# In-line combination of LC with MS, NMR, UV and IR in drug analysis

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## Abstract

Mass spectrometry (MS), nuclear magnetic resonance (NMR) spectrometry and Fourier transform infrared (FTIR) spectrometry have for many years been three of the analyst's main tools for the characterisation of unknown compounds. Their complementary nature is well known - NMR can provide unequivocal data for structural isomers (something not possible by mass spectrometry) while mass spectrometry and infra red can often identify moieties which lack a proton NMR resonance. For mixtures, high performance liquid chromatography (HPLC) coupled with any of these spectrometric techniques would provide a better solution than analysing the sample mixture directly, where spectral deconvolution would be extremely difficult. Similarly this combination would avoid the time-consuming isolation of individual components followed by off-line spectrometry. This work addresses the problems of bringing chromatography together with these detectors simultaneously, to provide an extremely powerful tool for the characterisation of mixtures

The first combination used was HPLC-NMR-MS. The conflicting requirements of both detectors in terms of flow and solvent composition were investigated. While a working combination was achieved choice of eluent remained difficult, principally because of eluent signals in the NMR recordings. Deuterated eluents were expensive and still contained residual proton signals. In chapter 2 the feasibility of LC-NMR-MS is demonstrated, while some limits on the types of solvents required are established, with the need for a compromise of the ideal eluents necessary for either LC-NMR or LC-MS being noted.

In an effort to seek a better eluent for the combination of multiple spectroscopic techniques, superheated water was investigated as an alternative eluent for LC-NMR-MS. This proved to be a good eluent for multiple hyphenation, especially for NMR, as few additional signals were introduced. The effects of superheated water on column lifetime were significant for silica based columns, and compound stability was also a potential problem for some compounds. It was found that superheated deuterated water could produce stable deuterated compounds in high yields, with deuteration possible at sites other than just those of labile protons.

The introduction of FTIR to the LC-NMR-MS system added additional constraints in terms of eluent, but IR spectra of adequate quality for library searching were obtained with an ATR flow cell. An automated flow injection system was constructed and a working sensitivity of 150-200 μg for a range of pharmaceutical compounds established for the IR part of the system.

Finally, a series of complex samples was investigated by LC-NMR-MS-IR with both conventional eluents and with superheated water. Good separations and good quality spectra were obtained for a range of compounds under different chromatographic conditions.

The work generated 18 peer reviewed papers on both the practicalities and the application of multiple hyphenation. The work also generated great interest from instrument manufacturers and shortly after the completion of the work both Varian and Bruker launched multiply hyphenated systems for purchase.

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# Chapter 1

# Complex mixture analysis utilising a multiply hyphenated approach.

## 1.1 Introduction

In many fields of chemistry, biology and medicine, progress is often limited by the inability to resolve complex analytical problems. Researchers and instrument companies have invested vast amounts of time and money in an effort to reduce the time and cost required to solve such problems. As a result, analytical techniques have been developed to provide an integrated approach to the separation of mixtures together with structural elucidation of unknown compounds. High Performance Liquid Chromatography (HPLC), Gas Chromatography (GC), Gel Permeation Chromatography (GPC), Supercritical Fluid Chromatography (SFC), Capillary Electrophoresis (CE) and Capillary Electrochromatography (CEC) are all used to separate complex mixtures. The advantages of the combination of these techniques with various spectroscopic techniques have been well recognised and have led to the development of Liquid Chromatography-Mass Spectrometry (LC-MS), Liquid Chromatography-Nuclear Magnetic Resonance (LC-NMR) and Liquid Chromatography-Infra Red (LC-IR), and the combination of HPLC with other "detectors" will undoubtedly continue to be attempted. However, little effort has been dedicated to the *simultaneous* combination of two or more of these spectroscopic techniques with HPLC. This thesis will explore and discuss the merits of such an

approach and demonstrate both the technical requirements and the application of such a multiply hyphenated approach to solving complex problems.

It is worth first reviewing the current state of the art with respect to each of the techniques to be studied and briefly placing them in some historical context.

## 1.2 LC-MS

By far the most mature of these hyphenated techniques is LC-MS. Initially thought to be extremely difficult if not impossible, LC-MS evolved from the work to marry GC with MS. The difficulties of this combination were originally thought too difficult to overcome, as the effluent from a GC could be measured in litres per minute whereas mass spectrometers required ultra low vacuums to be able to operate. However, the development of momentum separators and improved vacuum pumping systems eventually saw these two techniques successfully brought together[1-3]. The task of joining an HPLC chromatograph with a mass spectrometer was far more challenging than for GC, as the output from a HPLC system is many orders of magnitude greater in volume than from a GC, when the liquid flow is vaporised. LC-MS really only started to become viable in the late 1970's and early 1980's, first with the development of moving wire and moving belt interfaces[4], then with Direct Liquid Injection (DLI) as proposed by Henion[5]. The moving belt and wire techniques involved the deposition of the liquid effluent onto a rotating system which was heated inside the mass spectrometer to volatilise the sample. DLI required very low flow rates typically of the order of 0.1 – 1.0 μL/min and as such was of limited use at a time when typical HPLC flow rates were of the order of 1-2 mL/min. Soon after

these came Particle Beam (PB) and Thermospray (TSP) both of which allowed the analyst to work at reasonable flow rates with conventional eluents. Particle beam was the commercialisation of the work of Browner and Willoughby[6] and was a desolvation technique which allowed the acquisition of electron ionisation (EI) or chemical ionisation (CI) spectra as were then common in mass spectrometry. Thermospray as proposed by Vestal[7] was, as the name suggests, a system which sprayed the eluent through a heated nozzle to produce ions which were subsequently analysed by the mass spectrometer. However, all of these techniques were superseded by the introduction of electrospray. Electrospray as described by Fenn[8] following the work of Dole[9], heralded an explosion in LC-MS as a technique as it allowed the use of only slightly modified eluents and it was relatively easy to use.

### 1.2.1 Electrospray Ionisation

The ESI source has undergone much development since the earliest examples, but the general arrangement has remained basically the same (see Fig. 1.1). The analyte is introduced to the source in solution either from a syringe pump or more usefully as the eluent flow from a liquid chromatograph. In early versions the flow rate was typically restricted to a few microlitres per minute, but nowadays flows of up to one millilitre per minute or more can be tolerated. The analyte solution flow passes from the chromatograph through the electrospray needle that has a high potential difference (with respect to a counter electrode) applied to it (this is typically in the range from 2.5 to 4 kV). This potential difference causes transfer of electrons from the needle to the surface of the liquid (in negative ion MS) or vice versa (in positive ion MS). In a static or very low flow system (typically 1-5μL/min) this effect forces the formation

of a Taylor cone and as the ions build up the surface tension of the liquid is eventually overcome and results in the spraying of charged droplets from the needle with a surface charge of the same polarity as the charge on the needle (see Fig. 1.2). The droplets are repelled from the needle towards the source sampling cone on the counter electrode. As the droplets traverse the space between the needle tip and the cone the solvent is evaporated. As the droplets reduce in size the ions get closer and closer together until the forces pushing them apart is sufficient to break the surface tension of the droplet. This is known as a Coulombic explosion. This process continues as the droplets pass from the capillary to the counter electrode until only ions reach the mass spectrometer. This is a very soft ionisation method as very little residual energy is transferred to the remaining ions. The resulting spectra therefore show predominantly an intact ionised molecular species.

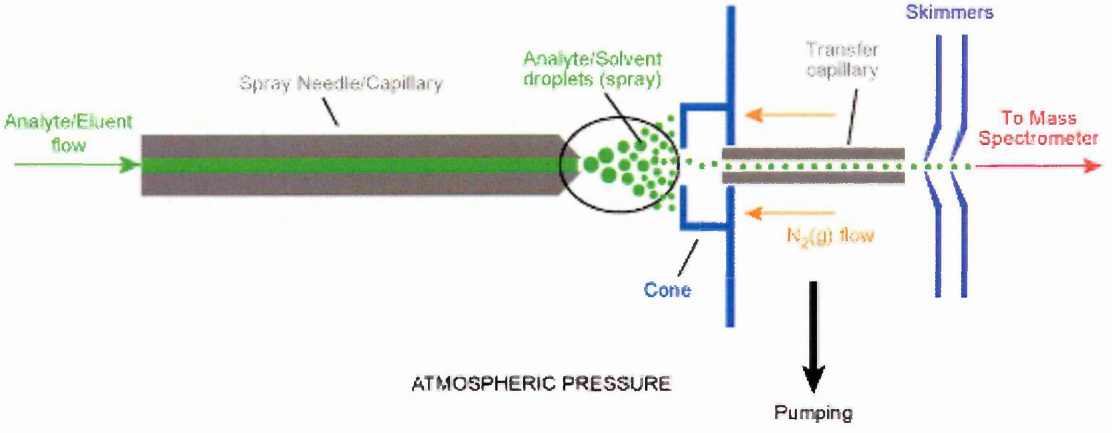



Fig. 1.1[10] *Schematic diagram of an electrospray source showing the relevant major components. The capillary is held at a potential difference to the sampling cone thus inducing droplet formation and charging. The droplets are desolvated to leave analyte ions which are sampled into the mass spectrometer.*

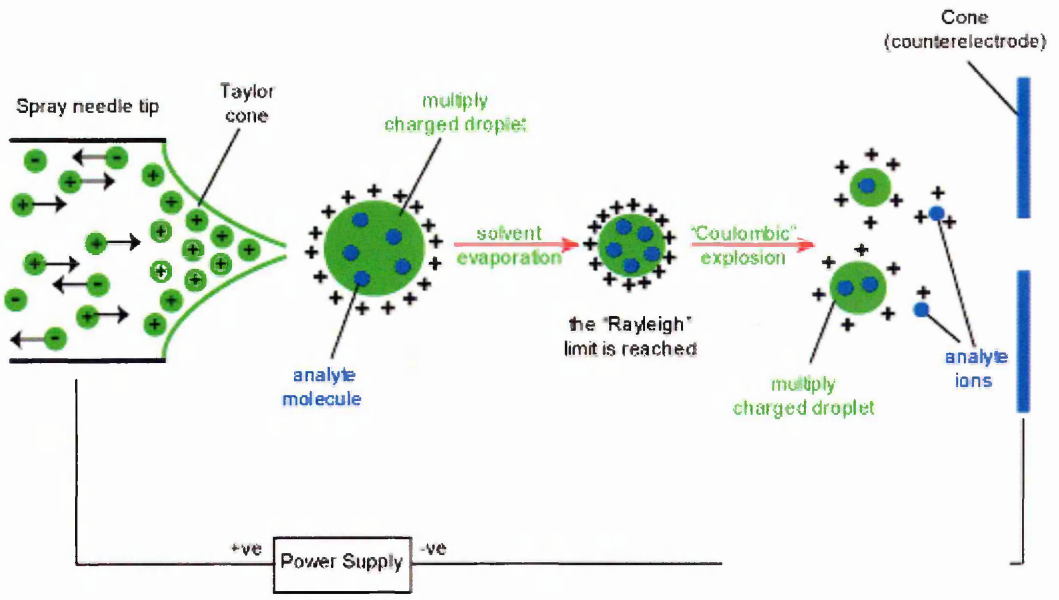



Fig. 1.2[10] *The ion production process in electrospray ionisation showing ion migration to the surface of the liquid at the end of the capillary resulting in the formation of a Taylor cone and the subsequent spraying of droplets from the tip. As the droplets migrate towards the counter electrode they are desolvated until the charges on the ions are sufficient to overcome the surface tension on the droplet resulting in a Coulombic Explosion. The process is repeated until eventually only charged ions reach the counter electrode.*

Commonly the spraying of droplets and their desolvation is enhanced by the flow of an axial sheath gas, usually nitrogen (see Fig. 1.3). Some source designs also incorporate a counter flow of gas as shown in yellow in Fig. 1.1. and/or a heated transfer capillary to provide further desolvation. The droplet production and initial desolvation zone is circled in Fig.1.1 and shown in greater detail in Fig.1.2.

The combination of HPLC and mass spectrometry is now a well established technique widely used in many fields. The electrospray interface and other similar atmospheric

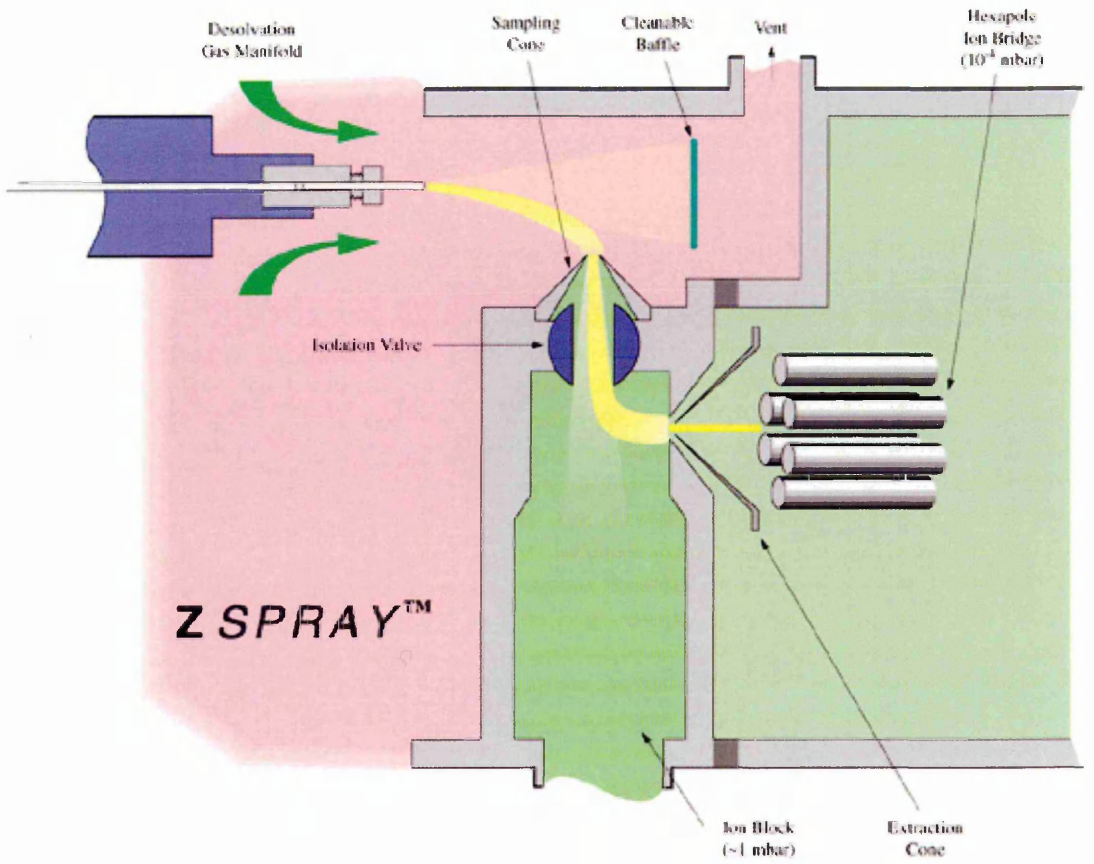



Fig. 1.3 *Schematic diagram of the Micromass Z-spray source*[11]. *This is a two stage source, the first stage of orthogonal sampling provides greater tolerance to biological matrices and involatile buffers, while software control of the cone gas improves the ruggedness and solvent adduct suppression. The second stage of orthogonal sampling allows more of the sample into the mass spectrometer without compromising the vacuum and therefore increases the overall sensitivity. This novel arrangement prolongs the lifetime of the source, thus increasing uptime.*

pressure ionisation techniques allow the facile joining of the two techniques. Although mass spectrometry is not the most information rich of the spectroscopic techniques it does provide much useful information, not least the molecular mass of the compounds present. Electrospray is a soft ionisation technique, and as such the

resultant spectra tend to be dominated by ions of the type $[M+H]^+$ and exhibit limited structural information. However, the use of either higher cone voltages or of $MS^n$ techniques can provide further fragmentation and hence structural information. In combination with more advanced computer techniques and a spectrometer capable of accurate mass measurement the molecular mass can be converted into a set of postulated empirical formulae.

One of the drawbacks of LC-MS is the constraint on the mobile phase composition. Traditionally, chromatographers had used involatile buffers such as sodium phosphate to control pH and thus the elution of compounds, but these types of buffers are incompatible with mass spectrometry over extended use. The use of volatile equivalents such as ammonium formate is usually a simple substitution procedure. Similarly the widely used acid trifluroacetic acid (TFA) can usually be replaced with formic acid which is both volatile and does not cause the ionisation problems associated with TFA.

### 1.2.2 Mass Analysers

Sources such as electrospray can ionise the types of molecules of interest in LC separations, and along with this mass analyser performance has improved with respect to speed, accuracy, and resolution. Quadrupoles, ion traps, and time-of-flight (TOF) mass analysers have undergone numerous modifications and improvements to function better with electrospray. The biggest challenge came in interfacing atmospheric pressure (760 torr) sources to analyzers maintained at $10^{-6}$ to $10^{-11}$ torr.

Mass spectrometers have variations in their capabilities as a result of their individual design and intended purpose. While all mass spectrometers rely on a mass analyser, not all analysers operate in the same way; some separate ions in space while others separate ions by time. In the most general terms, a mass analyser measures gas phase ions with respect to their mass-to-charge ratio (m/z), where the charge is produced by the addition or loss of proton(s), cation(s), anion(s) or electron(s). The creation of a charge allows the molecule to be affected by electric fields thus allowing its mass measurement. It is important to remember that mass analysers measure the m/z ratio, not the mass. Therefore, if an ion has multiple charges, the m/z will be significantly less than the actual mass. Multiply charged ions are common in electrospray especially when large (greater than 600 Daltons) molecules are involved. The performance of a mass analyser can typically be defined by the following characteristics: accuracy, resolution, mass range, $MS^n$ capabilities, and scan speed.

### 1.2.3 Quadrupole Analysers

Quadrupole mass analysers are the most common mass analysers currently in use. They offer three main advantages as mass analysers. Firstly, they can tolerate relatively high pressures, secondly, they have a significant mass range with the capability of analyzing up to an m/z of 4000, and finally, quadrupole mass spectrometers are relatively low cost instruments. Considering the mutually complementary features of ESI and quadrupoles, it is not surprising that the first successful commercial electrospray instruments were coupled with quadrupole mass analysers.

A quadrupole mass analyser consists of four parallel rods (see Fig. 1.4) that have fixed DC and alternating RF potentials applied to them. Ions produced in the source of the instrument are then focussed and passed along the middle of the quadrupoles. Their motion will depend on the electric fields so that only ions of a particular m/z will be in resonance and thus pass through to the detector. The RF is varied to bring ions of different m/z into focus on the detector and thus build up a mass spectrum. The trajectory of the ions through the quadrupole is actually very complex - the figure shows a very simplified version!

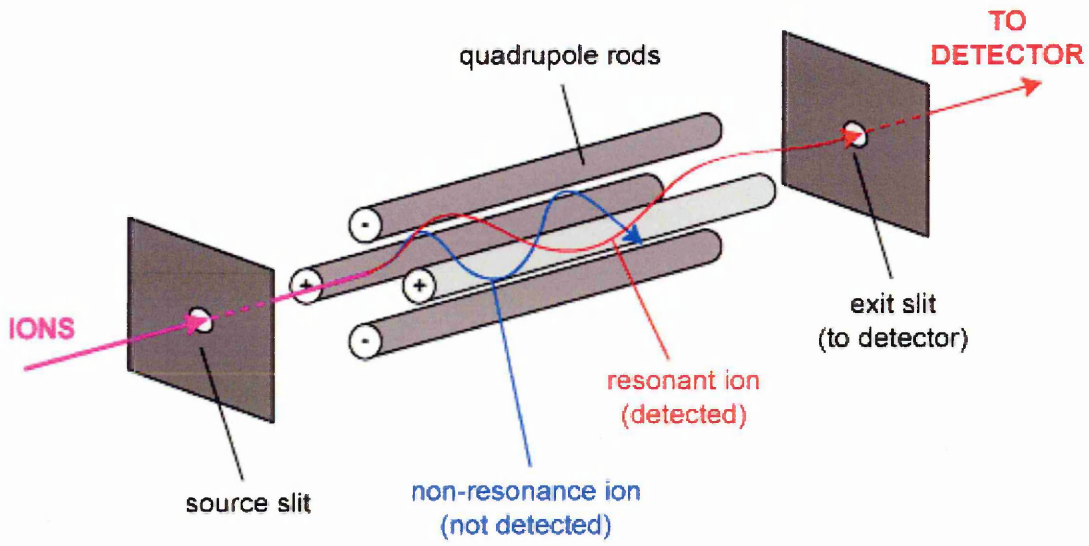



Fig. 1.4[10] *Schematic of a quadrupole mass analyser showing the path of a selected ion and the path of an ion of another m/z*

The two opposite rods in the quadrupole have a potential of $+(U+V\cos(\omega t))$ (labelled '+' on the Fig. 1.4) and the other two $-(U+V\cos(\omega t))$ where 'U' is the fixed potential and $V\cos(\omega t)$ is the applied RF of amplitude 'V' and frequency '$\omega$'. The applied potentials on the opposed pairs of rods varies sinusoidally as $\cos(\omega t)$ cycles with time 't'. This results in ions being able to traverse the field free region along the central axis of the rods but with oscillations amongst the poles themselves. These oscillations

result in complex ion trajectories dependent on the *m/z* of the ions. Specific combinations of the potentials 'U' and 'V' and frequency 'ω' will result in specific ions being in resonance creating a stable trajectory through the quadrupole to the detector. All other *m/z* values will be non-resonant and will hit the quadrupoles and not be detected (see Fig. 1.4). The mass range and resolution of the instrument is determined by the length and diameter of the rods.

Quadrupole mass spectrometers generally have two configurations in the modern laboratory. They are very commonly used in conjunction with either gas-chromatography or liquid-chromatography as a simple high throughput screening system. Quadrupoles can also be placed in tandem to enable them to perform fragmentation studies - the most common set-up is the triple quadrupole (QQQ) [3] mass spectrometer which enables basic ion fragmentation studies (tandem mass spectrometry MS/MS) to be performed.

### 1.2.4 Quadrupole Ion Trap Analysers

The ion trap mass analyser shown in Fig. 1.5 was conceived at the same time as the quadrupole mass analyzer by the same person, Wolfgang Paul. The physics behind both of these analyers is similar, however, in an ion trap, rather than passing through a quadrupole analyser with a superimposed radio frequency field, the ions are trapped in a radio frequency quadrupole field. Ions are generated externally in the ESI source and using ion optics they are injected into the trapping volume. The quadrupole ion trap typically consists of a ring electrode and two hyperbolic endcap electrodes (Fig. 1.5). The motion of the ions, induced by the electric field applied to the electrodes, allows ions to be trapped or ejected from the ion trap. In the normal mode, the radio

frequency is scanned to resonantly excite and therefore eject ions through small holes in the endcap to a detector. As the RF is scanned to higher frequencies, higher m/z ions are excited, ejected, and detected.

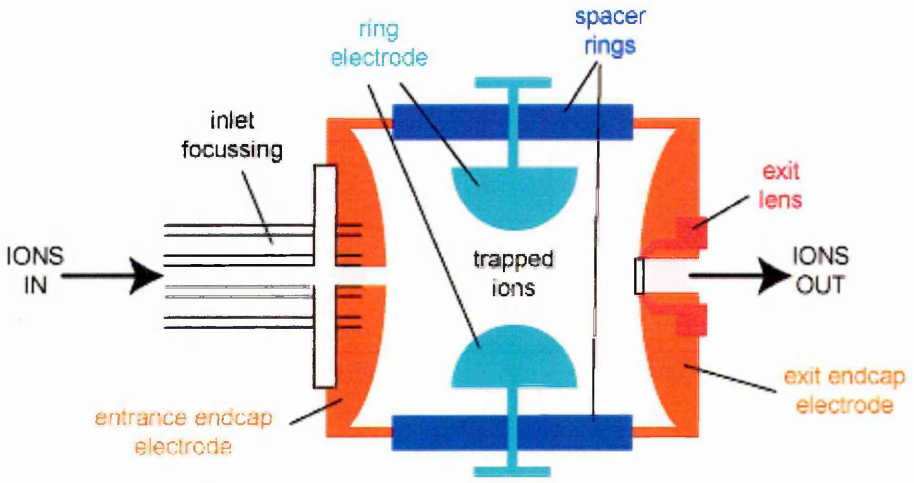



Fig. 1.5[10] *Schematic of a quadrupole ion trap mass analyser showing the electrodes, entrance slit and exit slit.*

One feature of ion traps is that it is possible to isolate one ion species by ejecting all other ions from the trap. The isolated ions can then be fragmented by collisional activation and the fragments detected. In doing so it is possible to create a fragmentation tree or pathway and thus gain valuable structural insight into the isolated ions. The primary advantage of quadrupole ion traps is that these multiple collision induced dissociation experiments can be performed quickly without the need for multiple analysers. Other important advantages of quadrupole ion traps include their compact size, and their ability to trap and accumulate ions to provide a better ion signal.

The mass range (~m/z 4000) of commercial LC-traps is well matched to m/z values generated from electrospray ionization and the resolution allows for charge state

identification of multiply-charged ions. Quadrupole ion trap mass spectrometers can routinely analyse samples down to the femtomole level.

The ion trap does have some significant limitations. Firstly, the ability to perform high sensitivity triple quadrupole-type precursor ion scanning and neutral loss scanning experiments is not possible with ion traps. Secondly, the upper limit on the ratio between precursor m/z and the lowest trapped product ion is ~0.3 (also known as the "one third rule"). An example of the one third rule is that product ions of a precursor ion of m/z 600 will not be detected below m/z 200. Finally, the dynamic range of ion traps is limited because when too many ions are in the trap, space charge effects diminish its performance. To get around this, automated scans can rapidly count ions before they go into the trap, therefore limiting the number of ions allowed to enter. However, this approach does not work when an ion of interest is accompanied by a large background ion population.

