (3) collision-induced dissociation (CID) with the TSP repeller[129,130]; varying the repeller position and voltage[131]; and (4) CID with a triple-stage-quadrupole (or other MS-MS system).[132]

3.3 LIQUID CHROMATOGRAPHY/TANDEM MASS SPECTROMETRY (HPLC/MS/MS)

The reduced fragmentation resulting from mild ionisation provides insufficient structural information for identification of unknown compounds. Fortunately, ionisation techniques that produce abundant molecular or pseudomolecular ions are well suited for tandem MS (MS/MS).

The key principle of MS/MS is collisionally activated dissociation (CAD), which produces a family of daughter ions.[133] For example a protonated molecule can produce a full-scan CAD mass spectrum characteristic of the structure of

the parent compound. When this is accomplished with HPLC sample introduction, any of the forms of direct liquid introduction described so far, including thermospray LC/MS, would benefit from this aspect of tandem MS. This is because all these forms of LC/MS provide minimal fragmentation information, and abundant solvent cluster ions, which are observed in the lower mass region overwhelm lower mass analyte fragment ions that may be of interest.

HPLC/MS/MS techniques can provide full scan mass spectra down to low mass that are rich in structurally significant fragmentation information. In addition, the mixture analysis capability of MS/MS may facilitate the identification of components in unresolved HPLC peaks, allowing the selection and fragmentation of each component of interest.

3.4 SUPERCRITICAL FLUID CHROMATOGRAPHY/MASS SPECTROMETRY (SFC/MS)

Supercritical fluids are gases (e.g., CO_2) which have been heated to a temperature above their critical temperature while simultaneously compressed to a pressure above their critical

pressure. Under these conditions the gas is converted into a single phase, dense fluid whose solvent properties resemble a gas in viscosity, a liquid in density, and are intermediate between these two phases in terms of diffusivity. Therefore it provides a favourable medium for the transport of solutes through a chromatographic column.

SFC is complementary to GC and HPLC for the analysis of organic compounds.[134] Unlike GC, it is not restricted by compound volatility or lability and if capillary columns are used, chromatographic efficiencies approaching those obtained by capillary GC are possible. SFC is more efficient than HPLC, even if packed columns are used, and thus the technique offers advantages in terms of speed of analysis and rapidity of method development. Capillary SFC offers optimal chromatographic efficiency,[135] whereas the packed column approach enables a wide range of modifiers to be used.[136]

A further attraction of SFC is that interfacing of the technique to a mass spectrometer should be easier than interfacing a high-performance liquid chromatograph to a mass spectrometer, since the

gas volumes generated from the mobile phase are lower.

A large portion of the literature on SFC is devoted to the analysis of relatively non-polar materials, such as polymers and natural fuel samples. The potential of the technique for the analysis of polar drugs was shown by Crowther *et al.*[137]

Recently Berry *et al.*[138] have shown the utility of combined SFC-MS to a mixture of ergot alkaloids, using a moving belt HPLC-MS interface with a modified thermospray deposition device. The technique, which has been shown to be effective in providing rapid chromatographic separation of a range of polar compounds, enables both EI and CI mass spectra to be obtained and allows a high proportion of the eluent from the chromatograph to be handled.

4. QUANTITATIVE MASS SPECTROMETRY

4.1 INTRODUCTION

Mass spectrometry is now widely accepted as an important quantitative technique in those areas where trace levels of organic compounds in complex mixtures have to be determined.

The basis of quantitative mass spectrometry is that the ion current obtained for any given compound in a mixture is linearly related to the amount of compound present, although the ionisation efficiencies of the various compounds in a mixture are rarely equal. Thus the use of an internal standard is generally required for accurate measurements. If the internal standard is chosen correctly, the ratio of the analyte to standard in the gas phase presented for ionisation is proportional to that in the mixture. Further, if the compounds of interest are separated from each other by physical methods, such as GC or HPLC, or if different *m/z* values are monitored, such as in isotope dilution analyses, the assumption that the abundance of an ion from one compound is independent of other components in the mixture is generally valid, although for some soft

ionisation methods such as FAB this may not be true.

The technique of selected ion monitoring (SIM), in which a mass spectrometer is used essentially as a highly specific chromatographic detector, is now generally recognized as the most accurate of all the sensitive methods currently available. The method originates from the published work of Henneberg, 1961[139] and Sweeley *et al.*, in 1966[140] and further developed by Hammar *et al.*, in 1968[141] for drug analysis under the name "mass fragmentography". This technique has been called "multiple ion detection" (MID), "multiple ion monitoring" (MIM) and various similar names.

The sensitivity of the method is very high as the instrument looks at only a few ion signals and can thus integrate ion currents for much longer than during a scanned spectrum.

The topic of quantitative MS in general has been widely discused in publications by de Leenheer *et al.*, in 1982,[142] Millard, in 1978,[143] Marshall *et al.*, in 1978,[144] Falkner, in 1981,[145] Beckner and Caprioli, in 1984,[146] and Gelpi, in 1985.[147]

4.2 METHODOLOGY

Since Sweeley's original publication,[140] methodology and technology have progressed and the number of applications of selected ion monitoring to quantitative analysis has increased very rapidly. Present day technology has made available laminated electromagnets which can be switched rapidly and precisely under data system control. Previously, magnetic sector instruments could only be used if the accelerating voltage was varied to monitor the selected ions, which had the following disadvantages:

- The ion source focusing conditions changed with the mass being monitored.
- The dynamic range was limited to about 30% of the highest mass

Quadrupole mass instruments are much more suitable than older magnetic instruments for quantitative work as they offer the advantages of rapid mass switching, ease of computer interface and linear scan. Thus these instruments have been much more popular for drug monitoring than magnetic instruments. Unlike a double focusing magnetic instrument, they are not capable of high resolution or metastable ion monitoring. However,

selected reaction monitoring (MS/MS) experiments can be perfomed on a triple quadrupole instrument.

4.3 INTERNAL STANDARDS

A suitable internal standard should allow the determination of mole ratios from the measured intensity ratios between analyte and standard. There are basically three types:

4.3.1 STABLE ISOTOPES

Chemically these are the same compounds as the analyte except for the introduction of an alternative stable isotope, therefore they have the same extraction efficiencies and derivatisation properties. Deuterium is widely used because of cost, although in some situations it may be labile and also the labelled analogue may have a different retention time than the sample and in extreme cases (large numbers of deuterium atoms) may separate entirely. Other isotopes such as ^{15}N and ^{13}C are also used. The labelled compound should be synthesised preferably by non-exchange techniques in order to produce the highest isotopic content, and it

should contain sufficient numbers of labelled atoms to avoid cross contamination and overlaps in the mass spectrum.

Disadvantages of standards labelled with stable isotopes:

1. They require a second channel for monitoring.
2. The label may be lost by exchange reactions.
3. They are often expensive and difficult to prepare.
4. Calibration lines may be non-linear due to isotopic contributions from one compound to the other.[14e]

4.3.2 HOMOLOGUES

These should be very similar in chemical structure and therefore, have similar extraction efficiencies. However, partition ratios may be somewhat different, even for very similar compounds.

Homologues may offer the advantage of single ion monitoring as ions may be present which are common with those of the compound to be analysed. As no channel switching is involved, this method can

lead to greater precision. Also the sensitivity is higher than when several channels are used. This advantage will not apply to analyses using soft ionisation methods in which the quasimolecular ions are monitored, as homologues will always have different molecular weights than the analytes. There will be no cross contamination at ions monitored as long as the compounds are chromatographically separated. However, when ions of the same mass are monitored, if separation is not achieved, significant errors will result. Calibration lines should go through the origin and should be linear.

4.3.3 ANALOGUES

This will usually be the least accurate method as extraction ratios and derivatisation can differ considerably and channel switching is usually involved. Chemical analogues may pose problems in FAB since the ion abundance may not increase proportionally to the amount of solute across the entire concentration range and internal standards may be ionised with different efficiencies if they are not isotopically labelled analogues of the analyte.[149]

4.4 SOURCES OF ERROR[150]

The analysis of a complex biomedical mixture by mass spectrometry requires more stages than a typical chemical assay. The latter may involve only the injection of liquid samples into the instrument, followed by analysis of the results, whereas a biomedical assay usually involves extraction, evaporation and derivatisation stages. Because of this a biomedical assay will be subject to more sources of error than a simple chemical assay. Errors can be divided into random and systematic errors, although some may fall somewhat between the two.

Random errors cause the individual results to fall both sides of the average value. Random errors affect the precision or reproducibility of results.

Systematic errors cause all the results to be in error in the same sense (i.e. all the results are too high or too low). Systematic errors affect accuracy of results. The presence of systematic errors in an analysis can be most readily confirmed by repeating the analysis using a different technique which is known to be as free

as possible of systematic errors. The most important sources of error are:

i. Exraction and clean-up:

The sample and the standard may not extract to the same extent. Extensive clean-up may introduce unacceptable losses which may differ between sample and standard. Removal of the solvent prior to derivatisation may cause loss of material.

ii. Derivatisation:

This is not always needed. The rate and thus the extent of derivatisation may be different between sample and standard.

iii. Sampling errors:

These may be caused by factors such as air bubbles in the syringe, leaky septum, and leaky syringe. Their effects will be minimised if an internal standard is used.

iv. Instrumental errors:

These are caused by fractionation in the inlet system, e.g. with a "split" capillary injector,

fractionation in interface, cold spots causing condensation, active metal surfaces, decomposition, variation in ion abundance caused by temperature differences, magnet drift, incorrect recorder response, and ion statistical noise.

Instrumental errors are usually small for well maintained instruments as long as the experimental conditions selected are appropriate for the particular assay.

4.5 SELECTIVITY

The scanning of the full mass spectrum with subsequent reversed library search techniques for identification purposes would be more selective than operation in the SIM mode where only a few selected masses are monitored. However, even though a great deal of MS information is lost in SIM due to the limited number of ions recorded, in practice this can be efficiently compensated by various means such as:

1. Consideration of the characteristic retention time in Chromatography-Mass Spectrometry operation.
2. Selection of those unique ions carrying the

highest structural information (e.g. molecular ions).

3. Enhancement of these ions by soft ionisation techniques whenever their relative abundance is low by electron impact ionisation.
4. Use of ion ratios relative to a standard.
5. Use of selective derivatisation techniques.
6. Use of high resolution mass spectrometry and tandem (MS/MS) techniques.

The above means, by themselves or in different combinations, afford a degree of selectivity compatible with high accuracy and precision in quantitative assays. Furthermore, some of these techniques not only increase selectivity but by doing so also lower the limit of detection as a result of the noise reduction, consequent on the elimination of interferences arising from chemical noise.

4.6 ACCURACY AND PRECISION OF MS MEASUREMENTS

The accuracy is the estimated proximity of values determined by the method to their true values and is not to be confused with precision, which is expressed as the coefficient of variation or standard deviation in the measurements.

The scope and limitation of MS as regards to the accuracy and precision of the experimental data, have to be considered from various points of view such as the introduction of the sample and its chemical pretreatment, the role and limitations of internal standards, choice of homologues,analogues or labelled variants, carrier effects, purity of isotopically labelled standards, interferences from chemical or instrumental noise, linearity of calibration curves, instrument calibration or contamination, and measurement of ion ratios.

i. Sample pretreatment:

A well known fact in the quantitation of trace amounts of organic compounds in multicomponent samples is that the more complex the sample manipulation, the lower the reproducibility in quantitative assays. Another possible source of error is the irreversible absorption of small but significant amounts of compound on GC columns and by active sites in interface lines to the mass spectrometer. These effects can be minimised by chemical derivatisation of polar functional groups. Proper selection of the most appropriate derivative type is essential for successful quantitative work.

ii. Internal standards:

The stable isotope labelled analogues of the analyte provide the lowest variance factors due to instrumental or procedural errors and being practically identical in physicochemical properties, effectively compensate for procedural losses of analyte. The isotopic purity of labelled standards is obviously of prime importance as any unlabelled material will be identical to the analyte.

iii. Carrier effects:

This is a well known factor that may adversely influence quantitation in MS assays.
A carrier is a compound which the absorbing system is unable to distinguish from the compound of interest. Although in principle the carrier compound should protect from degradative or adsorptive losses of the analyte it may also introduce unwanted contaminants or generate interfering byproducts upon its derivatisation for GC-MS.

iv. Chemical or instrumental noise:

In any quantitative assay, blanks have to be run to assess the contributions of background noise arising from interferences due to solvents, reagents, sample matrix, GC stationary phases, carrier gases, etc. These effects can be minimised by elimination of the contributing factor, if known, increasing the MS resolution, changing either to other ions in SIM or to other derivatives or by using a different ionisation mode.

v. Calibration curves and instrument calibration:

The usual procedure is as follows. The analyst prepares in addition to the samples, each of four or five, and possibly several more, different concentrations of internal standard equivalent to the median concentration of the test samples. A calibration line is constructed using mole and response ratio. This should be linear and pass through the origin. Lines intersecting the concentration axis usually indicate loss by adsorption or decomposition of low concentration samples. Lines intersecting the response axis ususally indicate the presence of co-channel interference.

All of the factors discussed above, together with instrument and software design, determine the ultimate precision and accuracy of the quantitative mass spectrometric methods and thus are critical for data reproducibility.

4.7. SENSITIVITY

The sensitivity of an assay is defined as the lowest concentration that can be reliably detected in the sample.

The development of new ionisation methods has made mass spectrometry one of the most sensitive analytical techniques available and this is very useful for quantitation of the exceedingly low levels of drugs, metabolites or endogenous compounds in body fluids and tissue samples. Nowadays a remarkable enhancement of both sensitivity and selectivity can be achieved by the use of very specific techniques such as negative CI GC-MS/SIM. Low detection limits can also be achieved by MS/MS, mostly due to the high S/N ratios derived from the extreme selectivity of these systems.

In addition to the instrumental developments mentioned above, better sensitivity assays are obtained by taking sensible precautions such as avoiding the use of plastics with organic solvents, and not using syringes with removable needles as analyte can penetrate the gap between needle and glass.

5. *CINCHONA ALKALOIDS*

5.1 INTRODUCTION

The genus *Cinchona* belongs to family Rubiaceae and thrives best in tropical climates. The most common *Cinchona* species yielding commercially important bark is *Cinchona ledgeriana*.

The alkaloids of commercial importance, quinine and quinidine, are still widely used pharmaceutically. Quinine which is used as an antimalarial[151] and for the treatment of night cramps[152] is also used extensively as a bitter flavouring in the soft drinks industry. Its stereoisomer, quinidine, is used clinically in the treatment of cardiac arrhythmias.

The *Cinchona* alkaloids that are present in the tree bark are usually mixtures of the individual stereoisomers of quinine and quinidine and of their desmethoxy analogues, together with their dihydro analogues.

By manipulating the physical and chemical environment in which the cells are growing, plant cell cultures may produce these secondary

metabolites at levels higher than that of the plant. One object of this work was to develop methods for optimising *C. ledgeriana* cultures (Fig. 5.1) conditions.

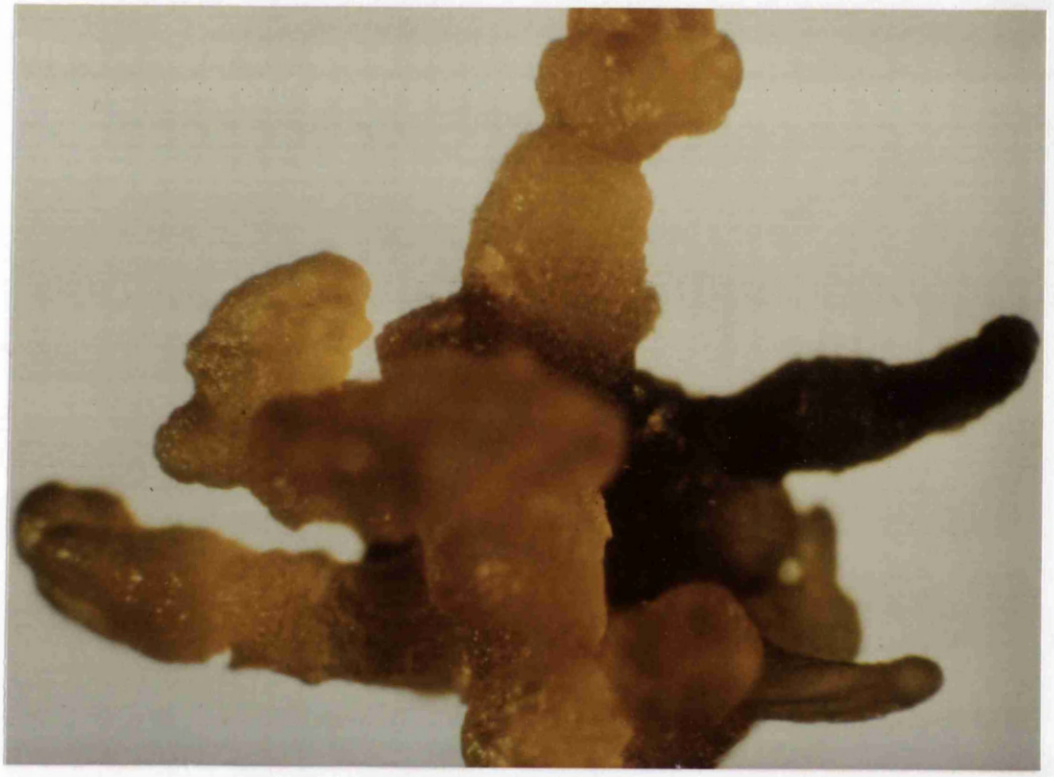


Fig. 5.1 *Cinchona ledgeriana* root organ cluster

The analysis of secondary products formed by these cultures can be very time-consuming , often entailing difficult separations of complex mixtures followed by chemical and spectroscopic analysis of many individual fractions. A rapid, reliable and sensitive method for determining the

composition of such mixtures would be of considerable assistance.

These alkaloids have been subjected to extensive studies. TLC[153] and GC[154] procedures had previously been developed. Since no single TLC or GC system would separate all of the compounds under consideration, multiple systems were used to characterise the samples.

Since the advent of HPLC, many proposed procedures for the determination of *Cinchona* alkaloids have been published.[155-159] These procedures include either adsorption or reversed-phase columns but do not adequately resolve all of the alkaloids of interest. Recent methods for the application of HPLC to the analysis of *Cinchona* alkaloids in botanical extracts and dosage forms also lack the desired specificity and sensitivity.[160-165]

Investigation of the HPLC procedures that were available served as the starting point in the development of a sensitive and selective assay for the simultaneous separation and identification of these alkaloids in complex mixtures using TSP HPLC/MS.

5.2 STEREOCHEMISTRY

The most important *Cinchona* alkaloids are the aminoalcohols (Fig. 5.2) consisting of quinoline and quinuclidine moieties linked by a carbinol group, and their corresponding di-hydro derivatives. Four asymmetric centres give sixteen possible stereoisomers, but only two pairs of stereoisomers occur as major components in the tree bark. The stereoisomers quinine and quinidine differ in configuration at C_8 of the quinuclidine ring and the carbinol C_9, quinine (Fig. 5.3) being 8(S), 9(R) and quinidine 8(R), 9(S). Cinchonidine and cinchonine are stereochemically analogues but they lack the quinoline C_{10}-methoxy group. These four erythro alkaloids are all pharmacologically active whereas their threo epimers are inactive.

The position of C_9 and N_1 influence strongly the antimalarial activity[166], while the position of C_9 and vinyl group influence the stability of the $M^{+\cdot}$ and the fragmentation in the ion source. Although quinidine and quinine are dextro and levorotatory respectively their optical powers are numerically different. They are not a pair of mirror-image enantiomers, do not form a racemate, and have different physical properties such as solubility.

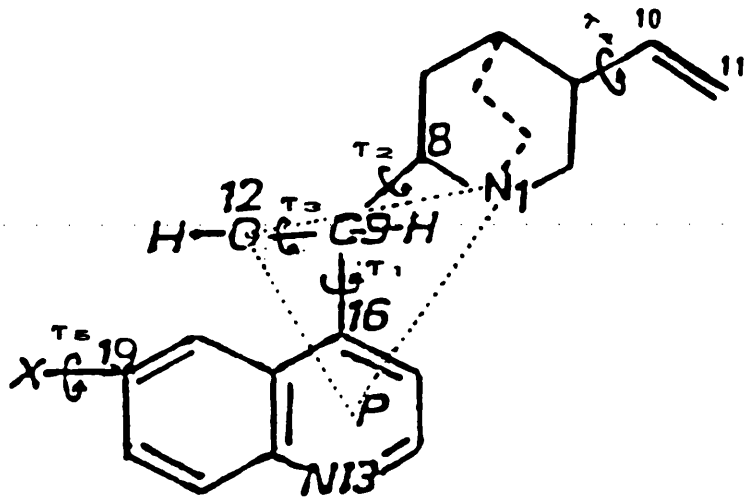
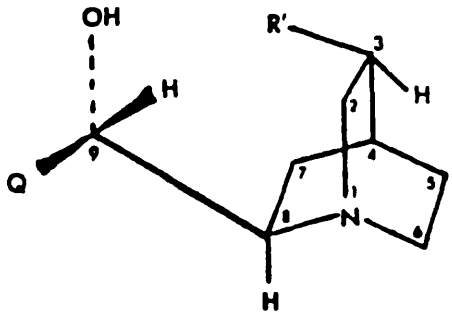


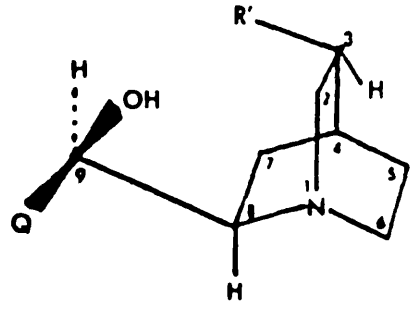
Fig. 5.2 Formula and absolute configuration of *Cinchona* alkaloids τ_1, τ_2, τ_3 τ_4, and τ_5 are torsion angles used in potential energy calculations. The dimensions of the pharmacophore are marked with the point-dashed line. Asymmetric carbon atoms are [C_3, C_4, C_8, and C_9]

Alkaloids	X
Quinine	OCH_3
Quinidine	OCH_3
Cinchonine	H
Cinchonidine	H

The dihydro analogues, (10,11) bond is saturated

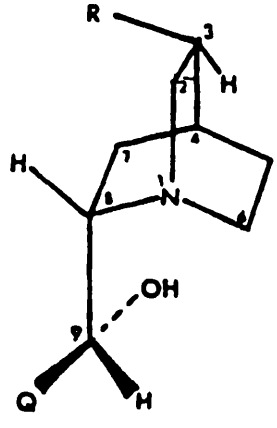
Erythro epimers (active) Threo epimers (inactive)

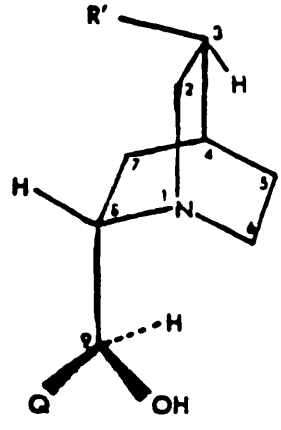




Cinchonine +224° Highly
Quinidine +254° dextrorotatory
8R 9S

Epicinchonine
Epiquinidine
8R 9R





Cinchonidine -111° Strongly
Quinine -158° levorotatory
8S 9R

Epicinchonidine
Epiquinine
8S 9S

Q= Quinoline
Order of priority of groups at C_8 and C_9

Asym. centre	Squence
C_8	N_1-- C_9-- C_7-- H
C_9	OH-- C_8-- Q-- H

Fig. 5.3 R-S Configuration at C_8-C_9

5.3 BIOLOGICAL ACTIVITY

The alkaloids of the *Cinchona* tree (Fig. 5.2) present a group of quite exciting compounds, very promising for a study of the relationship between molecular structure and biological activity. The problem of antimalarial activity of *Cinchona* alkaloids in relation to the configuration of C_8 and C_9 atoms, as well as to the presence of the methoxy group at C_{19} has been studied by Oleksyn.[167] It seems that one of the most essential factors of the activity of these and similar antimalarials is their conformational behaviour, which should influence the possibility of intercalation of drug molecules with DNA and RNA of malaria parasites.[168]

Their antimalarial activity is sharply determined by the absolute configuration as all threo epimers are inactive, while erythro epimers are active,[168] it is the environment of atom C_9 which plays the most important role in the activity. The role of the substituents at C_9 and C_{19} is not obvious.

i. THE MODE OF ACTION

The mode of action of the *Cinchona* alkaloids has not yet been established, but the generally accepted hypothesis assumes the intercalation of their quinoline moiety between base pairs of the DNA of the parasite and formation of a H-bond between the C_9 hydroxyl group and one of the DNA bases. The alcoholic hydroxyl group is probably essential for hydrogen bonding to DNA. Its elimination destroys antimalarial activity.[169] Similarly, epimerisation of this hydroxyl, yielding epiquinine, may introduce steric hindrance to hydrogen bond formation and does, in fact, result in a drastic reduction of antimalarial potency.

ii. CALCULATIONS AND OBSERVED DATA

The *Cinchona* alkaloid epimers were modelled by molecular graphics using the program QUANTA (Polygen Corp.), running on a Silicon Graphics IRIS 2D/20 personal workstation. The molecular dimensions determined for cinchonine were taken as a starting point for designing the molecular geometry of the other alkaloids. For each of these models the geometry has been assigned and the minimum energy conformations were found using the CHARM Energy Program.

The conformation of the investigated molecules can be described by five torsion angles about the rotation axes of the rigid parts of the molecules, the axes and the corresponding torsion angles being shown in Fig 5.2. It was found that variation of τ_4 only slightly affects the energy of the molecule. The effect of τ_5 is negligible; therefore the influence of the methoxy group at C_9 on the conformational behaviour of the *Cinchona* alkaloids was found to be negligible. τ_1, τ_2, and τ_3 significantly affect the energy of the molecule, therefore the potential energy of the molecule (Tab 5.1) was calculated as a function of τ_1, τ_2, τ_3, and τ_4. The energy of the inactive epimer was always higher than that of the active epimer.

iii. HYDROGEN BONDING

A. INTRAMOLECULAR H-BONDING

In none of the active alkaloids (erythro isomers) (Fig. 5.4 and 5.5) has an intramolecular H-bond been found. For most of the preferred conformers of active alkaloids the N_1-O_{12} distances were too long to allow an intramolecular H-bond to be formed. While the inactive alkaloids (threo

Table 5.1 Observed dimensions

Alkakoid	τ_1	τ_2	τ_3	τ_4	Optimal Charm E
Cinchonine	-20.19	-25.20	-25.12	-25.25	44.47
Epicinchonine	-15.81	-20.03	-21.75	-21.81	47.14
Cinchonidine	-20.38	-27.28	-27.08	-28.21	35.95
Epicinchonidine	-26.35	-26.01	-27.12	-33.12	43.63
Quinine	-24. 7	-27.11	-25.11	-28.00	31.63
Epiquinine	-19.58	-26.17	-28.63	-31.34	36.75
Quinidine	-24.36	-27.95	-27.51	-28.07	38.62
Epiquinidine	-22.59	-24.10	-28.00	-28.65	42.20

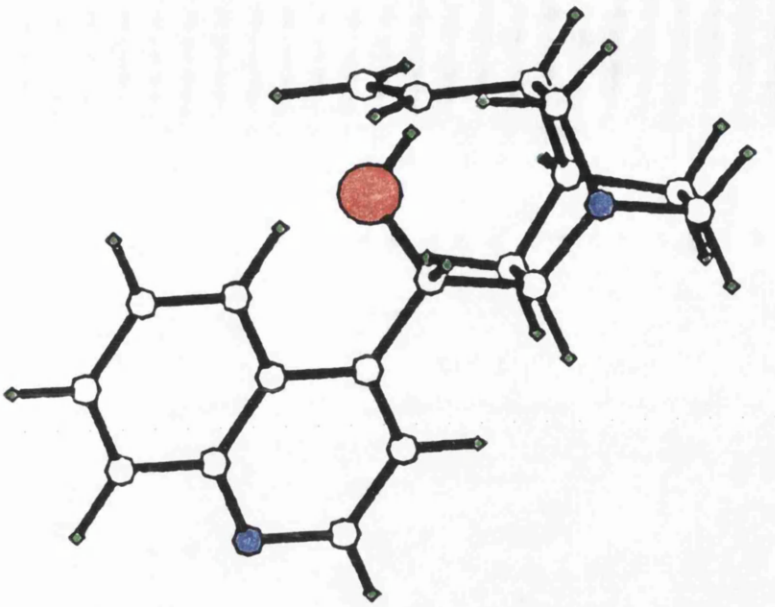


Fig. 5.4 QUANTA modeling of cinchonine (8)R (9)S

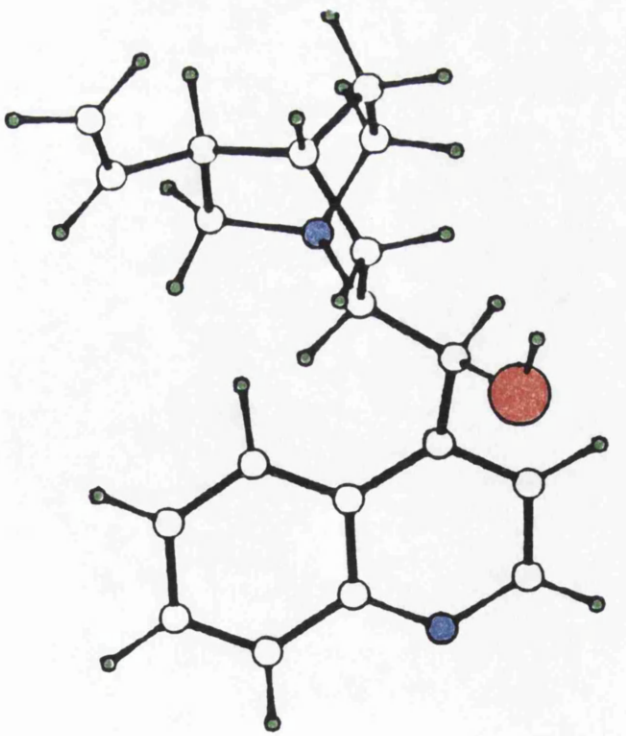


Fig. 5.5 QUANTA modeling of cinchonidine (8)S (9)R

isomers) e.g. epiquinine (Fig. 5.6), showed intramolecular H-bonding between the hydroxyl H and quinuclidine N. This is because the position of the hydroxyl group and the hydrogen atom in the threo isomers creates conditions favourable to formation of the intramolecular H-bond. This conclusion is in agreement with the crystal structure data[170] and IR spectra for solutions.[171]

B. INTERMOLECULAR H-BONDING

When two molecules of erythro epimers (e.g. cinchonidine) were packed (Fig. 5.7) two intermolecular H-bonds were found. By contrast, packing of two molecules of threo epimers did not favour the formation of intermolecular H-bonds.

iv. PHARMACOPHORE

The pharmacophore which occurs in all antimalarial aminoalcohols is represented by the triangle N-O-P (Fig. 5.2), where P is the centre of the heterocycle part of the quinoline ring. This pharmacophore was proposed by Cheng.[172] In order to compare the shape and dimensions of Cheng's

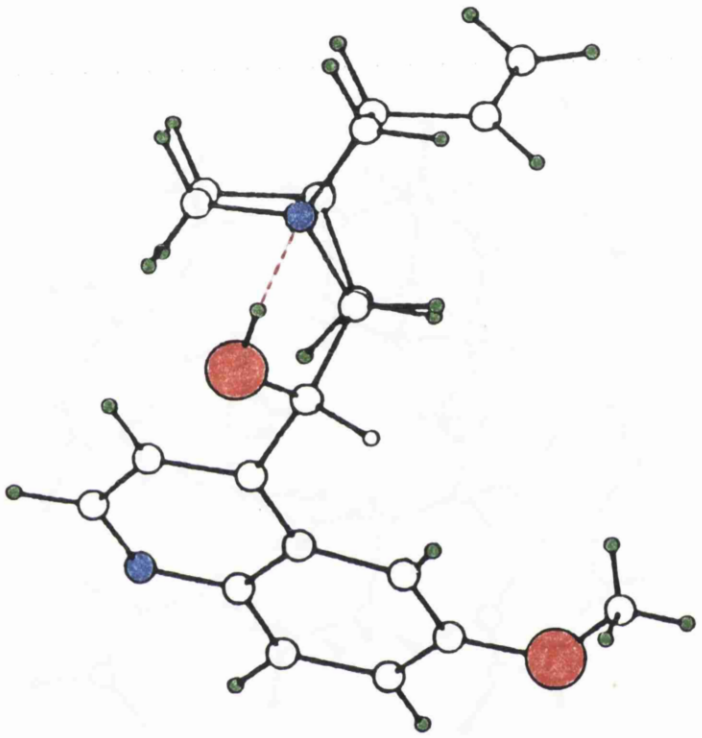


Fig. 5.6 QUANTA modeling of epiquinine (8)S (9)S

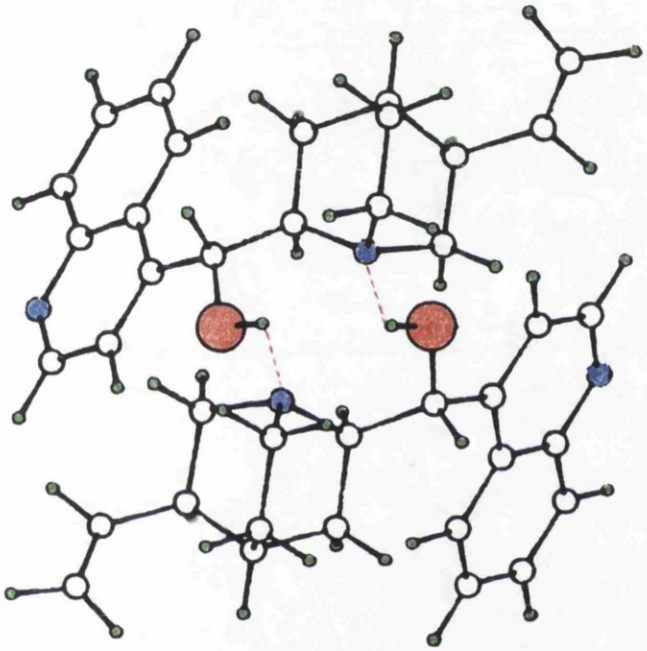


Fig. 5.7 Packing of two erythro epimers

pharmacophore, the lengths of the triangle sides were calculated (Tab. 5.2).

Table 5.2 Distances in the pharmacophore (A°)

Alkaloid	N-O	N-P	P-O
Quinine	3.04	5.17	3.67
Quinidine	3.02	5.13	3.68
Cinchonine	3.10	5.18	3.60
Cinchonidine	3.09	5.10	3.69
Epiquinidine	2.58	5.12	3.75

The active erythro epimers show very similar dimensions. The inactive threo epimer, epiquinidine has the shortest N-O distance of all the compounds studied. These observations lead to the general conclusion that the most important feature of the active molecules is the lack of intramolecular H-bonding, which makes the atoms O_{12} and N_1 accessible for interaction with a receptor via intermolecular H-bonding.

5.4 EXPERIMENTAL

i. REAGENTS AND MATERIALS

Methanol was of HPLC grade, all other reagents were of analytical grade. The solvents used for HPLC were filtered and vacuum-degassed using a 0.2 μm Anotop filter (Analchem, Luton, U.K) to remove particulate matter and then continuously purged with helium. Quinine, quinidine, cinchonine and cinchonidine were obtained from Sigma, Poole, U.K.

ii. MASS SPECTROMETRY

A. EI AND EI MIKES CONDITIONS

All EI experiments were performed on a VG Masslab VG 12-250 quadrupole mass spectrometer, operating under EI conditions, electron energy and emission current were 70 eV and 200 μA respectively. Samples were introduced by the direct insertion probe with a source temperature of 200°C.

All EI-MIKES experiments were carried out using a VG Analytical ZAB-2F instrument operating under EI conditions (70 eV, 200 μA) and at resolution Rs = 1000. MIKES spectra were obtained by mass selecting a specific ion and following its unimolecular decomposition between the two sectors (FFR2) by scanning the electric field voltage.

B. FAB AND FAB CID-MIKES CONDITIONS

All FAB experiments were performedon a VG ZAB-SE, operated at low resolution (Rs = 1000). The alkaloids were dissolved in a small quantity of appropriate liquid matrix (e.g. glycerol or 3-nitrobenzyl alcohol) which was coating a metal surface on the end of the probe. The liquid surface was bombarded in the source with a beam of 20 keV CS^+ ions at room temperature.

All FAB CID-MIKES experiments were carried out using a VG Analytical ZAB 2F instrument. The protonated molecular ions were collided with helium in the 2FFR. The pressure in the collision cell was such as to reduce the main beam intensity to 50% of its original value.

C. TSP CONDITIONS

HPLC/MS was carried out on a VG MassLab 12/250 quadrupole mass spectrometr fitted with a commercial thermospray source and VG 11-73 data system. The TSP ion source conditions were optimised with approximate temperatures as follows: source 200°, nozzle 190° and chamber 230°C. Scanned data were acquired between masses

100 to 500 to avoid the high background resulting from solvent cluster ions.

5.5 EI MASS SPECTRA

Although a few papers dealing with the mass spectral fragmentation of *Cinchona* alkaloids have appeared,[173-176] they are concerned only with the characterisation of these alkaloids.

The main goal of this part of the study was to compare the mass spectra and the MIKE spectra of (8)R and (9)S-epimers, to study the effects of the change in stereochemistry at C_8 on the fragmentation of these compounds. The EI-MS of the four epimers are shown in Fig. 5.8-5.11, the *m/z* values of the peaks observed for the four isomers are common, but the relative intensities of many of them are characterstically different. Therefore, these compounds are easily distiguishable from each other. The differences in intensities observed for the mass spectra of the (8)S and (8)R epimers are very large. This is very interesting because the fragmentations of *Cinchona* alkaloids has been assumed to start with opening the C_8-C_9 bond, i.e. with destroying the epimeric centre at C_8. On the basis of this

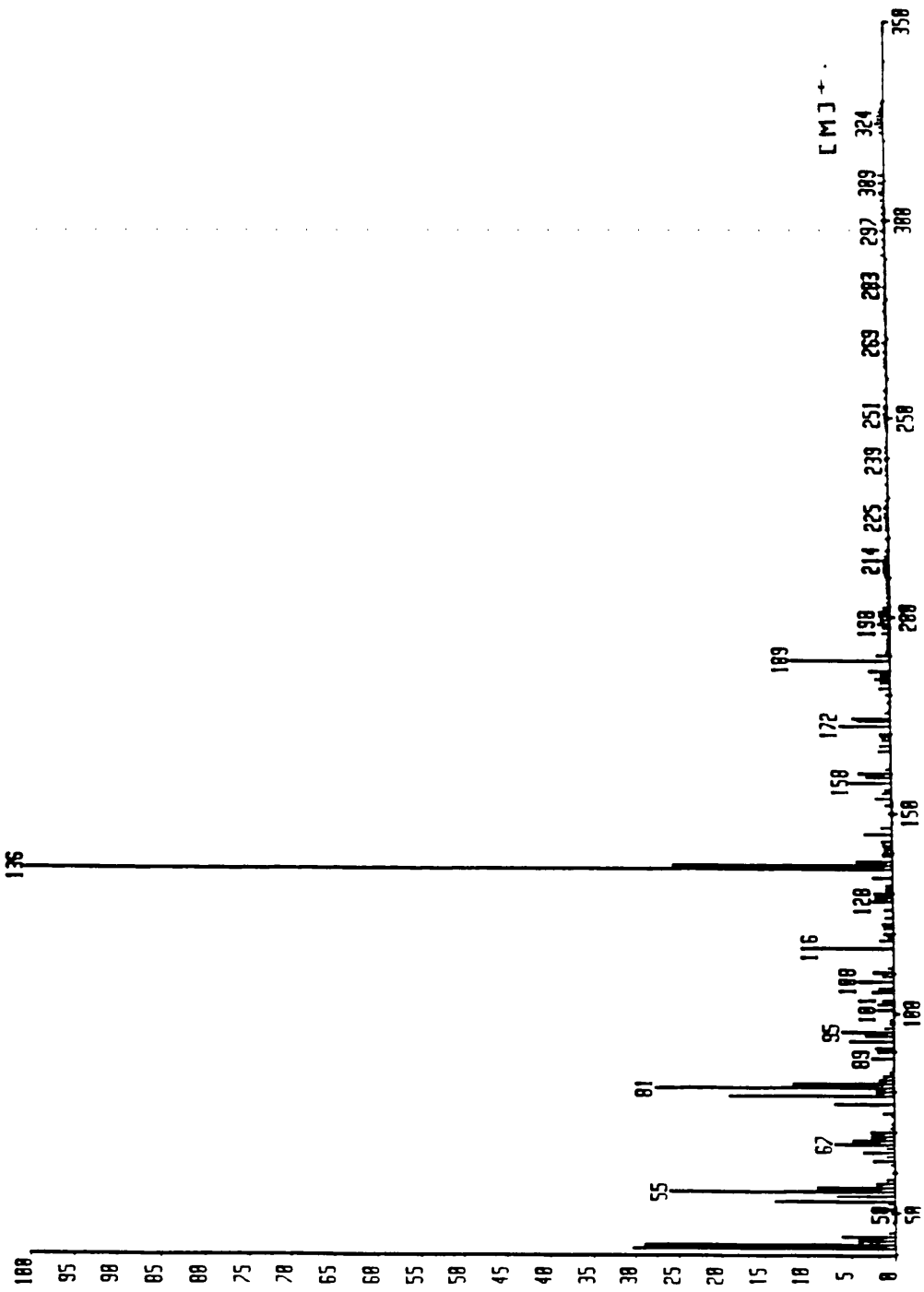


Fig. 5.8 EI-MS of quinine

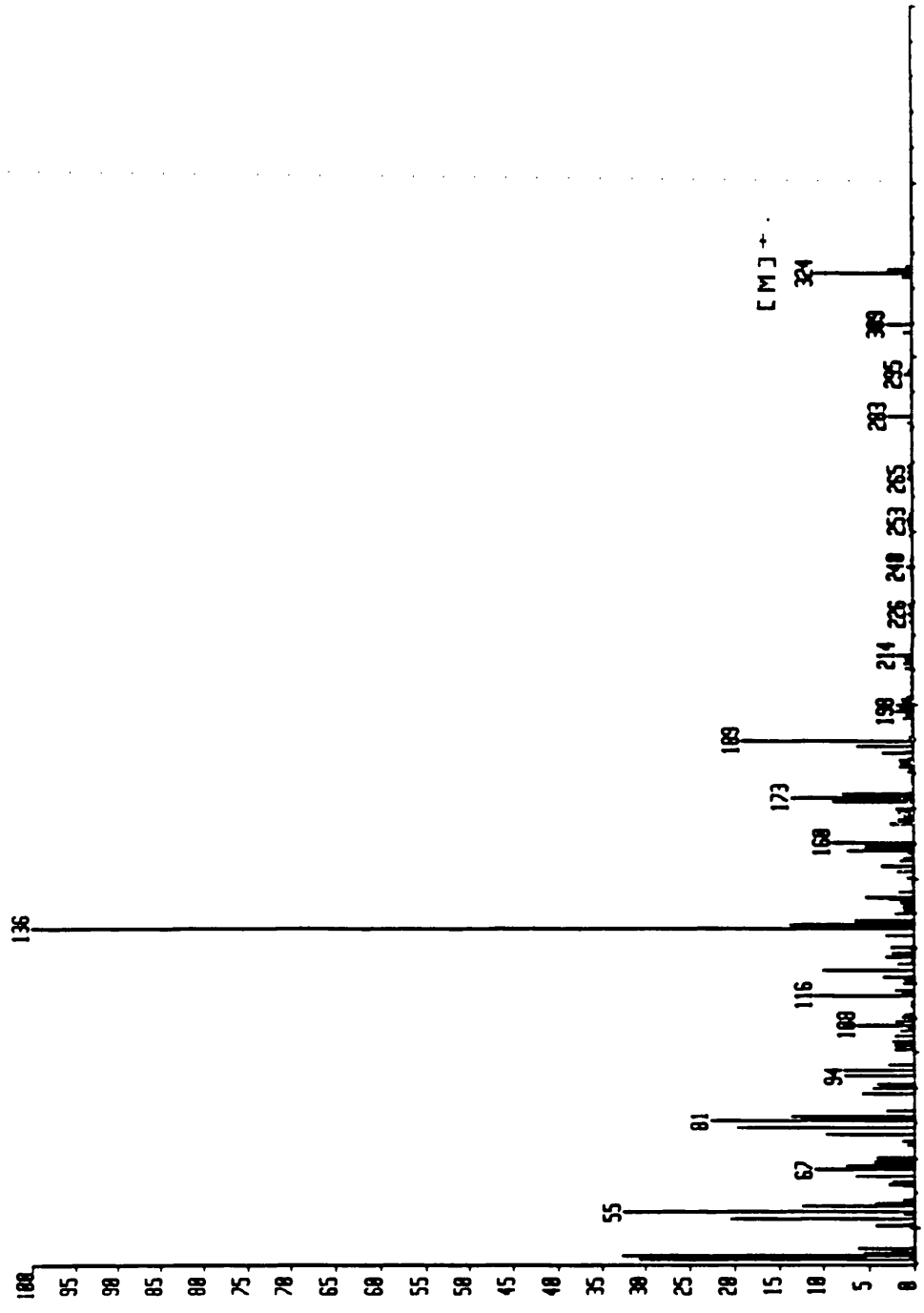


Fig. 5.9 EI-MS of quinidine

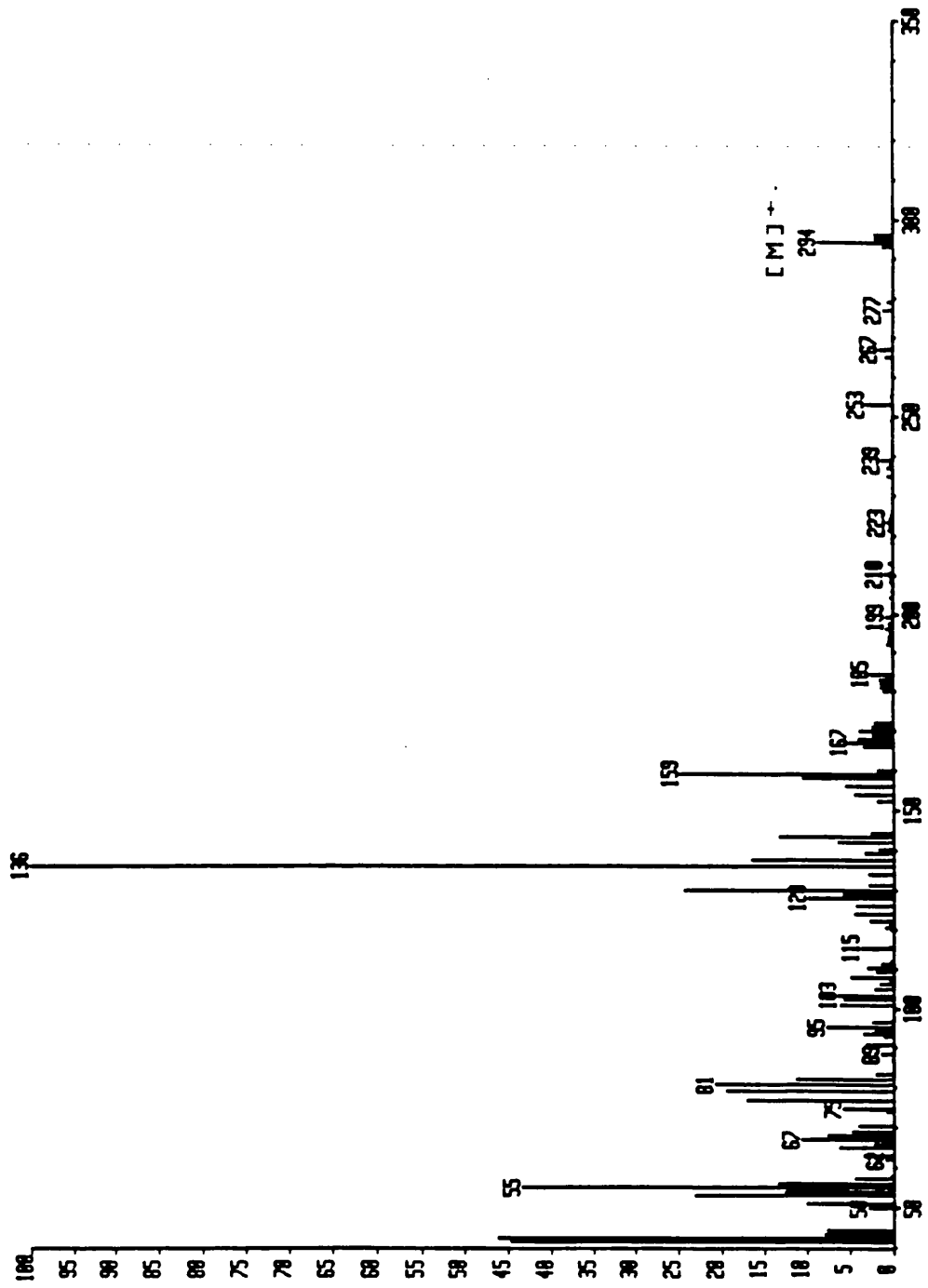


Fig. 5.10 EI-MS of cinchonine

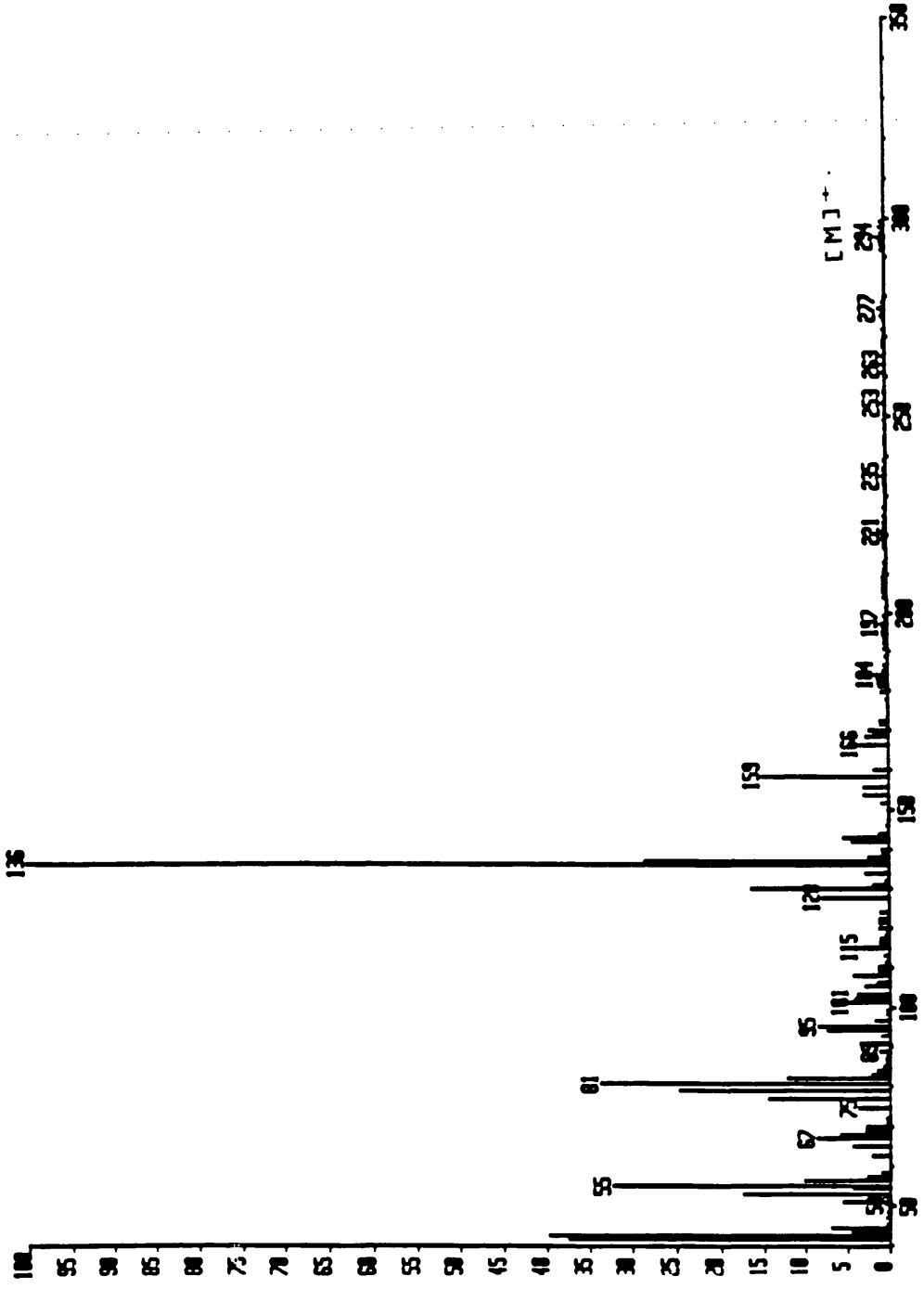


Fig. 5.11 EI-MS of cinchonidine

assumption significant differences would be expected between (8)S and (8)R epimers. The significant types of ions are discussed below:-

i. MOLECULAR ION STABILITY

The stereisomers of the series studied here show significant differences in the stability of their molecular ions. For instance in the case of quinine and quinidine (Fig. 5.8 and 5.9); (Tab. 5.3), the molecular ion of quinidine is more stable than that of quinine. This is in accordance with the stereochemical stability of these compounds: a ^{13}C NMR spectral study has shown a close proximity of the vinyl double bond to C_9-OH in quinidine.[177]

An examination of molecular models also shows that in the stable conformation with the quinuclidine and quinoline rings far apart, the vinyl double bond and C_9-OH are in close proximity in the stable isomer (Fig. 5.4). Thus, it appears that the $M^{+\cdot}$ ion is stabilised by the presence of H-bonding between the C_9-OH and the double bond. A similar correlation was also obtained for cinchonine and cinchonidine (Fig. 5.10 and 5.11).

ii. *M/Z* 309 $[M-15]^+$

Table 5.3 Partial EI-MS of *Cinchona* alkaloids

Alkaloid	$[M]^{+\cdot}$	$[M-Quinoline]^{+}$	$[M-15]^{+}$	$[M-Quinuclidine]^{+}$	$[M-41]^{+}$
Quinidine	(m/z 324)12	(m/z 136)100	(m/z 309)4	(m/z 189)17	(m/z 283)3
Quinine	(m/z 324)0.8	(m/z 136)100	(m/z 309)0.7	(m/z 189)12	(m/z 283)0.5
Cinchonine	(m/z 294)11	(m/z 136)100		(m/z 159)25	(m/z 253)3
Cinchonidine	(m/z 294)0.7	(m/z 136)100		(m/z 159)15	(m/z 253)0.5

The ion at *m/z* 309 is formed by loss of a methyl radical from the molecular ion. The origin of this 15 mass unit is the methyl group of the methoxy function in quinine and quinidine. The fragmentation mechanism is shown in Fig. 5.12A. The fragment ion intensity of *m/z* 309 in the stable isomer quinidine is larger than that in the unstable isomer quinine (Tab. 5.3). The results accord with the interpretation that the fragment ion intensity of the unstable compound is stronger than that of the stable compound. This indicates that the fragment ion intensity is affected by the stability of the fragment ion itself more than the stability of the molecular ion. Accordingly, the ion intensity at *m/z* 309 is directly proportional to the intensity of the molecular ion.

iii. *M/Z* 283 $[M-41]^+$

The $[M-41]^+$ ions of quinine and quinidine are formed by loss of an allyl radical from the molecular ion, although this peak is relatively weak in the mass spectrum of quinine. Spiteller *et al.*[174] have shown that the formation of the ion at *m/z* 283 involves the participation of the vinyl double bond and the bond cleavage of the quinuclidine skeleton (Fig. 5.12B). The ion at m/z 253 in cinchonine and cinchonidine may be

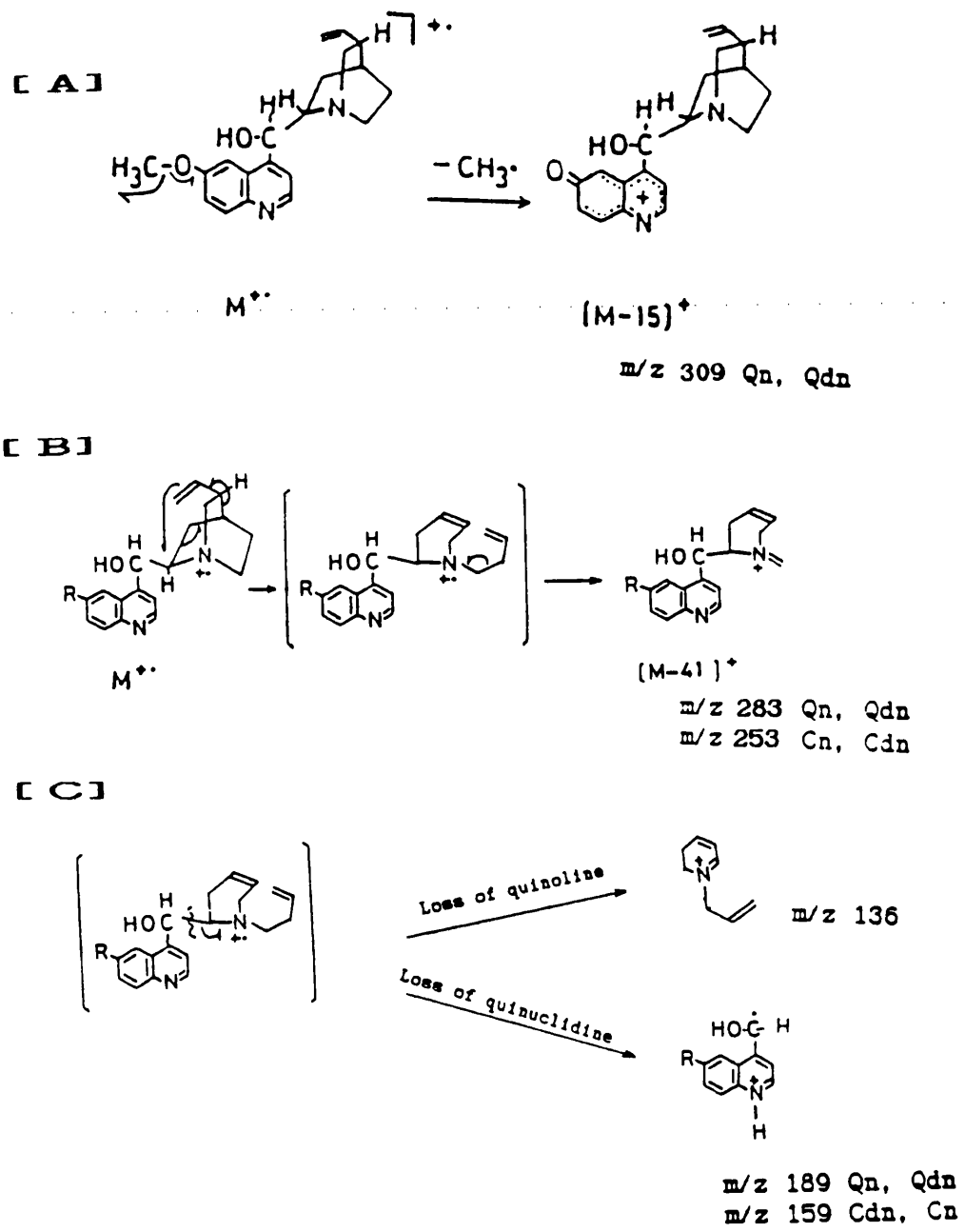


Fig. 5.12 EI fragmentation modes of *Cinchona* alkaloids

A. Mechanism of formation of [M-15]+ ion.

B. Mechnism of formation of [M-41]+ion.

C. Mechanism of formation of [M-quinoline]+, and [M-quinuclidine]+ ions

formed through the same process. Again these results show that the influence of the fragment ion stability is larger than that of the molecular ion stability. Therefore, the ion intensities at $[M-41]^+$ in quinidine and cinchonine are stronger than those in quinine and cinchonidne.

iv. *M/Z* 189 [M-QUINUCLIDINE]$^{+\cdot}$

This ion is formed by the loss of the quinuclidine moiety from the molecular ion (Fig. 5.12C). It was also found that the fragment ion intensity for quinine and quinidine is directly proportional to the corresponding molecular ion intensity.

v. *M/Z* 136 [M-QUINOLINE]$^+$

This ion is the base peak in all these alkaloids. It is formed by the loss of quinolinemethanol radical (Fig. 5.12C), which involves cleavage of the C_8-C_9 bond, and the formation of the N_1-C_8 double bond in the quinuclidine skeleton.