### 1.2.5 Time of Flight Analysers

The time-of-flight (TOF) is, at least conceptually, the simplest mass analysers. Thanks to improved electronics allowing much higher and more accurate sampling TOF has become more widespread and instruments allowing the coupling of electrospray, MALDI and gas chromatography electron ionisation mass spectrometry (GC/MS) with TOF are all commercially available. Time-of-flight analysis is based on accelerating a group of ions to a detector. All the ions are given the same amount of kinetic energy ($^1/_2\ mv^2$) through an accelerating potential and because the ions have the same energy, but a different mass, the lighter ions reach the detector first because

of their greater velocity, while the heavier ions take longer due to their heavier masses and lower velocity. Hence, the analyser is called time-of-flight,

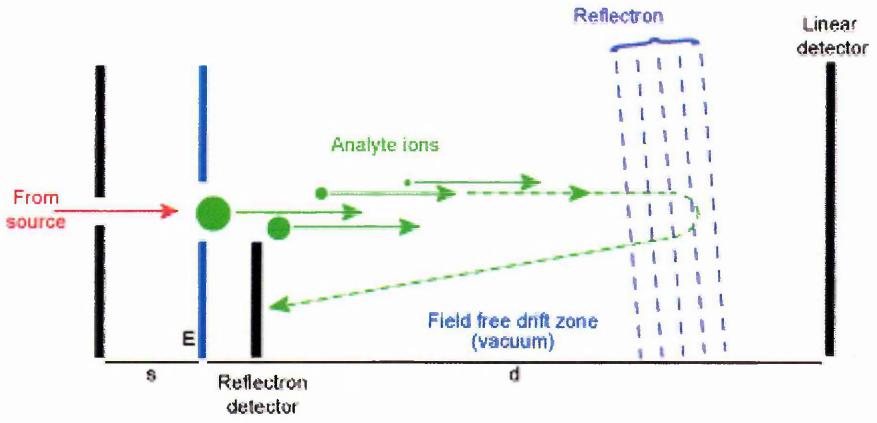



Fig. 1.6[10] *Schematic of a Time of Flight mass analyser showing the placement of the detector for either linear flight or reflectron operation.*

because the mass is determined from the ion's time of arrival. In order for TOF to be coupled with electrospray the ions produced by the electrospray must be converted from the constant stream in which they are produced into ion packets. This is done by

equation 1
$$\frac{m}{z} = 2eEs\left(\frac{t}{d}\right)^2$$

m/z is mass-to-charge ratio of the ion

E is the extraction pulse potential

s is the length of flight tube over which E is applied

d is the length of field free drift zone

t is the measured time-of-flight of the ion

| | Quadrupole | Ion Trap | Time-of-Flight |
|---|---|---|---|
| **Mass Accuracy** | 0.01% (100 ppm) | 0.01% (100 ppm) | 0.001 to 0.01% (10 ppm) |
| **Mass Resolution** | 4,000 | 4,000 | 8,000 |
| **m/z Range** | Up to 4,000 | U to 4,000 | >300,000 |
| **Scan Speed** | ~1 second | ~1 second | milliseconds |
| **Tandem MS** | $MS^2$ (triple quad) | $MS^n$ | MS |
| **Tandem MS Comments** | Good accuracy<br>Good resolution<br>Low-energy collisions | Good accuracy<br>Good resolution<br>Low-energy collisions | Not generally applicable |
| **General Comments** | Low cost<br>Ease of switching pos/neg ions | Low cost<br>Ease of switching pos/neg ions<br>Well-suited $MS^n$ | Low cost |

Table 1.1 *A summary of the qualities of each type of mass analyser to be used during the experiments*

trapping the ions in an electric field to accumulate them before pulsing the accumulated ions. Typically this trapping and pulsing occurs in a time that is of the order of milliseconds and consequently has no noticeable effect on chromatographic resolution. Although all of the ions receive nominally the same accelerating pulse in reality they experience a range of energies and hence ions of the same mass are accelerated at a range of velocities. This has the effect of producing a range of flight times for any particular m/z and therefore reducing the resolution of the analyser. This effect can be reduced by reflecting the ions in an electrostatic field (see Fig. 1.6). The equation governing separation in a TOF analyser is given below. The properties of each type of analyser are summarised in table 1.1.

## 1.3 LC-NMR

The combination of HPLC with NMR offers the potential of unparalleled chemical information from analytes separated from complex mixtures. In 1978 Watanabe[12] reported the coupling of an HPLC eluate to NMR using a stopped flow approach and within one year an on-line system[13] had also been reported. However, LC-NMR only began to take off as instrumental developments such as high field magnets (>500MHz), better coil designs and solvent suppression techniques yielded readily attainable and useful detection limits. Clearly the ability of NMR to distinguish between isomers whether structural, conformational or optical together with its non-destructive nature makes it an extremely powerful tool for the analyst.

### 1.3.1 The Principles of NMR

In order to better understand the nature of the developments of LC-NMR it is necessary to understand the principles of NMR itself. There are many useful and detailed texts treating NMR either from a physics point of view focusing on the mathematics of the principles or simplified texts aimed at non-specialists such as chemists and bioscientists[14]. However, a brief outline of Fourier Transform NMR is given here.

Unlike older continuous wave NMR techniques, FT-NMR offers short acquisition times for individual signals, with signal to noise (S:N) ratio being improved by successively summing a number of these signals. In recent years, a combination of stronger signals (from higher field strength magnets) and lower noise levels (from

better electronics) has made the prospect of time-resolved NMR realisable in the chromatographic range, with acquisition times of tens of seconds.

When a sample is placed in a magnetic field the magnet induces a magnetisation of the nuclei within the sample. This magnetisation can be perturbed or excited by a second field which is oscillating at an appropriate radio frequency perpendicular to the main field. The perturbation field is generated from a coil wound around the sample, and both changes the orientation of the nuclei in the z-direction and brings them into alignment (phasing) in the xy plane. Once the excitation pulse ceases, nuclei experience the force of the initial field and, because they are now in phase, precess about the direction of this field. This precession induces an oscillating current in a receiver coil, with the same orientation as the perturbing radio frequency field. As the nuclei return to alignment and dephase this precession signal decays. The rate of precession is both very high (hundreds of MHz) and very slightly different for each nuclear environment. In order to measure these small frequency differences the induced radio frequency (r.f.) signal is mixed with a constant r.f. signal of nearly the same frequency. This yields a signal at the difference frequency (an interferogram or beat signal) which is of low enough frequency to be accurately digitised. This is known as the free induction decay (FID): *free* of the influence of the radio frequency field, *induced* in the coil and *decaying* back to equilibrium. As the nuclei dephase and return to equilibrium the FID signal amplitude decreases, usually relaxing in seconds, after which another pulse can be applied and the FID signals averaged to enhance the signal to noise ratio. In $^{1}H$ Fourier Transform spectroscopy the entire spectrum of proton frequencies is stimulated simultaneously by a pulse of radio frequency energy

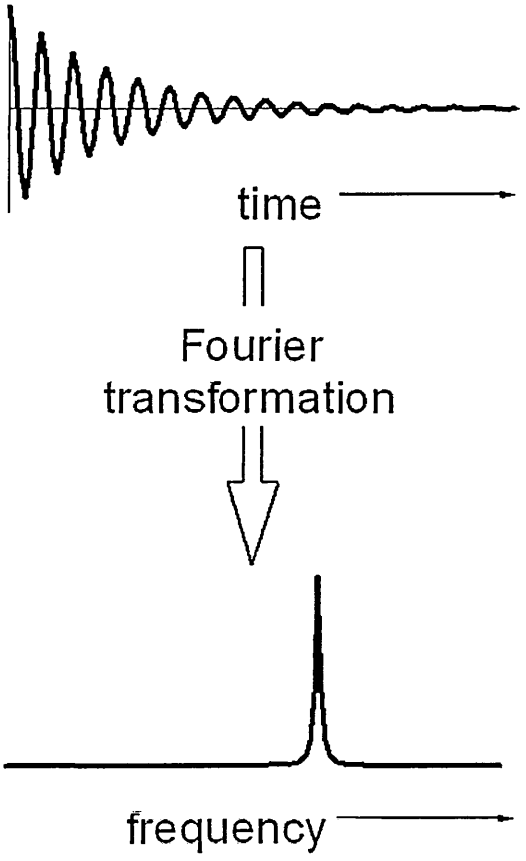



Fig. 1.7 *The FID signal as detected for a single nucleus (top) and the chemically more useful transformed signal below.*

and the response of the nuclei is measured as a function of time, as the FID. The time domain data are converted into the more useful frequency domain spectrum by the mathematical deconvolution of *Fourier Transformation* (see Fig. 1.7)[15].

### 1.3.2 NMR probe design

The first design of NMR probe for LC work was really just a slightly modified tube (see Fig. 1.8). The flow from the HPLC passed into the bottom of a NMR tube and back up and out of the tube. Later designs use a capillary around which the coils of the radio frequency generator are tightly wound as in Fig. 1.9. There are several types of probe design now available but in each case the eluent from the HPLC passes to a probe that sits within the NMR magnet. The key feature of each design is to wind the coil directly around the cell itself to reduce the gap between the cell and the coil.

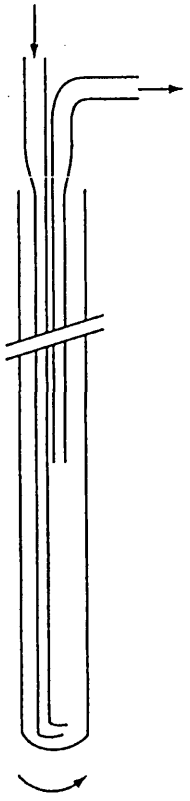


Fig. 1.8 *Early design of a modified NMR tube to allow the effluent from an HPLC to pass in and out of the rotating tube*[16].

This maximises the ratio between the volume of the cell and the volume of the coil, known as the filling factor, and increases the signal to noise ratio. Standard NMR flow probes have detection volumes of between 40 and 120 μL, much larger than conventional UV detector volumes which are in the order of 10μL. The NMR cells require much greater volumes for two reasons. Firstly, in flowing systems there is a distinct residence time of the nuclei within the cell. This residence time is defined by the ratio of the detection volume to the flow rate of the eluent. Because NMR is the

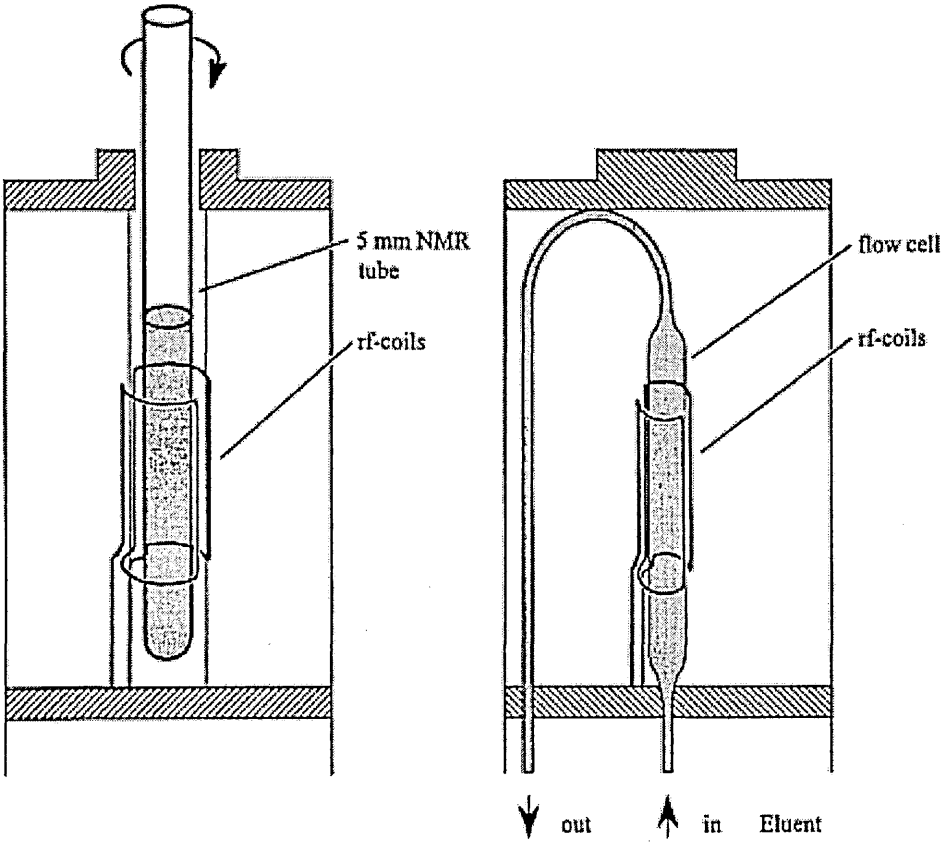



Fig. 1.9 *Diagrams of a standard tube NMR probe (left) and a typical design for a flow probe (right). The flow probe features a blown capillary with the r.f. coils wound as closely around the capillary as possible*[16].

least sensitive of the spectroscopic techniques used, a longer data collection time is needed to achieve an adequate signal to noise ratio. If the residence time is too low (e.g. below 5 seconds with current instrumentation) then weak signals and flow induced NMR signal line broadening will result in a reduction of the spectral resolution. Secondly, NMR spectrometry is a volume sensitive technique and requires the sample volume to be sufficient to fill the detection volume one peak at a time. This is difficult in chromatographic separations where the peak becomes broadened with elution time, and the sample (peak) volume increases with elution time due to diffusion. In NMR this may mean incomplete resolution of early peaks, and poor detection of late eluting peaks. The volumes of the cells therefore have to represent a compromise between the needs of the chromatography and the needs of the NMR.

### 1.3.3 Modes of operation in LC-NMR

There are three main modes of operation for a LC-NMR system; on-flow, stopped flow and loop collection (off-line). In on-flow the column eluent flows through the cell continuously during the chromatographic run and the NMR spectra are collected over the whole time frame. One obvious advantage of this approach is that all components are sampled, including those that do not contain a UV chromophore. However, the relative insensitivity of this approach limits the useful working limits of such a system and sample amounts in the 10-100μg range are commonly needed. In the stopped-flow mode the HPLC pump is stopped while NMR spectra are recorded for a particular peak. Once an adequate spectrum has been acquired the flow is restored and the chromatography continues until further components of interest are

reached. Some prior knowledge of the retention times is clearly required to be able to stop the flow at the appropriate time with the peak inside the NMR cell. This is usually directed by prior detection by UV and calibrating the time from the UV detector to the NMR with a known compound under the same flow conditions. By stopping the flow, longer NMR acquisition times and traditional NMR techniques such as 2D-NMR can be employed. Stopping the flow also allows for the measurement of lower concentration components. In the off-line mode the HPLC fractions are collected into loops during the HPLC run. Similar to stopped flow NMR, the collection is usually triggered by UV detection although it is possible to collect "blind". After the chromatographic run is completed the loops are subsequently eluted into the NMR for analysis. This approach is particularly useful when longer analysis times are required e.g. when only small amounts of sample are present or if 2D experiments are required. The loops can also be removed and stored for later analysis. A more simplistic variation of this approach is to collect the eluent in time fractions as they elute from an HPLC and to store them for later NMR analysis.

### 1.3.4 NMR solvent suppression

In most experiments the solvent present may also give signals in the spectrum. To overcome this, NMR invisible solvents such as deuterated water and deuterated DMSO are employed. However, in flowing systems the use of deuterated solvents is not always possible due to their greater expense and even when deuterated solvents are used there maybe still be residual signals from non-deuterated impurities. To minimise these unwanted signals so that they do not mask important signals from the

sample they are removed using solvent suppression techniques. After excitation, nuclei relax at different rates and this can be used to help remove unwanted signals. Water proton relaxation is slower than most organic proton relaxation, so water signals can be diminished by first irradiating the sample to excite the solvent and then again, after the organic protons have relaxed but before the water protons have, at the frequencies of the nuclei of interest. This is known as presaturation and the pulse sequence most often used is called a NOESY presat[15].

### 1.3.5 Problems due to the NMR stray field

One problem with LC-NMR is the physical location of each of the parts i.e. the LC and the NMR. The field generated by a super-conducting magnet can be detected several metres from the magnet body. Metal or other objects which can be magnetised will have an effect on the shape of the magnetic field and so their location must be considered. Worse still are moving objects within this stray field as they will constantly alter the magnetic field. For this reason the LC must be kept some distance from the NMR magnet. Interaction of the magnetic fields generated by the NMR and the mass spectrometer was also a concern, potentially affecting results from both instruments. Adequate physical separation and alignment were the only options available when this work was done. Newer shielded magnets in which the stray field is kept very small or even within the magnet were only just being offered by the manufacturers and were not available for use during these experiments. Clearly they would offer significant advantage in terms of the distance required between the LC, the NMR and the MS, potentially reducing both band broadening and inter-instrument interferences.

## 1.4 LC-FTIR

As with NMR, the development of Fourier-Transform instruments has improved attainable signal to noise (S:N) ratios in IR spectrometry to the extent that aquisition times short enough for chromatographic work are now possible. As in FT-NMR, an interferogram is generated quite rapidly (in this case from a Michelson interferometer), and the S:N ratio is improved by successive summing of these interferograms. Cooled detectors greatly reduce spectral noise, and aquisition times short enough for both GC and LC are now possible[17].

Probably the least advanced and utilised of the hyphenated techniques certainly within the arena of aqueous samples, LC-FTIR can yield significant amounts of useful information - most importantly the easy identification of many functional groups and of molecular conformations. There are two main possibilities for LC-FTIR sampling, either on-line or off-line analysis. The on-line approach is more elegant than the off-line and has the advantage that such instruments are simpler and therefore cheaper as well as providing spectral information in real time. High flow rates and the presence of non-volatile buffers and salts do not generally cause problems. However, a great deal of effort has been devoted by the manufacturers and research groups to eliminating the eluent, by applying a semi on-line or off-line approach, rather than using a flow cell in an on-line system. The obvious reason for eliminating the eluent is that in most cases it absorbs intensely in the IR region, obscuring the absorption of the analytes. The complete and reproducible evaporation of a non-volatile aqueous solvent is not always straightforward, and rather sophisticated elimination procedures are often needed[18]. Another important requirement for maximising the sensitivity is to

limit the size of the evaporated spots to the minimal size of the FTIR probe area. Nevertheless, the solvent evaporation approach has grown to be the more accepted and more utilised. Examples of both systems are outlined in the later chapters.

Disadvantages of the flow cell arrangement include difficulties in subtracting the eluent's absorption bands from the IR signals and this is further complicated when gradient elution is employed. Another problem is the need to use short optical path lengths, typically 5-50μm, depending on the solvents used and the spectral regions of interest. Short optical path lengths inevitably reduce the sensitivity of the analysis. As with NMR the option to work in either an on-flow or stopped flow mode exists with the same consequences.

The development of a suitable flow cell which can simultaneously meet the demands of both the spectrometer and the chromatograph is another commonality with LC-NMR. Two types of flow cell are commonly in use. These are the transmission flow cell and the Attenuated Total Reflectance (ATR) flow cell; only the latter of these was available during these studies but the operation of both is described below.

### 1.4.1 Transmission flow cells

As their name suggests transmission flow cells allow infrared light to pass through them. Therefore the windows must be made of materials transparent to IR. As well as being transparent to IR the cells must also not react with potential sample materials or in the case of LC-FTIR with the eluents used. For non-aqueous eluents this is fairly simple and cells fabricated from NaCl and KBr can be employed. They can be

made with long path lengths and the materials also have excellent refractive index properties. For aqueous eluents either zinc selenide or diamond windows are often used. Zinc selenide is economical but imposes a cutoff below 700cm$^{-1}$, while diamond is expensive.

### 1.4.2 Attenuated Total Refectance (ATR) flow cells.

The alternative to transmission flow cells is to use a cell based on ATR. Here the optical path is dependent on the number of reflections, the materials used and the geometry of the cell, so very short effective path lengths can be achieved. These types of cell are particularly useful where strongly absorbing materials are present.

An ATR system works by measuring the changes in transmitted intensity that occur in a totally internally reflected infrared beam when the beam comes into contact with a sample. An infrared beam is directed into an optically dense crystal with a high refractive index at an angle low enough to cause internal reflection within the crystal. This internal reflectance creates an evanescent wave that extends beyond the surface of the crystal and into the sample, which is in direct contact with the crystal (see Fig. 1.10). The evanescent wave protrudes only a few microns (0.5 -5 μm) beyond the crystal surface and into the sample, the depth of penetration being frequency dependent (see equation 2). In the regions of the infrared spectrum where the sample absorbs energy the evanescent wave will be attenuated. This attenuated energy is taken from the infrared beam which then exits the opposite end of the crystal at lower intensity and is passed to the detector. Fig. 1.10 shows a schematic of a standard ATR cell and a modified version to allow its use in LC-FTIR.

$$d_p = \frac{\lambda}{2\pi n_r \sqrt{\sin^2(\theta) - \frac{n^2}{n_r^2}}}$$

equation 2

$d_p$ = penetration depth

$\lambda$ = wavelength of the incident evanescent wave

$\theta$ = angle of incidence of the evanescent wave

$n$ and $n_r$ = refractive indices of the sample and crystal

One of the main problems associated with LC-FTIR is the masking of absorption bands due to the signals associated with the solvents used. Band positions in IR spectrometry depend on the bond strength and on the masses of the atoms at each end of the bond (see equation 3 Hooke's Law). As will be seen in later chapters, the use of deuterated solvents for NMR compatability had the beneficial effect of shifting the solvent absorption bands to lower frequencies, allowing the sample bands to be seen more clearly.

$$\nu = \frac{1}{2\pi c}\sqrt{\frac{k}{\mu}}$$

equation 3 Hooke's Law

$\nu$ = frequency

$c$ = speed of light

$k$ = force constant (bond strength)

$\mu$ = reduced mass = $(m_1 \times m_2) / (m_1 + m_2)$

Thus strong (short) bonds absorb more energy (vibrate faster) and bonds to light atoms also absorb more energy (vibrate faster)

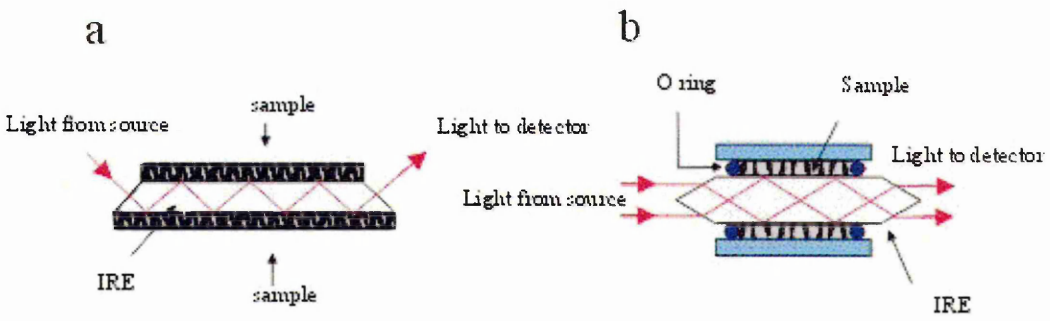



Fig. 1.10 *Schematics of a) a standard ATR cell and b) a cell modified for use in LC-FTIR experiments*[17]. *The IRE is the Internal Reflection Element (i.e. the ATR crystal). Typically 10 or 11 internal reflections occur as the infrared beam travels through the crystal.*

## 1.5 The Reasons for Hyphenation

Mass spectrometry, NMR and FTIR have for many years been three of the main tools in an analytical department's suite of techniques for the characterisation of unknown compounds. Their complementary nature is well known and it is most often demonstrated by referring to the ability of NMR to provide unequivocal data for structural isomers - something not possible by mass spectrometry - while conversely mass spectrometry and infra red can often identify moieties which lack a proton NMR resonance.

Chromatography coupled with any of the above mentioned spectroscopic techniques would provide a better solution for mixture analysis than trying to analyse the sample mixture directly, where spectral deconvolution would be extremely difficult. The alternative would be to isolate the individual components of any mixture and then analyse these components off-line. This is very time-consuming and can be

inherently difficult when many components are present; there is also the risk of chemically altering the compounds during the isolation process. Sequentially running the sample by LC-MS then LC-NMR and LC-FTIR poses problems in ensuring that exactly the same peaks are studied, particularly when chromatographic conditions are changed to accommodate the individual spectrometers. If the problems of bringing these detectors together could be overcome then an extremely powerful tool for the structural determination of mixtures would be the result. The results of such an approach are presented here.

### 1.6 Outline of the work planned

At the outset few papers had been published on the use of multiple hyphenation[19-23] and these had focused on the results obtained. Pullen et al.[19] showed how a mixture of triazoles could be separated and identified by a system which utilised a simple split to divert the eluent from a chromatographic separation to both a mass spectrometer and an NMR. They successfully demonstrated the use of both electrospray and particle beam interfaces for the mass spectrometer and demonstrated the complementary nature of both NMR and MS indicating how a combined approach had promise in terms of saving precious samples and in terms of throughput in busy environments. They also noted the positioning of the two spectrometers relative to each other had required some thought as they had seen a significant impact on the mass spectrometer performance leading to a reduction in sensitivity of about one order of magnitude. They noted the need to minimise or shield the fields from each spectrometer to enable closer placement and the minimisation of any tubing to prevent significant band broadening. Other important features of their work were the ability to suppress

solvent signals in the NMR and the necessity for better data handling with multiple spectrometers. Wolfender et al.[21] did not use a combined approach but rather a system that allowed for a simple switch after chromatographic separation to either the mass spectrometer or the NMR. They demonstrated the successful separation of plant metabolites and how their switch approach could be used to divert to different types of mass spectrometer. They utilised three different ionisation techniques, thermospray, continuous flow FAB and electrospray. Like Pullen et al they showed the problems of using non-deuterated solvents in NMR studies and the need for better solvent suppression. Strohschein et al.[22] studied non-polar compounds from palm oil extract using a $C_{30}$ column and only methanol as a solvent. This approach enabled them to employ high loadings to facilitate the acquisition of NMR while limiting the number of signals generated in the NMR by only using methanol as the mobile phase. They were thus able to obtain both 1D and 2D NMR spectra for their extracts. As with Wolfender et al. they did not combine the NMR and mass spectrometers in a single system, and like the other groups they noted a significant loss of sensitivity in the NMR due to the use of solvent suppression techniques which removed parts of the sample signal. In order to obtain electrospray data on the non-polar compounds being studied they added $AgClO_4$ to the eluent to form Ag adducts. Finally, Wilson et al.[23] employed a combined approach for the study of ecdysteroids. They noted the usefulness of being certain that the same peak was being examined by both the NMR and mass spectrometer when using a combined system. They were also able to identify a compound not previously found when employing LC-NMR and LC-MS separately. However, like all the other authors they provided little information on the practicalities of constructing such a system or how it could be made more routine.

The first objective of the present work was therefore to address the practicalities of joining multiple spectroscopic techniques, in particular any difficulties encountered when joining mass spectrometry and NMR with HPLC in a single system. The experience gained from developing and modifying LC-UV systems for LC-MS would be central to addressing the problems of multiple hyphenation. It was hoped that the lessons learned could be adopted to initially add NMR into the system. The difficulties envisaged were both instrumental (e.g. co-ordinated control, instrument placement and flow balancing) and chemical (e.g. the harmonisation of solvent system compositions). Physical constraints of the available laboratory and the placement of services within it were added challenges: as an example, before any work could begin a suitable exhaust system for the waste from the mass spectrometer had to be constructed and connected to the building's main extraction system.

The mass spectrometers used were quadrupole instruments which operate at radio frequencies and generate moving magnetic fields and the effects of these on the NMR (and vice versa) were unknown, so the placement and orientation of the mass spectrometer in relation to the NMR would also require consideration.

The project was therefore planned in three initial phases:

1. To address any laboratory and safety issues e.g. the piping in of high pressure gases and the removal of gaseous waste

2. To consider the physical location of the equipment and its orientation and in particular to assess the impact of the magnetic fields produced by the various spectrometers on the other instruments. At the same time as minimising any magnetic

effects it would be necessary to consider any implications any changes would have upon the chromatography.

3. Once the first two issues had been addressed it would be necessary to study the effects of solvent compositions and to find solvents which would be compatible with all parts of the system.

If these could be completed successfully, then the way would be clear to realise the use of multiply hyphenated systems in the study of complex samples such as compound libraries and biological fluids. As will be seen, these and further unexpected problems prompted solutions which in several cases yielded equally unexpected benefits.

# CHAPTER 2

# LC-NMR-MS INITIAL SET UP AND EVALUATION

## 2.1 Aims and Introduction

**The aims of the work described in this chapter were:**

1) **To investigate the feasibility of LC-NMR-MS by flow injection analysis of a series of test compounds.**
2) **To identify mobile phases suitable for combined LC-NMR and LC-MS.**

LC-MS has been employed for many years but only since the advent of electrospray ionisation has it become a truly robust and routine method for the analysis of mixtures. Previously techniques such as flow fast atom bombardment (FAB), thermospray and particle beam had been used with some success, but they had failed to allow transfer of the technique from the expert into the hands of the chemist. LC-NMR is by comparison a relatively recent introduction to the arsenal of the analyst. However, technological advances in NMR have resulted in LC-NMR becoming commonplace, certainly within the pharmaceutical industry.[24,25] Now that both these spectroscopic techniques have been successfully coupled with HPLC, it has for the first time become possible to acquire NMR and MS data simultaneously from a single chromatographic analysis. Many groups,[19,26-34] especially in the pharmaceutical industry, have published work on the analysis of mixtures and in particular of

impurities and drug metabolites. The nature of this work has meant that these papers concentrated on the results obtained[20-23], with little or no reference to the practical aspects of this technique, and hence the need for the investigation described in this chapter.

## 2.2 Initial experimental conditions

### 2.2.1 HPLC conditions

Chromatography was performed using a variety of $C_{18}$ 250 x 4.6 mm columns, attached to a Bruker LC system which comprised a Bruker LC22 pump, autosampler, UV detector and a BPSU-12 collector (Bruker Spectrospin, Coventry, UK). The eluents consisted of deuterated water ($D_2O$) (99.9 atom % Fluorochem, Glossop, UK) modified with 0.1% formic acid or trifluroacetic acid and acetonitrile (Riedel de Haan, Seelze, Germany). The flow rate was 1.0mL/min with UV detection at 254nm. The splitter (see section 2.2.4) was a simple Valco t-piece and all connecting tubing was in PEEK.

### 2.2.2 NMR spectrometry

NMR was performed using a Bruker DRX 500 spectrometer equipped with a dedicated $^{1}H/^{19}F$ flow probe with a cell volume of 120 µL. Stopped flow experiments were conducted using a double solvent suppression pulse sequence (1D-NOESY) to remove the residual $^{1}H$ signals from the acetonitrile and water. Data were acquired with between 32 and 160 transients into 16K data points with a pulse repetition time of approx 3s.

### 2.2.3 Mass spectrometer

The mass spectrometer was a Platform LC (Micromass, UK) fitted with an electrospray probe and a standard source. Data were acquired over the range m/z 100-600 with a scan time of 1 s and an interscan delay of 0.1 s. The capillary voltage was set to 3.5kV and the cone voltage varied from 25 to 90V. Scans were acquired in both positive and negative ion modes.

A series of easily obtainable compounds (see table 2.1) was chosen to assess any unforeseen problems with the coupling of LC to both NMR and MS. A selection of mass spectra obtained is shown in Figs. 2.1-2.4 to illustrate typical results from these test compounds. Caffeine (Fig. 2.1) showed a $(M+D)^+$ at m/z 196, and also an ion at m/z 237 $(M+D+CH_3CN)^+$. This adduct is not present in the lower spectrum, which was obtained at a higher cone voltage. Also shown are the negative ion electrospray spectra of indomethacin (Fig. 2.2) showing a $(M-H)^-$ at m/z 356 in the low cone voltage spectrum; the high cone voltage spectrum shows significant fragmentation noticeably the ion at m/z 312 due to the loss of $CO_2$ from the ion at m/z 356. The next spectra (Fig. 2.3) are of antipyrine. The ion at m/z 190 represents $(M+D)^+$ and the higher m/z ions are sodium adducts. These sodium adducts are reduced in the high cone voltage spectrum (shown in the lower of the two spectra). Finally there is a negative ion spectrum of Diclofenac (Fig. 2.4) with the ion at m/z 295 representing $(M-H)^-$.

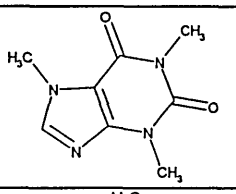
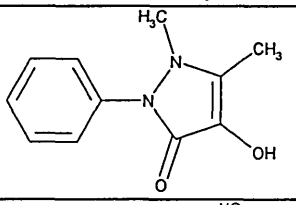
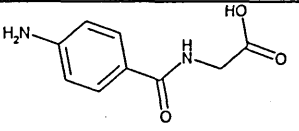
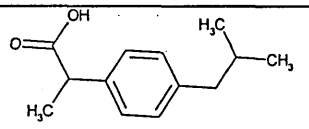
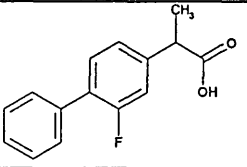
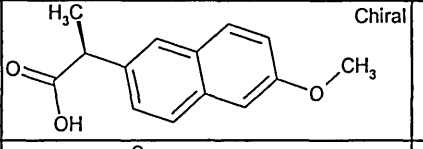
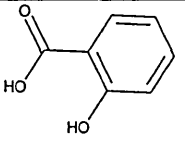
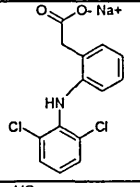
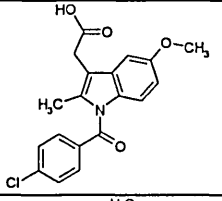
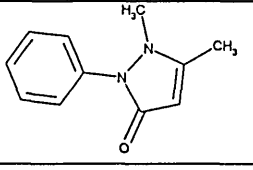
| Compound | Structure | Mol. Wt | Formula |
|---|---|---|---|
| CAFFEINE |  | 194.1930 | $C_8 H_{10} N_4 O_2$ |
| 4-HYDROXYANTIPYRINE |  | 204.2280 | $C_{11} H_{12} N_2 O_2$ |
| 4-AMINOHIPPURIC ACID |  | 194.1890 | $C_9 H_{10} N_2 O_3$ |
| IBUPROFEN |  | 206.2830 | $C_{13} H_{18} O_2$ |
| FLURBIPROFEN |  | 244.2640 | $C_{15} H_{13} F O_2$ |
| NAPROXEN |   | 230.2620 | $C_{14} H_{14} O_3$ |
| SALICYLIC ACID |  | 138.1210 | $C_7 H_6 O_3$ |
| DICLOFENAC SODIUM |  | 318.1340 | $C_{14} H_{10} Cl_2 N O_2 . Na$ |
| INDOMETHACIN |  | 357.7910 | $C_{19} H_{16} Cl N O_4$ |
| ANTIPYRINE |  | 188.2290 | $C_{11} H_{12} N_2 O$ |

Table 2.1 *The structures formulae and molecular weights of the test compounds used*

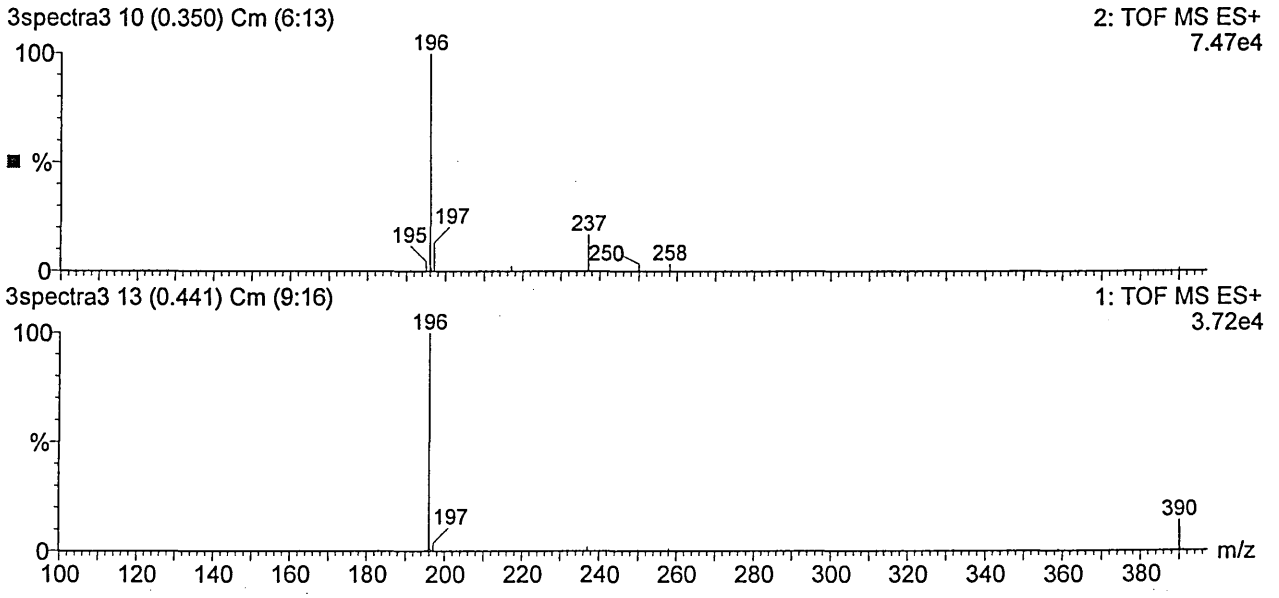



Fig. 2.1 *The low (30V) (upper spectrum) and high (60V) (lower spectrum) cone voltage spectra of caffeine. The upper trace shows the* $(M+D)^+$*, m/z 196 along with* $(M+D+CH3CN)^+$*, m/z 237 and* $(M+Na+CH3CN)^+$*, m/z 258 which are removed in the high cone voltage spectrum shown in the lower spectrum.*

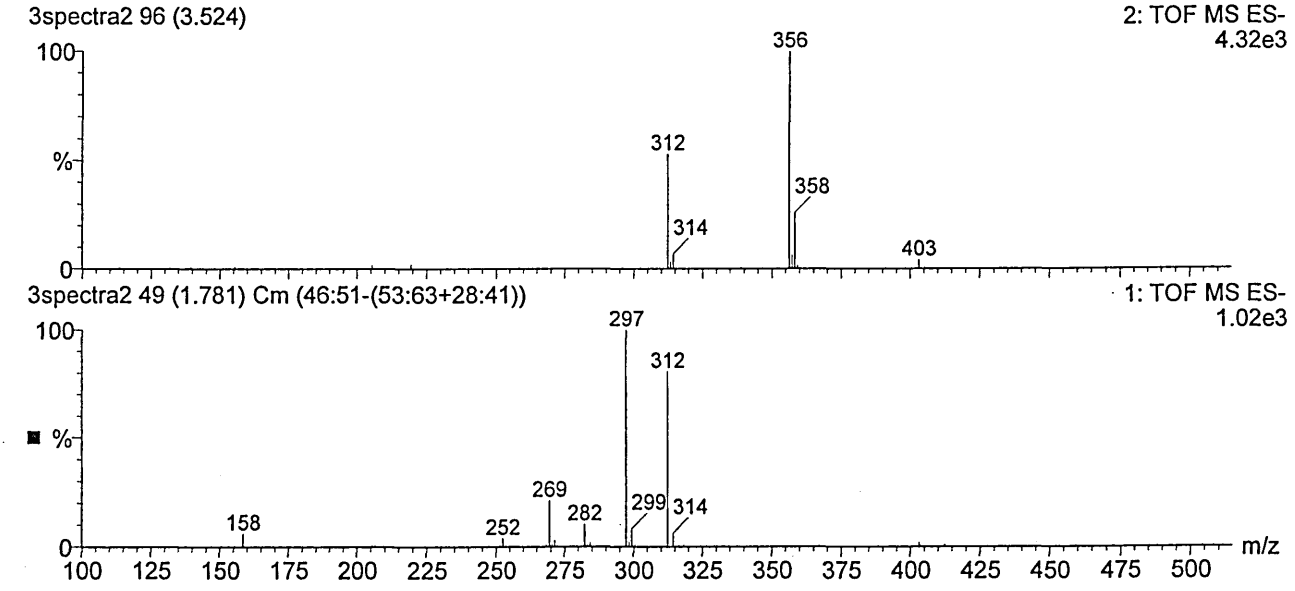



Fig. 2.2 *The low (30V) (upper spectrum) and high (60V) (lower spectrum) cone voltage spectra obtained for indomethacin. The upper trace shows a* $(M-H)^-$ *but this is absent in the lower trace which shows only product ions.*

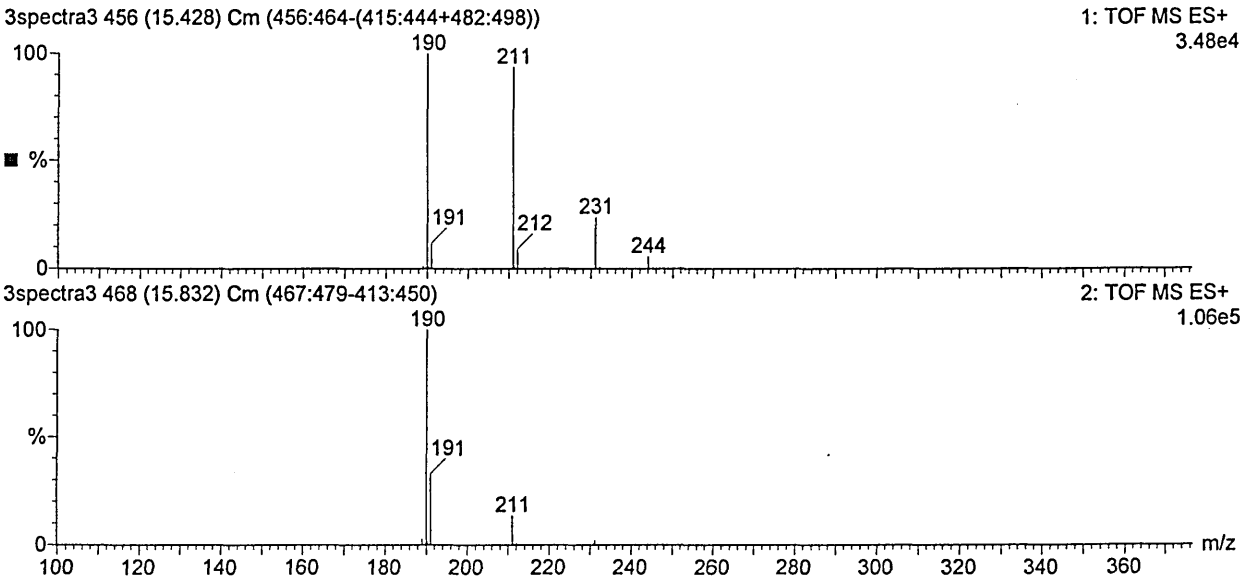



Fig. 2.3 *The low (30V) (upper spectrum) and high (60V) (lower spectrum) cone voltage spectra obtained for antipyrine. The low cone voltage spectrum shows significant sodium adduction compared to the higher cone voltage spectrum in the lower trace.*

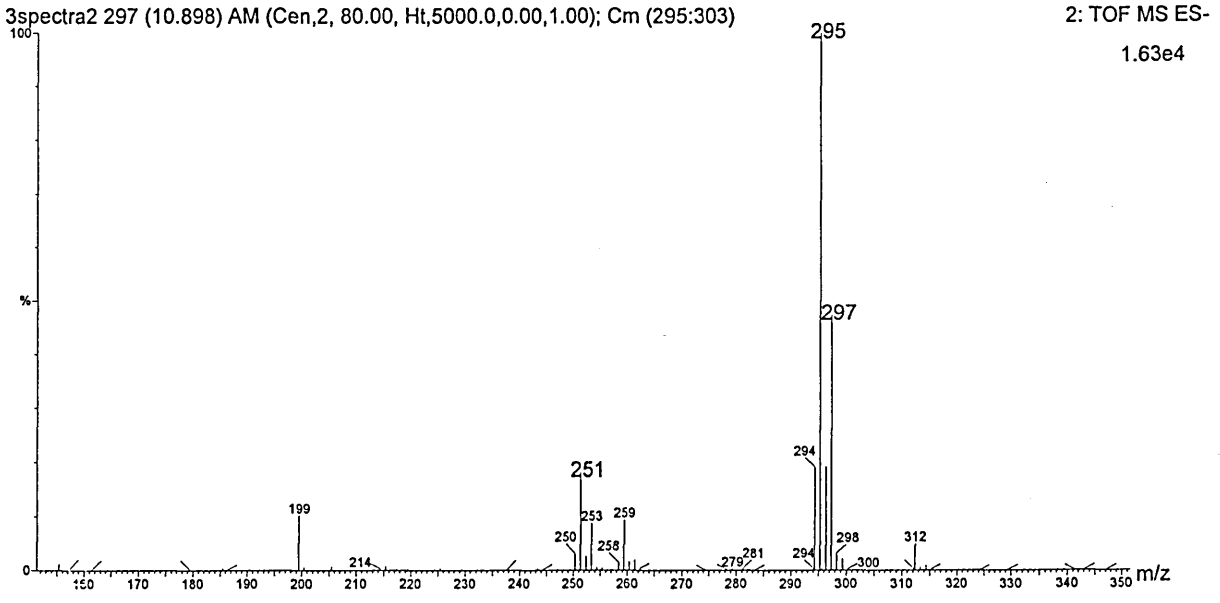



Fig. 2.4 *The negative ion electrospray spectrum(30V) of dichlofenac showing $(M-H)^-$ m/z 295 which represents the free acid not the sodium salt.*

### 2.2.4 Instrument Layout

The system was developed from the LC-NMR system already in place as supplied by Bruker Instruments (see 2.2.2). There were principally two ways to link the NMR spectrometer and mass spectrometer with the LC system, either in parallel or in series. NMR is a relatively insensitive technique, so large volumes and high concentrations of analytes were used which required the use of 4.6mm i.d. LC columns to supply sufficient material for the NMR spectrometer while avoiding problems of overloading the LC system (which would be encountered had columns of a smaller i.d. been used). This meant that flow rates in the range 0.5 to 1.0mL/min were needed to meet the requirements of the NMR spectrometer without compromising the chromatography. Such high flow rates can be accommodated by modern mass spectrometers but as electrospray is a concentration dependent technique as opposed to a mass sensitive technique, splitting the flow to the mass spectrometer had no effect on the sensitivity in the short term. However, splitting the flow greatly enhanced the source lifetime and hence allowed the mass spectrometer to be operated at optimum sensitivity over a much longer period of time. When the flow from the LC was split prior to the NMR spectrometer the lengths of the tubing to the mass spectrometer should ideally be adjusted such that the chromatographic peak has just passed through the mass spectrometer as it fills the NMR spectrometer flow cell, so that the MS data can be used to with the UV data to direct NMR experiments. Splitting in this manner (Fig. 2.5) would also enable the use of stop flow NMR with minimum degradation of the integrity of the chromatography.

Running with the spectrometers in series and splitting the flow after the NMR spectrometer would allow for the completion of all NMR experiments whether on flow or stop flow prior to the commencement of mass spectrometric analysis. However, this arrangement increases the possibility of peak dispersion before the mass spectrometer, for peaks trapped between

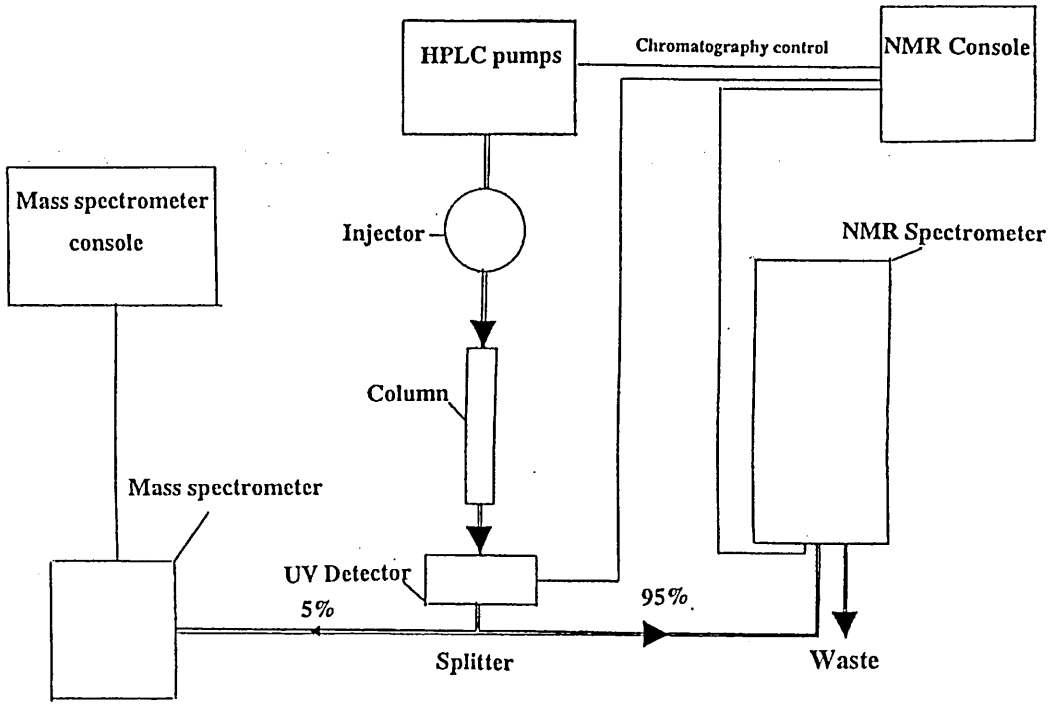



Fig. 2.5 *A schematic of the LC-NMR system modified to include an in line mass spectrometer.*

the spectrometers during stopped flow experiments. Series operation also raised the backpressure in the NMR spectrometer flow cell beyond its normal operating limits as these types of experiments had not been foreseen during the design of the flow probe. The result of this was leakage of the mobile phase and analytes from the NMR spectrometer flow probe, which caused considerable downtime. Furthermore running

in this configuration would not allow use of the MS as an extra flag to direct the NMR in stopped flow experiments, relying solely on UV for this purpose. Thus peaks which have no chromophores but are amenable to mass spectrometry would not be captured in the NMR. Finally the adoption of a series mode of operation of the hyphenated LC-NMR-MS meant the MS would sit idle for long periods of time when the NMR was performing long experiments, some of which could last for many hours. In series mode the MS must lie idle whereas in parallel mode there was the option for the MS to be re-employed elsewhere. Therefore all experiments contained within this thesis were acquired using the parallel approach.