5.6 MIKE SPECTRA

Fig. 5.13 and 5.14 show the MIKE spectra of $M^{+\cdot}$ (*m/z* 324) for quinine, and quinidine respectively. They show mainly :

-Loss of CH_3 leading to an ion at *m/z* 309

-Loss of H_2O leading to an ion at *m/z* 306

-Loss of an allylic radical leading to an ion at *m/z* 283

-Loss of quinuclidine leading to an ion at *m/z* 189

-An abundant loss of the quinoline moiety, giving rise to an ion at *m/z* 136

The intensity of the peak at *m/z* 309 in the two spectra indicates that the elimination of H_2O from quinine is much more facile compared with that from quinidine. A similar difference was found with the peak at *m/z* 306.

The intensities of the peaks at *m/z* 283, 189, and 136 in the two spectra show that the cleavage of the C_8-C_9 bond occurs much more easily for quinine than for quinidine. These results are supported by the differences in the relative intensities of the respective molecular ions.

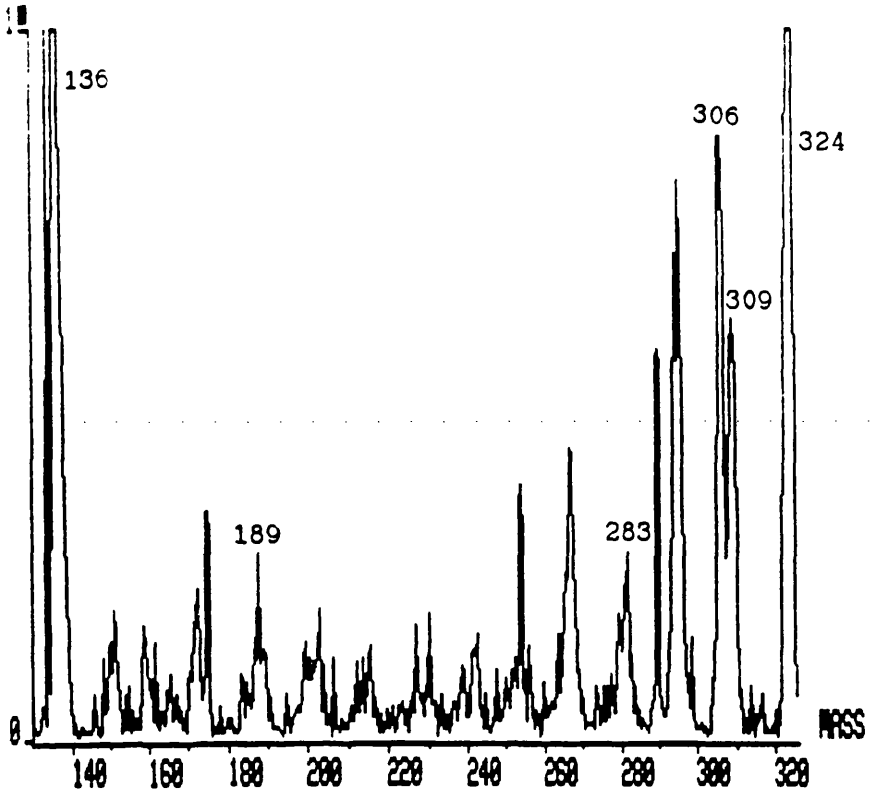


Fig. 5.13 MIKE spectra of quinine

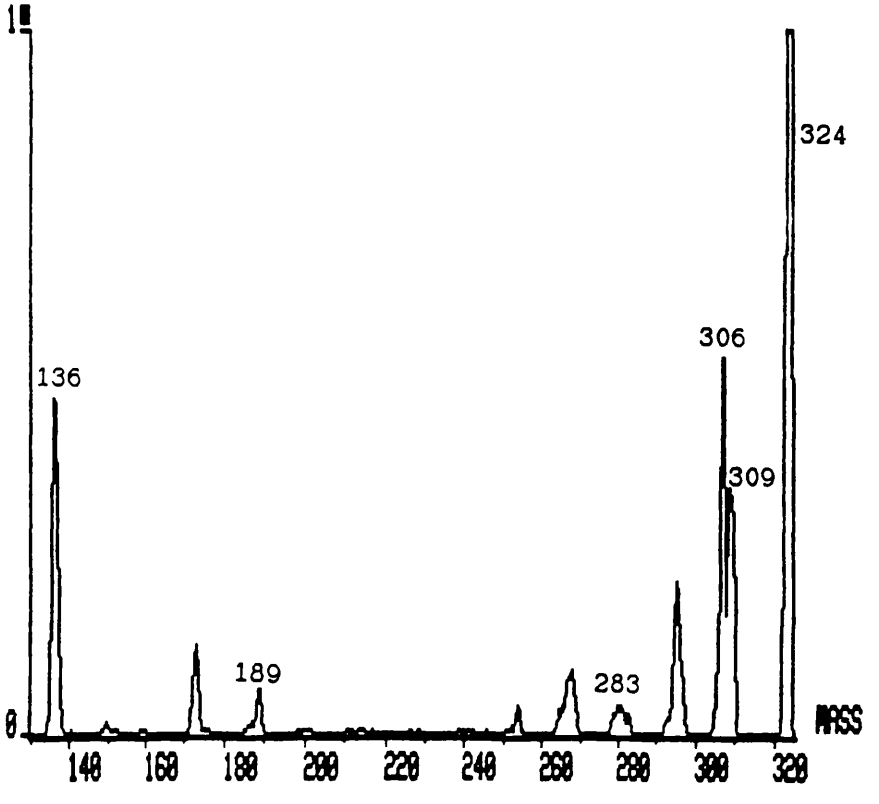


Fig. 5.14 MIKE spectra of quinidine

Therefore the determination of the C_8 configuration is possible from mass spectra and MIKE spectra.

5.7 FAB MASS SPECTRA

Cinchona alkaloids showed abundant protonated molecular ions, and little fragmentation (Fig. 5.15 and 5.16), individual isomers showing virtually identical spectra. Therefore, these isomers are not easily distinguishable from each other.

These results show that FAB and EI-MS complement one another well, e.g. as is shown by the case of quinine, whose EI spectrum is shown in Fig. 5.8, and its FAB-spectrum in Fig. 5.15. In the case of EI-MS the predictable lability toward electron bombardment of the C_8-C_9 bond leads to the formation of the base peak at *m/z* 136, relative to which the abundance of the molecular ion at *m/z* 324 is only 0.8%. However, with FAB-MS the base peak is the protonated molecular ion, while the peak at *m/z* 136 has a relative abundance of only 22%.

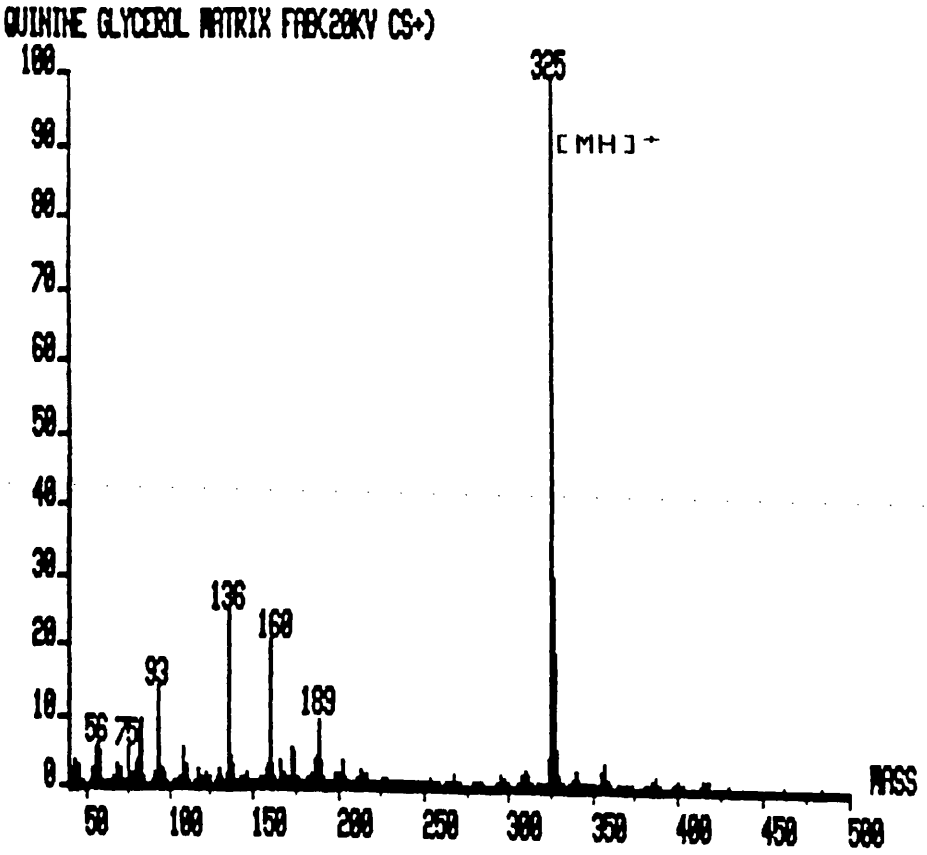


Fig. 5.15 FAB-MS of quinine

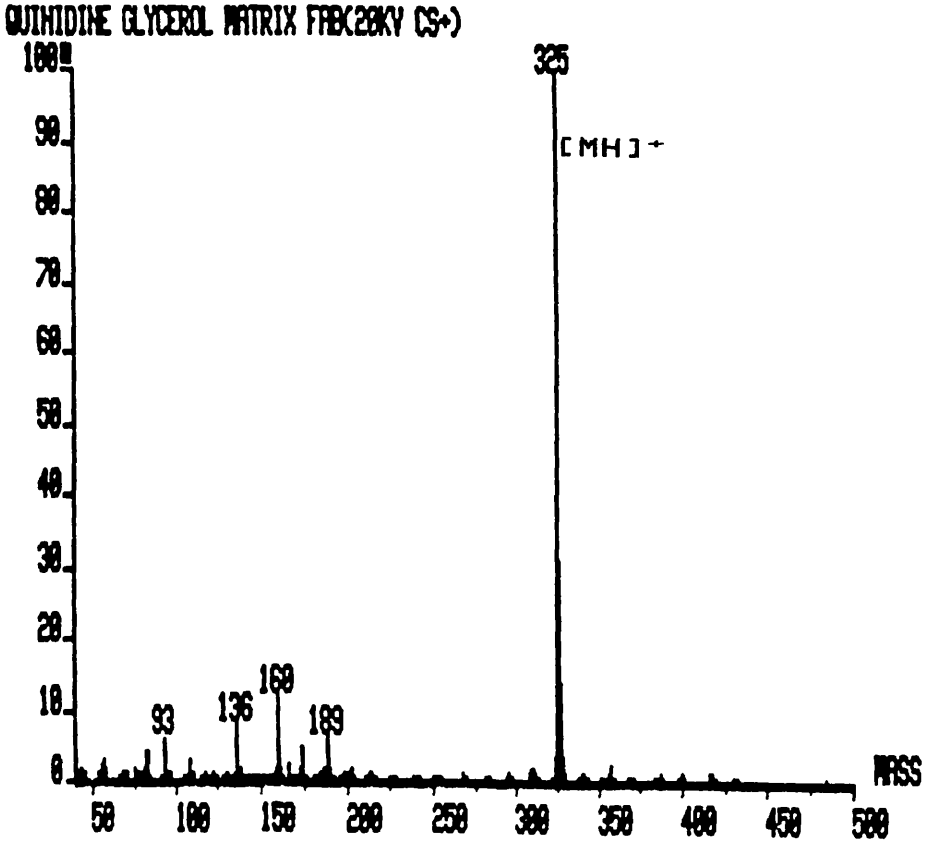


Fig. 5.16 FAB-MS of quinidine

5.8 ANALYSIS OF PLANT MATERIALS

5.8.1 EXTRACTION OF PLANT CELL CULTURES

The cell cultures were prepared by Hay *et al.*,[4] six separate cell cultures being studied in this work. Extraction of the alkaloids was by the following modified version of a procedure described by Robins *et al.*,[178] 3 g of freeze dried cell sample from *Cinchona ledgeriana* suspension cultures were ground, homogenised in 18 ml 0.2 M H_2SO_4 and 18 ml $CHCl_3$, (15 min sonication bath, then left to stand for 30 min) 2x, to allow the emulsion to break. The cell debris was filtered off and washed with 0.2M H_2SO_4, which was combined with the extract. The $CHCl_3$ was decanted and the aqueous phase was made alkaline with NH_4OH to pH 9.5-10 and poured onto three Extrelut columns (Merck-Darmstadt, FRG) and allowed to equilibrate for 30 min. The lipophilic components were eluted with 5-6 column volumes of $CHCl_3$. The combined organic extracts were dried over anhydrous Na_2SO_4 and concentrated to dryness under reduced pressure. The residue was dissolved in methanol and passed through a C_{18} Sep Pak (Waters Associates), washing with further methanol. The final concentration corresponded to 1 g of cells per 1 ml of methanol.

5.8.2 OPTIMISATION OF CHROMATOGRAPHIC SEPARATION

HPLC was carried out on a Du Pont model 870 under isocratic conditions using a Waters μBondapak C_{18} (25cm * 4.6 mm, I.D., 10 μm) column. Sample loading was via a Rheodyne injector with a 10 μl loop. Detection was with a Kratos FS970 fluorimeter (excitation 250 nm, emission 418 nm).

During the optimisation of the composition of the methanolic aqueous mobile phase for the separation of principal *Cinchona* alkaloids, on the C_{18} column, it proved to be advantageous to adjust the pH of the aqueous component to ≃3 with glacial acetic acid, in order to reduce tailing, and analysis time.

To achieve optimal conditions, changes in the pH and ammonium acetate concentration were investigated (Tab. 5.4), using eluent containing H_2O:CH_3OH (75:25).

Table 5.4 Mobile phase composition and pH

Mobile phase	H_2O	CH_3OH	CH_3COOH	pH
System A	75	25	-	7.0
System B	71	25	4	2.8
System C	71	25	4	3.0
	0.01M A.A.			
System D	71	25	4	3.9
	0.1M A.A.			

A.A. = Ammonium Acetate

Using solvent system A (Tab 5.5), peak tailing and broading were observed and the alkaloids were retained for too long to be rapidly determined.

Table 5.5 Dependence of alkaloid retention times on the mobile phase composition

Mobile phase	retention time (min)	
	Quinidine	Quinine
System A	44	48
System B	12	15.9
System C	13.1	17.1
System D	27.5	36.5

On reducing the pH to 2.8 (system B) with acetic acid, the chromatogram improved dramatically, the

alkaloids were adequately retained, tailing was drasticaly reduced, more symmetric and sharper peaks were observed. This led to resolution of the alkaloids within a reasonable time (≃ 16 min).

The effect of introducing ammonium acetate to the mobile phase are shown in system C and D. It was found that an ammonium acetate concentration of 0,01 M (system C) did not significantly alter the resolution and retention times, but this effect was found significant with 0,1 M ammonium acetate (system D).

System C (Fig. 5,17) was found to be the most favourable in terms of peak shape and overall

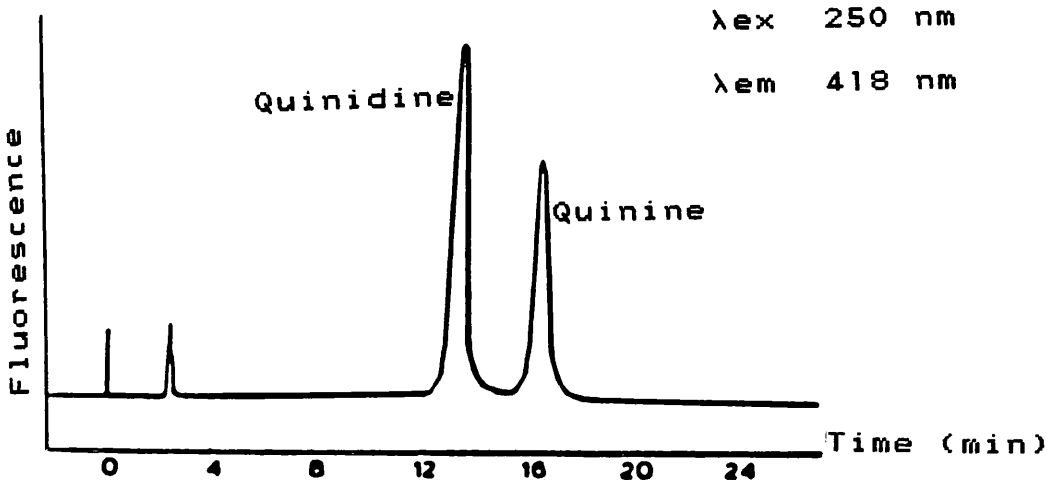


Fig. 5,17 Fluorescence chromatogram of authentic *Cinchona alkaloids* on C_{18} Water Bondapak. Mobile phase (system C).

analysis time. Subsequently , we successfully used this mobile phase for the separation of the principal alkaloids in extracts from *Cinchona ledgeriana* cell cultures using on-line HPLC/TSP-MS.

5.8.3 HPLC AND HPLC/MS

The rather weak molecular ions and their widely differing intensities means that EI ionisation is not ideal for detecting or assaying these alkaloids in complex mixtures, although the use of a moving belt interface with EI ionisation has been described (Eckers *et al*, 1980).[179] A soft ionisation method such as TSP is preferable as the protonated molecular ion is likely to be the base peak and TSP is directly compatible with HPLC, allowing selected ion monitoring (SIM) to give the optimum selectivity and sensitivity. TSP analysis of *Cinchona* alkaloids has been described previously by Mellon, 1988.[180] In the current study it was established that the system described by Mellon was able to separate quinine from quinidine but it failed to separate the other two stereoisomers (cinchonine and cinchonidine). Furthermore the mobile phase was not readily compatible with TSP. Hence the chromatographic

conditions needed to be optimised. Our development of a suitable system for the HPLC/MS assay has recently been reported.[18]

i. PROCEDURE FOR OPTIMISING OF THERMOSPRAY

Compared with GC/MS, the interfacing of HPLC to a mass spectrometer is not a trivial process. A careful regime for heating the interface and incrementing the flow rate of mobile phase needs to be used. The source must be heated to about 200°C and the chamber and nozzle temperature allowed to equilibrate at about 150°C before any eluent is introduced into the system. The rotary backing line valve is opened immediately prior to introduction of the mobile phase. The flow rate of the latter is increased slowly in steps of 0.1 ml/min, each increment being accompanied by a subsequent increase in chamber and nozzle temperatures, until a flow rate of 1 ml/min is achieved. The temperatures of the different zones of the interface must be allowed to equilibrate before each temperature increment.

In this study the HPLC eluent ions (m/z = 33 amu, protonated methanol or m/z = 61 amu, protonated

acetic acid) were used to optimise the lens voltages and tuning parameters of the source. Standard solutions of quinine, quinidine, cinchonine and cinchonidine of approximately 10 µg/ml were prepared in 10 ml of methanol, and 10 µl aliquots of these were injected directly into the mass spectrometer i.e. not through the column. The interface temperatures were then varied until a strong signal for the protonated molecular ion was seen on the oscilloscope. Repeat injections were made at higher repeller voltages in an attempt to observe some fragmentation in the mass spectrum. Despite the use of a repeller, no fragmentation occured, irrespective of the voltage that was applied,[182] and the NH_4^+ adduct ions were weak and variable in intensity.[131]

TSP spectra of quinidine and cinchonidine obtained by averaging the ion current for 5 scans are shown in Fig. 5.18 and 5.19.

ii. SELECTIVE ION MONITORING CALIBRATION

Prior to an SIM acquisition, it is necessary to have the instrument calibrated, such that a channel can be accurately positioned on a designated mass. A "cocktail" of polyethylene

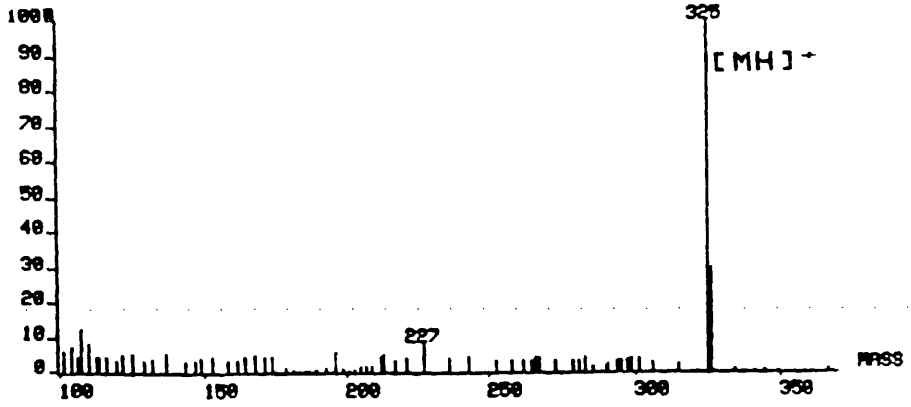


Fig. 5.18 TSP-MS of quinidine

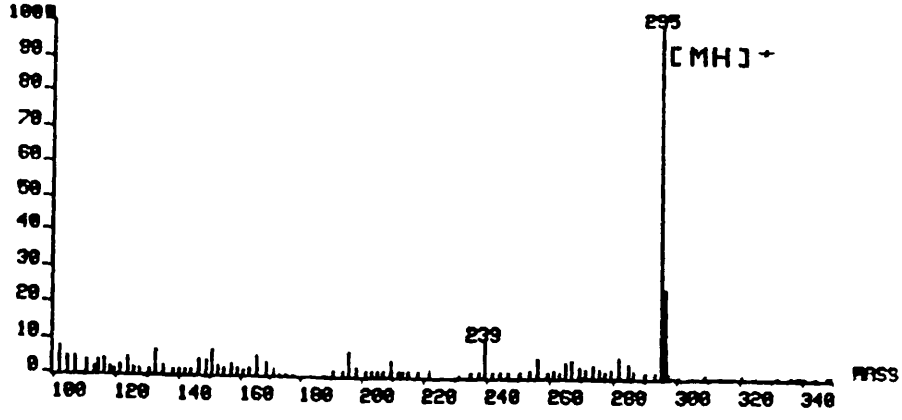


Fig. 5.19 TSP-MS of cinchonidine

glycols (PEG234) at 1 µg/ml total concentration was used as a calibrant. This cocktail is a mixture of solution of PEG-200, PEG-300 and PEG-400 in 70:30 methanol:water (0.1 M ammonium acetate).

The calibration process was activated by setting up the data system as follow:

- The calibration scan (CLS) = 1, this represents the maximum number of separate calibration scan attempts per group.

- The channel parameters were specified as follow:
  1. The masses to be monitored:
     A. 325 (protonated quinine and quinidine)
     B. 295 (protonated cinchonine and cinchonidine).
  2. Sampling time = 80 milliseconds
  3. Inter-channel time = 20 milliseconds

SIM aquisition was then started by injecting 10 µl of the PEG234 and the positions of the two identified channels and peaks were improved by adjusting the span as necessary (typically using the + command) until both references were wholly (or nearly so) on the display. Then the peaks were centroided using the comand ESC, this had the

effect of storing any adjustments made to peak positions for later use during data acquisition following a successful group's calibration. At this point the data acquisition was halted , the CLS was set to zero for sample acquisition and the HPLC column was switched on line.

iii. SELECTIVE ION MONITORING ANALYSIS

The optimum separation and TSP ionisation for quinine and quinidine monitored at *m/z* 325 and cinchonine and cinchonidine at *m/z* 295 was achieved with $H_2O/CH_3OH/CH_3COOH$ in the ratio 71:25:4 with 0.01 M ammonium acetate (pH 3.0) and a C_{18}-RP column. This is illustrated in the SIM chromatograms in Fig. 5.20A for a mixture of 250 ng each of the authentic alkaloids. Fig. 5.20B illustrates the corresponding chromatograms for a typical example selected from six samples extracted from cultured cells equivalent to 10 mg of the original dry cells. The six separate *Cinchona* cell culture samples studied in this work all gave essentially identical data and therefore only one example is presented.