### 2.2.5 The effect of mobile phase composition

Solvent selection for any system involving both NMR and mass spectrometry must be a compromise between the ideal requirements of each instrument. In NMR inorganic buffers such as sodium phosphate are preferred for pH modification because no additional signals are observed in standard proton or carbon NMR experiments. However, this type of buffer is incompatible with long term LC-MS as the buffer becomes deposited on the MS source and eventually blocks the source completely. Even when a Z-spray source (Fig. 2.6) is utilised these buffers will still ultimately reduce the lifetime of the MS source. An alternative for NMR studies is TFA, which has no protons to cause interference. Experiments using propranolol as a model compound showed that 0.1% TFA could be used with LC-MS for a limited range of analytes present at high concentration (>1 μg on column) in positive ion mode. This is illustrated by the LC-NMR and LC-MS results shown in Figs. 2.7. However, with acidic analytes such as ibuprofen, ion suppression was complete,[35] and even at high

sample concentrations where good quality $^{1}H$ NMR data were readily obtained MS data could not be acquired. Formic acid was found to provide a suitable compromise between the needs of the MS on the one hand and those of the NMR on

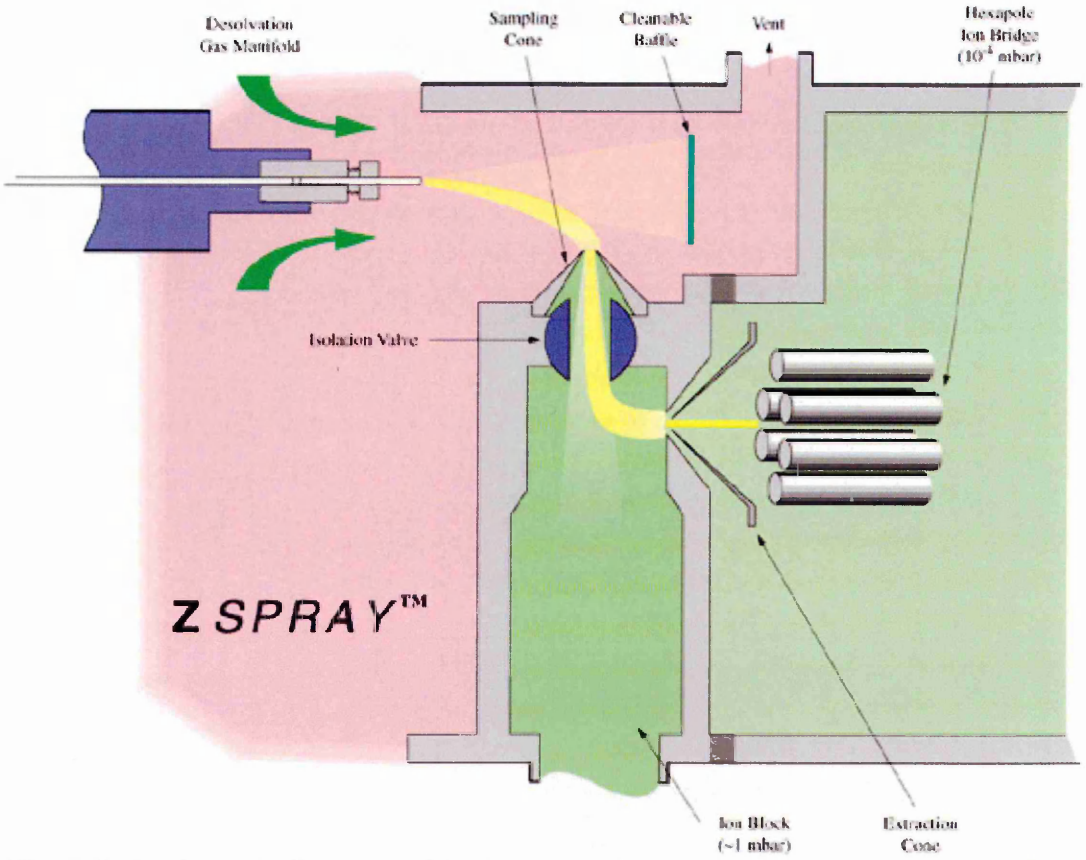



Fig. 2.6 *A schematic diagram of the Micromass Z-spray source. This novel design minimises the effects of insoluble analytes and hence prolongs the lifetime of the source, thus increasing uptime.*

the other whilst still giving acceptable chromatography. The single proton of formic acid, which gives a sharp singlet at 8.5 ppm, gave minimal interference in the resulting NMR spectra and enabled MS data to be acquired for acid analytes such as glucuronides. In general, formic acid is preferred for MS because of the well-known loss of sensitivity caused by TFA in both negative and positive ion modes. Up to

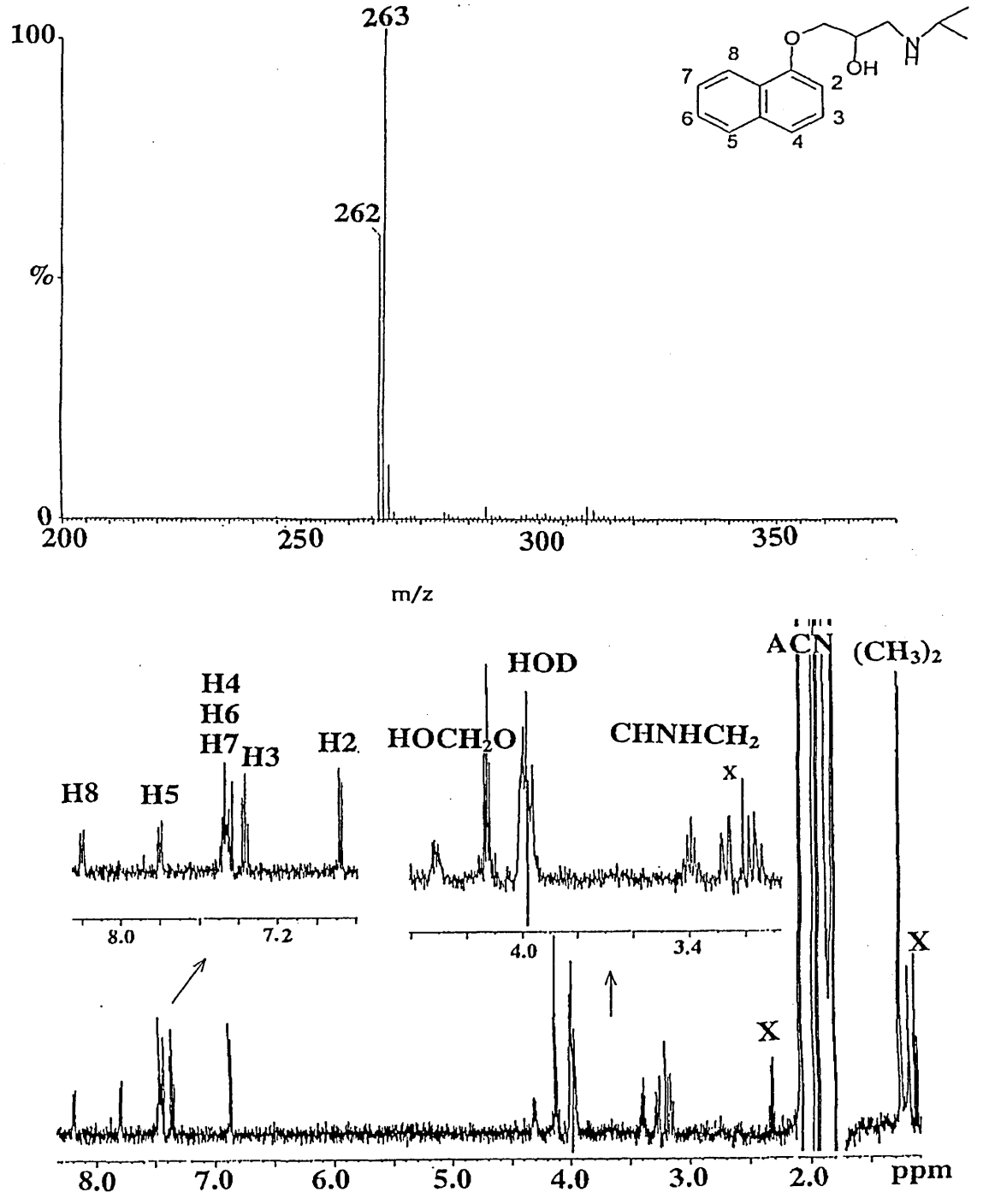



Fig. 2.7 *The stopped flow HPLC-NMR (lower trace) and the on flow mass spectrum (upper trace) obtained for propranolol (10 μg) during the same run with a mobile phase of $D_2O$:acetonitrile:TFA (40:60:0.1 v/v/v) at 1.0 mL/min. The mass spectrum shows ions at m/z 262 $(M+H)^+$ and m/z 263 $(M+D)^+$*

0.5% formic acid was necessary to give satisfactory chromatography. The purity of solvents is always important in any chromatographic technique but especially so in LC-NMR, for the simple reason that the impurities can produce a significant contribution to the background. Clearly the fewer number of components present whether as modifiers or contaminants, the cleaner the resultant background and hence the smaller the chance of any interference with any analyte signals. Whilst the $D_2O$ employed here was free from NMR detectable contaminants, the acetonitrile used

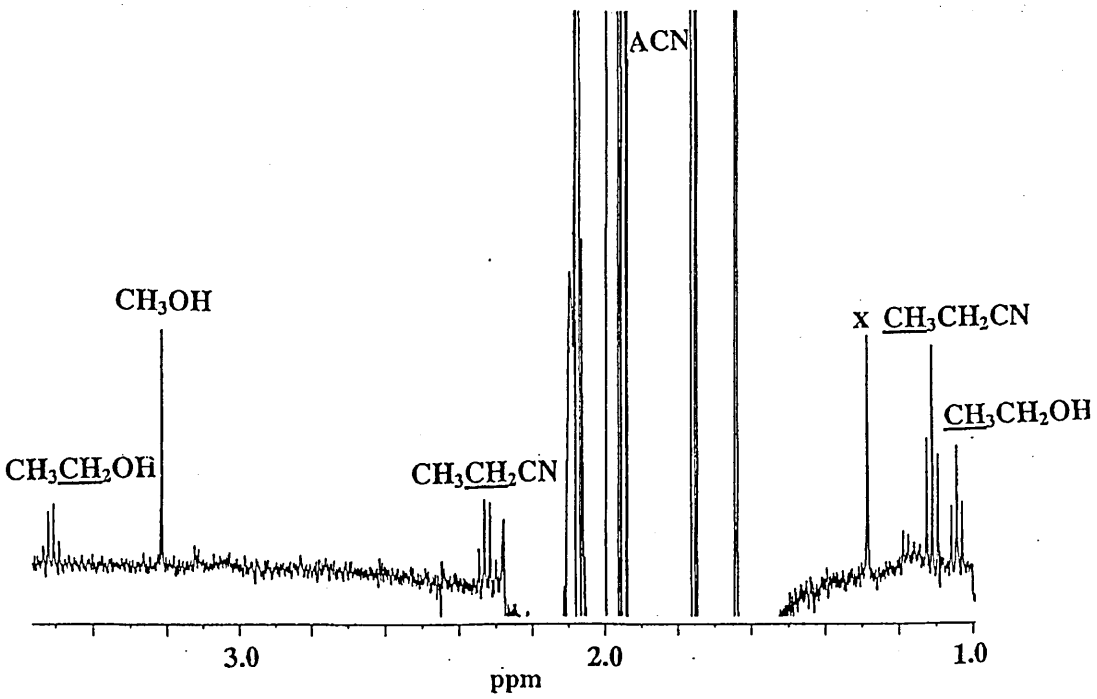



Fig. 2.8 *A typical LC-NMR solvent background spectrum (for acetonitrile: 0.1M ammonium acetate) due to the impurities in the "Pestanal" grade acetonitrile. Peaks marked X are unknown impurities.*

(Pestanal grade) as the organic modifier contained detectable quantities of proprionitrile (see Fig. 2.8). The use of specially prepared NMR modified solvents such as deuterated acetonitrile and deuterated methanol reduces the number of signals,

which must be suppressed, but these solvents are very expensive and their use was therefore limited.

### 2.2.6 Proximity and angle of the mass spectrometer to the NMR field

Given the strength of the magnetic field surrounding a 500MHz NMR magnet there was obviously the potential for interference with the operation of the mass spectrometer. There was also the potential for the mass spectrometer to interfere with the operation of the NMR spectrometer. In the studies that follow the mass spectrometer was sited outside the ten gauss line of the NMR spectrometer with its axis radial to the NMR spectrometer and hence the NMR field. Varying the angle of the mass spectrometer to the NMR spectrometer had no effect on the data either from the mass spectrometer or the NMR spectrometer. The LC, NMR spectrometer and various mass spectrometers were situated approximately at the corners of an equilateral triangle but this was dictated largely by the size and shape of the room and access to supplies for the mass spectrometer. The mass spectrometer needed to be recalibrated as the NMR stray field caused a mass shift, but this was done utilising the software rather than a physical change in the instrument. No effect on the operation of the NMR spectrometer was observed due to the presence of any of the mass spectrometers. Ideally the LC, NMR spectrometer and mass spectrometer would be placed in a line with the LC at the mid point. This would ensure the mass spectrometer and NMR spectrometer are as far apart as possible whilst minimising the distance from the LC to either spectrometer, and therefore minimising the effect on the chromatography during transfer to the spectrometers.

### 2.2.7 Stopped Flow NMR

NMR is inherently many orders of magnitude less sensitive than MS but it gives, in general, far more structural information. Injecting relatively large amounts of sample onto the column compensated for this lack of sensitivity. Where it was possible to obtain large quantities (150-300 μg) it was possible to acquire spectra on flow. Where the amount of material on the column (15-30 μg per analyte) was insufficient for on flow acquisition, spectra were obtained by stopping the flow with the analyte in the NMR spectrometer flow cell to increase the number of FIDs that were obtained. Using this stopped flow technique interpretable spectra could be obtained on 10-20μg of analyte in 1-2 hours.

During the initial stopped flow experiments a gradual decrease in the signal from the analyte during NMR acquisition was observed. This was traced to the mass spectrometer which, because the mass spectrometer source works at slightly positive pressure in electrospray mode, caused the flow to the NMR spectrometer to continue after the LC pump had been stopped. This pressure was sufficient when splitting post column for the eluent to be slowly forced through the tubing and flow cell of the NMR spectrometer (N.B. this was also a problem when running in series). To overcome this it was necessary to switch off the nebulizing gas of the mass spectrometer when the LC pump was stopped and only re-establish it once the flow was re-instated. In an ideal integrated system this would be done automatically. When using stopped flow NMR, the type of LC pump can affect the detectability of closely eluting components. The most modern LC pumps are designed for optimum chromatographic performance and as such they usually raise and lower the pressure

(and hence the flow) gradually to protect the column from pressure pulses which could cause damage. This was less than ideal when trying to precisely trap components in the NMR flow cell as it could result in some drifting of peaks; however, the older Bruker pumps used here did not suffer from this problem.

When using LC-NMR the chromatography is often developed off line from the NMR spectrometer using standard non-deuterated solvents. It was not always simply a matter of replacing these solvents with their deuterated equivalent to reproduce the chromatography for LC-NMR or LC-NMR-MS. Switching to deuterated solvents could occasionally give rise to changes in retention times or even to the change of elution order of peaks. For this reason it was standard practice to carry out an initial chromatographic run with a small injection volume (e.g. 10 μL) and then scale up (e.g. to 50 μL) for stop flow NMR, once the optimum conditions had been established. It was found that during this initial run it was usually possible to acquire all of the MS data, which could then be analysed and used to direct the selection of peaks for the subsequent NMR acquisitions. Additionally, this also allowed the second run to be acquired while mixing the eluent just prior to the mass spectrometer with a non-deuterated solvent to back exchange the deuteriums for protons. In this way, if the initial data could not be readily understood then the number of exchangeable protons could be counted for any analyte, sometimes adding valuable additional information. This was best illustrated in the "Hot Water" experiments described in chapter 3.

### 2.2.8 The Mass Spectrometer

Although NMR generally gives far more structural information than mass spectrometry, the mass spectrometer could generate much useful information to aid in the identification of analytes. Obviously the molecular weight could be identified but as most chromatographic peaks were of the order of 30-60 seconds wide there was ample opportunity to acquire multiple channels of mass spectrometric data simultaneously. The simplest form of this was to acquire at multiple cone voltages to generate pseudo MS/MS data, or to switch between acquiring in positive ion mode and negative ion mode. Simply by monitoring for specific masses e.g. m/z 80 and 97 for sulphate and the loss of 164 Da for glucuronide fragments in metabolism studies it was possible to supplement the UV data and thus aid the identification of peaks requiring NMR. Similarly, when compounds contained elements such as bromine which have distinct spectral finger prints, the spectrometer could be used to look specifically for this occurrence and again direct the NMR for stopped flow evaluation of the identified peaks.

## 2.3 The identification of the metabolites of Ibuprofen in human urine by LC-NMR-MS

The known phase 1 metabolites of Ibuprofen were chosen to show how LC-NMR-MS could be used to identify unknowns in complex mixtures. The structure of Ibuprofen is shown in Fig. 2.9

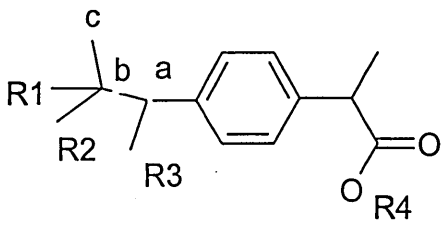



Fig. 2.9 *The general structure for Ibuprofen and its metabolites. For ibuprofen R1, R3 and R4 = H and R2 = methyl.*

### 2.3.1 LC Conditions[34]

HPLC was performed using a Hypersil BDS C18 column (5μm, 250 x 4.6 mm i.d.), attached to a standard Bruker LC system comprising a Bruker LC22 pump, autosampler, UV detector and BPSU-12 collector (Bruker Spectrospin, Coventry, UK). Gradient chromatography was used with 0.2% formic acid in $D_2O$ (99.9%, Fluorochem) and acetonitrile (99% Riedel de Haen). A linear gradient from 20 % to 60 % acetonitrile was employed over 45 min. at a flow rate of 1.0 mL/min with UV detection at 254 nm. Under these conditions the transfer time for peaks from the UV detector to the NMR probe was 52s and to the mass spectrometer 36s.

### 2.3.2 NMR Spectrometry

The NMR spectrometry experiments were conducted on a Bruker DRX500 Spectrometer equipped with a dedicated $^{1}H/^{19}F$ flow probe with a cell volume of 120 μL. Stopped flow experiments were carried out using a 1D-NOESY pulse sequence for double solvent suppression. Data were generally acquired with between 32 and

160 FIDs into 16K data points with a pulse repetition time of ca 3s. Chemical shifts were referenced to acetonitrile at 1.93 ppm.

### 2.3.3 Mass Spectrometry

The mass spectrometric analysis was performed on a Micromass Platform mass spectrometer (Altrincham, UK) using negative ion electrospray ionisation. Alternate spectra were recorded using low (30V) and high (60V) cone voltages to give spectra that essentially contained $(M-H)^-$ (30V) and product peaks (60V). The low voltage spectra were scanned from m/z 200 to 600 and the high voltage spectra from m/z 100 to 600.

### 2.3.4 Sample preparation

A urine sample was obtained from a normal healthy adult volunteer for the period 0-3 hours following the oral administration of 400 mg of Ibuprofen. The sample was stored frozen at –20C until required for analysis. An aliquot of the sample (20mL) was adjusted to pH 2.0 using 0.1 M HCl. This aliquot was then applied to a 3 mL solid phase extraction cartridge containing 500 mg of C18 sorbent (BondElut, Jones Chromatography, UK). The cartridge had previously been conditioned using 5 mL of methanol followed by 5 mL of 0.1 M HCl. Following the application of the sample the cartridge was washed with 2 mL of 0.1 M HCl and the metabolites and other retained material eluted with 5mL of methanol. The volume of this eluate was reduced to 1 mL under a stream of nitrogen. For LC-NMR-MS 40 μL of this sample

were taken and mixed with 160 μL of $D_2O$ (99.9% Fluorochem) and the whole of this sample was injected onto the HPLC column.

### 2.3.5 Metabolite Profiling

The gradient employed gave a good separation of the ibuprofen-related material. The bulk of the co-extracted endogenous contaminants remaining after SPE generally eluted in the early part of the run. The various components were identified by a combination of NMR and mass spectrometry. The metabolism of Ibuprofen is complex and results in the formation of a range of phase 1 oxidised metabolites and their corresponding phase 2 glucuronide conjugates. In addition, as the drug was administered in the form of a racemate, the possibility existed for the formation of diastereomeric metabolites via metabolic attack at chiral centres, or conjugation to D-glucuronic acid.

The metabolites were identified by a combination of stopped flow NMR of peaks detected by UV and on flow MS. In this way it was possible to identify 9 compound related peaks (listed with spectroscopic data in table 2.2). It can be seen from both the UV trace (Fig. 2.10) and the total ion current (TIC) chromatogram generated from the MS data (Fig. 2.11A), acquired at low cone voltage, that there were a large number of components in the urine sample. These included both endogenous compounds and ibuprofen metabolites. If such a chromatogram, acquired using either UV or MS, were used in isolation to trigger acquisitions on the NMR, much superfluous data would be generated. However, under high cone voltage conditions glucuronides give a diagnostic product ion at m/z 196 and as several of the metabolites of ibuprofen, including the drug itself, are excreted as glucuronides, this provided a suitable

| Structure | MS m/z $[M-H]^-$ | $^1$H-NMR Chemical Shift Data in PPM | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Me-CH | Me-CH | a-$CH_n$ | b-CH | c-Me | R1, R2 | gluc $H_1$ | RT (min) |
| 1<br>R1 = Me<br>R2 = OH<br>R3 = H<br>R4 = gluc | 401 | 3.81 | 1.38 | 2.65 | – | 1.05 | 1.05 | 5.45 | 8.7 |
| 2<br>R1 = $CH_2OH$<br>R2, R3 = H<br>R4 = gluc | 401 | 3.80 | 1.37 | 2.63<br>2.23 | ? | 0.72 | 3.28<br>3.35 | 5.44 | 9.9 |
| 3*<br>R1 = $CO_2H$<br>R2,R3 = H<br>R4 = gluc | 415 | 3.80 | 1.37 | 2.83<br>2.62 | 2.66 | 1.03 | – | 5.44 | 10.5 |
| 4*<br>R1 = Me<br>R2 = H<br>R3= OH<br>R4 = gluc | 401 | 3.83 | 1.38<br>1.40 | 4.23<br>4.37(?) | 1.9(?) | 0.834<br>0.829 | 0.62 | 5.42<br>5.44 | 13.3 |
| 5<br>R1 = Me<br>R2 = OH<br>R3, R4 = H | | 3.68 | 1.34 | 2.65 | – | 1.06 | 1.06 | – | 14.5 |
| 6<br>R1 = $CH_2OH$<br>R2, R3 = H<br>R4 = H | | 3.66 | 1.33 | 2.63<br>2.33 | 1.9(?) | 0.72 | 3.28<br>3.35 | – | 16.9 |
| 7<br>R1 = $CO_2H$<br>R2, R3 = H<br>R4 = H | 236 | 3.68 | 1.35 | 2.86<br>2.63 | 2.69 | 1.05 | – | – | 17.6 |
| 8<br>R1, R2 = CH2<br>R3 = H<br>R4 = gluc | 382 | 3.79 | 1.37 | 3.23 | – | 1.56 | 4.72<br>4.65 | 5.43 | 27.2 |
| 9<br>R1, R3 = H<br>R2 = Me<br>R4 = gluc | 384 | 3.78 | 1.36 | 2.37 | 1.73 | 0.78 | 0.78 | 5.42 | 31.2 |

Table 2.2 *Metabolites of ibuprofen and their NMR and MS data, as identified from one individual's urine sample.*

prompt to search for drug related compounds. A single ion chromatogram of this ion, derived from the high cone voltage data (Fig. 2.13B), highlights a number of peaks due to the presence of glucuronides in the extract. These data were therefore used to

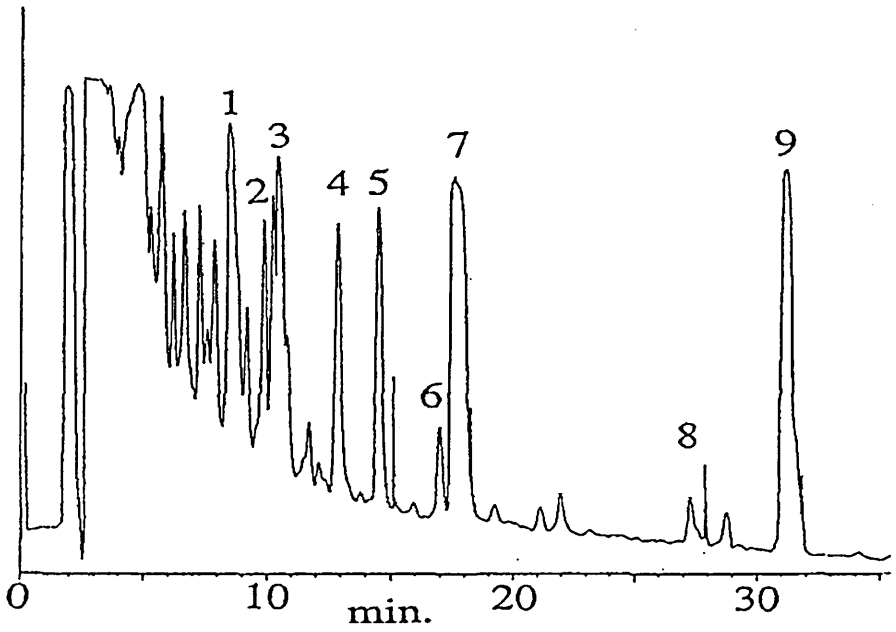



Fig. 2.10 *The UV absorbance (254nm) HPLC chromatogram of the extracted human urine containing the ibuprofen metabolites referred to in table 2.2*

select peaks subsequent to NMR analysis. This was particularly valuable in the case of very minor peaks e.g. metabolite 8, eluting at 27.2 min which would otherwise have been missed, while additionally showing which peaks were not glucuronides. In addition, for each glucuronide identified by the high cone voltage data, the $(M-H)^-$ could be determined from low cone voltage data. Such data enabled some peaks to be discounted as not drug related. The data did, however, highlight one limitation of the MS data in this context in that the sample contained three glucuronide metabolites, at 8.7, 9.9 and 13.3 min (metabolites 1, 2 and 4 respectively, table 2.2), which have the same molecular weight. It may, in some cases, be possible to predict isobaric metabolites from the product ion spectra, but this is not always so. Thus, the MS data may only be able to indicate the molecular mass of the compound and point to the fact that it contains a specific sub-group, in this case a glucuronide. As will be seen later

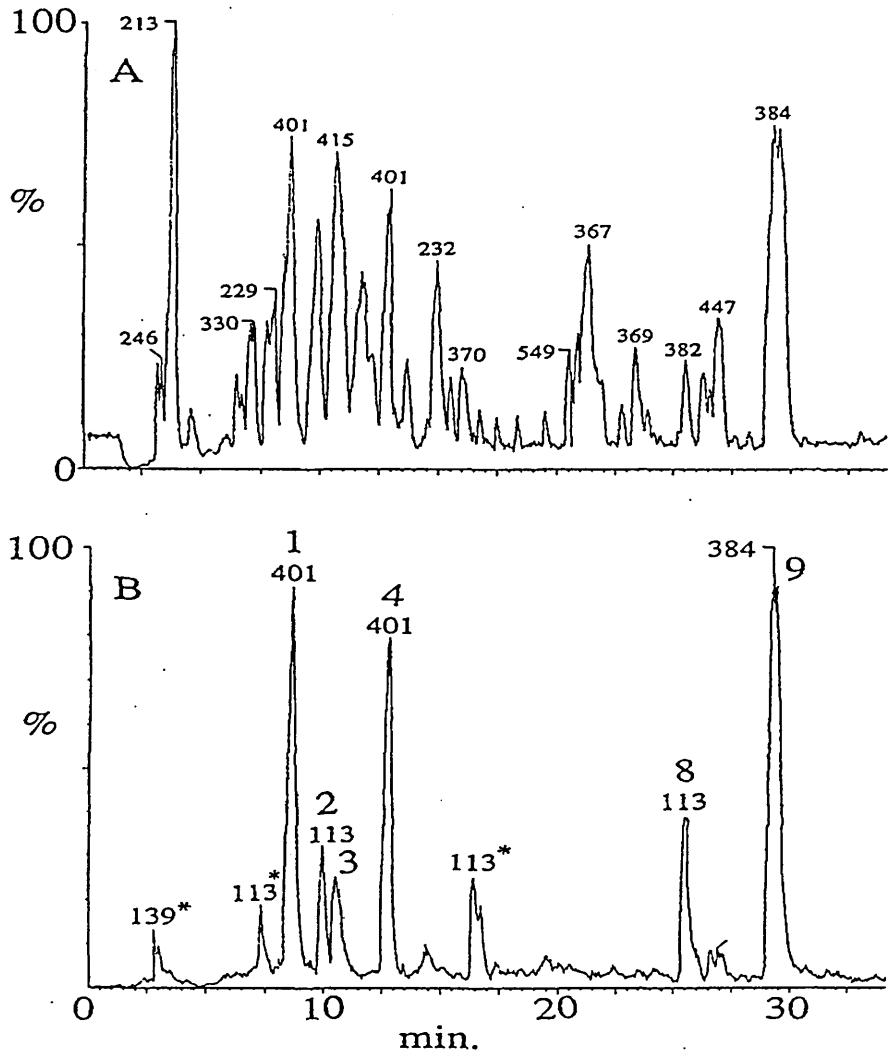



Fig. 2.11 *Mass chromatograms for the extracted urine. A:- the total ion chromatogram is highly complex showing all the compounds present including any endogenous materials. B:- the trace highlighting those compounds that showed a loss of glucoronic acid. The numbering is the same as for table 2.2 and the text.*

the use of a mass spectrometer capable of accurate mass measurement would have allowed for improved identification.

Under these circumstances the NMR spectrometer gave much more information about the structure of the metabolites than MS. For example the NMR spectra of

metabolites 1,2 and 4 showed signals typical of an ester glucuronide conjugate. The observation of a singlet methylene and singlet C-dimethyl peaks in metabolite 1, eluting at 8.7 min, indicated that the iso-butyl side chain had been hydroxylated at the β-carbon as shown in Fig. 2.12A). A quite different NMR spectrum was observed for the isomer peak at 13.3 min (metabolite 4) where the presence of a doublet at 4.23 ppm shows that hydroxylation has occurred at the α-carbon of the isobutyl side chain (Fig. 2.12B). Interestingly the introduction of a further unresolved chiral centre, as occurred in the case of metabolite 4 as a result of the introduction of a hydroxyl group into a chiral centre, created a pair of diastereoisomers. The individual diastereoisomers are no longer equivalent and thus can be distinguished from each other. Hence most NMR signals from metabolite 4 are duplicated. The spectrum also reveals that both diasteriosomers are present in roughly equal proportions (Fig. 2.12B). No indication of this could be obtained from MS data alone. Similarly the conjugation of ibuprofen and its metabolites to D-glucuronic acid would also be expected to produce pairs of diastereoisomers. In the case of metabolite 3, the glucuronide of the dicarboxylic acid metabolite of Ibuprofen, there is some evidence for the presence of a pair of diastereoisomers, but one of these predominates. The lack of duplication of the signals in the NMR spectrum obtained from metabolites 1 and 9 (Ibuprofen glucuronide itself) would imply that these were present largely as single diastereoisomers despite the dosing of racemic drug. This observation is consistent with the known metabolic chiral inversion of Ibuprofen *in vivo*.

On the basis of the stopped flow NMR data, 6 of the 9 Ibuprofen related compounds detected in this study were confirmed as ester glucuronides (compounds 1,2,3,4,8 and 9, respectively). In these metabolites the signal for the anomeric proton occurred at a

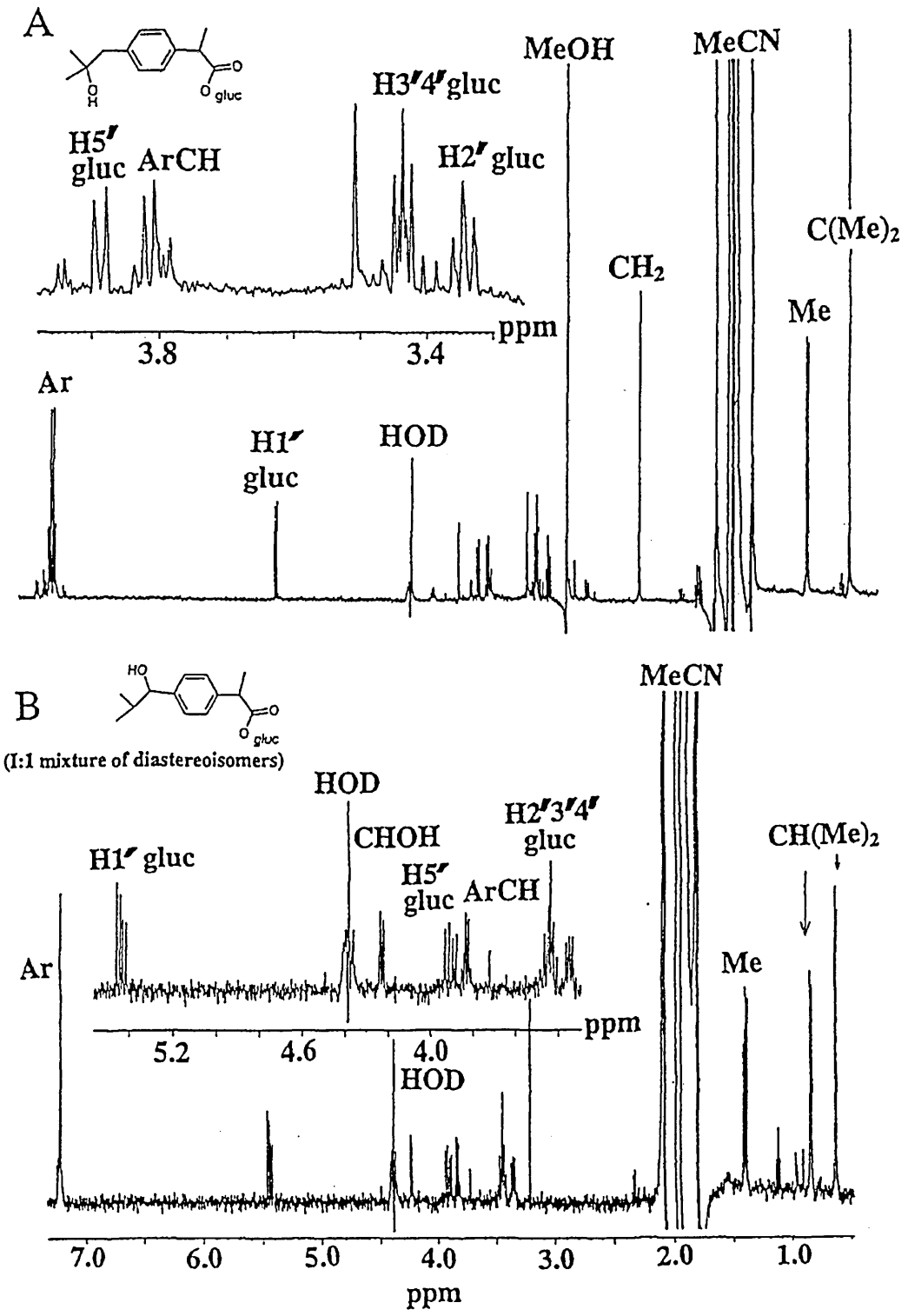



Fig. 2.12 A:- *The stopped flow $^1$H NMR spectrum of the ester glucuronide of the side chain β-hydroxylated metabolite of ibuprofen (metabolite 1) B:- The stopped flow $^1$H NMR spectrum of the α-hydroxylated metabolite of ibuprofen (metabolite 4) See table 2.2 and text for further details*

very similar chemical shift, accompanied by a diagnostic downfield shift of the α methine signal (see table 2.2). In addition to confirming the presence of glucuronic esters of these metabolites, the sites of hydroxylation on the iso-butyl side chain were identified at the α (metabolite 4), β(metabolites 1 and 5), and γ(metabolites 2 and 6) carbons as well as oxidation of one γ-methyl group to give the dicarboxylic acid (metabolites 3 and 7). A very minor peak in the chromatogram at 27.2 min (metabolite 8), highlighted by MS as a glucuronide 2 mass units down from ibuprofen glucuronide, was shown to be a novel side chain dehydration product, probably of the tertiary hydroxyl metabolite. The key feature of the NMR spectrum (Fig. 2.13) was the two broad singlets at 4.65 ppm and 4.72 ppm typical of a vinyl group and the downfield shift of the γ-methyl group to 1.56 ppm again consistent with a methyl group attached to an olefinic bond. Although this was an unlikely metabolite, and had probably been formed in the work up of the sample, it emphasises the strength of the combined techniques for without MS detection this minor chromatographic peak would not have been examined by NMR.

As these results show, by using the mass spectrometer with a high cone voltage it was possible to highlight specific classes of metabolite within a sample. In this case the glucuronides of ibuprofen and its various metabolites were detected and then analysed by NMR.

On a single quadrupole MS instrument this technique could only highlight metabolites which give rise to a consistent fragment for either part of the parent compound or a conjugate. In other metabolites where part of the molecule or the conjugate is eliminated as a neutral loss, a triple quadrupole neutral loss scan would be ideal to

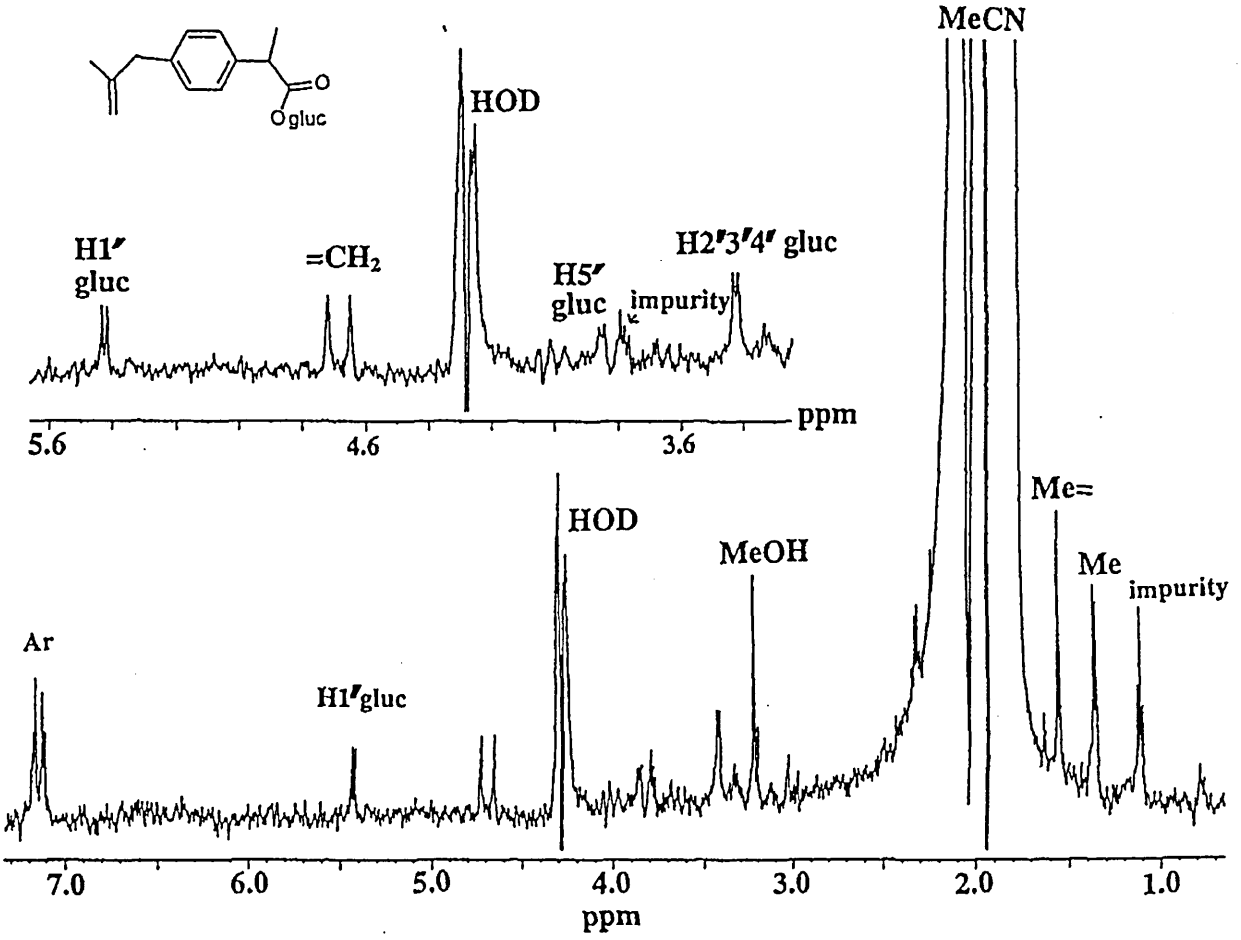



Fig. 2.13 *The stopped flow $^1$H NMR spectrum of the probable dehydration product (metabolite 8) of a hydroxyl-ibuprofen glucuronide metabolite (see table 2.2 for details). This metabolite was probably formed during the sample work up but illustrated how acquiring NMR and MS data simultaneously could identify otherwise unobservable compounds.*

look for unknowns (see sections 2.4 and 2.5). An automated data comparison of low and high voltage data for different mass losses would be almost as efficient and would allow for other neutral losses to be identified post acquisition.

Despite the success of MS at highlighting the chromatographic peaks resulting from the presence of glucuronides in the extract, it would be wrong to assume that it would

be sufficient to rely on MS data alone to direct the NMR spectral acquisitions. This is because, if the ionisation conditions were unsuitable, the mass spectrometer might not have detected compound-related peaks. In this case the mass spectrometer was extremely sensitive at detecting the conjugated metabolites identified, but this was not the case for the aglycones. Three compound related peaks (5,6 and 7), which were readily detected by UV and identified by their NMR spectra as hydroxy, and carboxy-metabolites of ibuprofen, were not observed as major components in the TIC. Plots of the appropriate single ion chromatograms (m/z 222 for peaks 5 and 6 and m/z 236 for peak 7) were able to show a peak at 17.04 min for peak 7 but no ion for peaks 5 and 6. Presumably the ionisation of the two mono carboxylic acids (5 and 6) was suppressed by either the formic acid or some other endogenous material present, whilst that of the glucuronides and the dicarboxylic acid were not.

## 2.4 LC-NMR-MS/MS for the identification of the known metabolites of *Hypericum perforatum* L extract

This section introduces the use of LC-NMR-MS/MS to identify known metabolites. Recent years have seen a resurgence in the interest in natural products in the search for pharmacologically active compounds. This interest has been focused mainly on plant extracts[36-38], but has also included marine organisms[39]. Plant extracts of *Hypericum perforatum* L are used in therapy as antidepressants. The major constituents of this extract have been known for many years, and structures have been elucidated by NMR spectroscopy. More recently LC-MS has been used to investigate this extract[40-42]. Some of the major constituents are given in Fig. 2.14

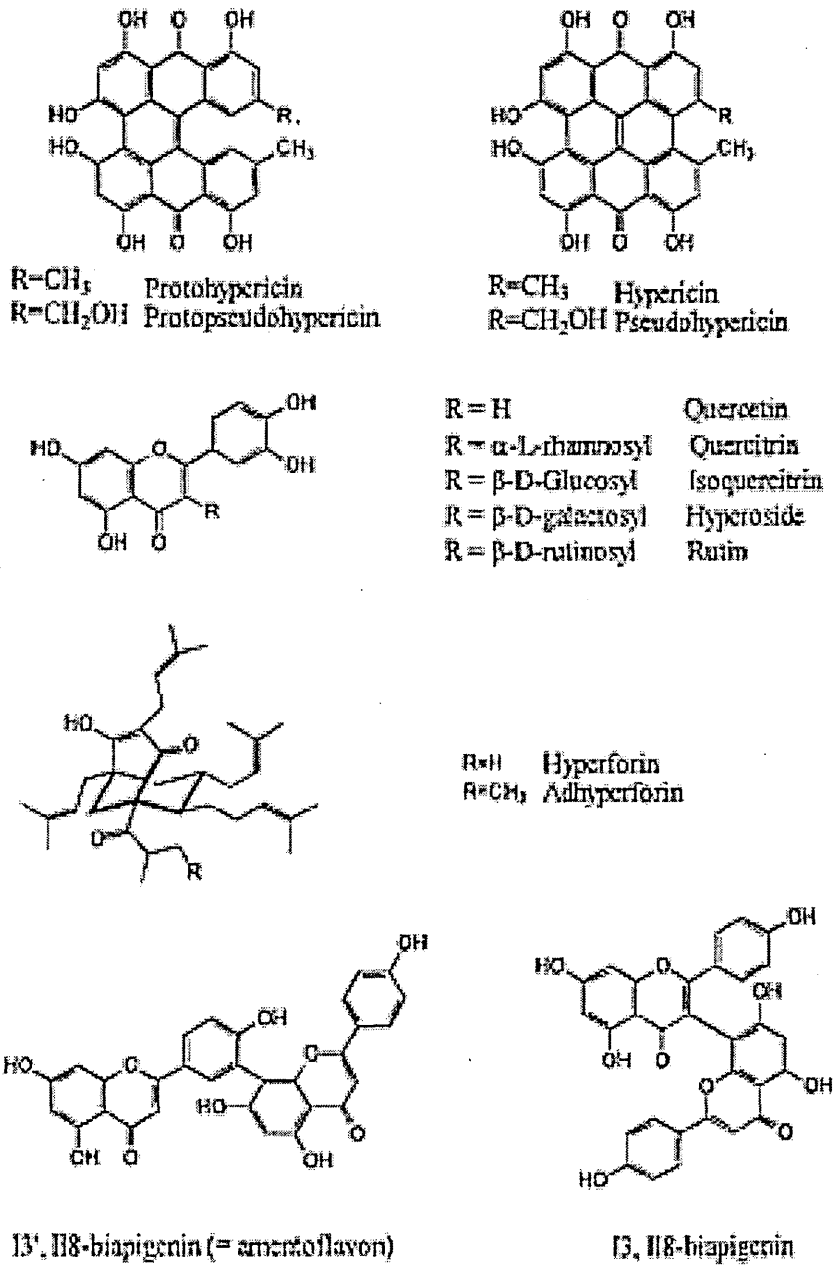



Fig. 2.14 *The structures of the major constituents of the extract of Hypericum perforatum L*

### 2.4.1 LC and NMR Equipment

The LC and NMR systems were unchanged from the earlier experiments in this chapter.

### 2.4.2 The Mass Spectrometer

Electrospray data were acquired using a Quattro LC (Micromass UK Ltd) utilising multiple cone voltages in the range 25-120V to generate molecular weight and fragmentation information simultaneously in MS only mode. The instrument was scanned from m/z 115 to 750, although in some instances a narrower mass range was also used to simplify the identification of components as they eluted in order to better direct NMR acquisitions. The source was held at 80C and the capillary at 3.4kV. Supplementary LC-MS and LC-MS/MS experiments using $H_2O$ and $D_2O$ were performed using a Finnigan (San Jose, CA) LCQ ion trap mass spectrometer. The electrospray interface of the LCQ was used in the negative ion mode with a sheath gas pressure of 50 instrument units and an auxiliary gas flow of 20 instrument units. A voltage of –4.25kV was applied to the electrospray needle. The temperature of the heated capillary was set to 250C and the voltage to –8V. A two-event scan was used. The first event was a full scan (m/z 200-1000). The second event was a dependent product ion scan (m/z 200-1000) of the most abundant ion from the first event. A collision energy of either 25 or 35V was used. Each scan event required 4.2s. Three microscans per full scan event and 5 microscans per dependent full product ion scan were performed.

### 2.4.3 Sample preparation

The plant extract of *Hypericum perforatum* L was a gift from SanoPharm A/S (Vedbaek, Denmark). 100 mg of the crude extract was dissolved in 1 mL of a mixture

of 80% *d*-methanol ($CD_3OD$) (Analar grade, Merck, Darmstadt, Germany) in $D_2O$ 99.9% Fluorochem), and 20 µL of this sample was injected onto the column.

To facilitate the identification of minor constituents, the extract was further fractionated and concentrated into a flavonoid fraction and a more non-polar fraction containing hypericin and other more lipophilic substances. This was done using the following procedure: 50 mg of crude extract dissolved in 75% methanol was applied to an OASIS (Waters, USA) 60 mg solid phase extraction cartridge which previously had been conditioned first with, 1.5 mL of methanol then with 1mL of water. The cartridge was eluted with 0.75 mL of 75% methanol giving a fraction consisting mainly of flavonoids. The cartridge was then eluted with 1.5 mL of a mixture of ethyl acetate + methanol + 25% ammonia (3:1:0.5 v/v/v). The total procedure could be repeated four times before the cartridge was discarded. The pooled flavonoid fractions from 1g of crude extract were evaporated to dryness at reduced pressure by rotary evaporation. The residue was dissolved in 250 µL of $CD_3OD + D_2O$ (1:1 v/v) before analysis by LC-NMR-MS. The pooled fractions of the lipophilic substances were treated similarly. 20 µL of these preparations were injected onto the HPLC system. This sample preparation made it possible to obtain NMR spectra of constituents at the 0.1% level by mass in the original extract.

### 2.4.4 Chromatographic conditions

A 120 x 4.6 mm i.d. Apex-1 ODS, 5 µm column (Jones Chromatography, UK) was used for the separation. Gradient elution was performed according to table 2.13. For LC-NMR-MS studies the diluted acetic acid and the ammonium acetate buffer were

| Time | % A | %B |
|---|---|---|
| 0 | 90 | 10 |
| 10 | 80 | 20 |
| 20 | 0 | 100 |
| 30 | 0 | 100 |
| 32 | 90 | 10 |

Table 2.3 *The gradient conditions used for the elution of the Hypericum perforatum L extract A = acetonitrile + 20 mM ammonium acetate (5:95 v/v) B = acetonitrile + 20 mM ammonium acetate (95:5 v/v) The total run time was 40 min.*

made up in $D_2O$. The flow rate was 1.0 mL/min. Additional LC-MS/MS experiments in which $D_2O$ was replaced by $H_2O$ in order to achieve knowledge of exchangeable protons in the molecules were performed using the same column but on a Finnigan LCQ mass spectrometer. Data were acquired in full scan mode with the capillary temperature at 200C, a capillary voltage of 25V and a spray voltage of 4kV.

### 2.4.5 Metabolite profiling

Fig. 2.15 shows the UV 254 nm absorbance chromatogram, and it is obvious that some peaks partly co-elute at a retention time of about 12 min. However, one of the advantages of NMR as well as MS was the possibility of individually measuring more than one substance at a time.

The present example also showed the advantage of the combination of NMR and MS as the partly co-eluting peaks (hyperoside and isoquercetin) at 11.7-12.0 min by MS showed the same m/z values for $(M-D)^-$ but very different NMR spectra of the sugar moieties, which make unambiguous identification possible.