The peaks illustrated in Fig. 5.20 show some tailing but they are sufficiently well resolved

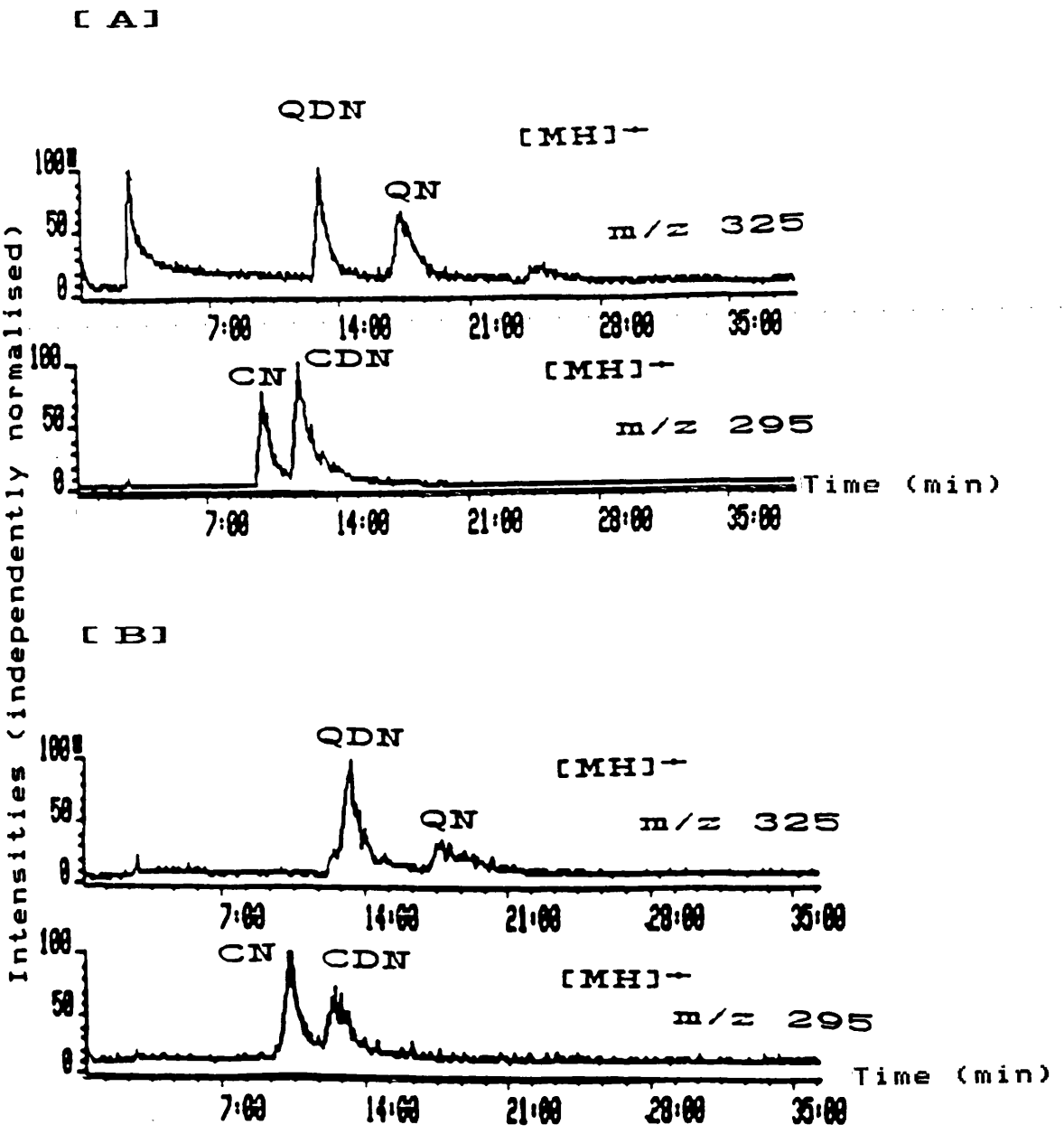


Fig. 5.20 HPLC selected ion TSP profiles of
A. Authentic *Cinchona* alkaloids
B. Alkaloids extracted from a *Cinchona ledgeriana* cell culture

Mobile phase (system C)

for their areas to be evaluated. Quinidine and cinchonidine both elute close to 13 min. and they would be unresolved in a total ion current chromatogram, but as they have different molecular masses the use of SIM separates them completely. This illustrates a major advantage of combined LC/MS for the analysis of complex mixtures, i.e. the ability to identify and resolve multicomponent chromatographic peaks.

The dihydro analogues of cinchonine, cinchonidine, quinidine and quinine were also detected in the same cultured cell extract (Fig. 5.21). These analogues have been detected previously in *C. ledgeriana* cell cultures (Mellon, 1988).[180]

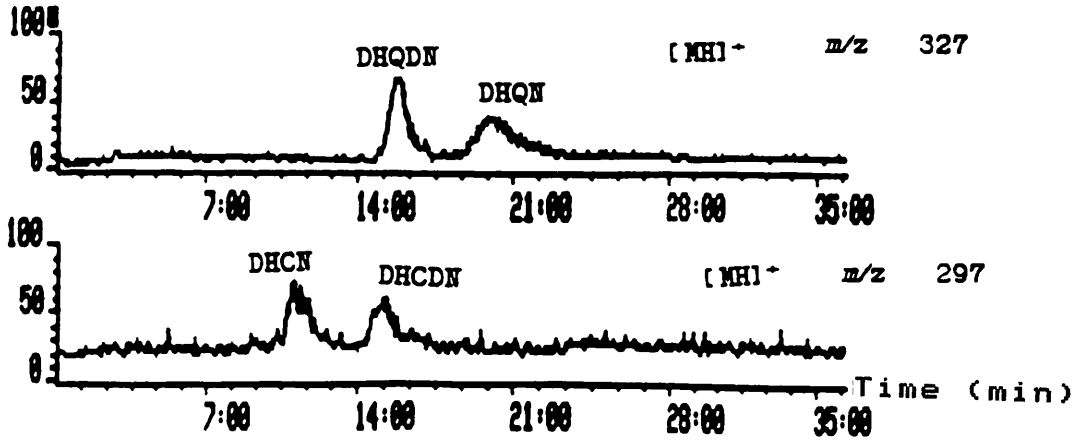


Fig. 5.21 HPLC selected ion TSP profiles of the same extract (Fig. 5.20 B), *m/z* (297 and 327) were monitored

iv. FLUORESCENCE CHROMATOGRAM

Comparing the fluorescence chromatogram (Fig. 5.22) on the same cultured cell extract, in the absence of ammonium acetate buffer, with that obtained by TSP-MS detection in the presence of the buffer (Fig. 5.20), it can be seen that the buffer degrades the resolution slightly.

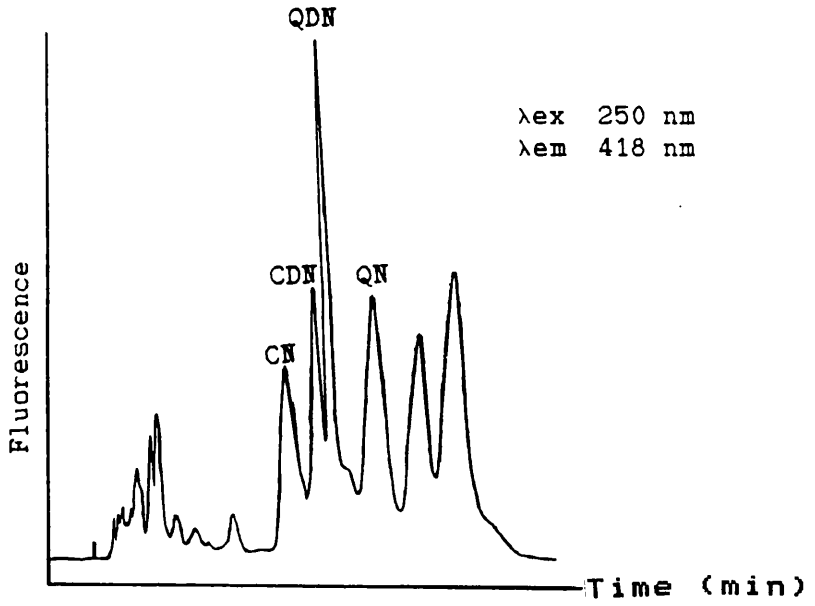


Fig. 5.22 Fluorescence chromatogram of alkaloids extracted from a *Cinchona ledgeriana* cell culture

Fluorescence detection gives superior signal:noise but cinchonidine and quinidine are not well resolved and it detects two other major components which are clearly not the target metabolites.

Furthermore, in the absence of other analytical information, it is impossible to assign the peaks to the individual alkaloids.

5.8.4 QUANTITATIVE ANALYSIS

In order to achieve a measure of the reproducibility and sensitivity of the TSP LC/MS system, a series of solutions of cinchonine in methanol were prepared containing 25, 50, 100, 150, 200 and 250 µg/ml of cinchonine respectively. A complete mass spectrum was obtained on 250 ng of cinchonine injected on column. When the SIM technique was used and the $(M+H)^+$ ion at *m/z* 295 monitored, 25 ng injected on column gave a very clearly defined peak (Fig. 5.23). There was a linear relationship between the area of the ion signal at *m/z* 295 over the range 50-250 ng of cinchonine injected on column (Fig. 5.24). The peak intensities were adversely affected by the peak broading associated with the HPLC column.

Higher sensitivity was achieved when samples were injected directly into the TSP source without an HPLC column. Fig. 5.25 shows a series of replicate injections of 500 pg of quinidine

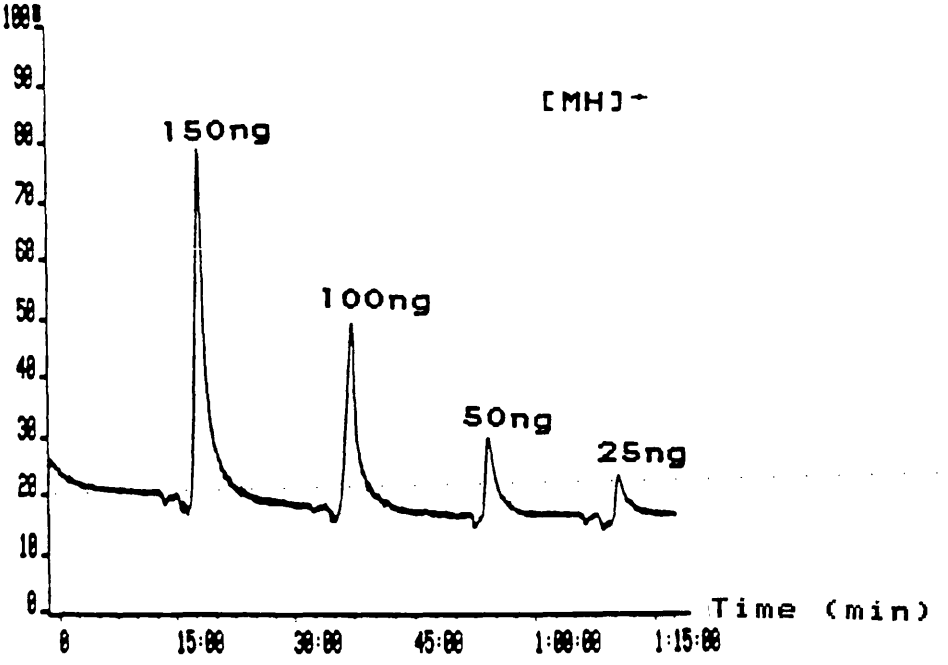


Fig. 5.23 HPLC selected ion TSP profiles of cinchonine [MH]+, injected onto the column at various concentrations

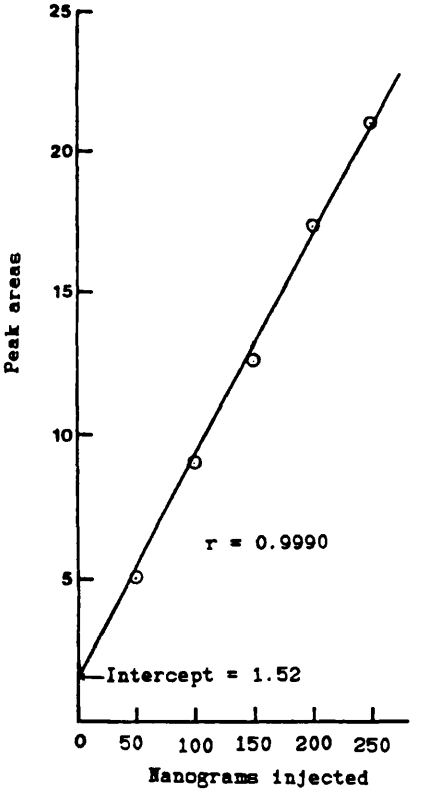


Fig. 5.24 The calibration graph obtained from cinchonine by HPLC/TSP-MS

monitored at *m/z* 325, with direct injection into the TSP source.

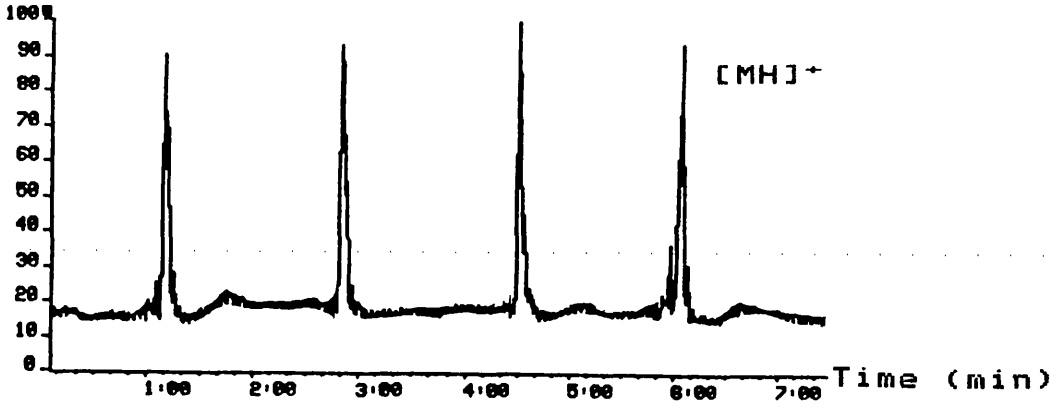


Fig. 5.25 A series of direct injections of 500 pg of quinidine

By decreasing the injected amounts to 10 pg (Fig. 5.26) the signal:noise was reduced to about 10:1.

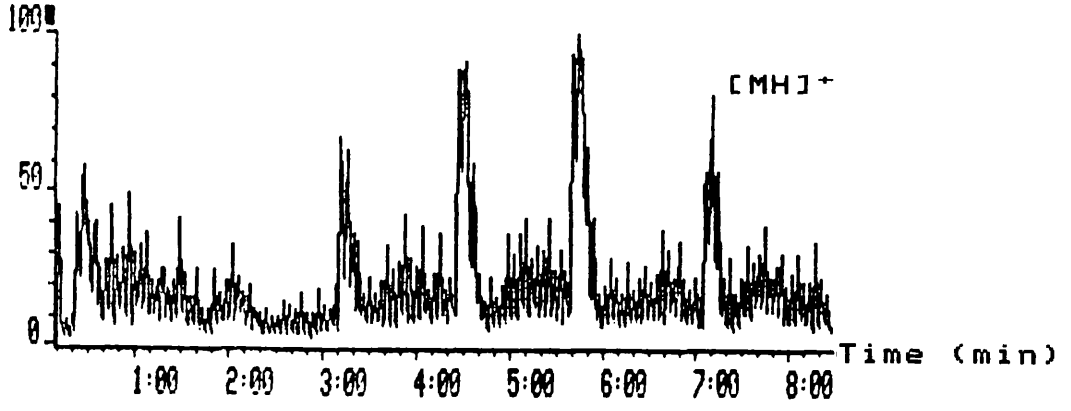


Fig. 5.26 A series of direct injections of 10 pg of quinidine

The standard deviation in the signal intensity is approximately 20%, which may be partly

attributable to injection technique, and partly due to pulsing of the flow and variations in ionisation efficiency. These variations may be worse for the narrow peaks arising from direct injection without HPLC (approximately 10 s) than for the wider HPLC peaks (approx 1 min.), for which the fluctuations may average out.

The similarity of the four alkaloids under study makes it likely that they would all give a similar response by TSP. This is broadly supported by the measured peak areas from (Fig. 5.20A) for the equimolar mixture which for quinine, quinidine, cinchonine and cinchonidine are in the ratio 1:1:1.5:1.7. The fact that these are not equal may be due in some measure to differences in the purities of the standards used. There is also some variability in the sensitivity of response in TSP.

Assuming that the individual alkaloids do give equal responses, the peak areas in (Fig. 5.20B and 5.21) show quinine, quinidine, cinchonine, cinchonidine and their dihydro analogues in this particular cell extract to be present in the approximate ratio 1:7:2:1:1:6:1:1. For true quantitation it is necessary to have a suitable internal standard to compensate for changes in

ionisation efficiencies between samples. Such a standard would also allow for sample losses in the extraction and work-up procedures.

5.8.5 POSSIBLE INTERNAL STANDARDS FOR *CINCHONA* ALKALOIDS

Possible internal standards for quantitative assays of the *Cinchona* alkaloids may be divided into three types, as follows:

Type A:

Stable isotope labelled analogues of one of the *Cinchona* alkaloids. These give different *m/z* values but similar or identical retention times on GC or HPLC. Starting from quinine, attempts were made by an undergraduate project student to synthesise 19-OCD_3-dihydroquinine (M.w. 329) by low temperature catalytic hydrogenation of the vinyl bond, demethylation of the 19-methoxy group with HBr, formation of the quinolinoxide salt and remethylation with CD_3I. The use of dihydroquinine without deuteration would have been unsatisfactory as this alkaloid can occur naturally and the mass difference of only two mass

units would be barely sufficient. However, the combination of hydrogenation and CD_3 incorporation would yield a mass difference of 5 Da compared with quinine. It seems unlikely that TSP ionisation efficiency would be affected by reduction of the vinyl group as protonation should occur at one or other of the nitrogen atoms. The presence of CD_3 rather than CH_3 would be unlikely to affect the retention time in HPLC although GC can be sensitive to such changes.

Type B:

Close homologues of the *Cinchona* alkaloids. These can be chosen to give the same *m/z* value, in which case they must have a different retention time on GC or HPLC. Alternatively if they have different masses they can be monitored by multiple ion monitoring (MIM) without the need for different retention times.

Type C:

Compounds from the same chemical class as the *Cinchona* alkaloids, as with type B these may have the same *m/z* value, in which case the retention

time must be different, or they may have a different *m/z* value, in which case the retention time may be the same or different.

The selection of an internal standard should be based on a consideration of its effect upon limiting the errors at each stage of the analysis, obviously with the greater weight being attached to the largest errors. For the mass spectrometric part of the assay, a Type B or C standard having the same mass as the analyte is superior. Types A, B and C are equally useful for the purpose of maintaining a constant ratio of sample to internal standard. However, the focus of our attention for selecting an internal standard lies primarily in its behaviour relative to the compound of interest, particularly during extraction and ionisation in the ion source, and in most respects a stable isotope labelled analogue would be ideal for this purpose.

5.9 TANDEM MASS SPECTROMETRY

An earlier study by Mellon *et al.*,[183] used EI ionisation without chromatographic separation but with tandem methods (unimolecular metastable fragmentation monitored by B/E linked scans) in an

attempt to monitor plant cell tissue culture of *Cinchona* alkaloids.

For the analysis of individual components in complex mixtures, the reliance on the observation of a single peak under SIM conditions may be considered inadequate for compound characterisation. For the *Cinchona* alkaloids, TSP gives only protonated molecular ions, even with a repeller electrode, and it may be desirable to carry out collision-induced dissociation (CID) in a tandem mass spectrometer. This facility was not available on the quadrupole mass spectrometer used for the HPLC/MS separations, but initial experiments were carried out on the protonated molecular ions formed by FAB and subjected to CID in the second field-free region of a double focussing mass spectrometer, i.e., CID-mass analysed ion kinetic energy mass spectrometry (CID-MIKES).

The spectrum of quinine given in Fig. 5.27 shows major fragment ions at *m/z* 309, 189, 173, 160 and 136, corresponding to the main collision dissociations of the molecule. It is noteworthy that these are essentially the same fragments observed under EI conditions. It is very probable that the same peaks would be obtained by TSP CID-

MIKES, which would allow multiple ion monitoring e.g., with a triple quadrupole mass spectrometer, thereby increasing the analytical reliability of the data.

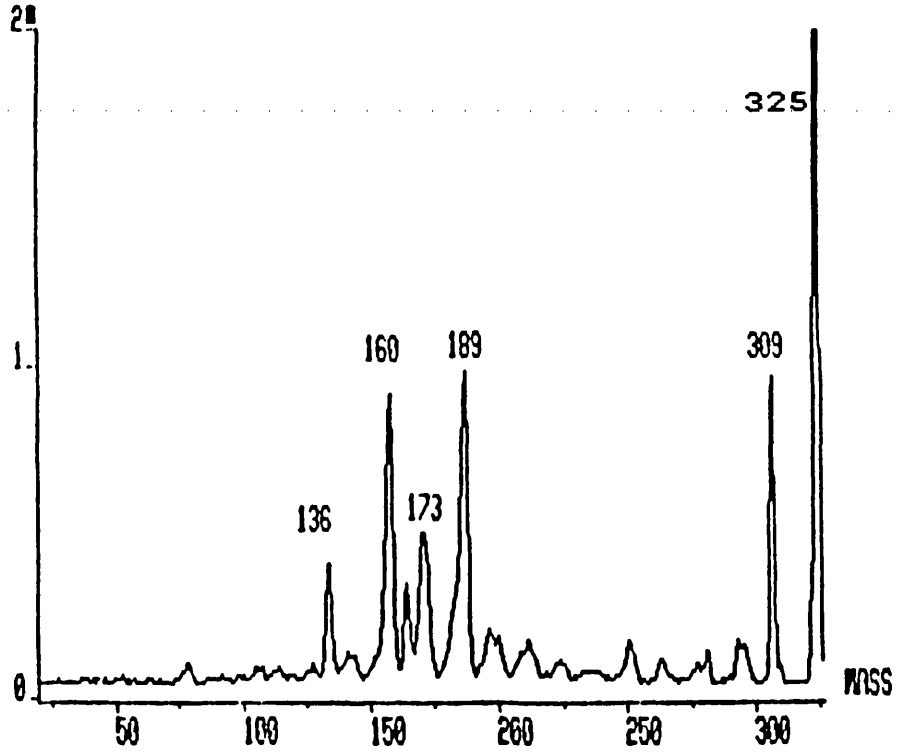


Fig. 5.27 FAB CID-MIKES spectra of $[MH]^+$ of quinine (*m/z* 325)

6. OPIUM ALAKLOIDS

6.1 INTRODUCTION

The poppy, *Papaver somniferum L.* (Papaveraceae) is an annual herb (Fig. 6.1) which continues to be the principal source of the pharmaceutically useful opiates as well as source of seed and seed oil for culinary purposes.[184]

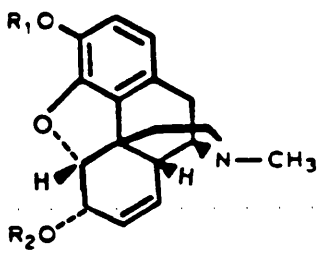
Fig. 6.1 *Papaver somniferum*

The alkaloids (Fig. 6.2) derived from the *P. somniferum* are important medicinally but morphine is also subject to abuse. It is recognised that the physical and psychological dependence on morphine and its diacetyl derivative, heroin, has resulted in international social and criminal problems.

Morphine, codeine and their derivatives are powerful analgesics, acting as central nervous system depressants, alleviating anxiety as well as pain. Morphine also reduces intestinal motility and is used in diarrhoea. Codeine is a milder analgesic without addictive properties and has an additional use as an antitussive.

Other pharmaceutically important opium alkaloids include thebaine, noscapine and papaverine. Thebaine is also used to produce other analgesics, whereas noscapine is a cough suppressant and papaverine a smooth muscle relaxant and cerebral vasodilator. The detection and analysis of these alkaloids from plant sources may provide valuable information to assist in the selection of suitable plant material for initiating cell organ cultures.

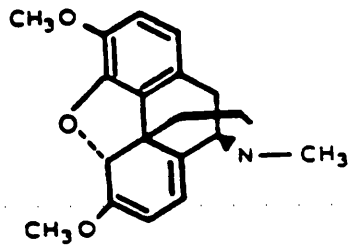
The presence of certain derivatives of morphine as metabolites of heroin in urine or blood may be



MORPHINE R_1 AND R_2 = H (M.W. 285)

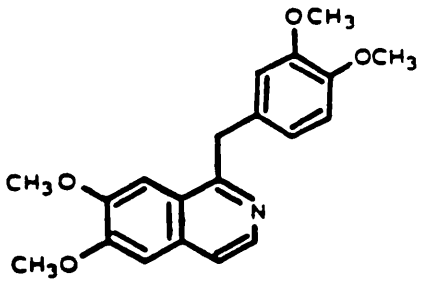
CODEINE $R_1 = CH_3$, $R_2 = H$

(M.W. 299)



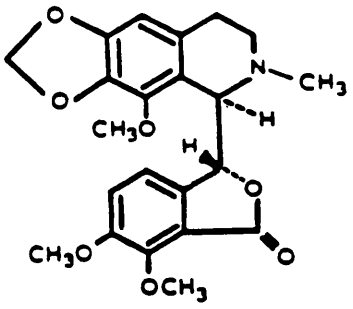
THEBAINE

(M.W. 311)



PAPAVERINE

(M.W. 339)



NOSCAPINE

(M.W. 413)

Fig. 6.2 The medicinally valuable alkaloids of *Papaver somniferum* L. plants

indicative of heroin use and this is therefore important from the viewpoint of forensic analysis.

Although opium has been in use for centuries, there is no consensus on a uniform method for the simultaneous quantitation of morphine and other related opium alkaloids.[185] The quest for better chromatographic methods for simultaneous separation and quantitation of the principal opium alkaloids such as morphine, codeine, thebaine, papaverine and noscapine still continues, as revealed by a survey of the recent literature, (see below).

The major problems associated with the analysis of opium alkaloids are: (i) complexity of the matrix itself; (ii) incomplete recovery of alkaloids from the matrix during extraction due to solubility limitations and (iii) strong adsorption of polar alkaloids like morphine on column packings.

Some classical methods are known to yield low assay results for morphine.[186] Further, pharmacopoeial methods involve a rather tedious preseparation of morphine from the matrix before it is quantitated. The limitations and difficulties of some classical methods for quantitation of opium alkaloids have been briefly

reviewed by Ziegler *et al.*,[187] A radioimmunoassay (RIA) method has been developed for the determination of morphine in *P. somniferum* capsules by Hsu *et al.*,[188] This assay is highly sensitive in the picomole range but not specific due to cross-reaction with the other opiates.

Several workers have observed that underivatized morphine is difficult to quantitate by gas chromatography. This is most probably due to its high polarity which favours strong adsorption at active sites of the column packings, resulting in deceptively low and non-quantitative results.[189]

More recently, attention has focused on the use of HPLC. The application of HPLC in straight-phase, reversed-phase, ion-pair and ion-exchange modes to the separation of opium alkaloids has been reviewed.[189-192] The reversed-phase mode using bonded cyano,[193,194] amino,[195] octyl,[196] phenyl,[197] and octadecyl[198,199] packings and the ion-pair mode[200-203] in combination with UV or fluorescence detection has been widely used for separation of the principal opium alkaloids, although the specificity of the techniques is often questionable. Recently Ayyangar and Bhide[204] have used gradient RP-HPLC for the separation of opium alkaloids. The use of

supercritical fluids as mobile phases with packed columns has recently become popular and this method is now an attractive one for opium alkaloids.[205] As far as the author of this thesis is aware, as yet no literature reports have appeared describing TSP studies of opium alkaloids.

In order to use MS analysis with chromatographic separation without prior derivatisation, TSP ionisation has been investigated and a reversed-phase isocratic separation has been developed. In this way several alkaloids have been identified in extracts of *P. somniferum L.* by HPLC/TSP-MS using single ion monitoring (SIM), and it has been demonstrated that this analysis can be applied to detection of the 3 and 6-monoacetyl metabolites of heroin, which are associated with heroin use.

6.2 STEREOCHEMISTRY

The steric structure for morphine is shown in Fig. 6.3. The morphine molecule is generally considered to be a phenanthrene derivative[206] consisting of five fused ring systems. Carbon atoms 1-4, 11, and 12 form an aromatic ring, and the oxygen bridge between carbon atoms 4 and 5

forms a furanoid-type ring. A methylamine chain connects carbon atoms 9 and 13, thus forming a N-methylpiperidine ring across the phenanthrene skeleton. This tertiary amino group accounts for the basic properties of the morphine molecule. Two hydroxyl groups, a phenolic one at carbon 3 and an alcoholic one at carbon 6, determine some of the chemical properties observed during the metabolism of morphine in man. Asymmetric centers at carbon atoms 5,6,9,13, and 14 account for the strong levorotation of morphine. The levo isomer is the pharmacologically active compound. The morphine dextro isomer is devoid of any of the significant pharmacological activity observed for the levo isomer.