Figs. 2.16 – 2.19 show examples of NMR and mass spectra of four of the substances (quercetin-galacturonide (tentatively), hyperoside, I3-II8-biapigenin, and hypericin) identified from the extract. In table 2.4 a list of all the constituents identified in this

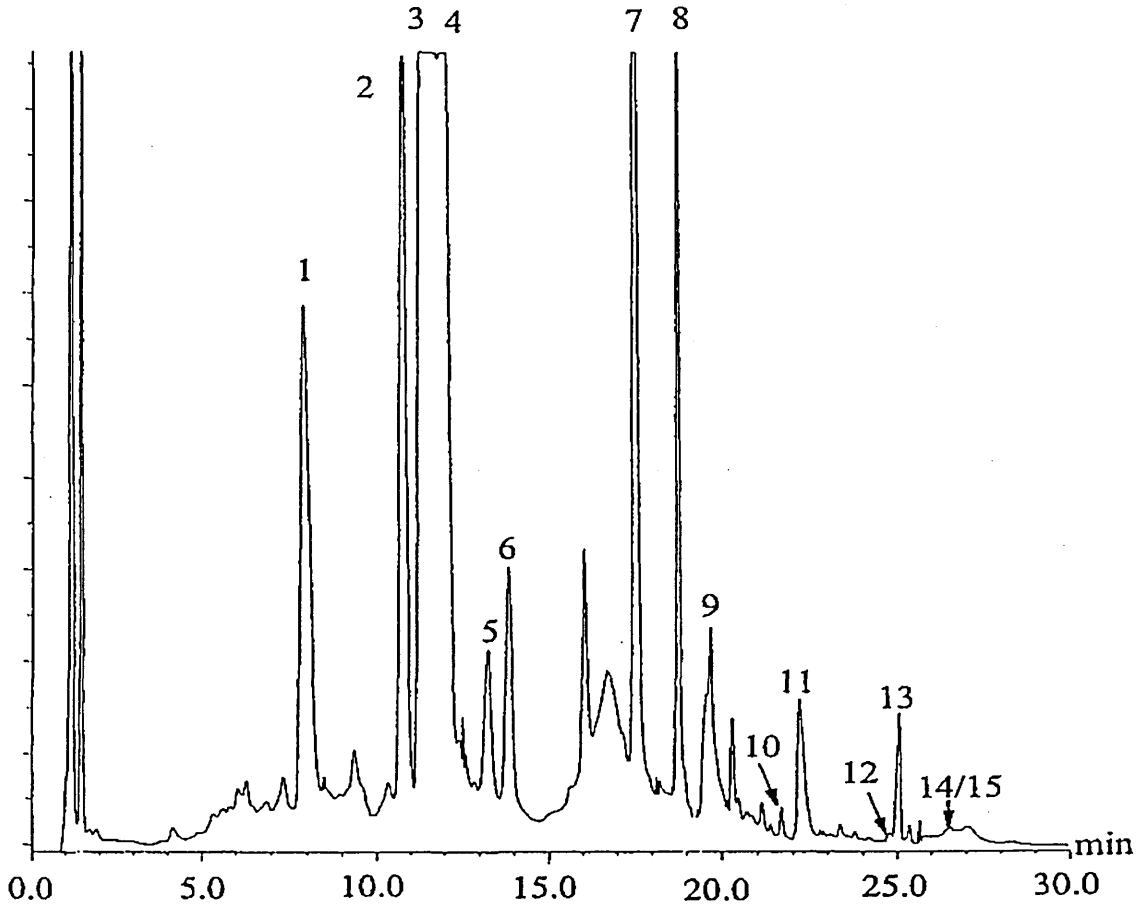



Fig. 2.15 *The UV chromatogram of the Hypericum perforatum L extract. The peak numbers correspond to the results shown in table 2.4*

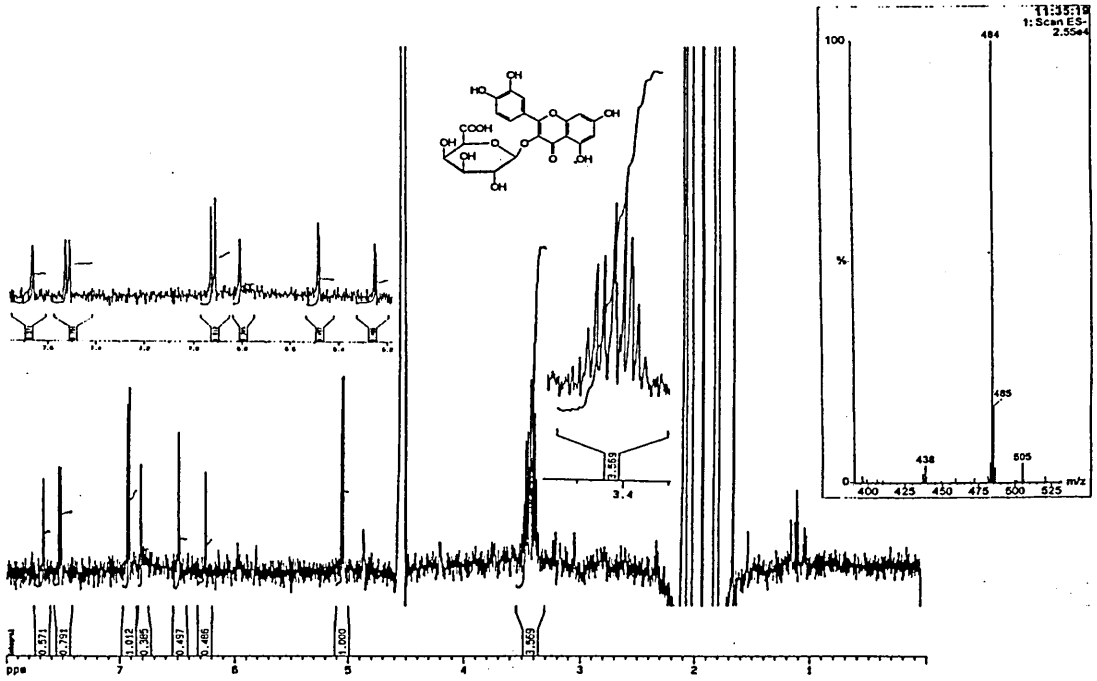


Fig. 2.16 *The NMR and mass spectra of peak 1 in Fig. 2.15 which was tentatively identified as quercetin-galacturonide.*

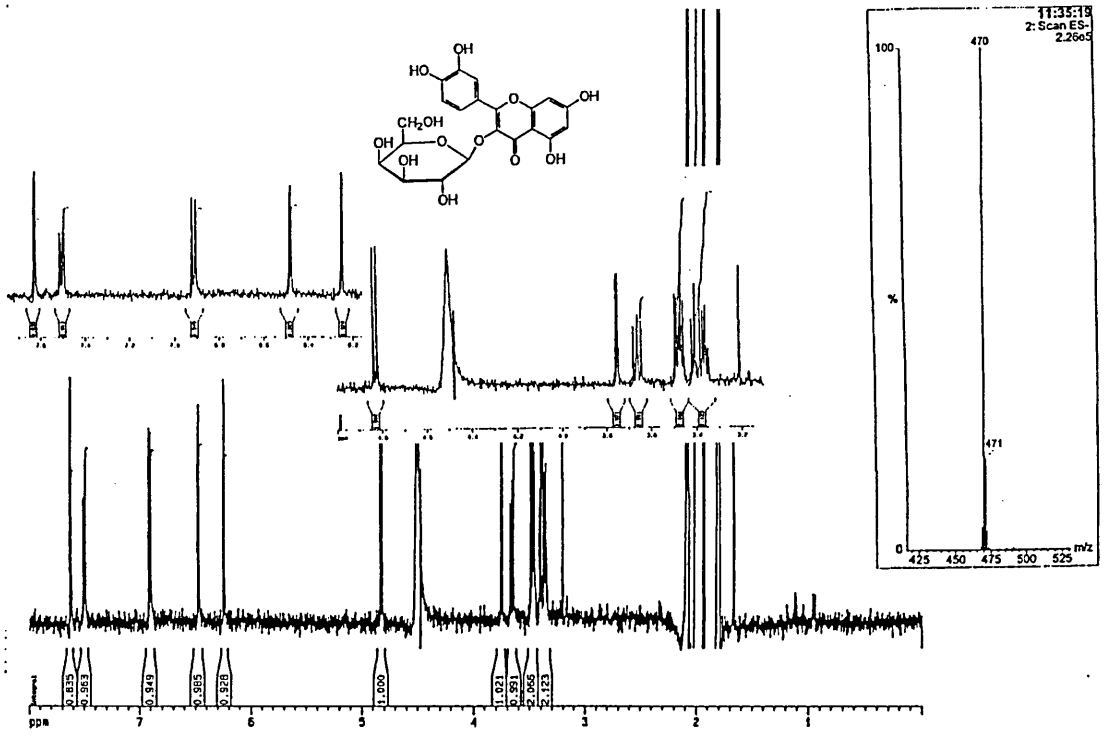


Fig. 2.17 *NMR and mass spectra of peak 3 (in Fig. 2.15) which was identified as hyperoside*

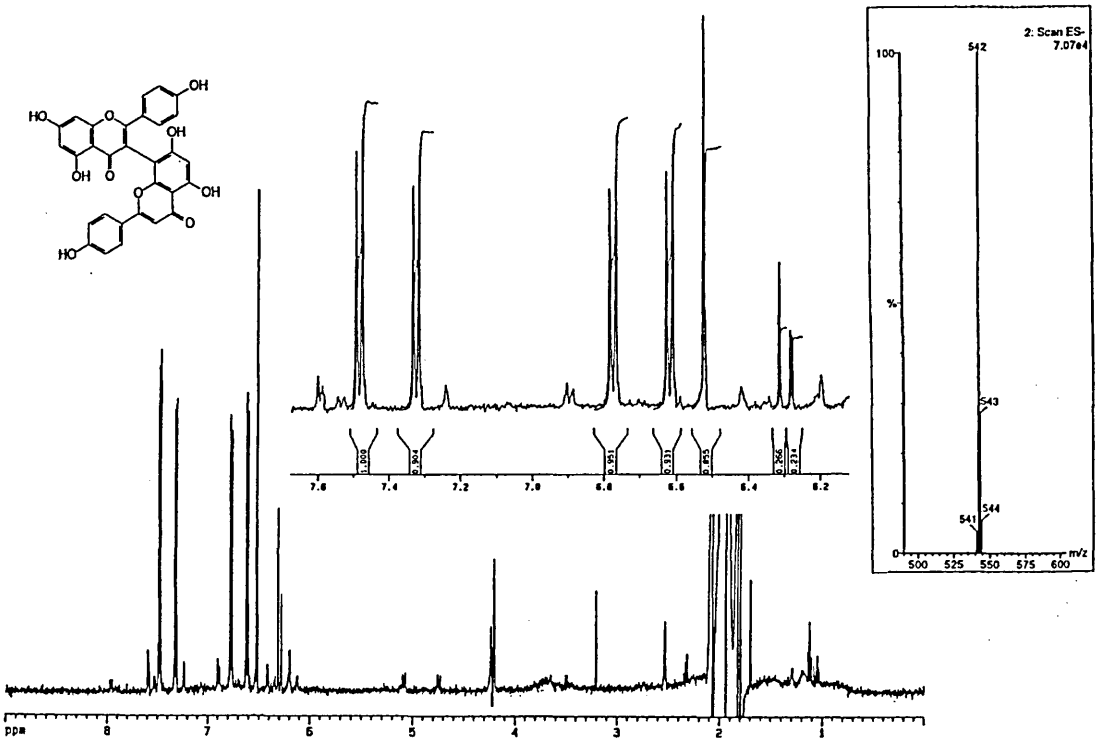



Fig. 2.18 *NMR and mass spectra of peak 8 (see Fig. 2.15) identified as I3-II8-biapigenin*

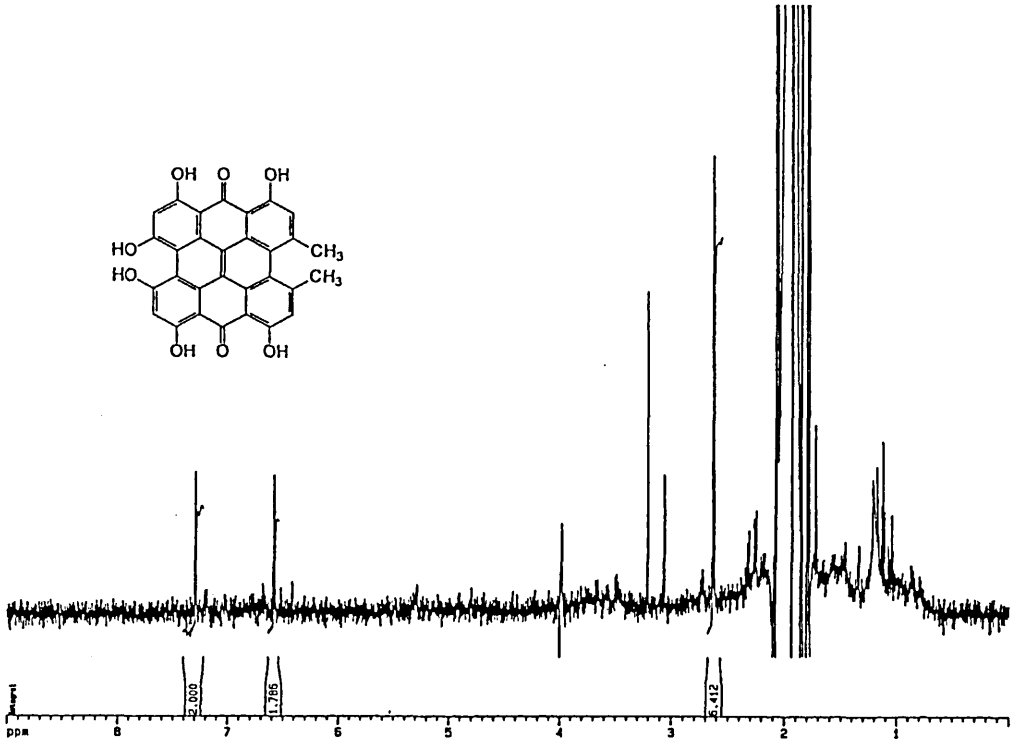



Fig. 2.19 *The NMR spectrum of peak 13 (see Fig. 2.15) identified as hypericin*

study is given. The retention times refer to the chromatogram in Fig. 2.15. This structure elucidation was based on the combined NMR and MS data. The presence of some known constituents at very low levels was verified by MS only. Further evidence was provided by the knowledge of the exchangeable protons achieved by the LC-MS/MS experiments performed in $H_2O$ and $D_2O$, respectively. In this case the number of exchangeables indicated the number of hydroxy groups present, these groups being NMR invisible. In Fig. 2.16 some additional signals are present. The signal at 6.8 ppm originated from an impurity, confirmed by a separate stopped flow experiment, stopped at a slightly different time, in a different chromatographic run. Two components detected here, quercetin-arabinoside and quercetin-galacturonide (tentatively), had not previously been described as extracts of *Hypericum perforatum* L. The tentative structure elucidation of the galacturonide was based on the following reasoning. The substance was much more polar than the other flavonoids and the NMR spectrum showed the characteristic signals for quercetin. The spectrum was analysed using the MIMER[43] NMR simulation program and the chemical shifts and (scalar) coupling constants are shown in table 2.4. The fact that there were only two large (aa type, 5-10 Hz) coupling constants proved that only the first three protons on the carbohydrate moiety were axial. The coupling constant from C(3')H to C(4')H was 1.9Hz corresponding to an axial-equatorial or equatorial-equatorial coupling constant. This corresponded well with the fact that an aromatic ether glucuronide would be expected to have a doublet (with a large coupling) at approximately 4.2 ppm[44]. Therefore the compound in question was not a glucuronide and was in fact likely to be a galacturonide. The number of exchangeable protons was consistent with this, and the major flavonoic constituent of the extract was hyperoside, so it would not be unexpected if a flavonoid containing a uronic acid as the sugar moiety turned out

to be quercetin-galacturonide. However, this was not proved conclusively. Fig. 2.18 shows the NMR spectrum of I3-II8-biapigenin. The signals near 6.3 ppm were assigned to the C(6)H protons of the A ring of the apigenin moieties. The integrals of these signals were smaller than expected as a result of exchange with deuterium from the $D_2O$ in the solvent.

| ber 3) | $m/z$ $(M-D)^-$ in eluent with $D_2O$ | | $m/z$ $(M-H)^-$ in eluents with $H_2O$ | | NMR signals[a] chemical shift in ppm (coupling constant in Hz) | substance |
|---|---|---|---|---|---|---|
| | MS | MS/MS | MS | MS/MS | | |
| | 484 | 305 | 477 | 301 | H2' 7.67(2), H6' 7.52(8.5, 2), H5' 6.92(8.5), H8 6.48(2), H6 6.25(2), H1" 5.05(8.0), H2" 3.47(8.0, 8.5), H4" 3.43(1.9, 1.0), H5" 3.41(1.0), H3" 3.38(8.5, 1.9) | quercetin-galacturonide |
| | 619 | 305 | 609 | 301 | H2' 7.57(2.2), H6' 7.52(8.5, 2.2), H5' 6.90(8.5), H8 6.48(2), H6 6.25(2), 4.89(7.8), 4.3(<2), 10 H 3.50–3.14, 3H 0.95(6.25) | quercetin-rutinoside rutin |
| | 470 | 305 | 463 | 301 | H2' 7.62(2), H6' 7.51(8.5, 2), H5' 6.91(8.5), H8 6.48(2), H6 6.25(2), H1" 4.83(7.9), H4" 3.75(3.3), H2" 3.66(10.0, 7.9), H3" 3.48(10.0, 3.3), H4" 3.47(11.9), H5" 3.41(6.5), H6"[a] 3.37(11.9, 6.5) | quercetin-galactoside hyperoside |
| | 470 | 305 | 463 | 301 | H2' 7.57(2), H6' 7.51(8.5, 2), H5' 6.91(8.5), H8 6.48(2), H6 6.25(2), 4.94(9.1), 6H 3.5–3.1 | quercetin-glucoside isoquercetrin |
| | 439 | 305 | 433 | 301 | H2' 7.63(2), H6' 7.52(8.6, 2), H5' 6.93(8.6), H8 6.49, H6 6.27, H1" 4.83(6.8), H4" 3.76(3), H2" 3.74(8.3, 7.1), H5"a 3.63(12.8, 1.5), H3" 3.53(8.5, 3.0) H5"[b] 3.32(12.8, 2.6) | quercetin-arabinoside |
| | 453 | 305 | 447 | 301 | H2' 7.30(2), H6' 7.27(8.6 2), H5' 6.95(8.6), H8 6.47(2), H6 6.25(2), H1" 5.17(3), H2" 4.12(3.2, 3), H3" 3.65(9.5, 3.2), H4" 3.22(9.8, 9.5), H5" 3.07(9.8, 6.3), H6" 3H 0.76(6.3) | quercetin-rhamnoside quercetrin |
| | 305 | | 301 | | H2' 7.61(2), H6' 7.56(8.6, 2), H5' 6.91(8.6), H8 6.46(2), H6 6.21(2) | quercetin |
| | 542 | | 537 | | H2–H6 2H 7.49,[b] H3–H5 2H 6.77,[c] H2–H6 2H 7.23,[b] H3–H5 2H 6.62,[c] II H3 6.53, I H8 6.53(2), II H6 6.31, I H6 6.29(2) | I3–II8-biapigenin |
| | 542 | | 537 | | nd | I3'-II8-biapigenin amentoflavon |
| | 527 | | 521 | | nd | protopseudohypericin |
| | 525 | | 519 | | nd | pseudohypericin |
| | 510 | | 505 | | nd | protohypericin |
| | 508 | | 503 | | 7.28, 6.56, 3H 2.66 | hypericin |
| | 535 | | 535 | | | hyperforin |
| | 549 | | 549 | | nd | adhyperforin |

Table 2.4 *Breakdown of the NMR and MS data for the chromatogram of the Hypericum perforatum L extract. The peak numbers correspond to those in Fig. 2.15*

## 2.5 LC-$^{1}$H NMR-MS/MS and LC-$^{19}$F NMR-MS/MS for the identification of metabolites of 5-trifluromethylpyridone in hydroponically grown plants

In these experiments the metabolism of 5-trifluromethylpyridone (5-TFMP) in hydroponically grown maize plants was studied using a combination of LC-$^{1}$H NMR-MS/MS and LC-$^{19}$F NMR-MS/MS, to see whether 19F-NMR would also work in the multiply hyphenated system and if so would it offer any further advantages. Hydroponically grown plants offer several advantages over either soil grown plants or tissue culture[45]. The conditions are more easily controllable than for plants grown in soil and allow the introduction of the xenobiotic directly into the nutrient solution. Unlike tissue culture, the hydroponically grown plant is a whole plant and so gives results that are similar to those that are likely to occur in soil grown whole plants. 5-trifluro-methylpyridone (TFMP) was chosen for study as it is a simple substrate to examine the possible occurrence of *N*- versus *O*-glycosylation in plants.

### 2.5.1 Growth and dosing of maize plants

The plants (maize seeds Zeneca Agrochemicals, UK) were germinated for 2 days then placed into pots. After two weeks the plants were placed in hydroponic units and left to acclimatise for one week prior to dosing. Approximately 100 mg in water of 5-TFMP (Flurochem, UK) was added to each hydroponic unit.

### 2.5.2 Sample preparation

At 1, 3, 7, 14, 28 and 38 days after dosing the plants were harvested. Roots and

shoots were separated below the first leaf and the roots rinsed to remove excess nutrient solution. Samples were then placed in a freezer at –20C until required for use. To prepare samples the plants were snap frozen in liquid nitrogen and homogenised with a mortar and pestle while still frozen. The resultant sample was placed in a centrifuge tube with distilled water (volume dependent on sample size) and was centrifuged for 30-45 min. to separate out the solid matter, after which the supernatant was drawn off. Typically 300 μL was used for the LC-NMR-MS studies.

### 2.5.3 HPLC, NMR and MS systems

The equipment used and the layout were as described in section 2.4 for the study of the *Hypericum perforatum L* extract. The NMR spectrometer, as previously mentioned, was a dual $^{1}H/^{19}F$ probe and so no changes in the layout were required.

For $^{19}F$ continuous flow studies spectra were acquired at 470.5 MHz using 32k data points with 200 time increments (8 scans per increment). The acquisition time was 0.58s with a delay of 1s between pulses. Spectra were referenced to trifluoroethanol at -77 ppm.

The gradient used for the chromatography is indicated below

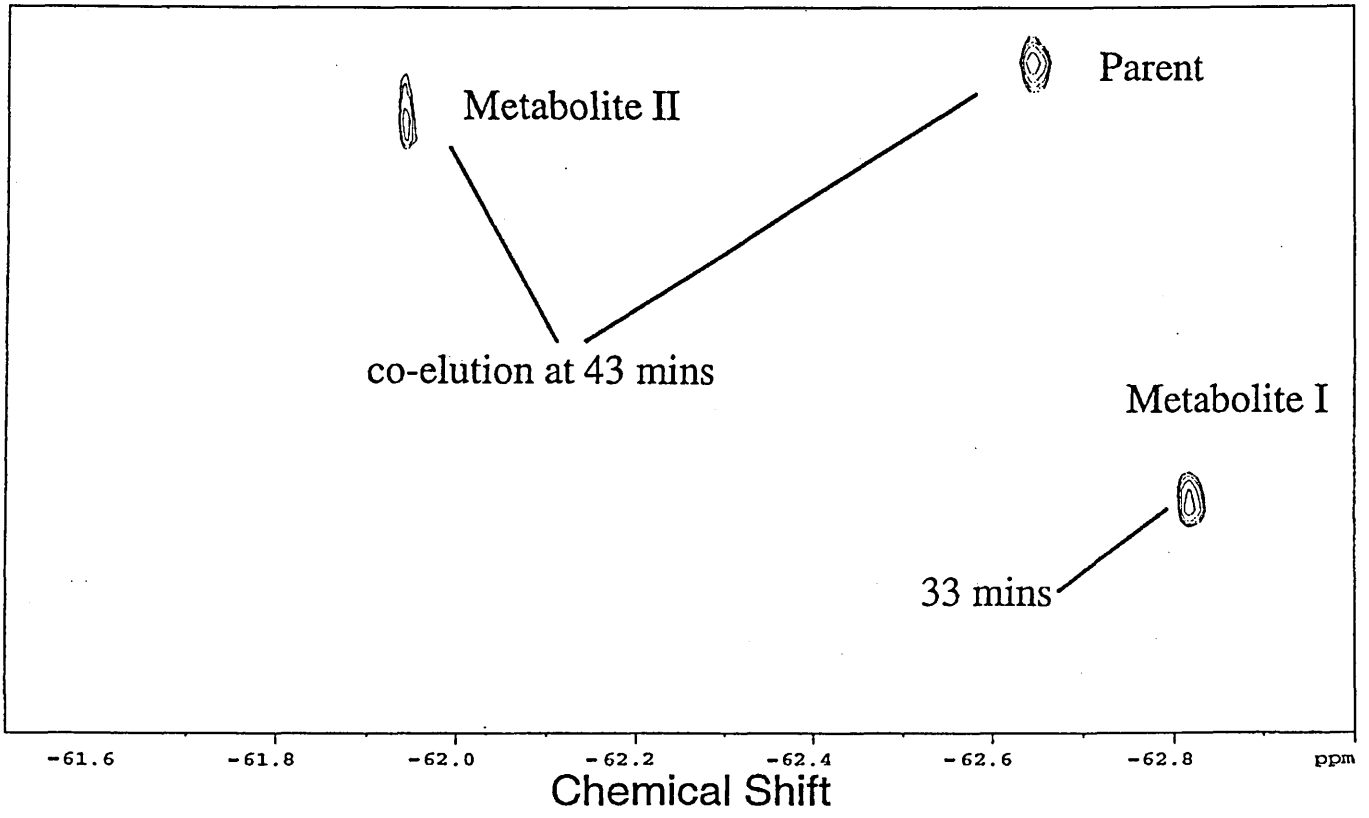
| Time | %A | %B |
|---|---|---|
| 0 | 100 | 0 |
| 20 | 98 | 2 |
| 60 | 80 | 20 |

where A = 0.01M pH 7.0 ammonium formate in $D_2O$ and B = acetonitrile. UV detection was at 254 nm and the flow rate was1.0 mL/min. The column used for these studies was a Hypersil BDS C18 250 x 4.6 mm i.d.

### 2.5.4 Metabolite profiling

The pseudo 2D LC-$^{19}$F NMR trace is shown in Fig. 2.20a. This trace indicated the retention times of the three most abundant compounds to be determined and correlated to the UV trace in Fig. 2.20b. Peaks of interest were identified at 33.9 min and (two co-eluting peaks) at ~ 43 min and confirmed by MS. Precursor ion MS/MS was employed to determine which peaks in the chromatogram contained the pyridone sub-structure by scanning for m/z 166 in positive ion mode and m/z 162 in negative ion mode. The resulting ion chromatograms indicated the presence of three major compounds at the retention times above.

Stopped flow $^1$H NMR (Fig. 2.21a) of the peak at 33.9 min indicated a compound containing the pyridone moiety (singlet at 8.1 ppm, doublets at 7.7 and 6.6 ppm), a conjugate containing an anomeric signal at 5.9 ppm, and signals consistent with a sugar between 3.4 and 3.8 ppm. Integration of the 3.4-3.8 ppm region indicated the presence of six protons, consistent with a glucose moiety, and the coupling constant of 8.8 Hz was consistent with a β-isomer. Overall, this spectrum was consistent with an N- or O-glucoside. MS data (Fig. 2.21b) supported this postulate. The number of exchangeable protons, calculated from repeating the MS experiments using protonated rather than deuterated solvents, provided further evidence for the structures of the metabolites, i.e. the product ion spectrum of the m/z 331 species



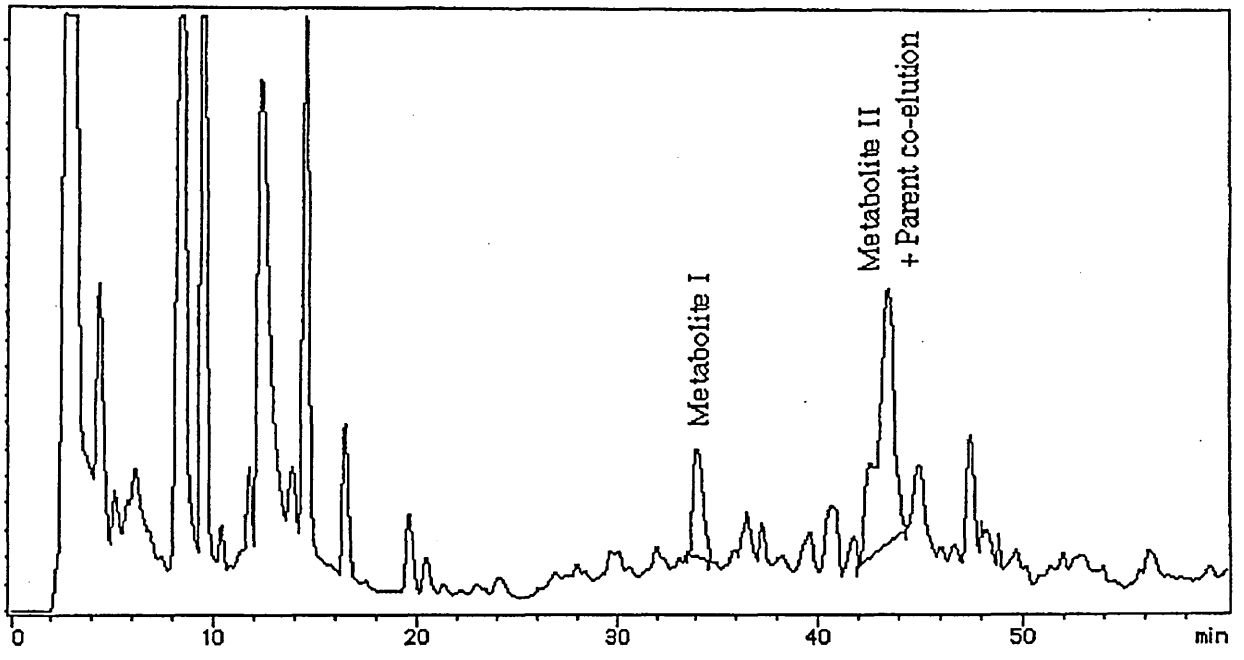




Fig. 2.20 a) *the on-flow 470.5 MHz $^{19}F$ NMR pseudo 2D chromatogram for the separation of the plant extract* b) *the UV chromatogram for the separation of the plant extract. Metabolite peaks are indicated at 33.9 and 43.3 min, the latter co-eluting with the parent compound.*

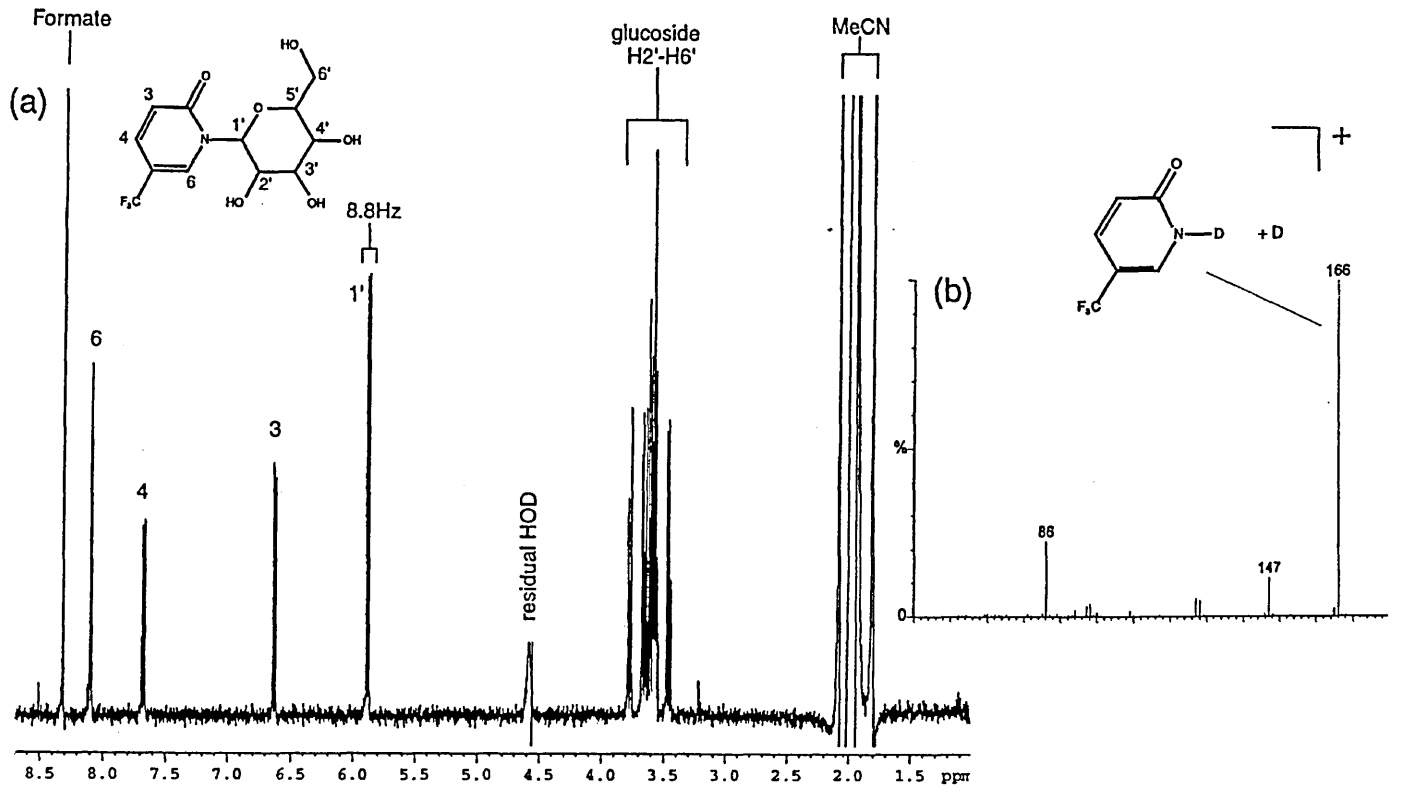



Fig. 2.21 a) *the stopped flow 500 MHz $^1H$ NMR of metabolite I and* b) *the corresponding mass spectrum taken from the peak at 33.9 min*

showed the characteristic loss of the glucose moiety to leave the deuterated pyridone at m/z 166 (the pyridone fragment has a m/z of 163; exchanging the NH for ND results in m/z 164, and then adding $D^+$ instead of $H^+$ gave an m/z of 166). Repeating with non- deuterated solvents gave an ion at m/z 326 a difference of 5 indicating 4 exchangeable protons plus the replacement of the protonating proton with a deuterium ion. This corresponds to the replacement of the hydroxyl protons of the glucoside conjugate with deuteriums.

By stopped flow $^1H$ NMR (Fig. 2.22a) it can be seen that there are two compounds present in the peak at 43 min. The major component was readily identified as the parent (labelled P in the Fig.), with the remaining signals suggestive of a sugar

conjugate, with an anomeric proton at 5.6 ppm and other signals between 3.0 and 4.4 ppm. The difference between this NMR spectrum and the one for metabolite I was an extra singlet at 3.1 ppm. This was consistent with an isolated methylene group such as in a malonyl structure, metabolite II. Further confirmation of this structure was provided by the downfield shift of ~ 0.7 ppm of the glucose methylene signal, H6', indicative of ester formation on the primary alcohol group.

Full scan MS data showed the major ions to be m/z 166 in positive ion mode and 162 in negative ion mode indicating the presence of the pyridone fragment. However, the precursor ion experiment indicated the presence of a further component of MW 415 (Fig. 2.22b), consistent with the fully deuterated *O*- or *N*- linked malonylglucoside conjugate of the parent pyridone. The product ion spectra of m/z 417 (positive ion) and m/z 413 (negative ion) typically show that the only major fragmentation is the loss of the malonylglucose unit. The non-deuterated mass spectrum (not shown) provided confirmation of this by the presence of an ion at m/z of 412. By comparing the chemical shifts of the aromatic protons, it can be determined that the glucoside conjugate is in the *N*-form, whereas the malonylglucoside is in the *O*-form.

| Compound | H3 | H4 | H6 |
|---|---|---|---|
| Parent | 6.6 | 7.7 | 7.9 |
| Metabolite I | 6.6 | 7.7 | 8.1 |
| Metabolite II | 7.0 | 8.0 | 8.4 |

Table 2.5 *A comparison of the important $^{1}H$ NMR chemical shifts for the parent compound and the two metabolites.*

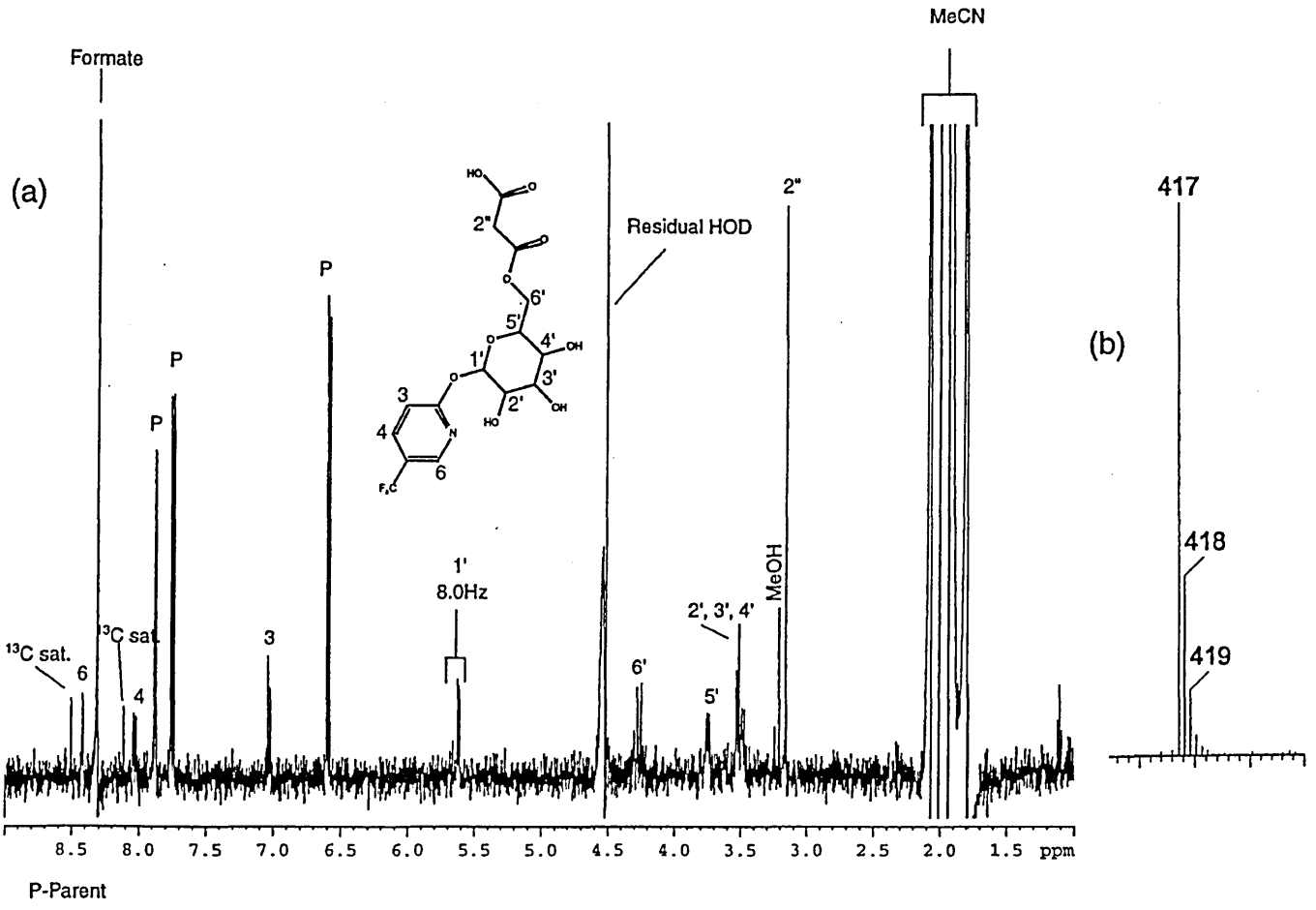
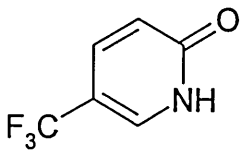
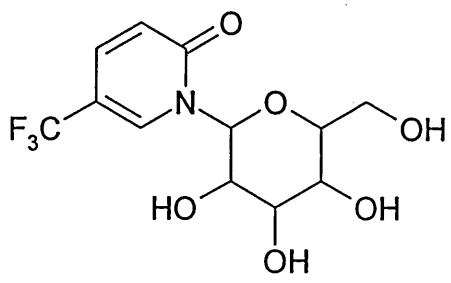



Fig. 2.22 a) *the stopped flow 500 MHz $^1$H NMR for the peak at ~ 43 min. The parent signals are marked P.* b) *the mass spectrum for metabolite II found in the peak at ~43 min*

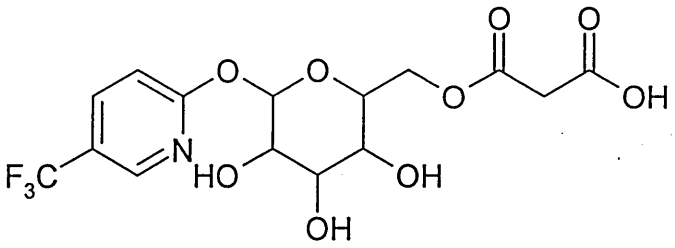
By comparing the chemical shifts of the parent, metabolite I and metabolite II (see table 2.5), it can be seen that the chemical shifts of metabolite I are more similar to those of the parent. It is well established that, in aqueous solution, the lactam/lactim equilibrium lies well over to the lactam side[46]. It follows therefore that metabolite I is the lactam form and metabolite II the lactim. The structures of the parent and the two confirmed metabolites are shown below.




**Parent**




**Metabolite I**




**Metabolite II**

## 2.6 Summary

- The feasibility of LC-NMR-MS was established and a working system established which accommodated several different mass spectrometers.
- The mobile phase limitations were established showing that a compromise between the ideal requirements for NMR and MS is required.
- A set of test compounds was established to further validate other modifications.
- It was shown that "real" problems could be addressed and solved by utilising this combined approach.

# CHAPTER 3

# THE USE OF SUPERHEATED WATER AS AN ALTERNATIVE ELUENT IN HYPHENATED SYSTEMS

## 3.1 Aims and Introduction

**The aims of the work described in this chapter were:**

1 **To investigate the feasibility of superheated water as an eluent in LC-NMR-MS studies by the analysis of some test compounds.**

2 **To assess any possible advantages or disadvantages of superheated water when used as an eluent in NMR and MS studies.**

Superheated deuterated water was used as the eluent for reversed phase chromatography with on line NMR and NMR-MS detection. A simple separation of model drugs was used to demonstrate the advantages and disadvantages of substituting organic additives with superheated water. Caffeine and a number of analgesics were separated on a polymer based column in isothermal and temperature programmed modes. Both one and two dimensional NMR spectra were obtained showing less interference than with conventional organic eluents. Unlike supercritical fluid chromatography-NMR (SFC-NMR)[47-52], the spectra could be obtained at room temperature and pressure. Further experiments show the inclusion of MS so as to simultaneously obtain NMR and mass spectra from a single HPLC run. Other

experiments show the separation of a series of sulphonamides which required the addition of a buffer and a temperature gradient to accomplish a satisfactory result.

Many of the practical problems of coupling HPLC to NMR or MS have been recently addressed[53,54] and although on line HPLC-NMR spectroscopy has become practical for routine applications in recent years[55-58], there are still complications arising from strong background signals for protons in the constituents of the mobile phase, which can overlap with resonances from the analyte. Additionally, even the purest conventional "HPLC grade" mobile phase constituents can also contain minor impurities, which contribute additional interfering signals. Although these interfering signals can be suppressed by the utilisation of a suitable pulse sequence, this can result in the loss of signals from the region of the spectrum and therefore potentially eliminating signals from analytes of interest. NMR or "spectroscopic grade" grade deuterated solvents with minimal interfering proton signals such as $D_3$ acetonitrile, can be used, but these are expensive (and can still contain interfering impurities). Alternatively proton free solvents such as supercritical carbon dioxide can be used but these often require the addition of a modifier for enhanced chromatography and in particular in this study to aid MS detection. Also, in supercritical carbon dioxide the spin lattice relaxation time is increased resulting in a longer sample acquisition time. The NMR flow cell must also be capable of operating at high pressures of up to 400 bar, and these must be maintainable even under stop flow conditions. It has also been reported that problems can be encountered when pressure gradients are employed due to changes in the chemical shift with eluent density. More importantly SFC-NMR is essentially a normal phase separation technique and is therefore more restricted in its application than reversed phase HPLC-NMR-MS. Unmodified supercritical carbon

dioxide is really only suitable for relatively nonpolar analytes, such as phthalate esters and vitamin A. This is particularly important in this project, which was aimed at aiding drug discovery and development in the pharmaceutical industry. Typically in the pharmaceutical industry the compounds are of a more polar nature and hence more amenable to separation by reversed phase chromatography than normal phase.

In a recent study, superheated water at temperatures from 100 to 220C and at pressures up to 50 bar was found to be an effective low polarity solvent and was initially employed for extraction[59,60]. Subsequently it has been used for the analysis of a range of analytes including phenols, amides,esters and barbiturates[61-64]. Under the conditions described above little hydrolysis or oxidation was observed, unlike supercritical water at 400C and 350 bar, which is known to be chemically aggressive. Superheated water chromatography has also been shown to be compatible with standard HPLC detectors (e.g. UV/diode array). The absence of any organic modifier means that it can also be used with a flame ionisation detector[59,63,65,66], thus providing a potential universal detector. This is extremely important when conducting impurity or metabolite analysis in order to both detect and accurately quantify all of the components present. Currently a host of different detectors are used to perform these analyses e.g. UV, chemiluminescence nitrogen detectors (CLND), light scattering detectors etc. Clearly the potential to use a single detector would therefore be of great interest and importance. The eluent strength can still be increased with superheated water by raising the temperature; thus it is possible to carry out gradient elution. This will be demonstrated by the description of the separation later in this chapter of a series of sulphonamides[67].

As the properties of deuterated water are very similar to those of normal protonated water it was considered worthwhile to test it as a mobile phase as it should in particular make an excellent eluent for NMR purposes, with potentially a zero background, though in reality this is not usually the case with trace amounts of protonated water being present. Compared with deuterated organic solvents it is comparatively cheap and is available in a high state of purity with significantly no organic impurities. The principal impurity as stated is non-deuterated water (~0.1%) and the signals for this can easily be suppressed using conventional NMR pulse techniques. Unlike supercritical fluids, only low pressures are required, and because water is virtually incompressible, small changes in pressure should have no effect on the chromatography or spectroscopy. It has been previously shown that superheated deuterated water can be used as a mobile phase for the HPLC-NMR separation of barbiturates in both on line and stop flow modes of detection[68].

## 3.2 Experimental Conditions

### 3.2.1 Chemicals and Samples

The work presented here involved the examination of a number of model drugs (analgesics and caffeine) with superheated $D_2O$ as the eluent to demonstrate that both 1D and 2D spectra could be obtained from the NMR. Furthermore, if successful, it was aimed to couple this to a mass spectrometer in order to obtain mass spectra from the same chromatographic run.

Salicylamide, salycilic acid, acetylsalycilic acid, phenacetin, paracetamol and caffeine were all obtained from Sigma (Poole UK). $D_2O$ (99.9%) was obtained from

Fluorochem (Glossop UK). Acetonitrile was HPLC grade obtained from Fisons (Loughborough UK).

Salycilamide was prepared at a concentration of approximately 10 mg/mL in acetonitrile. A mixture of the model drugs was prepared from 10 mg each of paracetamol, caffeine and phenacetin in 80:20 acetonitrile: $D_2O$ (1 mL). The phosphate buffer pH 3.0 (correctly pD as the buffer was dissolved in $D_2O$) mobile phase was prepared from $H_3PO_4$ and $KH_2PO_4$ at approximately 1-3 mM in $D_2O$ and then adjusted by adding a solution of concentrated $Na_3PO_4$ in $D_2O$.

### 3.2.2 Superheated water chromatograph

The superheated water chromatographic system (Fig. 3.1) consisted of a Shimadzu LC 10AD pump (Kyoto, Japan), which delivered $D_2O$ at 1.0 mL/min to the column through a preheating coil made of 1m x 0.01in. i.d. stainless steel tubing. The column and preheating coil were placed inside a gas chromatographic oven (Series 104 Pye Unicam, Cambridge UK), the temperature of which was controlled by a programmer/controller (Series 104 Pye Unicam). The samples were injected at room temperature using a Rheodyne 7125 valve (Cotati, CA) fitted with a 20 μL loop that was mounted outside the oven. The analytes were separated on either a PLRPS (PS-DVB) (150 x 4.6 mm, Polymer Labs Church Stretton, UK) or a 5 μm NovaPak $C_{18}$ (150 x 4 mm, Waters, Milford, MA) column. A set of copper cooling fins (3 cm x 12 cm x 0.05mm) was attached to the tubing exiting the column and connecting the column to a Jasco UV detector (model 870, Tokyo, Japan) operating at 254 nm. The pressure at the outlet of the column was held high enough to keep the eluent above the boiling point of the water by either a Jasco back pressure regulator (model 880/81)

that was set at 20 bar or a restrictor coil of PEEK tubing (3m x 0.13mm id.). The chromatograms were collected on a Hewlett Packard model 3396a integrator.

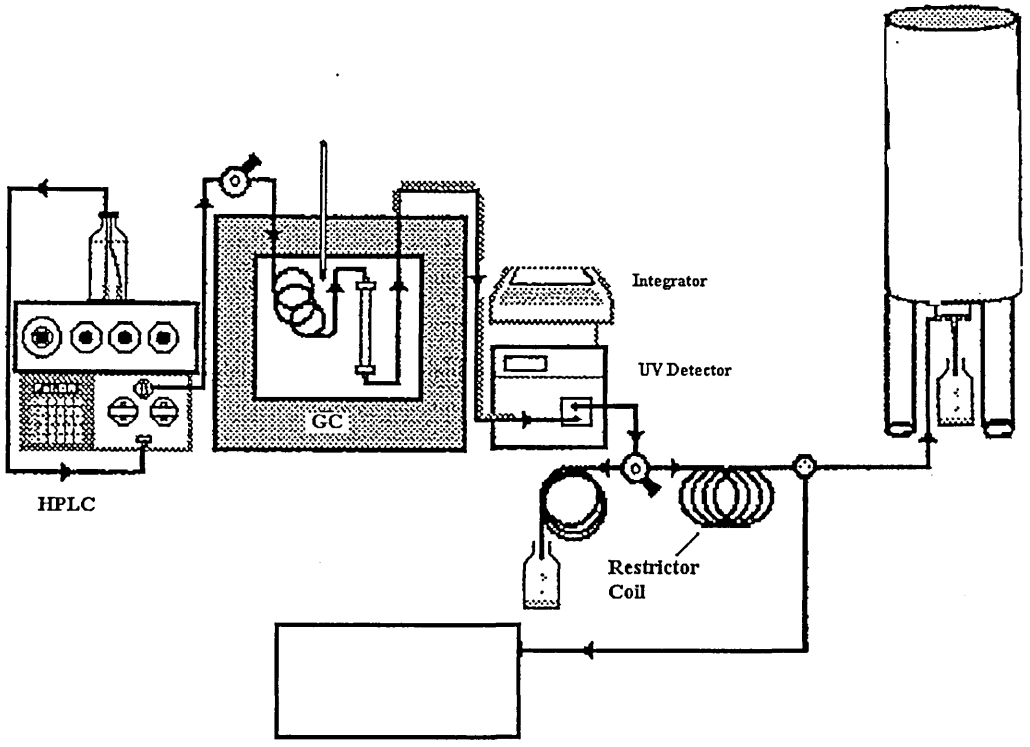



Fig. 3.1 *Schematic of the system used for superheated water separation of model drugs and the recording of NMR and mass spectra.*

### 3.2.3 LC-NMR and LC-NMR-MS

For the NMR and NMR-MS studies an additional Rheodyne 7125 injection valve was introduced after the UV detector so the flow could be directed either to the back-pressure regulator or the coil of PEEK, or alternatively via a 3m x 0.13mm length of PEEK tubing to the flow cell of the NMR spectrometer. For stop flow experiments, free induction decays (FIDs) were collected over a spectral width of 8278 Hz into 16384 data points with an acquisition time of 0.99s using the NOESYPRESAT pulse sequence (Bruker). The residual water resonance was suppressed using pre-

irradiation during the relaxation delay of 2.0s and the mixing period of 0.10 s. The delay time between the UV and the NMR peak was approximately 33s.

For the two dimensional correlation spectroscopy (2D COSY) experiment, 1K data points were used in the F2 domain with the number of experiments set to 256 (TD1). The 90° pulses were employed over a sweep range of 4990 Hz in both dimensions with a relaxation delay of 1.5s. Data were zero filled in the F1 dimension and Fourier Transformed after application of a sinebell window function in both dimensions, followed by symmetrization about the diagonal.

For LC-NMR-MS studies a T-piece was placed in the tubing leading to the NMR spectrometer approx 30cm before the magnet. The flow was split through a second 3m x 0.13mm id length of PEEK tubing to a Quattro LC mass spectrometer (Micromass Ltd Altrincham, UK) fitted with a z spray source acquiring at high and low cone voltages in positive electrospray mode. The cone voltages were 25 and 60V, respectively. At 25V the mass range was scanned from m/z 80 to 450 over 1s with a 0.1s interscan delay. At 60V, the mass range was scanned from m/z 35 to 450 over 1s with the same interscan delay. The capillary voltage was 3.45kV, and the source block temperature was maintained at 80C. The desolvation temperature used was 150C. The nebuliser gas flow was 80 L/h and the desolvation gas flow 564 L/h.

### 3.3 Results

The configuration of the superheated water chromatograph employed in these experiments is shown in Fig. 3.1. A preheating coil was placed in the GC oven prior

to the column to ensure good temperature control. Conventional HPLC UV or fluorescence detectors could be used as the pressure required to maintain superheated water as a liquid was less than 50 bar (approx. 700psi) at 200C. The actual pressure employed was not critical as the water under these conditions was barely compressible and any changes in pressure had no effect on the retention times. The eluent was cooled prior to it reaching the UV detectors so as to minimise any refractive index fluctuations. The majority of the studies involving superheated water were conducted using polymer (polystyrene divinylbenzene PS-DVB) columns due to their greater thermal stability than standard ODS-bonded silica columns.

In an initial experiment carried out to determine suitable chromatographic conditions, salicylamide was examined on a PS-DVB column connected to on-line UV and fluorescence detectors with the back-pressure regulator set at 15 bar, which was sufficient to maintain the superheated water in the liquid state throughout the system. The salicylamide was readily eluted from the column at 180C in 5.22 min and was easily detected by the UV detector at 254nm and the fluorometer using an excitation wavelength of 300nm and a detection wavelength of 430nm. By the time the eluent reached the fluorometer, it was close to room temperature. Using similar conditions at 190C, it was possible to separate paracetamol (acetaminophen) (2.5min), caffeine (5.77min), and phenacetin (14.04min) within a reasonable time. However, aspirin (acetylsalicylic acid) was eluted rapidly as salicylic acid and represented the first compound then encountered to be hydrolysed during superheated water chromatography.

Having demonstrated that these model drugs could be separated by superheated water chromatography, the fluorometer was replaced and the connection made to the NMR instead. As with previous experiments the connection to the NMR spectrometer was via a 3m length of PEEK tubing. This acted both as a restrictor to keep the water liquid by creating sufficient back-pressure, and also allowed the eluent enough time to cool to room temperature before it reached the NMR flow cell. Thus, the spectra were measured at ambient temperature and pressure under conditions that were essentially independent of the separation conditions.