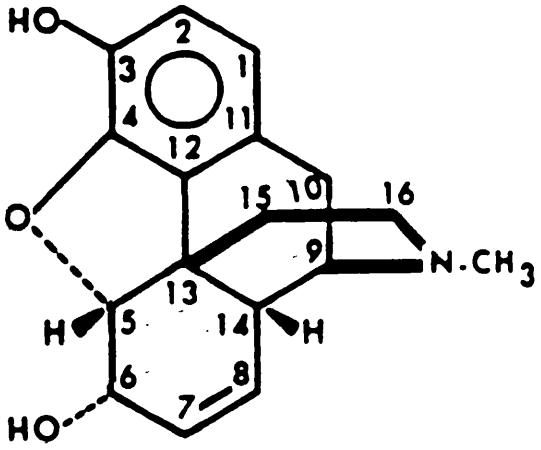


Fig. 6.3 Morphine

6.3 EXPERIMENTAL

i. REAGENTS AND MATERIALS

Acetonitrile was of HPLC grade (BDH, Poole, U.K.), all other reagents were of analytical grade. Deuterated acetic anhydride, morphine, codeine, thebaine, papaverine and noscapine were obtained from Sigma Poole, U.K. 6-Monoacetylmorphine was generously provided by Dr. C. Curry (The Metropolitan Police Forensic Sci. Lab. London). 3-[$^{1}H_3$] and [$^{2}H_3$] monoacetylmorphine were synthesised from morphine.

10 mg of each alkaloid was dissolved in 10 ml methanol with a trace of dilute acetic acid where necessary to aid dissolution. 20 µl aliquots of these solutions were injected when UV was used as a detector. For TSP/MS detection, the solutions were further diluted in methanol to a concentration of 100 µg/ml, and 10 µl aliquots were injected on the column

ii. INSTRUMENTATION

Mass spectrometric experimental conditions were as previously described in section 5.4 ii, except

that the TSP conditions were optimised with approximate temperatures: source 200°, nozzle 200° and chamber 250°C.

6.4 OPTIMISATION OF CHROMATOGRAPHIC SEPARATION

Separations were carried out using a Gilson 714 HPLC, as described in chapter 5 the system was interfaced to a VG MassLab 12/250 quadrupole mass spectrometer fitted with a commercial TSP source and VG 11-73 data system. Chromatographic conditions were:

Column:	C_{18} Waters Bondapak 25cm * 4.6 mm
UV detection	254 nm
Flow rate:	1.0 ml/min

Mobile phases based upon ammonium acetate buffer-acetonitrile of varying proportions (Tab. 6.1) were investigated:

Table 6.1 Mobile phase composition

Mobile phase	1% Ammonium acetate	Acetonitrile
System A	70	30
System B	65	35
System C	60	40
System D	55	45

The buffer was prepared by adjusting the pH of a 1% solution of ammonium acetate to pH range (5.5-6.0) with acetic acid.

Two operating parameters were studied

i. Variations in the pH within the (5.5-6.0) range had no effect on the separation of the five alkaloids. Therefore the pH 5.8 was held constant.

ii. Variations in the mobile phase composition on retention times of the alkaloids (Tab. 6.2)

Table 6.2 Dependance of alkaloid retention times on the mobile phase composition

Mobile phase	Retention time (min)				
	Mor	Cod	Theb	Pap	Nos
System A	4.3	5.3	12.3	24.6	52.4
System B	4.2	5.0	10.0	15.1	33.2
System C	4.0	4.6	9.2	11.8	25.5
Syatem D	3.9	4.0	8.9	10.5	22.5

Mor = Morphine, Cod = Codeine, Theb = Thebaine, Pap = Papaverine, Nos = Noscapine

When system A (Tab. 6.2) was used as a mobile phase, the five alkaloids were completely

separated but noscapine was retained too long to be determined simultaneously. The retention times were found to be shorter with increasing percentages of acetonitrile in the mobile phase (system B, C and D). System D failed to separate morphine from codeine as the difference in retention times was not high enough to permit the two peaks to be resolved. Therefore system C was considered to be the most favourable in terms of resolution and overall analysis time.

6.5 ANALYSIS OF STANDARD ALKALOIDS

Separation of an equimolar mixture of authentic samples (Fig. 6.4) of morphine (1), codeine (2), thebaine (3), papaverine (4) and noscapine (5) was carried out with an isocratic analysis using the above HPLC conditions which had been optimised for TSP detection.

Morphine ran close to the solvent front and as detected by UV (254 nm), the peak shape was unsatisfactory. However, HPLC/TSP-MS run under identical conditions with SIM of morphine detected at *m/z* 286 showed no such peak shape degradation. The SIM traces illustrated in the Fig. 6.5A show significant variation in sensitivity for the

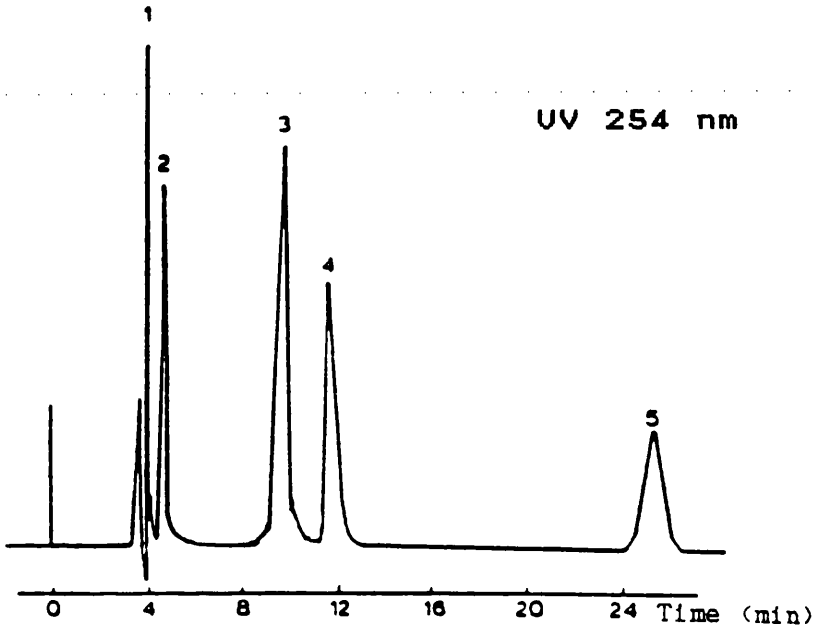
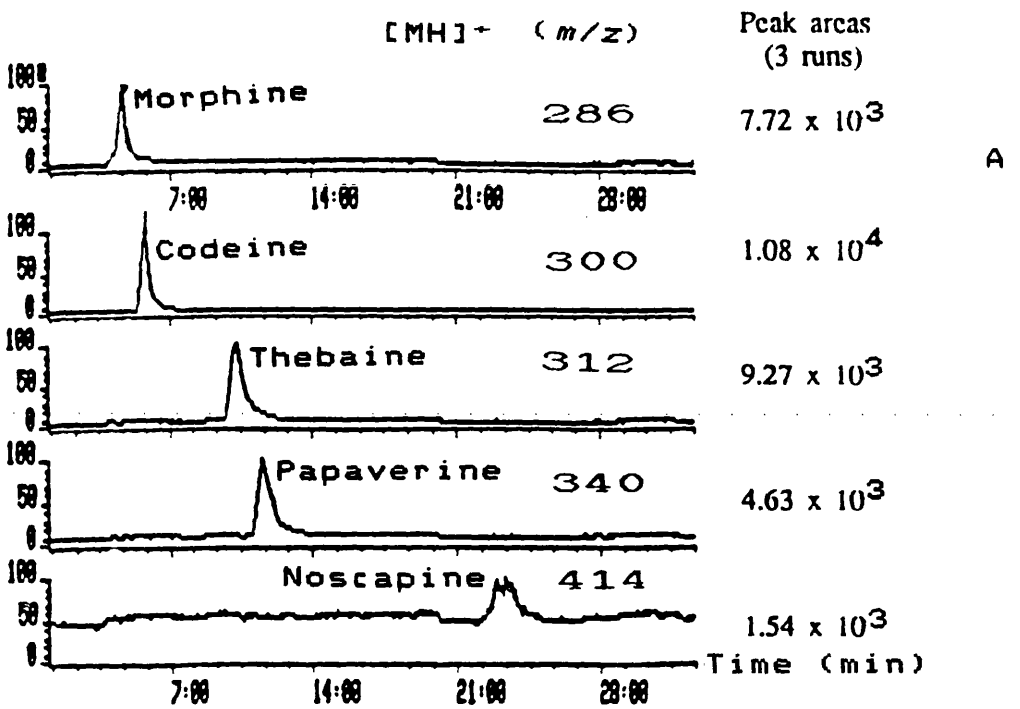


Fig. 6.4 UV chromatogram of five authentic alkaloids on C_{18} Water Bondapak. 1=Morphine; 2=Codeine; 3=Thebaine; 4=Papaverine; 5=Noscapine.

Mobile phase (system C): 1% ammonium acetate (PH 5.8)-acetonitrile (6:4).



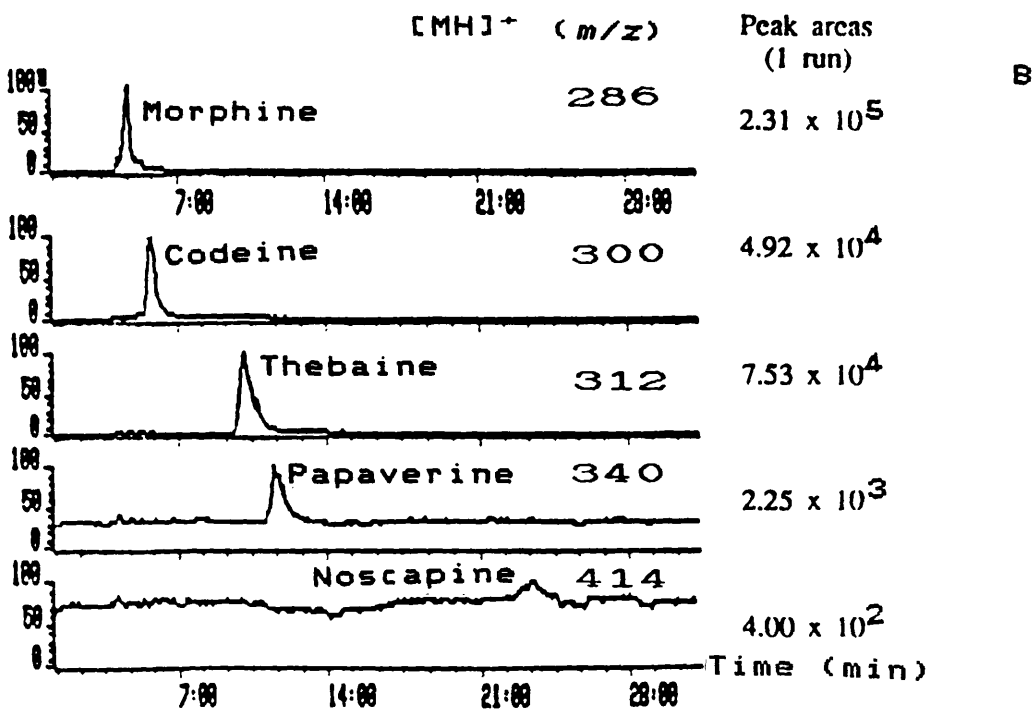


Fig. 6.5 HPLC selected ion TSP profiles of

A. Authentic opium alkaloids

B. Alkaloids extracted from a *Papaver somniferum*

various alkaloids, peak areas for 200 ng each of morphine, codeine, thebaine, papaverine and noscapine monitored for $(M+H)^+$ ions at *m/z* 286, 300, 312, 340 and 414 being in the approximate ratios 5:7:6:3:1 respectively.

6.6 ANALYSIS OF PLANT MATERIALS

6.6.1 ALKALOID EXTRACTION

The extraction method used was developed by M.F.Roberts and T.Pham, Department of Pharmacognosy, School of Pharmacy. Four separate samples were analysed in this study.

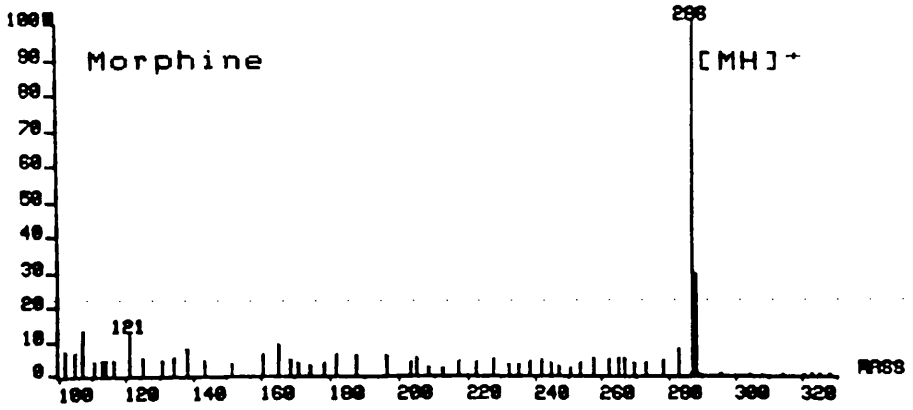
1 g of finely powdered (*Papaver somniferum L.*) plants (capsules with 8 inch of stems) was exhaustively extracted with 5% acetic acid. The extract was evaporated to dryness. The residue was dissolved in 2% H_2SO_4 (total 20 ml) and then made alkaline with NH_4OH to PH 9.5. This solution was placed on a standard Extrelut column (Merck-Darmstadt, FRG) and allowed to equilibrate for 30 min. The alkaloids were then removed from the column with 5-6 column volumes of chloroform : isopropanol (4:1). The combined organic extracts

were dried over anhydrous Na_2SO_4 and concentrated to dryness under reduced pressure. The residue was dissolved in a portion of the mobile phase used for HPLC analysis.

6.6.2 HPLC/TSP-MS

This work has established that opium alkaloids display very similar characteristics to the *Cinchona* alkaloids under TSP conditions, producing protonated molecular ions and no detectable fragments. Fig. 6.6 shows typical mass spectra of some standard alkaloids. Fig. 6.5B shows typical SIM traces obtained for the HPLC/TSP-MS analysis of the alkaloids derived from the equivalent of 2 mg dried plant material of four extracts. By comparing the peak areas with triplicate runs of the standards one can roughly estimate the amount of each component (in ng) to be as follows:

Alkaloid	Amount (ng)/2 mg plant material
Morphine	6,000
Thebaine	1,600
Codeine	900
papaverine	100
Noscapine	50



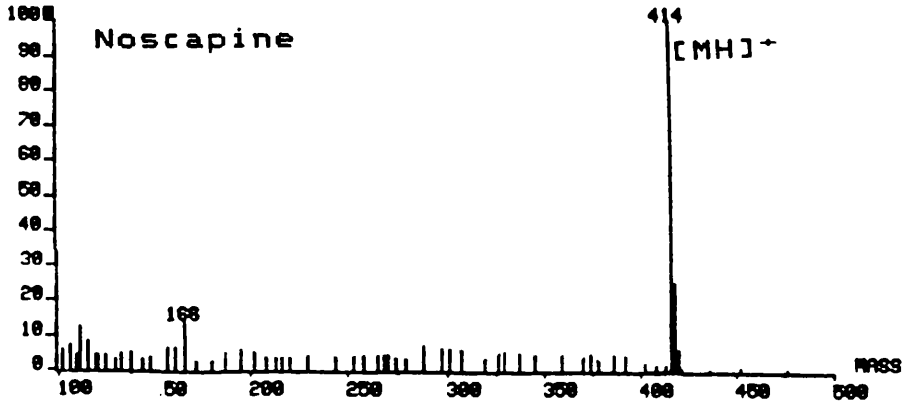


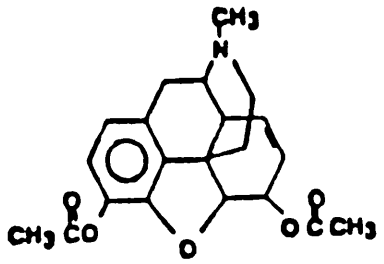
Fig. 6.6 TSP-MS of morphine and noscapine

Other opium alkaloids were also detected in the plant extracts. Some of these are isomeric e.g., neopinone, codeinone and oripavine, and can not be distinguished on the basis of molecular mass alone. Standards would be required to determine the differences in retention times. Furthermore, it is likely that some differences in structure might be revealed with collision induced dissociation, e.g., with a triple quadrupole mass spectrometer.

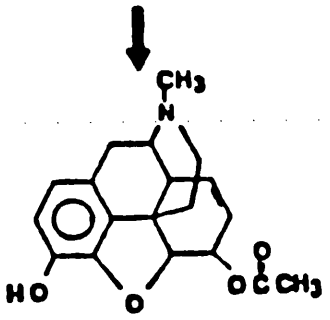
6.7 DRUG ABUSE

6.7.1 METABOLISM OF HEROIN AND CODEINE IN HUMANS

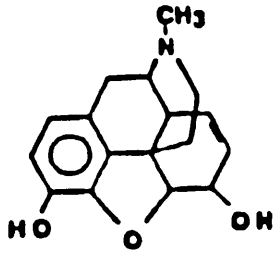
Heroin is metabolised to 6-acetylmorphine and then to morphine, whereas codeine is metabolised directly to morphine[207] (Fig. 6.7). Although the ingestion of opium alkaloids in foodstuffs is widespread, e.g., through eating poppy seeds, human metabolites of morphine do not include 3 and 6-monoacetylmorphine, and the presence of these is indicative of heroin use. For this reason, it would be useful to have a procedure that is capable of separating heroin and 6-monoacetylmorphine from morphine and codeine.



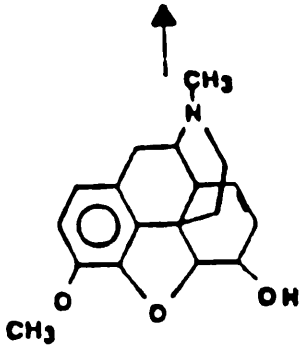
Heroin
369



6-Monoacetylmorphine
327



Morphine
285



Codeine
299

Fig. 6.7 Metabolism of heroin and codeine in humans[207]

Until recently, most studies of the metabolism of these drugs in man relied on RIA. Although the method is capable of detecting picogram amounts of heroin, it lacks specificity due to cross-reactivity of RIA antisera, and it therefore cannot distinguish between heroin and its metabolites.

GC methods[207,208] require extensive sample clean-up and derivatisation procedures prior to assay, making such methods very time-consuming.

During the past ten years, HPLC methods for the measurement of various drug metabolites have been introduced using a combination of electrochemical and UV detection in order to improve the detectability.[209] Recently Venn and Michalkiewicz[210] have assayed morphine and its metabolites using HPLC and a native fluorescence detection to increase the reliability of the method. However, these methods are complicated and tedious, owing to the combined detector approach.

The optimum separation and TSP ionisation for morphine, codeine, 6-monoacetylmorphine, and heroin monitored at *m/z* (286, 300, 328 and 370) respectively was determined in this work, as is

illustrated in the SIM chromatogram in Fig. 6.8 for a mixture of 250 ng each of the authentic alkaloids. Codeine and 6MAM, which have very similar retention times, are easily distinguishable because of their different molecular masses.

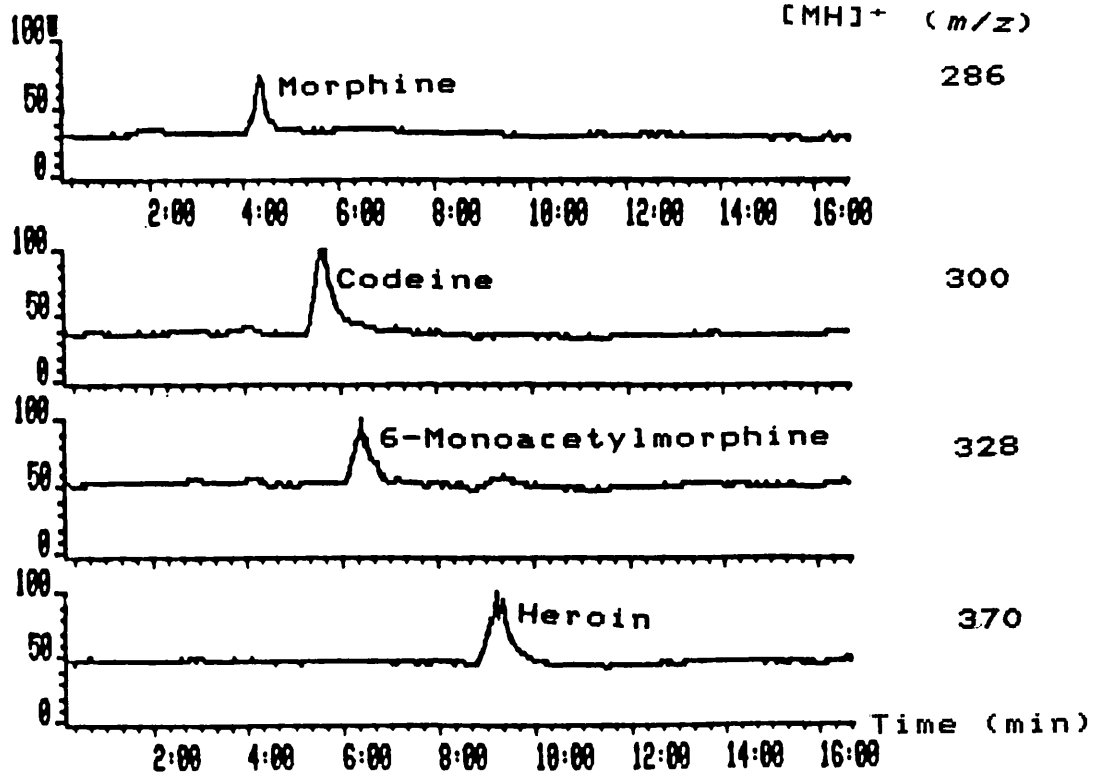


Fig. 6.8 HPLC selected ion TSP profiles of authentic opium alkaloids

6.7.2 SYNTHESIS OF 3-MONOACETYLMORPHINE

The phenolic hydroxyl group of morphine was selectively acetylated following the procedure described by Welsh[211] by reacting acetic anhydride and morphine in equimolar portions in the presence of aqueous sodium bicarbonate.

Procedure:-

0.05 g of morphine base was dissolved in 5 ml of distilled water contained in a 50 ml separatory funnel. 0.5 g sodium bicarbonate was added and the funnel was swirled until the bicarbonate had dissolved. 0.25 ml acetic anhydride was rapidly injected into the funnel by means of a calibrated 1 ml syringe, the funnel was immediately stoppered and vigorously shaken until the evolution of carbon dioxide had ceased (4-5 minutes, shaking was interrupted momentarily several times to release pressure in the system). After the reaction mixture had been allowed to stand for 5 minutes, it was extracted with 10 ml $CHCl_3$ x 6. The combined extracts were evaporated to dryness initially under vacuum until the volume was reduced to approximately 1 ml and then with nitrogen.

The HPLC trace showed that 100% of the morphine was converted to 3-monoacetylmorphine. The TSP mass spectrum (Fig. 6.9) showed only one peak at *m/z* 328, corresponding to the protonated monoacetylmorphine.

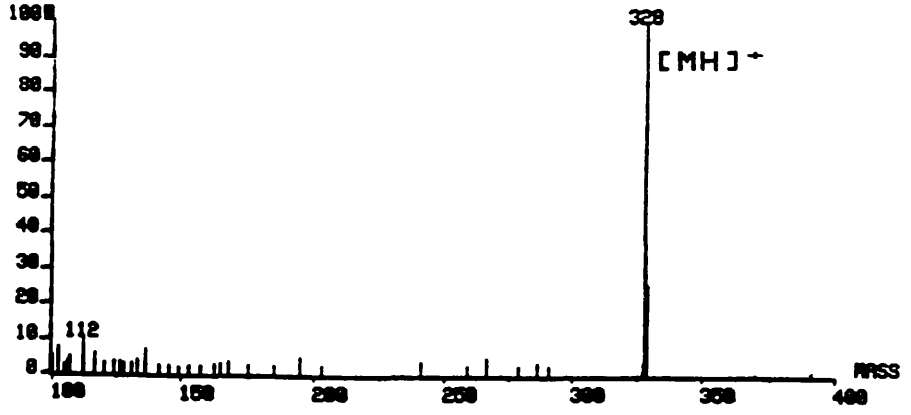


Fig. 6.9 TSP-MS of 3-monoacetylmorphine

Furthermore the EI-MS (Fig. 6.10) showed an intense molecular ion at *m/z* 327 and $(M-CH_2CO)^+$ at *m/z* 285 as the base peak.

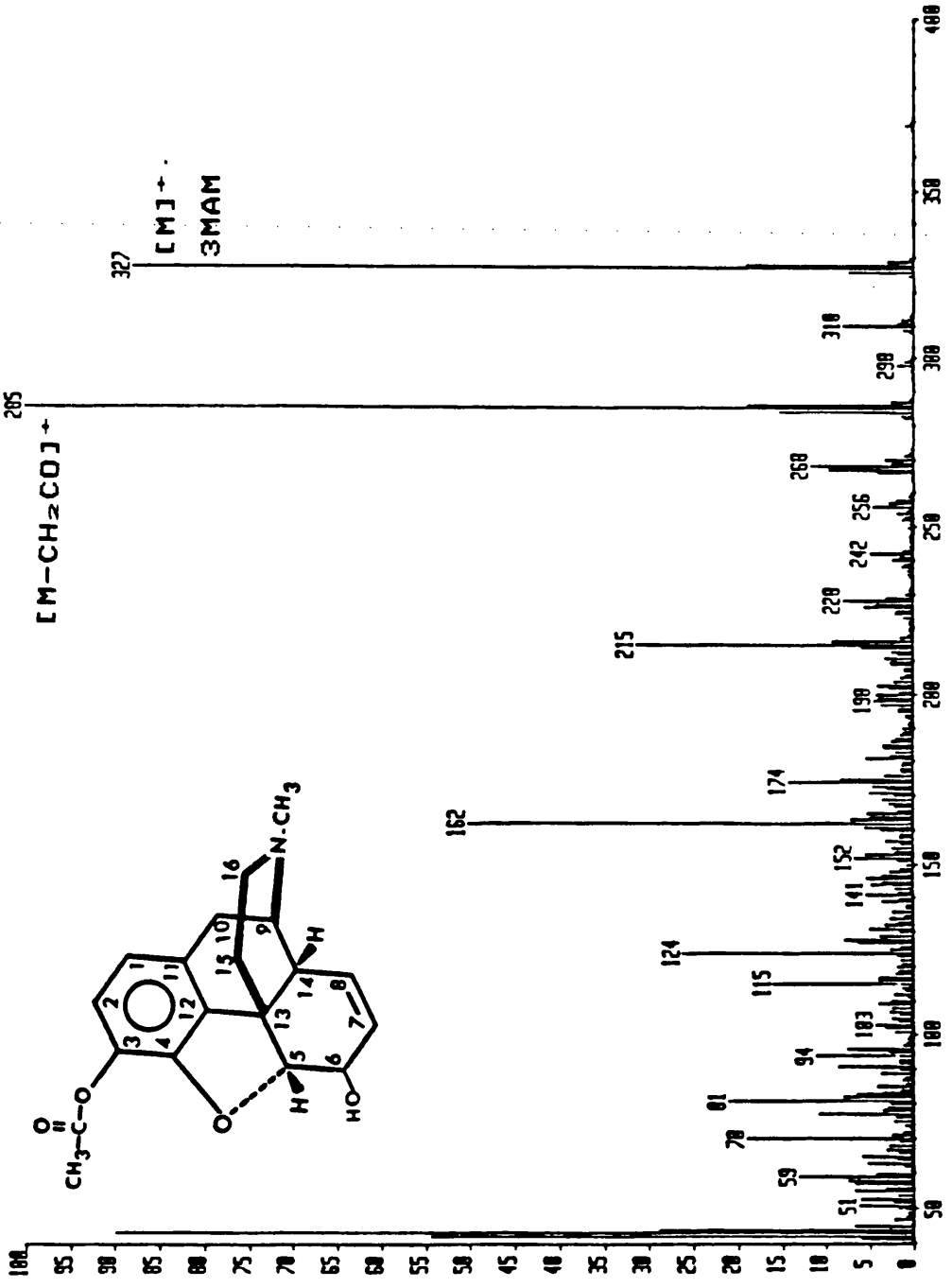


Fig. 6.10 EI-MS of 3-monoacetylmorphine

6.7.3 SEPARATION OF 6 AND 3-MONOACETYLMORPHINE

Morphine and its diacetyl derivative heroin have attracted considerable interest from analytical scientists who have been concerned with the identification and quantification, often of minute amounts, of these two potent narcotic analgesics which are widely utilised as drugs of abuse. Surprisingly there appears to be little published information on the separation of the monoacetylated morphines (6-acetyl from 3-acetyl).

It has been reported that morphine-6-glucuronide[212] and morphine-6-sulphate[213] have greater analgesic potency than morphine itself whereas the corresponding 3-glucuronide and 3-sulphate have little or no analgesic potency. If substitution of the phenolic hydroxyl at C_3 in morphine results in substantial loss of analgesic activity then it might be expected that the 3-acetyl morphine (3MAM) would be inactive and that 6-acetyl morphine (6MAM) would be active. It is therefore of some importance to establish sensitive analytical procedures in order to differentiate 3MAM and 6MAM.

Recently Mule and Casella[214] have used GC/MS to measure the 6MAM metabolite of heroin in human

urine, and confirmed that this metabolite is not present in poppy seeds or urine after the ingestion of products containing poppy seed. The application of the method to the 3MAM metabolite of heroin was not described.

The separation and TSP ionisation for 6 and 3-MAM monitored at *m/z* 328 is illustrated in the SIM chromatogram in Fig. 6.11 for a mixture of 500 ng each of the authentic alkaloids. The two isomers are distinguished by their characteristic retention times.

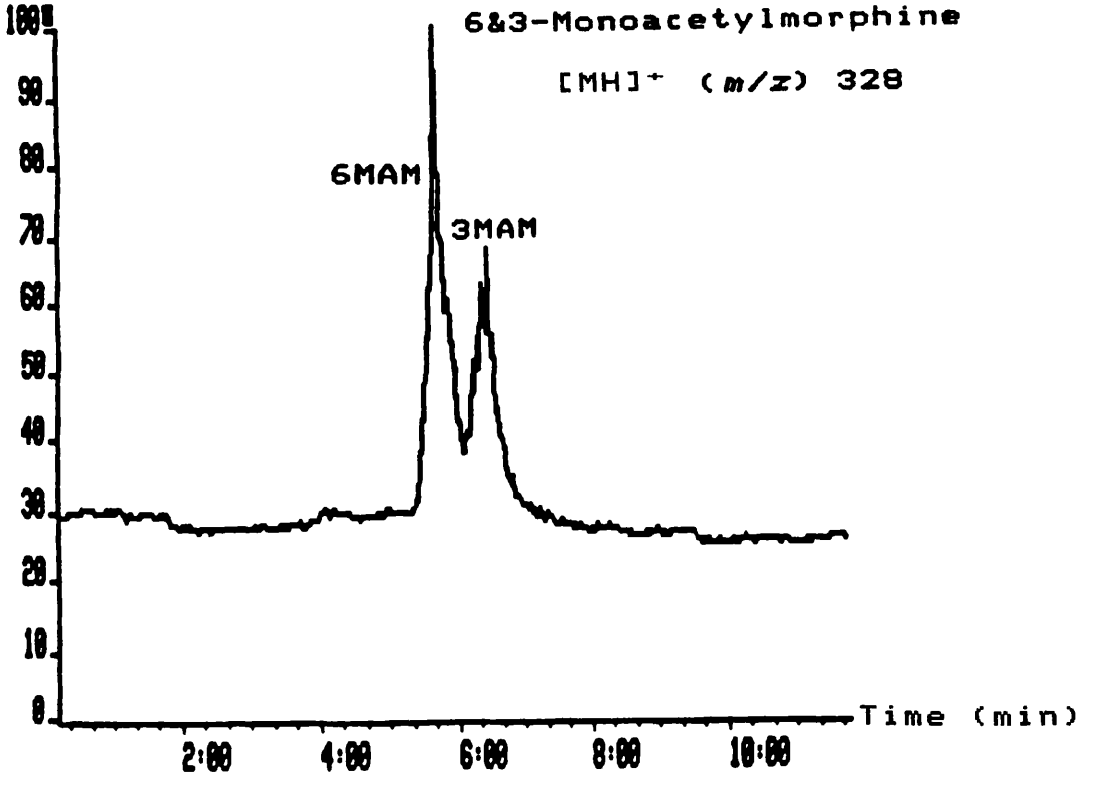


Fig. 6.11 HPLC selected ion TSP profiles of 6 and 3-monoacetylmorphine

6.7.4 SYNTHESIS OF 3-[$^2H_3$] MONOACETYLMORPHINE

The phenolic hydroxyl group of morphine was selectively acetylated to 3-trideuterated monoacetylmorphine (CD_3CO) following the procedure described by Welsh[211] by reacting labelled acetic anhydride [$(CD_3CO)_2O$] and morphine in equimolar portions in the presence of aqueous sodium bicarbonate see (6.7.2).

The 3-trideuterated MAM was characterised by EI-MS (Fig. 6.12) which showed an intense molecular ion at *m/z* 330 and $(M-CD_2CO)^+$ at *m/z* 286 as the base peak. The acetic anhydride was 99% labelled with deuterium, so the presence of unlabelled peaks was not significant.

Comparison of the labelled 3MAM spectrum (Fig. 6.12) with the unlabelled 3MAM (Fig. 6.10) showed that the molecular ion, $(M-OH)^+$ ion and the methyl acetate ion are clearly shifted from *m/z* 327, 310 and 59 in the unlabelled molecule to *m/z* 330, 313 and 62 subsequently in the labelled molecule, indicating the incorporation of three deuterium atoms into these structures. The $(M-CH_2CO)^+$ ion at *m/z* 285 and a few other ions at *m/z* 268, 256, 242, 228 and 215 are increased by one Da due to

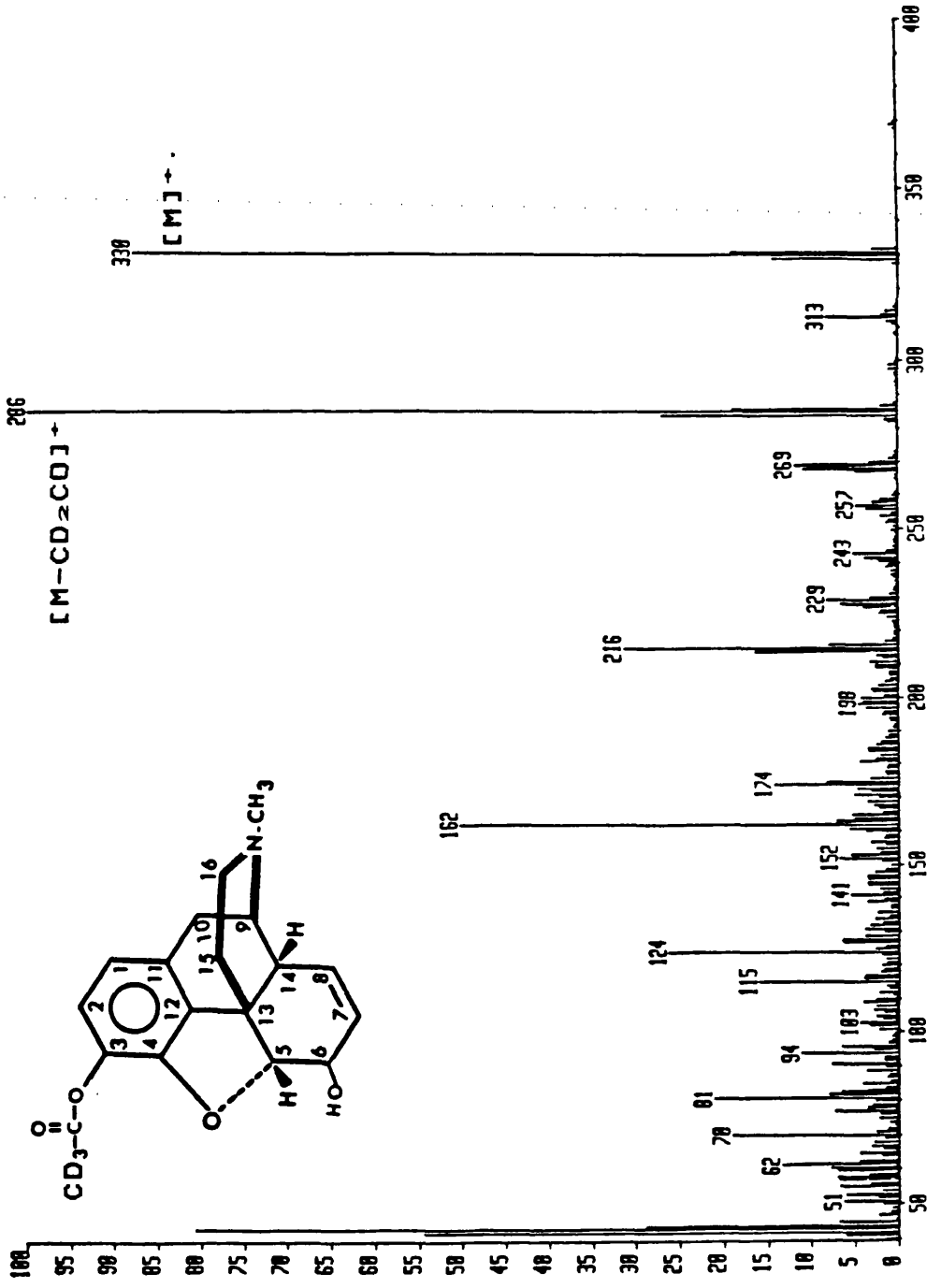


Fig 6.12 EI-MS of 3-[$^2H_3$] monoacetylmorphine

retention of one deuterium in their structures, while the remaining ions did not shift in mass.

Furthermore the TSP mass spectrum (Fig. 6.13) showed only one peak at m/z 331, corresponding to the protonated 3-[$^2H_3$] monoacetylmorphine.

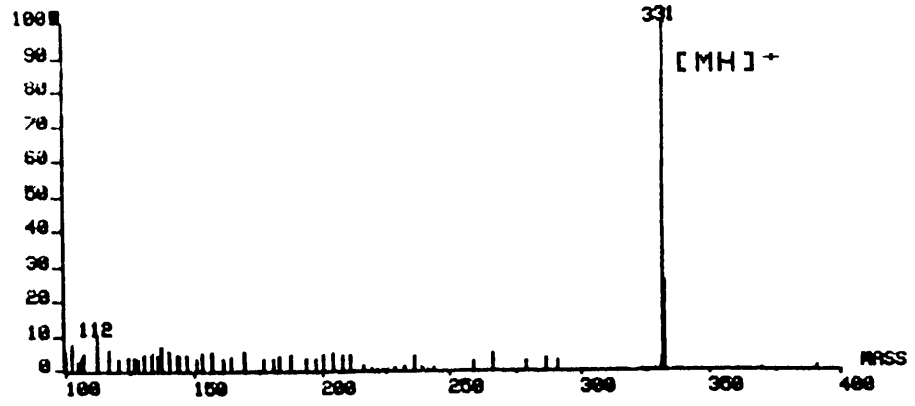


Fig. 6.13 TSP-MS of 3-[$^2H_3$] monoacetylmorphine

The presence of CD_3 rather than CH_3 did not affect the retention time in HPLC, this is illustrated in the SIM chromatograms in Fig. 6.14 for a mixture of 500 ng each of the unlabelled and labelled alkaloids.

The prepared deuterium labelled internal standard would allow to quantify the heroin metabolites.

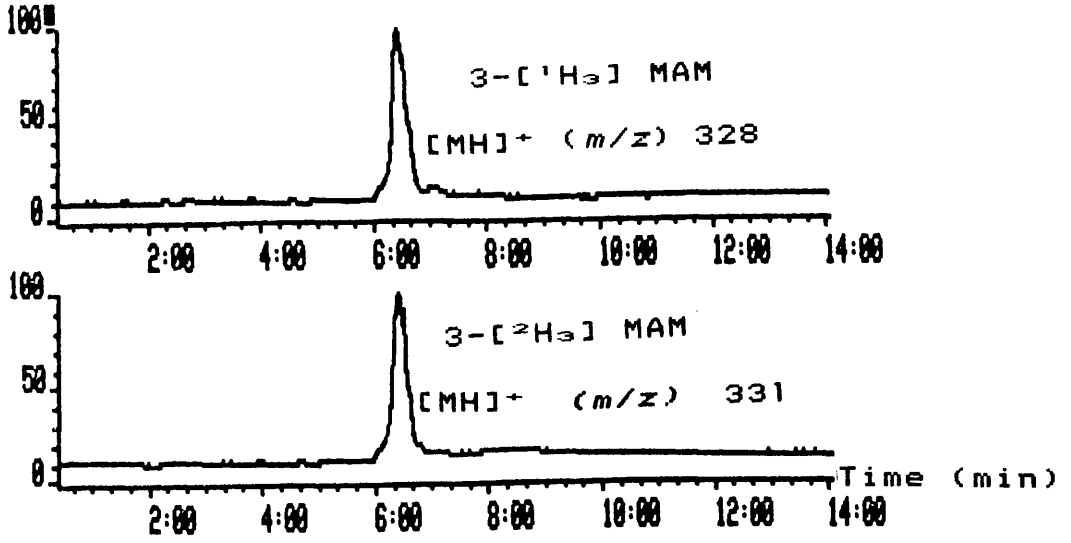


Fig. 6.14 HPLC selected ion TSP profiles of 3-[$^1H_3$] and [$^2H_3$] monoacetylmorphine

6.8 EI AND FAB SPECTRA

The direct inlet EI and CI mass spectra of illicit drugs have been reported by Ohno *et al.*,[215] Pelli *et al.*,[216] have described the CID (B/E=const) linked scans and MIKES spectra of morphine under EI conditions in the hair of heroin addicts. The potential of tandem MS as a rapid screening technique for drugs and metabolites in racing animals has been described by Brotherton and Yost.[217] The methane chemical ionisation mass

fragmentography of opium alkaloids in urine of opium eaters has been studied by Cone *et al.*.[218]

The direct injection of morphine into the EI source of the quadrupole mass spectrometer showed an intense molecular ion at *m/z* 285 as the base peak Fig. 6.15. The fragmentation as described by Pelli *et al.*[216] is illustrated in Fig. 6.16, most of the fragmentation processes being related to the cleavage of the nitrogen-containing ring, leading to ions at *m/z* 256, 242, 228 and 215. Losses of $CH_3\cdot$, $OH\cdot$ and H_2O give rise to the ionic species at *m/z* 270, 268 and 267 respectively. Finally an interesting fragmentation process leads to an ion at *m/z* 162, which is particularly abundant in the EI spectrum.

The FAB-MS of morphine (Fig. 6.17) provides an intense protonated molecular ion at *m/z* 286 as the base peak. The spectrum does not show a peak at *m/z* 162 which was abundant in the EI spectrum.

CID-MIKES experiments were carried out on the protonated molecular ions formed by FAB, because of the absence of fragment ions in the TSP mass spectra of these alkaloids, even with a repeller electrode. The FAB CID-MIKES spectra of morphine is shown in Fig. 6.18, the major fragment ions at

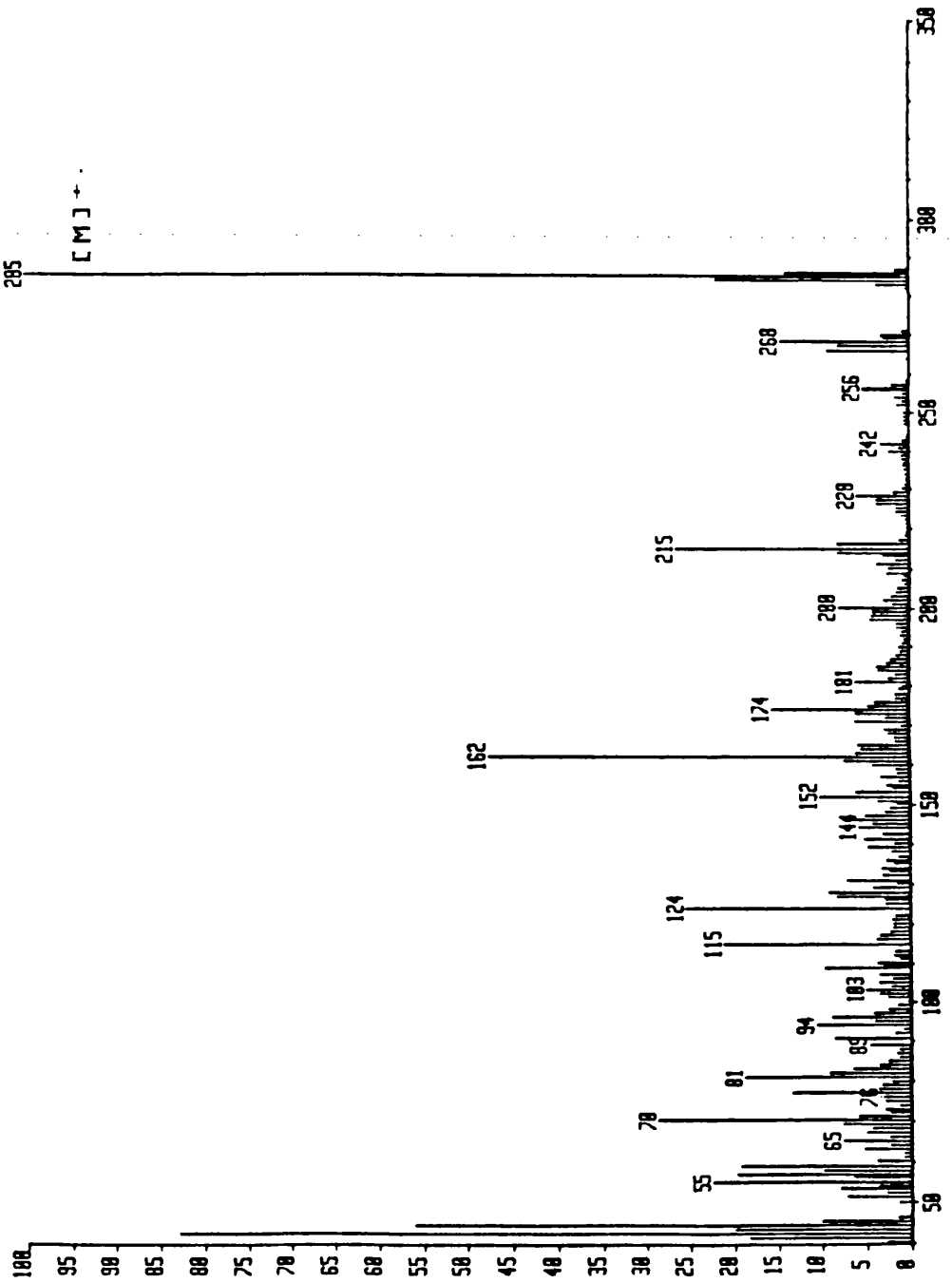


Fig. 6.15 EI-MS of morphine

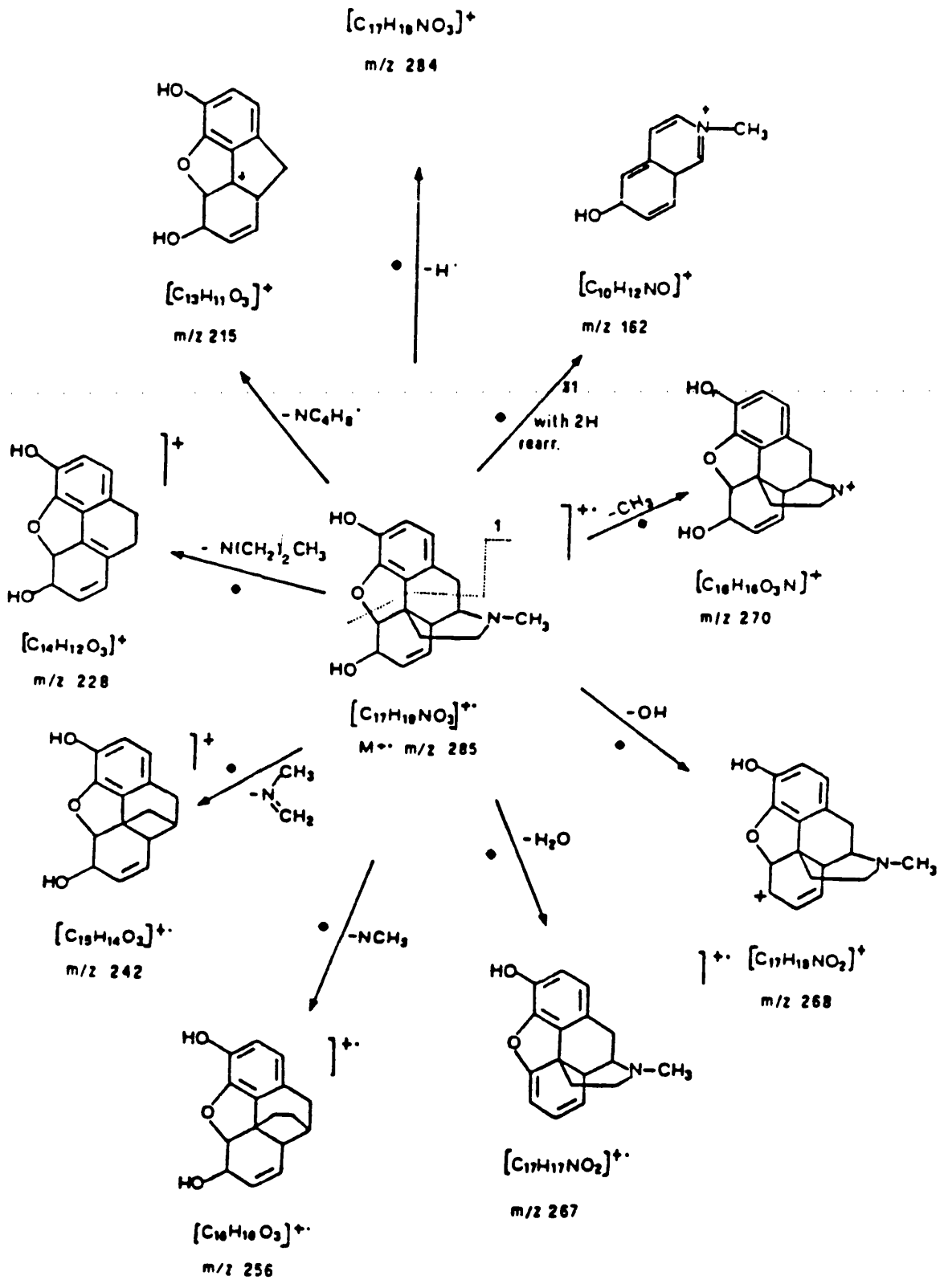


Fig. 6.16 EI fragmentation modes of morphine (Pelli *et al.*, 1987)[216]

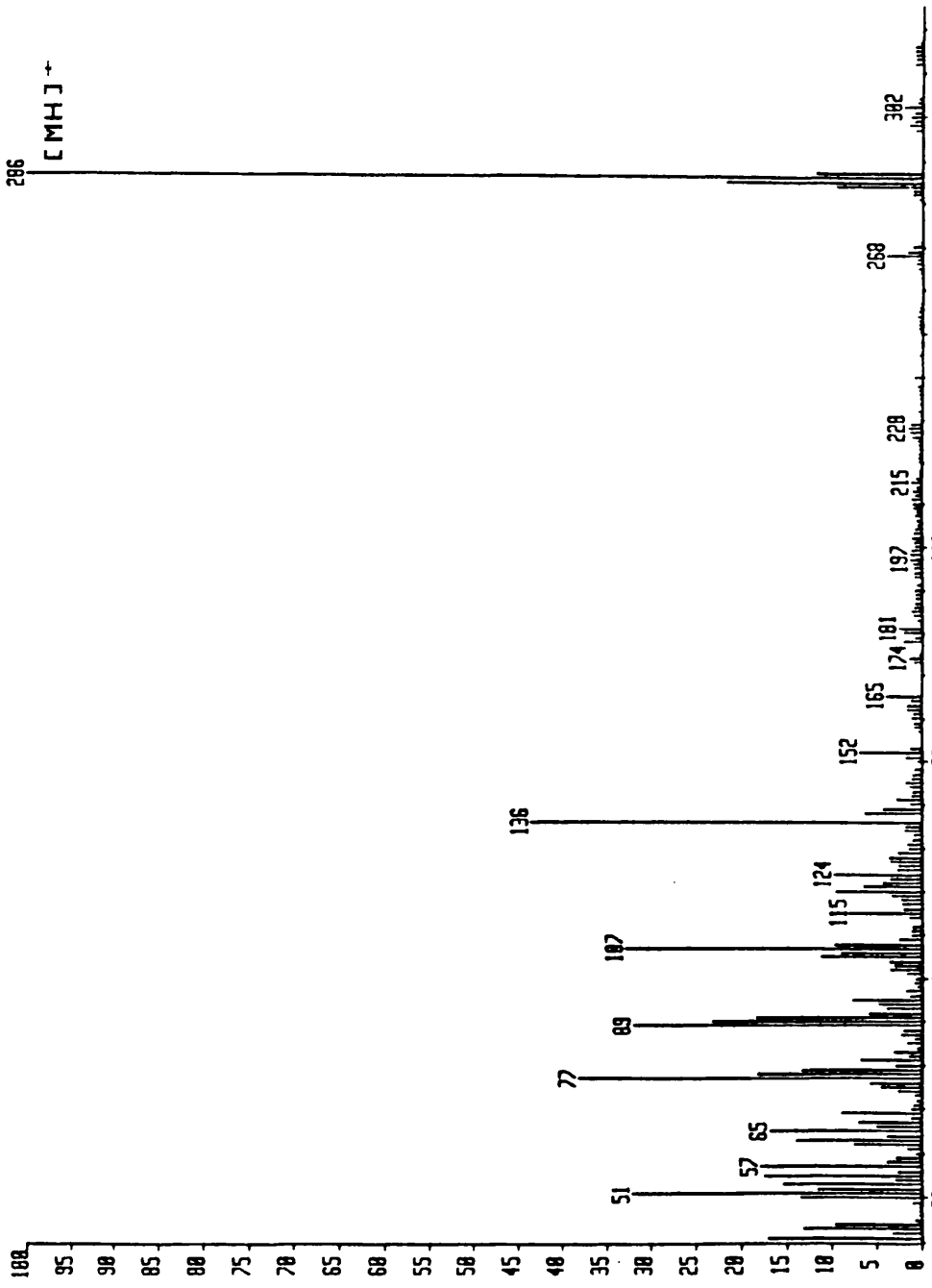


Fig. 6.17 FAB-MS of morphine

m/z 268, 228 and 215, corresponding to the main collision induced dissociations of the molecule.

The ion at *m/z* 228 is considered to be a characteristic fragment ion of the compounds having morphinan skeltons. It is very probable that the same peaks would be obtained by TSP CID-MIKES, which would allow multiple ion monitoring e.g. with a triple quadrupole mass spectrometer, thereby increasing the analytical reliability of the data. As with the *Cinchona* alkaloids, CID-MIKES on the protonated molecular ion produces a number of ions that are observed in the EI mass spectrum.

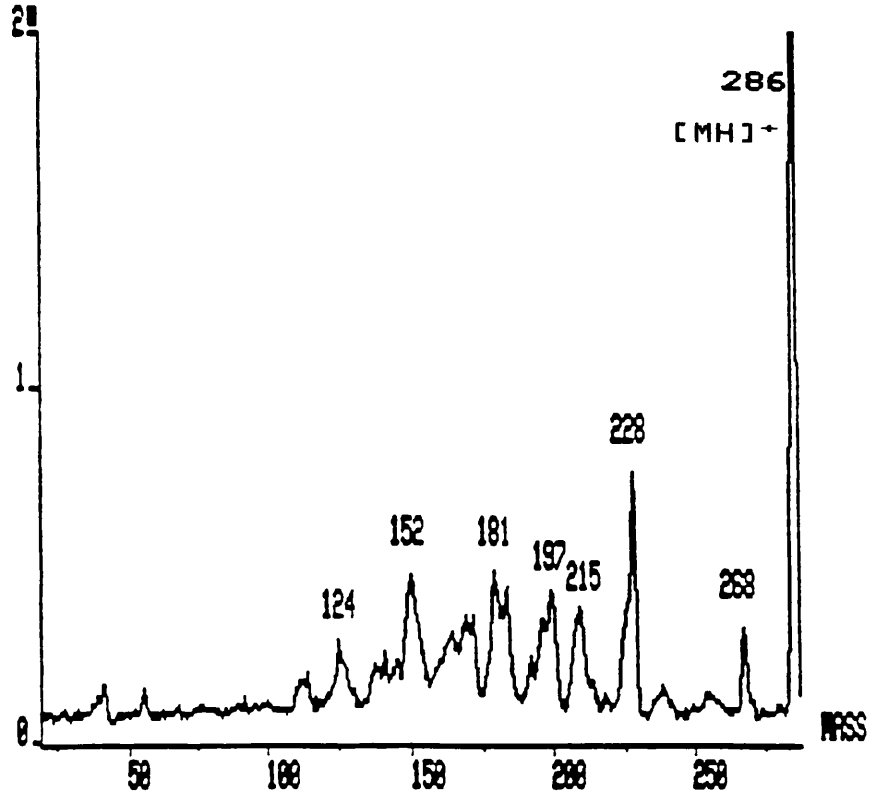


Fig. 6.18 FAB CID-MIKES spectra of [MH]+ of morphine (m/z 286)

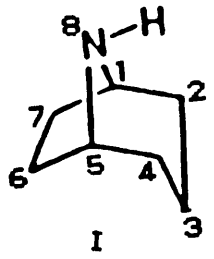
7. *DATURA* ALKALOIDS

7.1 INTRODUCTION

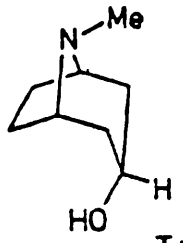
Within the family of Solanaceae, many genera- including *Datura, Atropa and Hyoscyamus*- are noted for their production of tropane alkaloids. Scopolamine and hyoscyamine are the major tropane alkaloids found in the genus *Datura* and in the hairy root cultures of these plants.[6] These alkaloids have been used in clinical applications (scopolamine as anaesthetics and hyoscyamine as spasmolytics) for a long time.[219]

Tropane alkaloids are characterised by the presences of a bicyclic amine octane ring system [I] (Fig. 7.1). The N-methyl derivative is trivially known as tropane. Many of the alkaloids are derivatives of tropine [II] which is tropan-3α-ol. Hyoscyamine [III] is an ester of tropane. Scopolamine [IV], an epoxide of hyoscyamine, is biosynthesised from hyoscyamine via 6β-hydroxyhyoscyamine.[220] Littorine [V] is an isomer of hyoscyamine.

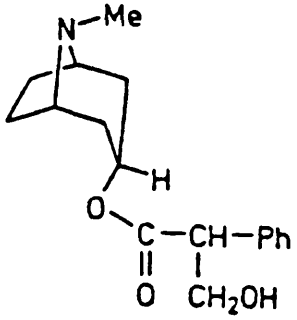
Tropane alkaloids have always been amongst the most difficult drugs to subject to trace analysis,



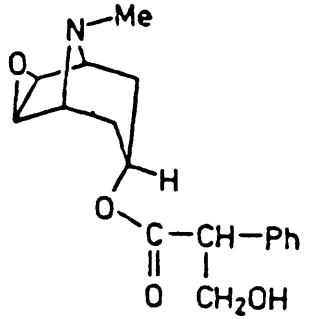
I



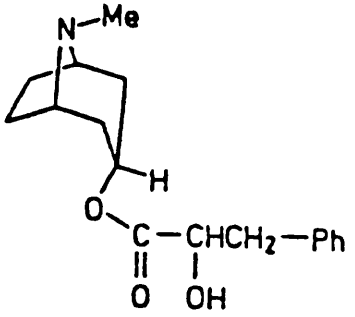
Tropine II



Hyoscyamine III



Scopolamine IV



Littorine V

Fig. 7.1 Structures of some tropane alkaloids

owing to their poor chromophoric activity, and instabillity to the heat and pH ranges normally encountered in suitable extraction procedures.

For the assay of tropane alkaloids, both in pharmaceutical preparations and in plant extracts, a number of chromatographic techniques have been employed, including thin-layer chromatography [221], gas chromatography[222-223], GC/MS[224,225] and HPLC.[226-239] Recently, ion-pair HPLC has been applied to the determination of four tropane alkaloids in Chinese Solanaceous plants.[240]

The combination of gradient HPLC with TSP-MS proved to be effective in the detection of eight tropane alkaloids in plant cell cultures by Mellon.[180] Recently Auriola *et al.*[241] have investigated the quantitative analysis of scopolamine and hyoscyamine in plant cell cultures using a polymeric reversed phase column and an isocratic alkaline eluent in combination with TSP-MS.

Studies were undertaken in our laboratory to modify several of the above mentioned HPLC systems to develop a suitable mobile phase for HPLC/TSP-MS analysis of *Datura* alkaloids using a C_{18} RP column. The most satisfactory system was found to

be 1% ammonium acetate/acetonitrile (60:40). In this way scopolamine and hyoscyamine were identified in extracts of *Datura candida* leaves by HPLC/TSP-MS using single ion monitoring (SIM). Scopolamine was the principal alkaloid and the scopolamine/hyoscyamine ratio was 6:1. We also analysed a large number of samples using GC/MS with EI ionisation, and we have compared the relative merits of GC/MS and TSP HPLC/MS.

7.2 EXPERIMENTAL

i. REAGENTS AND MATERIALS

Acetonitrile was of HPLC grade (BDH, Poole, U.K.), all the other reagents were of analytical grade. Hyoscyamine base and scopolamine hydrobromide were purchased from Sigma (Poole, U.K.) 10 mg of each alkaloid was dissolved in 10 ml methanol, the solutions were further diluted in methanol to a concentration of 100 µg/ml, and 10 µl aliquots were injected on the HPLC column.

ii. INSTRUMENTATION

FAB and tandem mass spectrometric experimental conditions were as previously described in Section (5.4 ii).

The TSP conditions were optimised with approximate temperatures: source 190°C, nozzle 195°C and chamber 240°C.

GC/MS analysis was carried out using a Hewlett-Packard 5890 A instrument in combination with a VG 12-250 mass data analysis system. A fused silica capillary column (15 m * 0,239 mm I.D.) coated with the methylsilicone phase DB-1 (J&W Scientific, U.S.A.) (film thickness 0.25 µm) was used with helium as carrier gas at 0.5 bar pressure.
Conditions: Injection temperature was 180°C; isothermal at 35°C during 2 min, 35-300°C, 30°/min; isothermal at 300°C; ionisation energy: 70 eV.

7.3 ANALYSIS OF PLANT MATERIALS

7.3.1 EXTRACTION OF LEAF MATERIALS

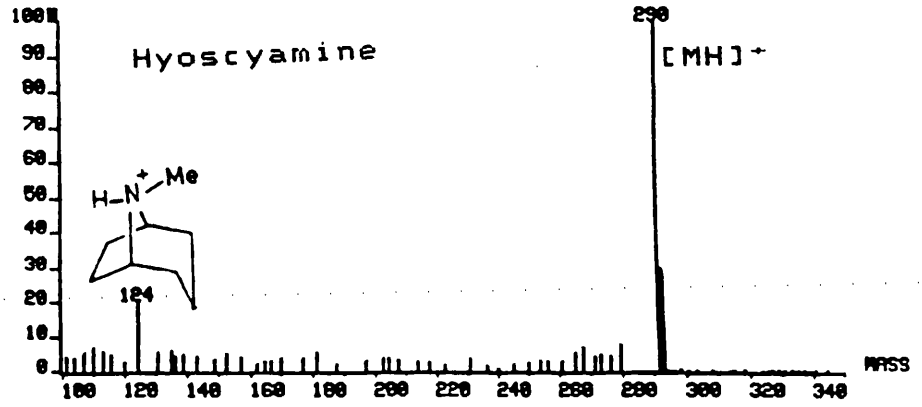
Datura candida hybrid leaf samples [produced by crossing *D. candida* (Persoon) Safford and an

orange-pink flowered cultivar of the same species] were extracted essentially according to Christen *et al.*,[6] by M.F.Roberts. The extraction solvents used were 0.2M H_2SO_4, 1M NaOH and $CHCl_3$. Five separate samples were analysed in this study.

7.3.2 HPLC/TSP-MS

Datura alkaloids exhibit intense protonated molecular ions in their TSP-MS, and a small fragment ion in the case of hyoscyamine, in contrast to the *Cinchona* and opium alkaloids. This is exemplified by the TSP-MS of hyoscyamine and scopolamine (Fig. 7.2). Hyoscyamine shows the protonated molecular ion at *m/z* 290 and the fragment ion at *m/z* 124. The structure of this fragment can be described in terms of cleavage of the C_3 ester function. Scopolamine shows only the protonated molecular ion at *m/z* 304.

The optimum separation and TSP ionisation for hyoscyamine and scopolamine monitored for $(M+H)^+$ ions at *m/z* 290 and 304 was achieved with 1% ammonium acetate (pH 5.8)/acetonitrile (60:40) and a C_{18} RP column. This is illustrated in the SIM chromatograms in Fig. 7.3A for a mixture of 500 ng each of the authentic alkaloids and Fig. 7.3B



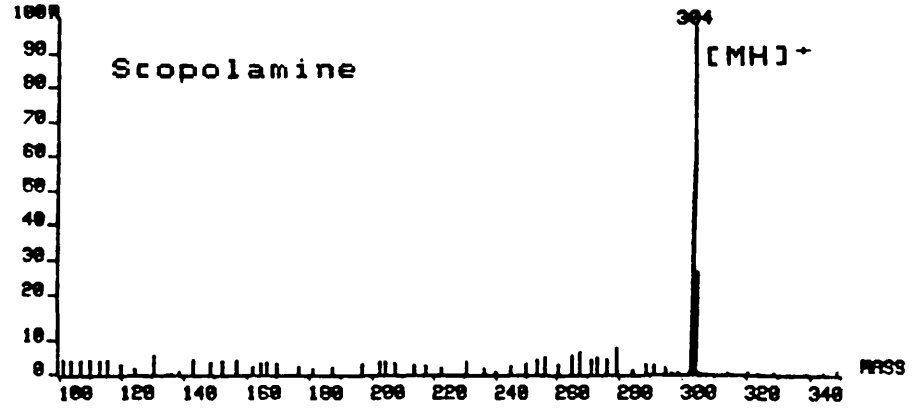
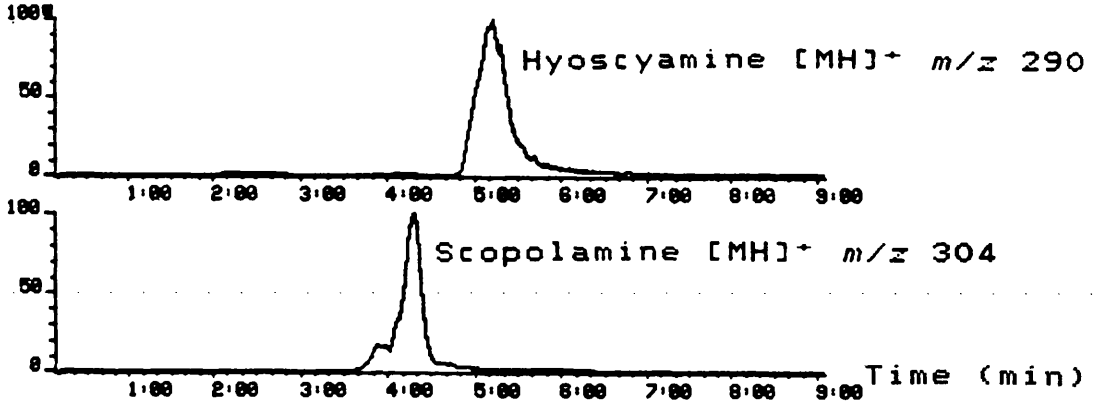


Fig. 7.2 TSP-MS of hyoscyamine and scopolamine

[A]



[B]

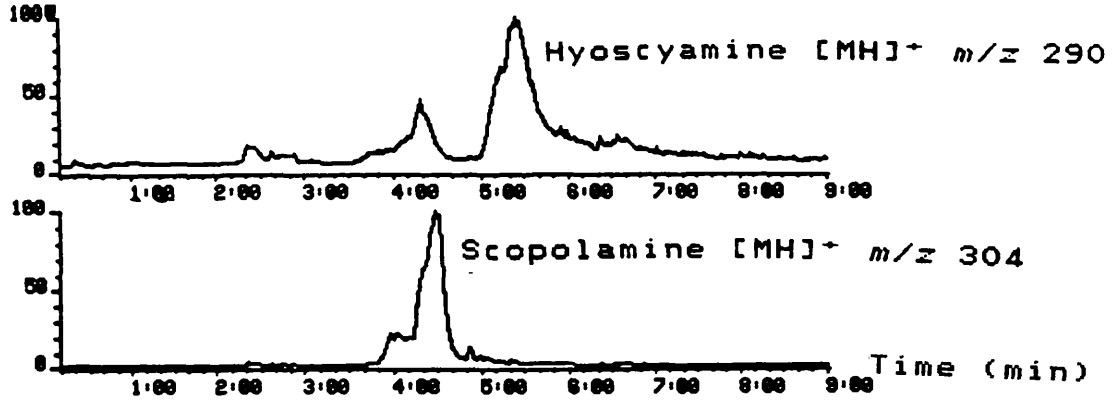


Fig. 7.3 HPLC selected ion TSP profiles of
A. Authentic tropane alkaloids
B. Alkaloids extracted from *D. candida* leaf

for a typical example of five samples extracted from *D. candida* leaves. An isomer of hyoscyamine (littorine) was also detected in the same leaf extract (Fig. 7.3B), as was distinguished on the basis of retention time. Littorine has been detected previously in *Hyoscyamus* root cultures.[242]

7.3.3 QUANTITATIVE ANALYSIS

The measured peak areas from Fig. 7.3A for the equimolar mixture of hyoscyamine and scopolamine is in the ratio 1:1.3. By comparing triplicate runs of these standards with that of the leaf extract (Fig. 7.3B) we can estimate the relative amount of hyoscyamine and scopolamine in the leaf extract to be present in the approximate ratio 1:6. For more accurate quantitation it would be necessary to have an appropriate internal standard.

7.4 FAB AND TANDEM MASS SPECTRA

The FAB mass spectrum of hyoscyamine (Fig. 7.4) showed an abundant protonated molecular ion at *m/z* 290 and a fragment ion at *m/z* 124, the same

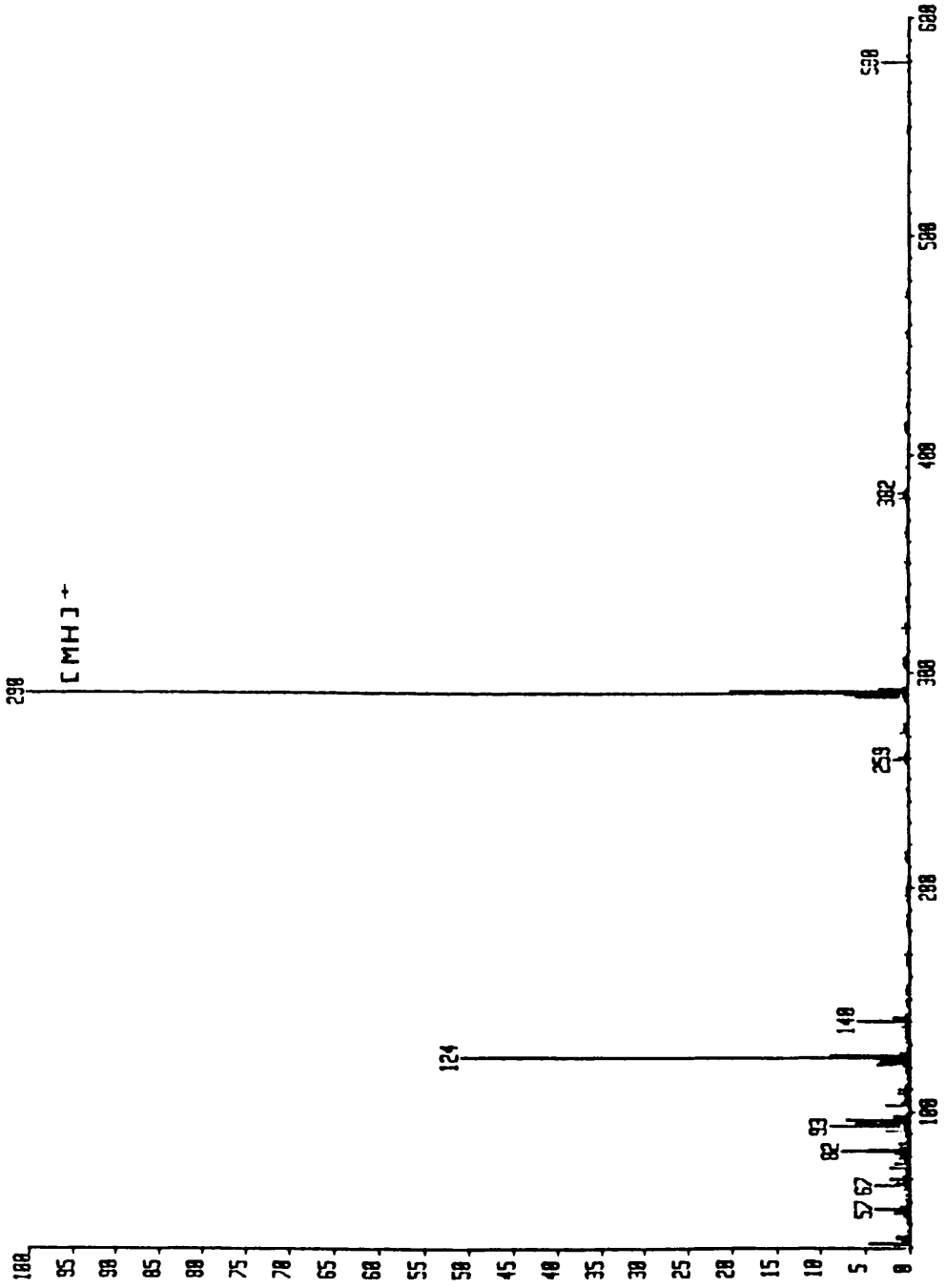


Fig. 7.4 FAB-MS of hyoscyamine

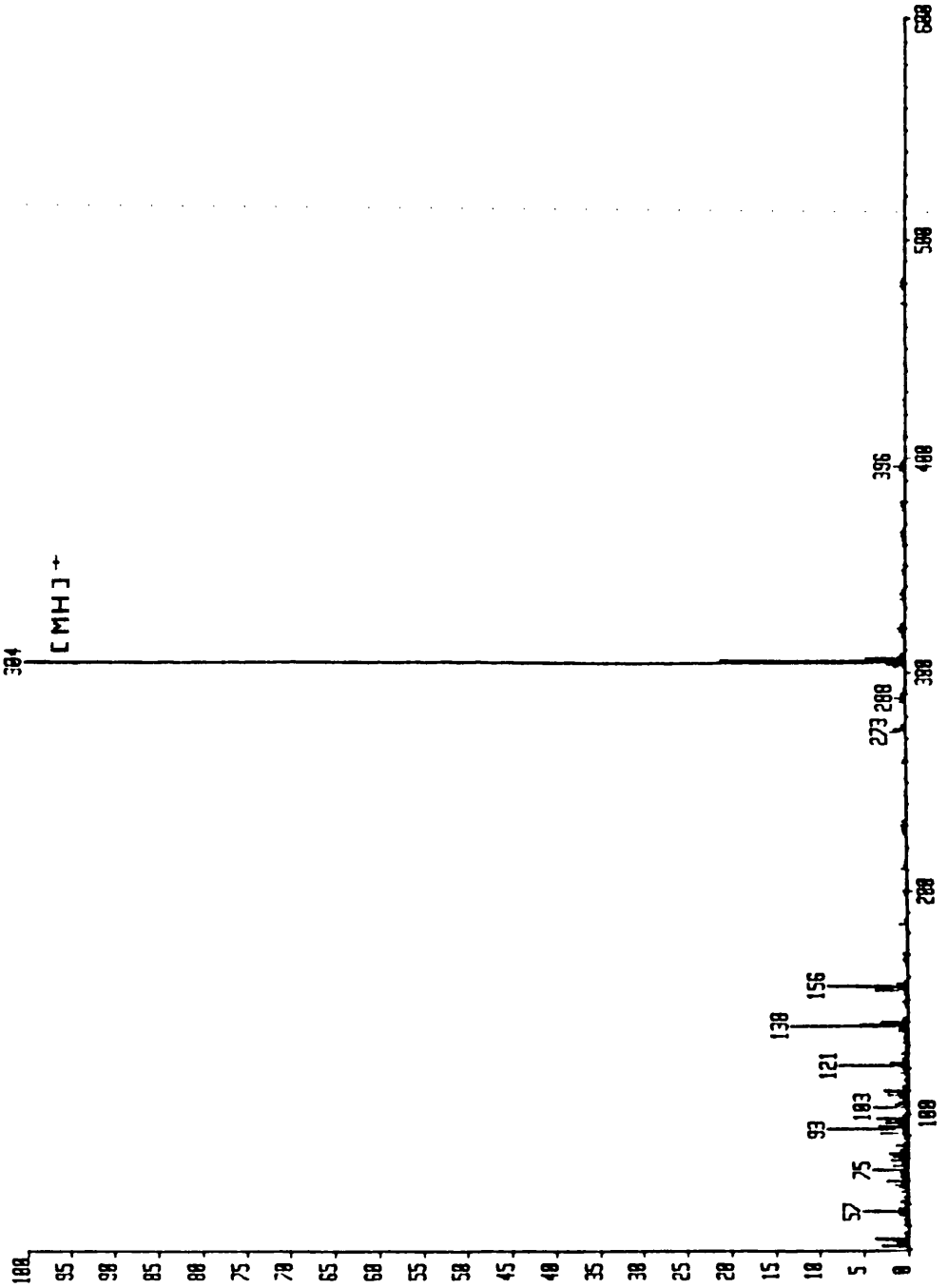


Fig. 7.5 FAB-MS of scopolamine

fragment as was seen in the TSP spectrum. Scopolamine (Fig. 7.5) also showed an abundant protonated molecular ion at *m/z* 304 and a fragment ion at *m/z* 138 which may be attributable to the same fragment but in this case with the C_6-C_7 epoxide.

The FAB CID-MIKES spectrum of hyoscyamine given in Fig. 7.6 shows major fragment ions at *m/z* 140,124 and 82 corresponding to the main collision dissociations of the molecule. It is very probable that the same peaks would be obtained by TSP CID-MIKES, which would allow multiple ion monitoring.

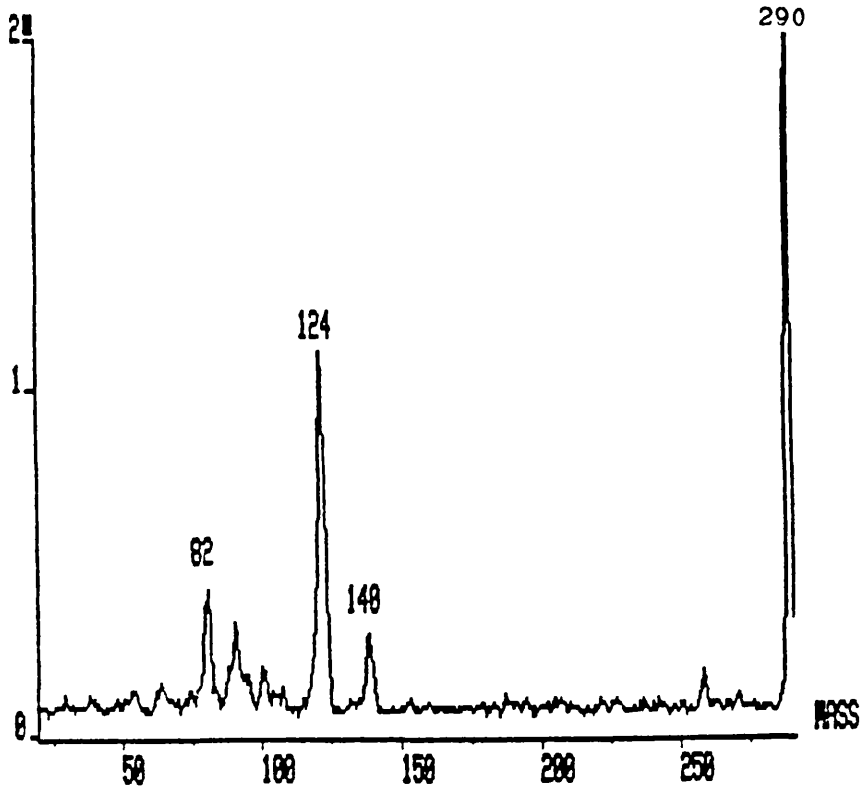


Fig. 7.6 FAB CID-MIKES spectra of $[MH]^+$ of hyoscyamine (*m/z* 290)

7.5 EI MASS SPECTRA

The EI and CI mass spectra of tropane alkaloids have been discussed in some detail[243-249] reflecting the importance of this class of substances. We have carried out further research in the EI spectra of tropine, hyoscyamine and scopolamine. The general fragmentation pattern of the 3-hydroxytropane esters has been described previously by Lounasmaa[250] in terms of five routes (A-E) (Fig. 7.7).

i. TROPINE

Tropine represents a relatively simple molecular structure and it provides an excellent model for the analysis of the tropane alkaloids. Its mass fragmentation was examined as a preliminary to that of more complex tropane analogues. Direct injection of tropine into the EI source of a quadrupole mass spectrometer (Fig. 7.8) showed a molecular ion at *m/z* 141, base peak at *m/z* 82 and an intense peak at *m/z* 96. One could expect localisation of charge to predominate on the N atom and to a lesser degree on the hydroxyl oxygen atom of tropine. Charge localisation on the nitrogen atom appears favoured and can account for

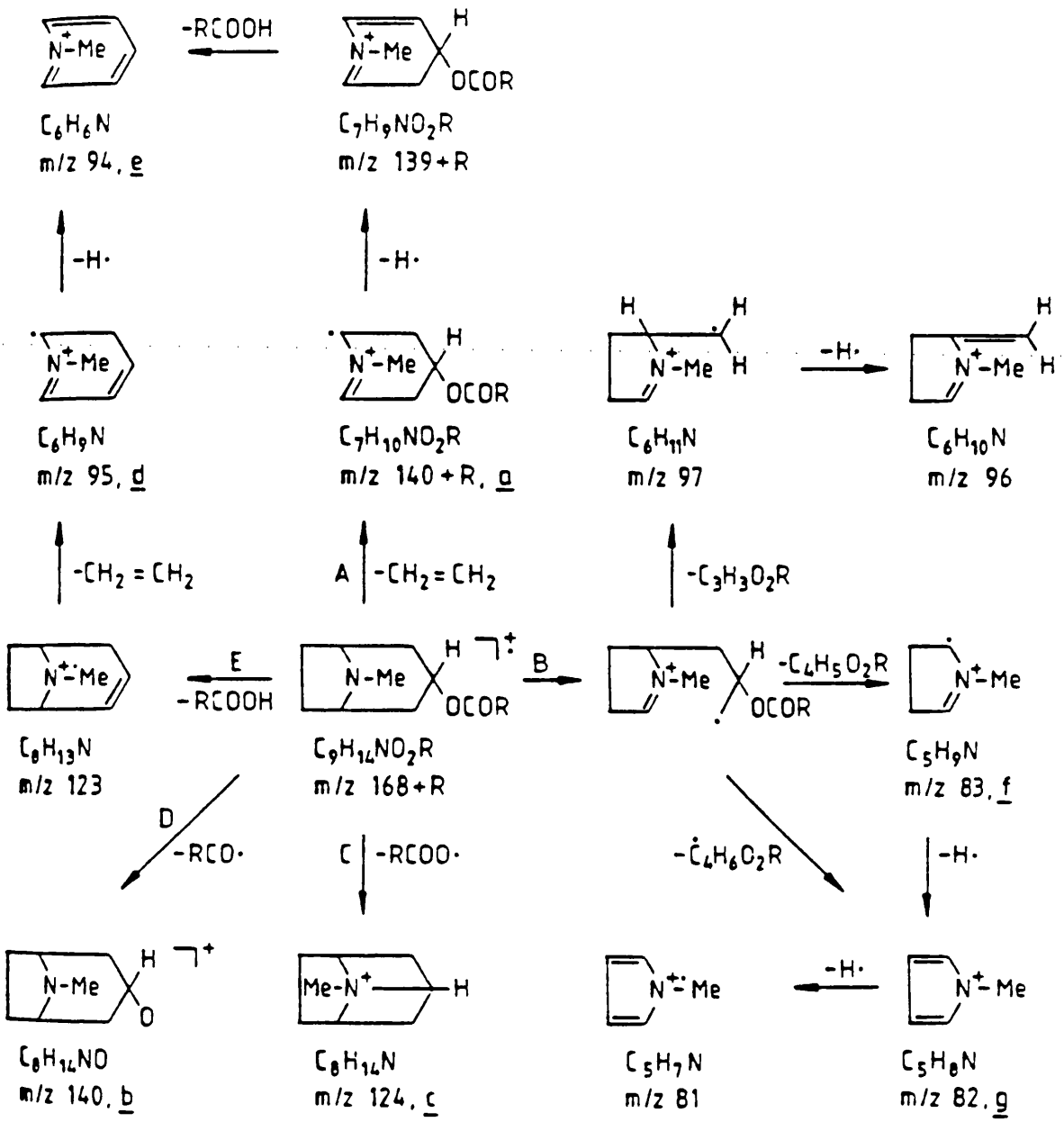


Fig. 7.7 General fragmentation pattern of 3-hydroxytropane esters[250]

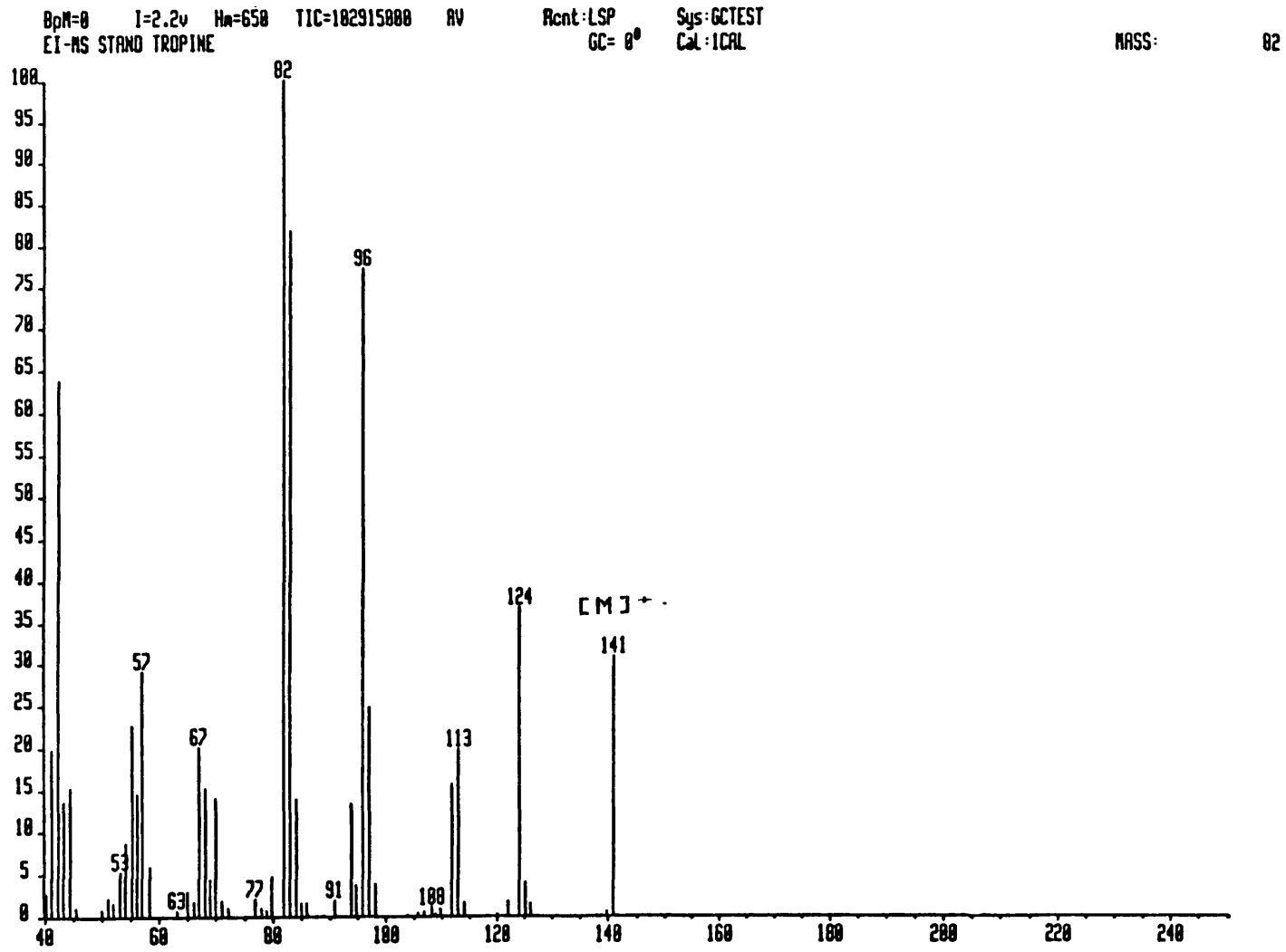


Fig. 7.8 EI-MS of tropine

α-cleavage and the initial loss of the hydroxyl radical OH· to give the ion at *m/z* 124. This mechanism has been proposed by Blossey *et al*,[245] to explain the same ion in the spectra of the tropane esters (Fig. 7.7, Route C), with the loss of RCOO· rather than OH·. The other major fragments may arise by route B, with initial cleavage of the C_{4-5} bond followed by several further fragmentations to give peaks at *m/z* 97, 96, 83 and 82.

ii. HYOSCYAMINE

The EI mass spectrum of hyoscyamine (Fig. 7.9) shows a weak molecular ion at *m/z* 289 with the base peak at *m/z* 124, (Fig. 7.7, Route C) arising through the loss of the C_3 ester function. The second most intense peak at *m/z* 82 can be described in terms of Route B. The third most intense peak at *m/z* 94 may be attributable to either route A or E.

(Route A): This involves cleavage of the 5,6 and 1,7 bonds with the elimination of an ethylene unit. This elimination is followed by the loss of a hydrogen radical and the ester function, leading to the relatively stable

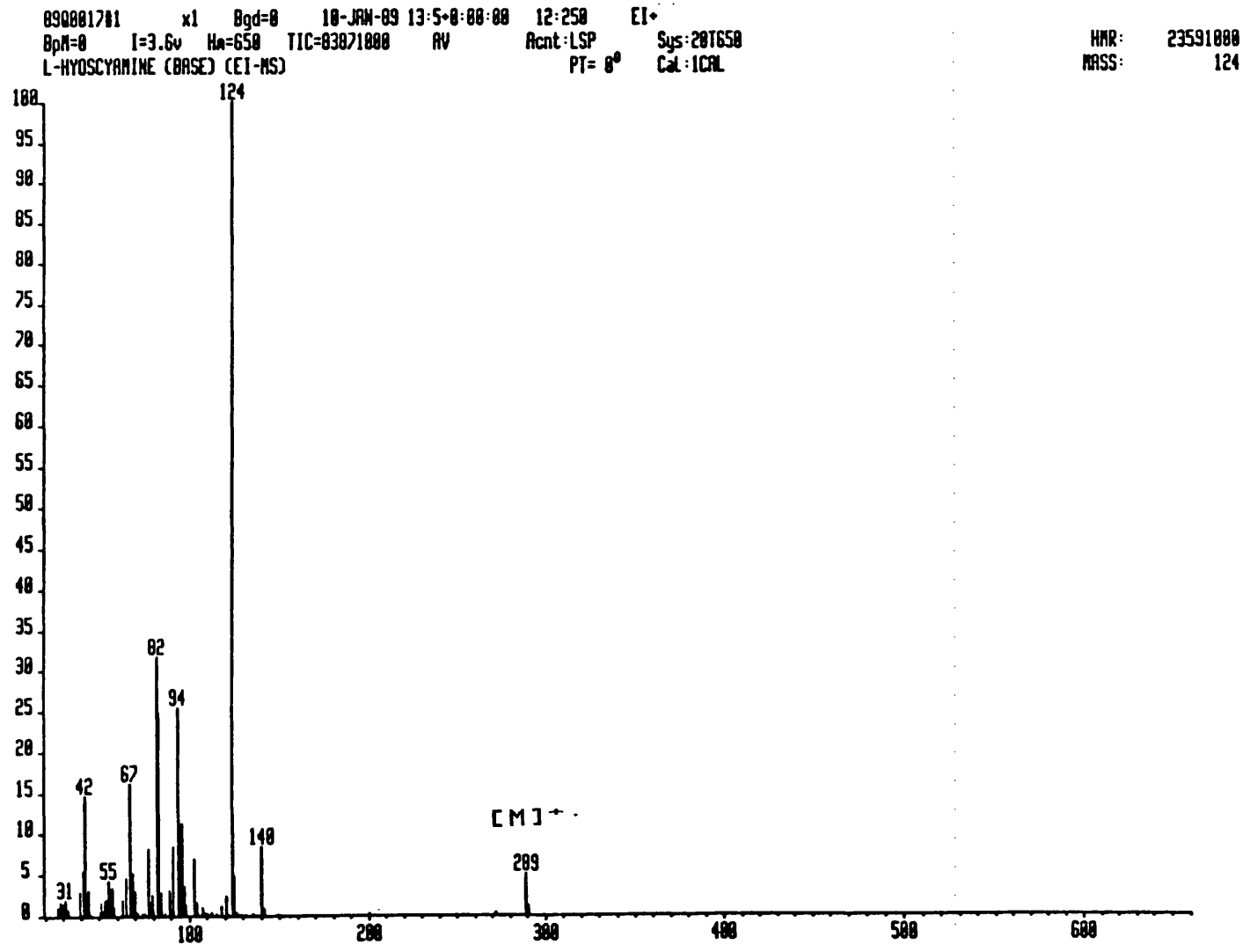


Fig. 7.9 EI-MS of hyoscyamine

N-methylpyridinium cation.

(Route E): In this route the C_3 ester function is cleaved with the loss of ROOH, forming ionised tropidine. Concomitant losses of ethylene and a hydrogen radical lead to the relatively stable N-methylpyridinium cation at *m/z* 94.

The fourth most intense peak at *m/z* 140 can be described in terms of route D, in which the C_3 ester fuction is lost as RCO· with retention of the C_3 oxygen.

The five routes presented in Fig. 7.7 give rise detectable fragments in the mass spectra of all 3-hydroxytropane esters, although their relative contributions to the general fragmentation patterns may vary widely.

iii. SCOPOLAMINE

The EI mass spectrum of scopolamine (Fig. 7.10) shows a weak molecular ion at *m/z* 303. The presence of the epoxide group in scopolamine appears to influence the course of fragmentation resulting in a shift of the base peak to *m/z* 94.

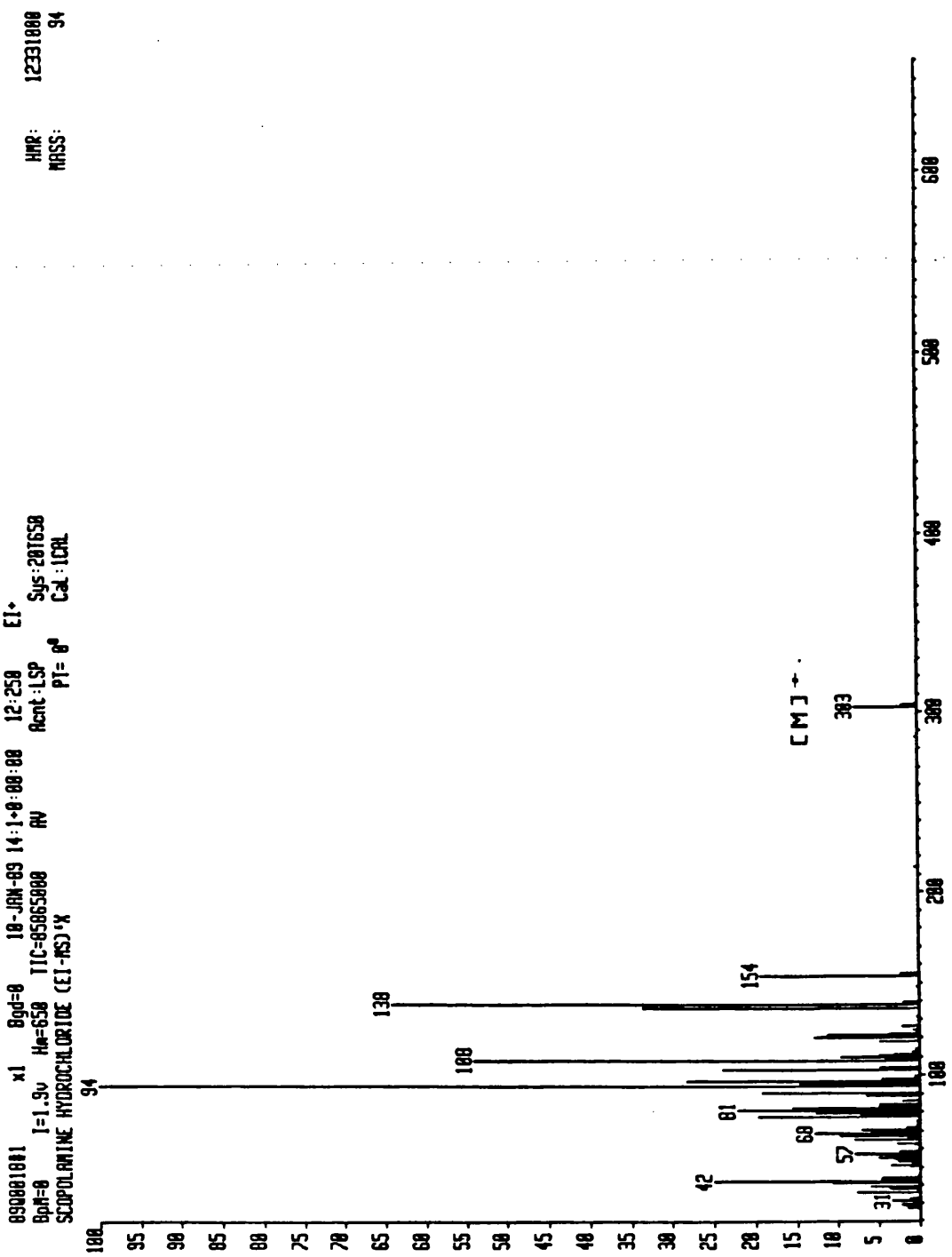


Fig. 7.10 EI-MS of scopolamine

The formation of this ion may be via Route D, but in this case with the C_6-C_7 epoxide, leading to the peak at *m/z* 154, this loss being followed by concomitant losses of H_2O and C_2H_2O to give the base peak at *m/z* 94. The second most intense peak at *m/z* 138 is directly analogous to *m/z* 124 from tropine and is formed via route C again with the C_6-C_7 epoxide. This is the same fragment ion as was observed in the FAB spectrum.

7.6 GC/MS OF LEAF MATERIALS

The GC/MS TIC chromatogram for a typical example of five samples extracted from *D. candida* leaves is shown in Fig. 7.11. The alkaloids were identified by (1), their retention times on GC, and (2), their mass spectra. These were compared to the corresponding values for authentic alkaloids.

Four alkaloids were identified:

Alkaloids found	Rt (min)	$M^{+\cdot}$
Tropine (TE)	10.19	141 (strong)
Oscine (OS)	11.40	155 (strong)
Hyoscyamine (HY)	20.20	289 (v. weak)
Scopolamine (SC)	21.41	303 (v. weak)

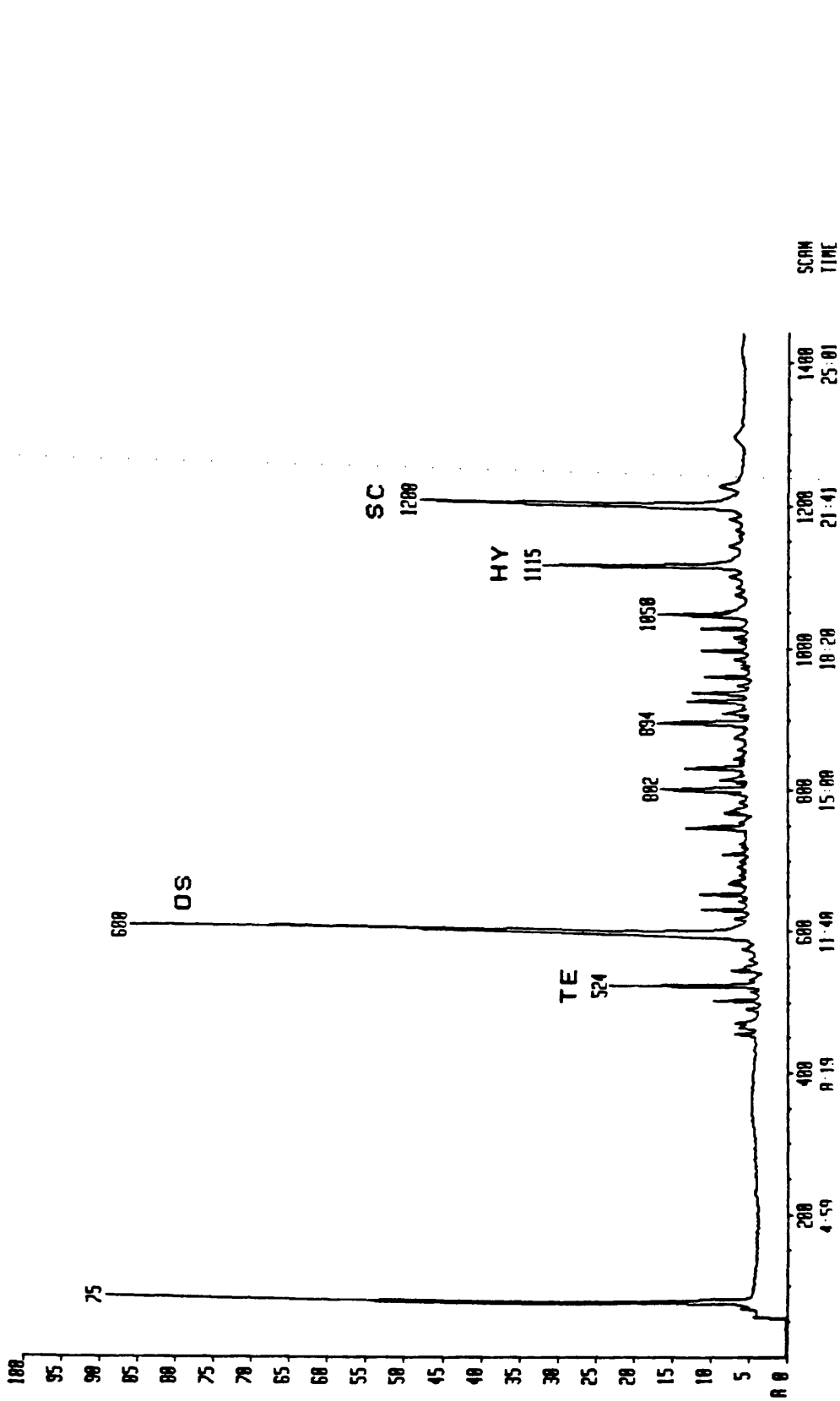


Fig. 7.11 GC/MS chromatogram of alkaloids extracted from *D. candida* leaf.

The mass spectra of the components identified as tropine, hyoscyamine and scopolamine were compared with authentic samples. Tropine showed a significant molecular ion whereas hyoscyamine and scopolamine showed very weak or absent molecular ions under EI GC/MS conditions.

Oscine was also identified by comparing its spectrum (Fig. 7.12) with published data. The compound showed a molecular ion peak at *m/z* 155 with the the base peak at *m/z* 96. Oscine was the most abundant alkaloid, but tropine, hyoscyamine and scopolamine were also present at relatively high amounts in these extracts.

7.7 COMPARISON OF GC/MS AND HPLC/MS DATA FOR *DATURA* ALKALOIDS

Comparing EI GC/MS and TSP HPLC/MS for the determination of the hyoscyamine and scopolamine contents in *D.candida* plant samples, it can be seen that with GC/MS the retention times of these two alkaloids are quite long (hyoscyamine = 20.20 min; scopolamine = 21.41 min) and they show rather weak or no molecular ion information under EI ionisation. With our new developed TSP HPLC/MS method, the analysis time is greatly reduced

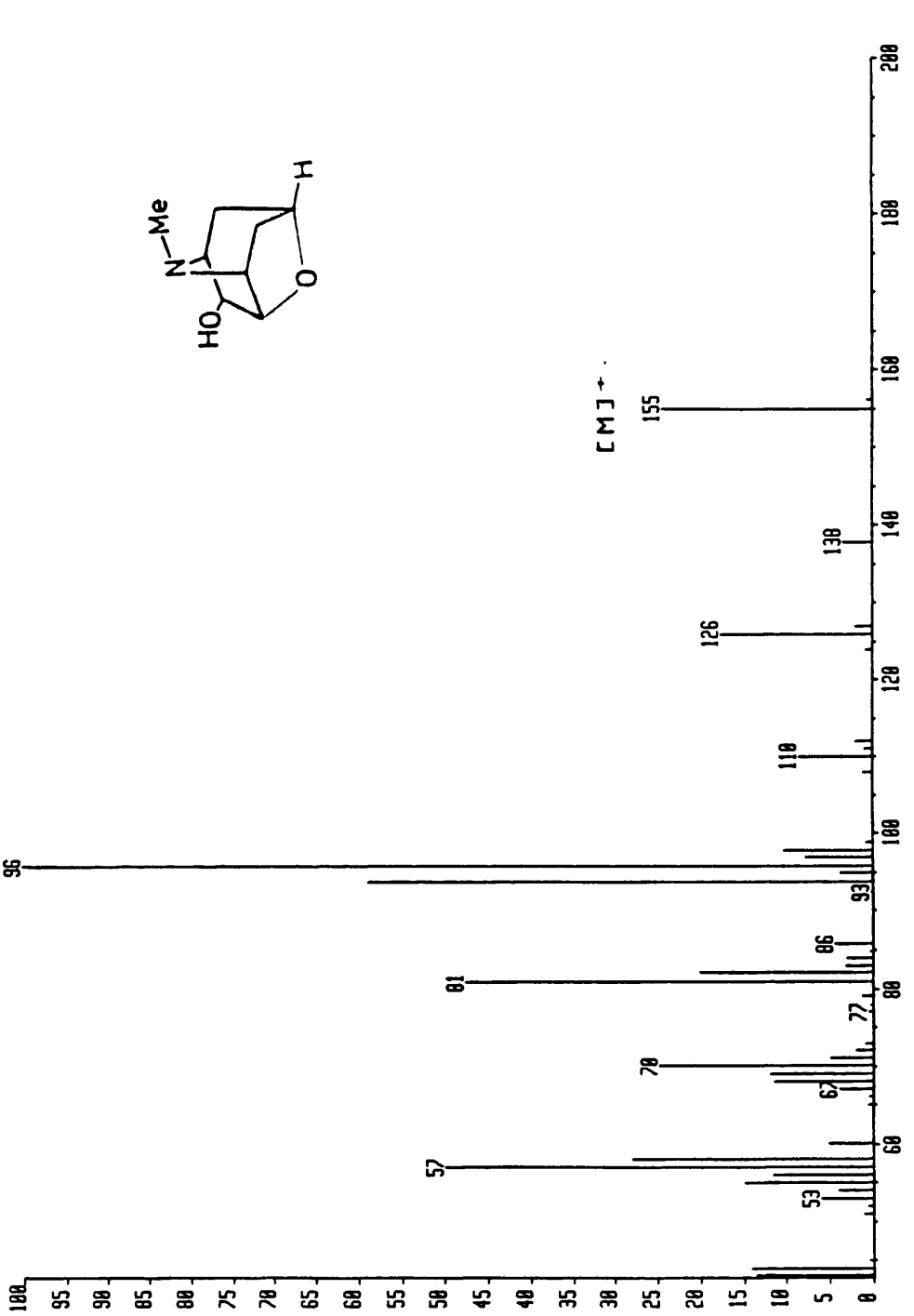


Fig. 7.12 EI-MS of oscine

(6 min) and the protonated molecular ions give the base peaks. Although the tropane alkaloids give well defined fragment ions with EI ionisation, many of the compounds of interest give the same peaks e.g. m/z 124, 92 etc. Thus multiple ion monitoring with GC/MS often fails to distinguish the particular alkaloids in the absence of adequate molecular ion signals. Therefore it can be concluded that the TSP HPLC/MS assay is preferable in many respects.

8. CONCLUSIONS

TSP LC/MS has been shown to provide a rapid, sensitive and selective assay for commercially important secondary metabolites of plant extracts. The technique minimizes sample handling and operator time.

The soft ionisation and chromatographic separation capability of TSP coupled with the generally simpler sample work-up for HPLC compared with GC suggests TSP as a tool for quantitative analysis. Detection limits for the alkaloids injected on column were better than 250 ng (full scan spectra) and 25 ng when the single ion monitoring (SIM) technique was used to monitor the MH^+ ion.

The utility of the method for the identification of quinoline alkaloids from *Cinchona ledgeriana* root organ suspension cultures, isoquinoline alkaloids from *Papaver somniferum* capsules, and tropane alkaloids from *Datura candida* hybrid leaf plants has been investigated.

The *Cinchona* results showed that analysis of quinine and quinidine obtained from plant sources would not give actual amounts of these alkaloids, unless they are free from their desmethoxy and

dihydro analogues i.e. complete resolution of all alkaloids should be achieved, otherwise interference results will be obtained.

UV detection was found to be inadequate as the level of quinoline alkaloids in these cell cultures is below the detection limit. Fluorescence detection gave superior signal: noise but it detected other major components which were not the target metabolites. These unwanted components were not detected by TSP/MS detection using the SIM technique. The assay developed in this study will have a useful role in defining the optimum tissue culture conditions for the production of the compounds of interest.

In order to evaluate the relative merits of the various ionisation modes, compounds were studied under TSP, EI and FAB conditions:

TSP-MS - MH^+ was the base peak.
- No diagnostic fragmentation was observed.

EI-MS - Weak $M^{+\cdot}$, some alkaloids did not show $M^{+\cdot}$
- Diagnostic fragmentation was obtained.
- The stereoisomers showed characteristically different spectra.

FAB-MS - MH^+ was the base peak.

- Little fragmentation was observed.
- The stereoisomers showed identical spectra.

Molecular graphics studies confirmed that intramolecular H-bonding between the hydroxyl H and the vinyl group is possible in cinchonine and quinidine but not in cinchonidine and quinine. This explains the significant differences in the stability of their molecular ions. We were also able to conclude that the most important feature of the active molecules is the lack of an intramolecular H-bond between the hydroxyl H and the quinuclidine N, which makes the atoms $O_{1,2}$ and N_1 accessible for interaction with a receptor via intermolecular H-bonding.

The assay developed for opiates is capable of providing a considerable amount of useful analytical data on complex mixtures of the *P. somniferum* plant alkaloids, this knowledge may provide valuable information to assist the selection of suitable plant material for initiating cell and organ cultures. TSP HPLC/MS showed significant differences in the sensitivity of detection for the five major alkaloids (morphine, codeine, thebaine, papaverine and

noscapine) because of the difference in their chemical structures and therefore different ionisation efficiencies. It has also been demonstrated that this analysis can be applied to detection of the 3 and 6-monoacetyl derivatives of morphine, which are associated with heroin use. The 3-[$^{2}H_{3}$] monoacetylmorphine synthesised would allow to quantify the heroin metabolites.

We have extended our investigations to *Datura candida* plants and as a preliminary step the hyoscyamine and scopolamine content of the leaves of these plants was examined. The two alkaloids did not show significant differences in their ionisation efficiencies. Scopolamine was the principal alkaloid, the scopolamine/hyoscyamine ratio being approximately 6:1. With UV detection we were not able to detect these alkaloids at their optimum absorption (210-215nm) because of the high UV cut-off value of ammonium acetate buffers in the mobile phase.

Comparing EI GC/MS and TSP HPLC/MS data for *Datura* alkaloids, the later showed many advantages over EI GC/MS including speed of analysis and the soft nature of the ionisation technique. For such reasons TSP HPLC/MS will find increasing use in alkaloid analysis.

A major advantage of the combined chromatographic-mass spectrometric studies for the analysis of complex mixtures is an ability to enable multicomponent peaks to be located and resolved.

The major problems with the TSP system involve difficulties in the day-to-day reproducibility of ionisation efficiency and fragmentation patterns, a severe dependence of the ion intensities on the liquid flow, and the fact that different compounds require different temperatures for efficient ionisation, and restrictions on the types of mobile phase compatible with TSP ionisation.

It may be possible to overcome the lack of diagnostic fragmentation in the mass spectra of many of the alkaloids by collisionally inducing dissociation in a tandem mass spectrometer[133] or by the filament-on or discharge ionisation techniques described by McFadden and Lammert, 1987.[182] An alternative ionisation method is continuous flow FAB. When this is linked to capillary HPLC operating at very low flow rates (5 µl/min) it provides a very sensitive analytical combination. It has been demonstrated in this study that the alkaloids investigated here all provide good FAB spectra, thus it can be anticipated that they will be amenable to

continuous flow FAB. As this technique does not require the use of a buffer and is apparently able to accept a wider range of HPLC mobile phases, it may prove to be superior to TSP analysis.

Another alternative analytical technique, packed column SFC/MS[139] also shows considerable promise and can be readily adapted to function with a TSP ion source. The method offers the prospect of greater sensitivity and shorter run and equilibration times than HPLC/MS.

Whether HPLC or SFC is used for the separation, SIM of the prominent MH^+ of these alkaloids is a very suitable method for screening these secondary metabolites.

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