Using this system, with the column at 180C and 35 bar back-pressure, salicylamide (200μg) on a PS-DVB column was detected as a homogeneous solution on-flow by the NMR spectrometer with a retention time of 4.7min. The spectrum showed the expected characteristic aromatic ring proton signals with no significant interfering signals. In order to increase the sensitivity, by allowing stop flow experiments, a switching valve was fitted after the UV detector. This diverted the flow to a back-pressure coil or regulator so that when the flow to the NMR spectrometer was stopped, the superheated conditions were maintained in the column and elution would continue.

To stabilise the system for other analytes that were being examined, the mobile phase was modified to a pD 3.0 phosphate buffer in deuterium oxide. Previous reported studies have shown that the pH of buffered solutions is effectively unaltered at high temperatures[68,69]and at this pH, small changes would have an insignificant effect on the ionisation of the analytes studied. When 100μg of salicylamide was injected using this system, a spectrum showing the aromatic protons was readily obtained (the

exchangeable $NH_2$ protons were not observed). A small interfering signal from traces of water (HDO) in the mobile phase was suppressed by a pre-irradiation step during the relaxation delay. By minimising the overall NMR solvent signal using $D_2O$, and eliminating organic modifiers, it should be possible to achieve a higher signal for the analytes and hence a better signal to noise ratio.

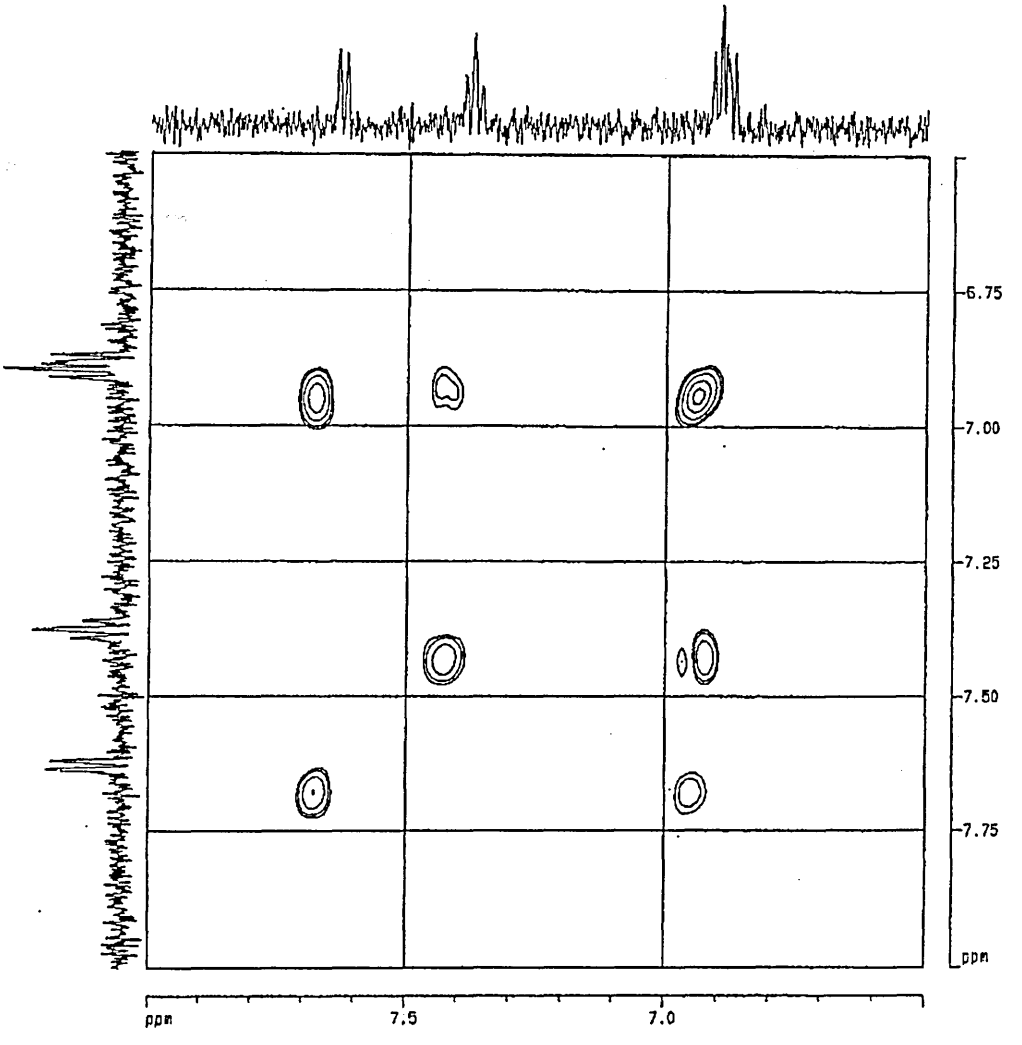



Fig. 3.2 *The COSY spectrum of salycilamide (100 μg) recorded off-line following superheated water separation at 190C on a 5μm PS-DVB column and with a deuterated phosphate buffer mobile phase.*

A 2D-COSY spectrum of the salicylamide sample was obtained (Fig. 3.2). All coupling correlations within the aromatic ring system were readily observed, such that a full assignment of these protons could be made. In contrast to SFC-NMR, there was no concern that a high pressure had to be maintained in the flow cell over a prolonged 2D measurement period, as after the superheated water separation the sample was at ambient pressure and temperature. In studies not included in this thesis, major problems in maintaining the pressure in the NMR flow cell during SFC experiments had been encountered, leading ultimately to the failure of the flow cell.

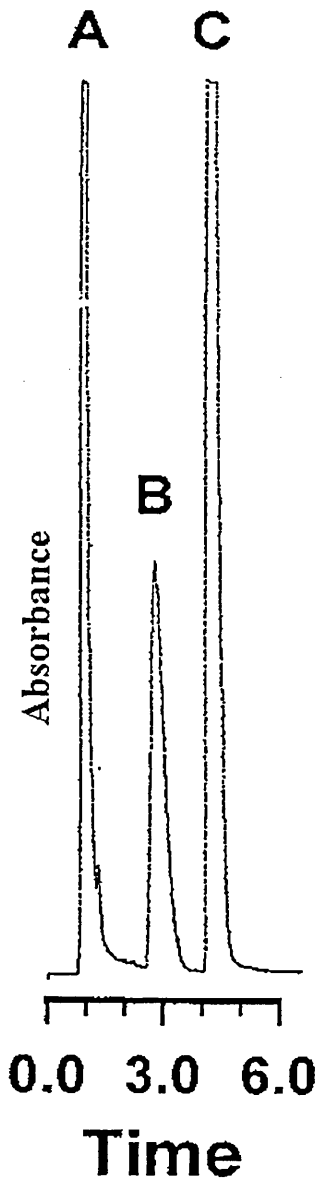



Fig. 3.3 *The superheated water (UV 254nm) chromatogram of paracetamol (A), cafeeine (B) and phenacetin (C)* 100 *μg each on column*

In further studies, a Novapak C18 column was used for the separation of some of the model compounds with an oven temperature program from 80 to 130C at 8C $min^{-1}$ to ensure the timely elution of the analytes (Fig. 3.3). This column gave reasonably shaped peaks for paracetamol (0.94min),

caffeine (3.06min), and phenacetin (4.58min). The change in oven temperature gave rise to an increase in the elution rate of the latter analytes, confirming that the column internal temperature was also increasing although there may be a small temperature hysteresis because of the thermal mass of the column. The NMR spectrum of each component could be measured by successive stop flow measurements (Fig. 3.4), confirming that the compounds had been separated without degradation on the column. Although this separation demonstrated successful separation on an "ordinary" ODS column, in subsequent runs the retention times of all of the components decreased indicating a degradation of the stationary phase. Similar problems had also been observed in other studies with a range of ODS-bonded silica materials, and the application of these phases seemed to be limited by their lability under these conditions.

### 3.3.1 LC-NMR-MS Detection

To demonstrate the suitability of superheated water as an eluent for this instrumental combination, preliminary experiments were again conducted using salicylamide. The superheated water chromatograph remained directly linked to the NMR spectrometer as previously but the flow was split a short distance before the NMR flow cell. As previously approximately 95% of the sample was directed to the NMR spectrometer and the remaining 5% to the mass spectrometer through a 3m length of PEEK tubing. This enabled the same sample to be examined by both NMR and mass spectrometry, in parallel, from a single injection as previously. However, on this occasion, the sample reached the

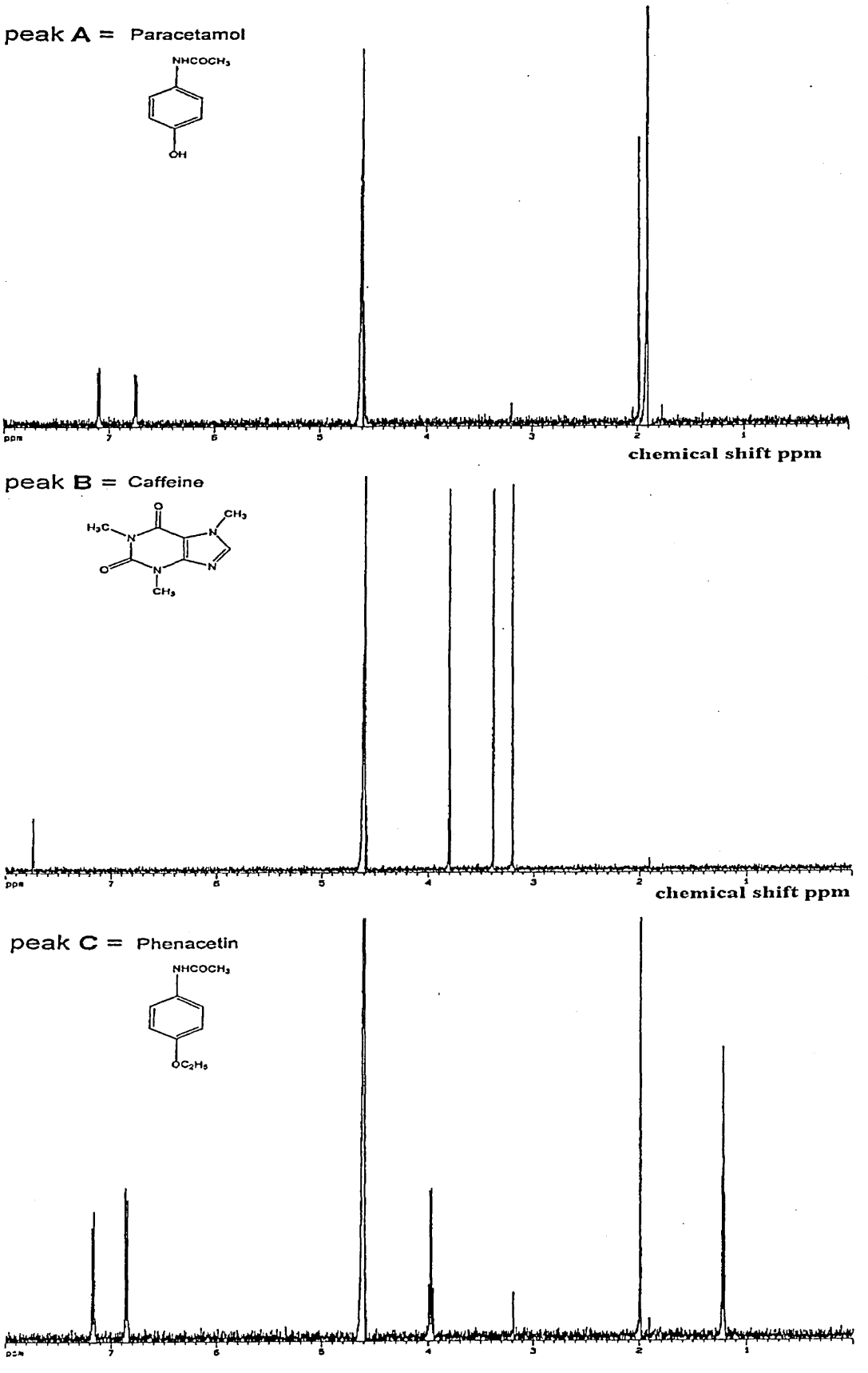



Fig. 3.4 *The 1D NMR spectra obtained in the stop flow mode for the three peaks in Fig. 3.3. A= paracetamol, B= caffeine and C= phenacetin*

NMR spectrometer before the mass spectrometer. Thus when the flow was stopped for stop flow experiments the sample was held in the PEEK tubing short of the mass spectrometer. Only once the stopped flow experiment was concluded and the flow re-established was the sample eluted into the mass spectrometer. When salicylamide was examined on this system using a PS-DVB column and a buffered eluent (pD3.0 potassium/ sodium phosphate) $^1$H NMR and mass spectra were readily obtained. The NMR spectrum was identical to that previously obtained while the mass spectrum (Fig. 3.5) showed the ions $[M-2H+3D]^+$ and the adduct ions $[M-2H+2D+Na]^+$ and $[M-2H+2D+K]^+$ at m/z 142, 163 and 179, respectively.

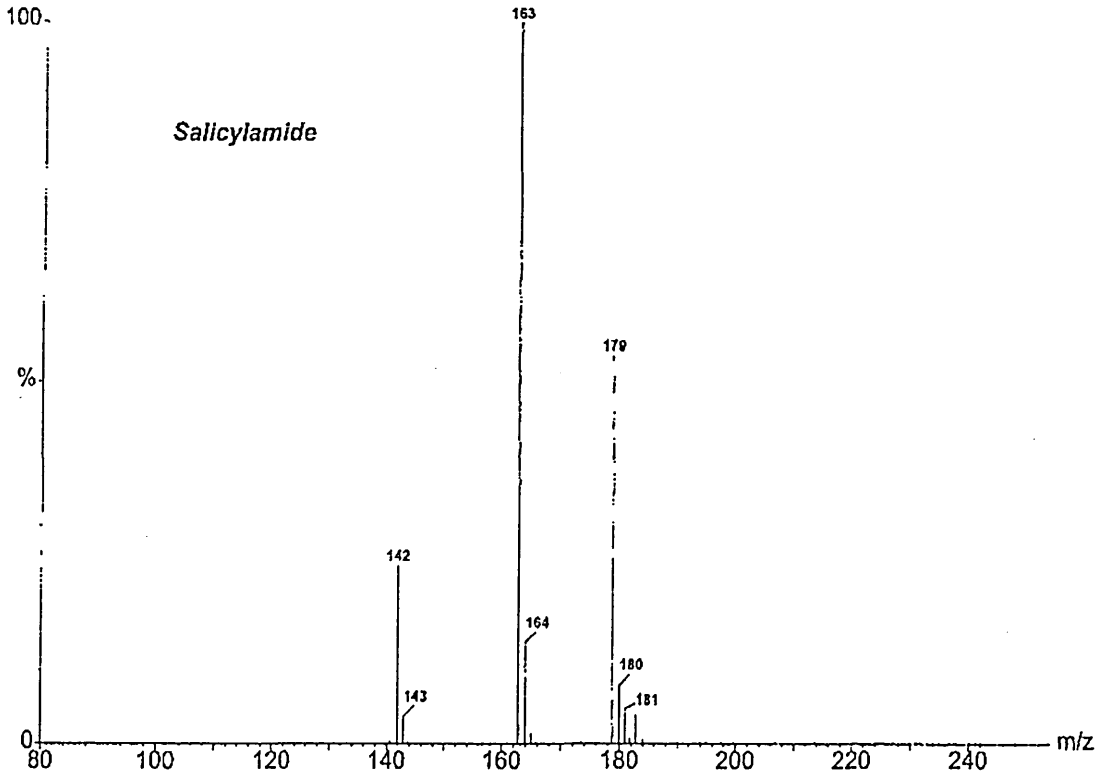



Fig. 3.5 *The mass spectrum of salicylamide obtained following elution with deuterated $KH_2PO_4$/ $NaH_2PO_4$ buffer.*

### 3.4 The Analysis of a series of Sulfonamides

Using the same conditions as above a series of sulfonamides were investigated. Initially this was planned as an extension of the previous experiments to further demonstrate the usefulness of superheated water as an alternative to conventional reversed phase HPLC analyses. However, when the resultant separated peaks were examined by NMR and mass spectroscopy an unexpected but highly efficient and selective deuterium exchange reaction appeared to have taken place.

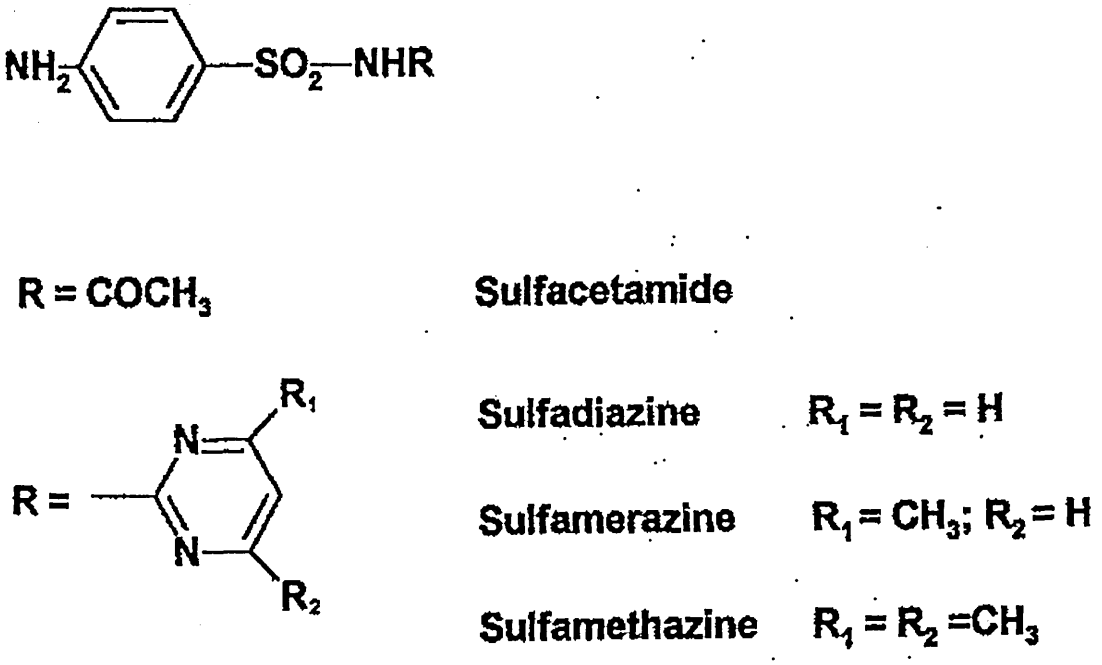



Fig. 3.6 *The structures of the four sulfonamides investigated by superheated water chromatography.*

#### 3.4.1 Results

A mixture of sulfacetamide, sulfadiazine, sulfamerazine and sulfamethazine (see Fig.

3.6 for the structures) was examined on a PS-DVB column using the buffered deuterated eluent described above, with a temperature program from 160 to 200C at 2C $min^{-1}$. The eluent was examined by stopped flow NMR and mass spectrometry. The four components were eluted in the order of decreasing polarity as would be expected (Fig. 3.7).

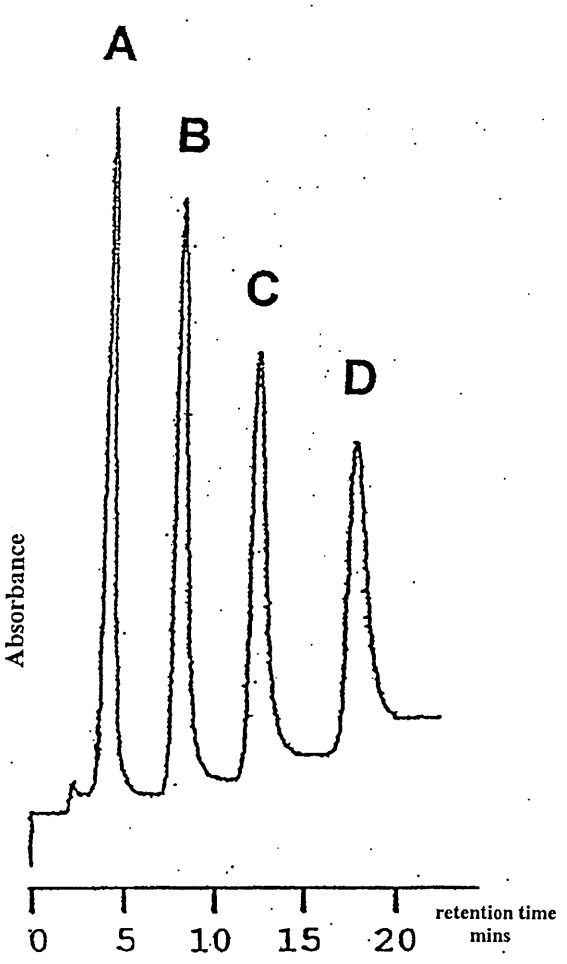



Fig. 3.7 *The UV chromatogram for the separation of the sulphonamide mixture A= sulfacetamide, B= sulfadiazine, C= sulfamerazine and D= sulfamethazine*

The NMR spectrum for sulfacetamide (Fig. 3.8A) was as expected, with a clear AA'BB' aromatic pattern and an acetyl methyl group (the second methyl signal was

identified as traces of residual methanol in the probe from earlier conventional LC-NMR studies). The labile amino and amido protons had exchanged as expected for all the sulfonamides. The second peak (Fig. 3.8B), corresponding to sulfadiazine, also contained the expected AA'BB' pattern, plus an $A_2B$ pattern for the three protons on the pyrimidine ring.

However, although the spectrum of the third peak, sulfamerazine (Fig. 3.8C) contained the AA'BB' aromatic ring signals and a pair of doublets for the two protons on the pyrimidine ring, unexpectedly there was no signal for the methyl group on the pyrimidine ring. Initially this appeared to suggest that there might have been degradation of the analyte in the superheated water. The spectrum of the last peak for sulfamethazine (Fig. 3.8D) contained the expected aromatic and pyrimidine proton signals, but again there was no peak for either of the heterocyclic methyl groups. A direct measurement of sulfamethazine dissolved in $D_2O$ confirmed that these peaks should be visible in the spectrum, and also confirmed that the sample was not degraded before the superheated water experiment.

The mass spectra of the sulfonamides (Fig. 3.9A-D) showed the expected spectra for sulfacetamide and sulfadiazine with base peak ions at m/z 256 and m/z 292 corresponding to the $[M+K]^+$ complexes of the trideuterated species (from exchange of the labile amino and amido protons). In contrast, the base peaks for sulfamerazine and sulfamethazine gave ions at m/z 309 and m/z 326, three and six mass units higher than expected, respectively. This corresponded to the substitution of the protons on the pyridinium methyl groups by deuterium atoms. It therefore appears these

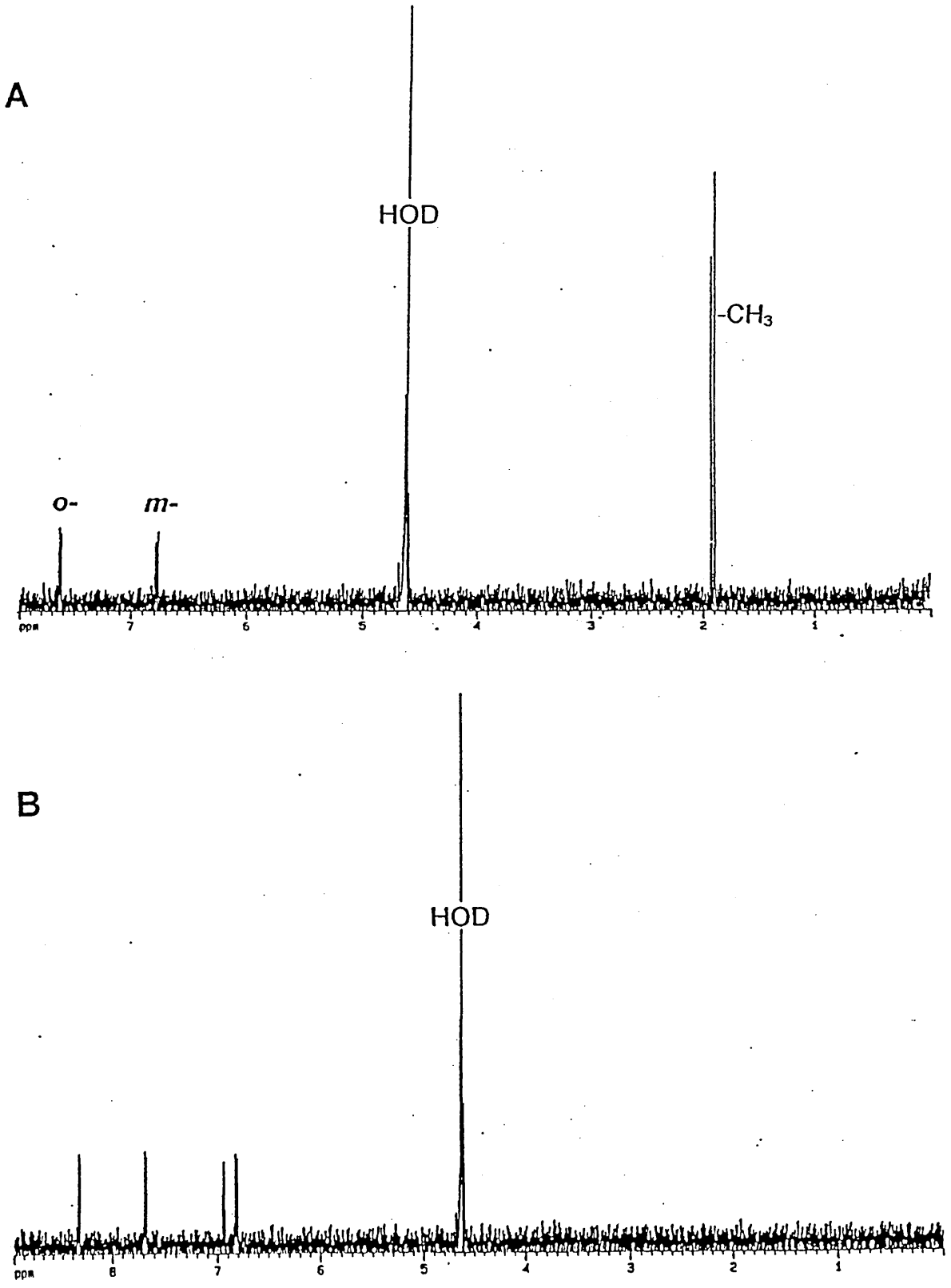



Fig. 3.8 *Stop flow $^{1}H$ NMR spectra of the sulfonamides following separation by superheated water chromatography. A= sulfacetamide and B= sulfadiazine*

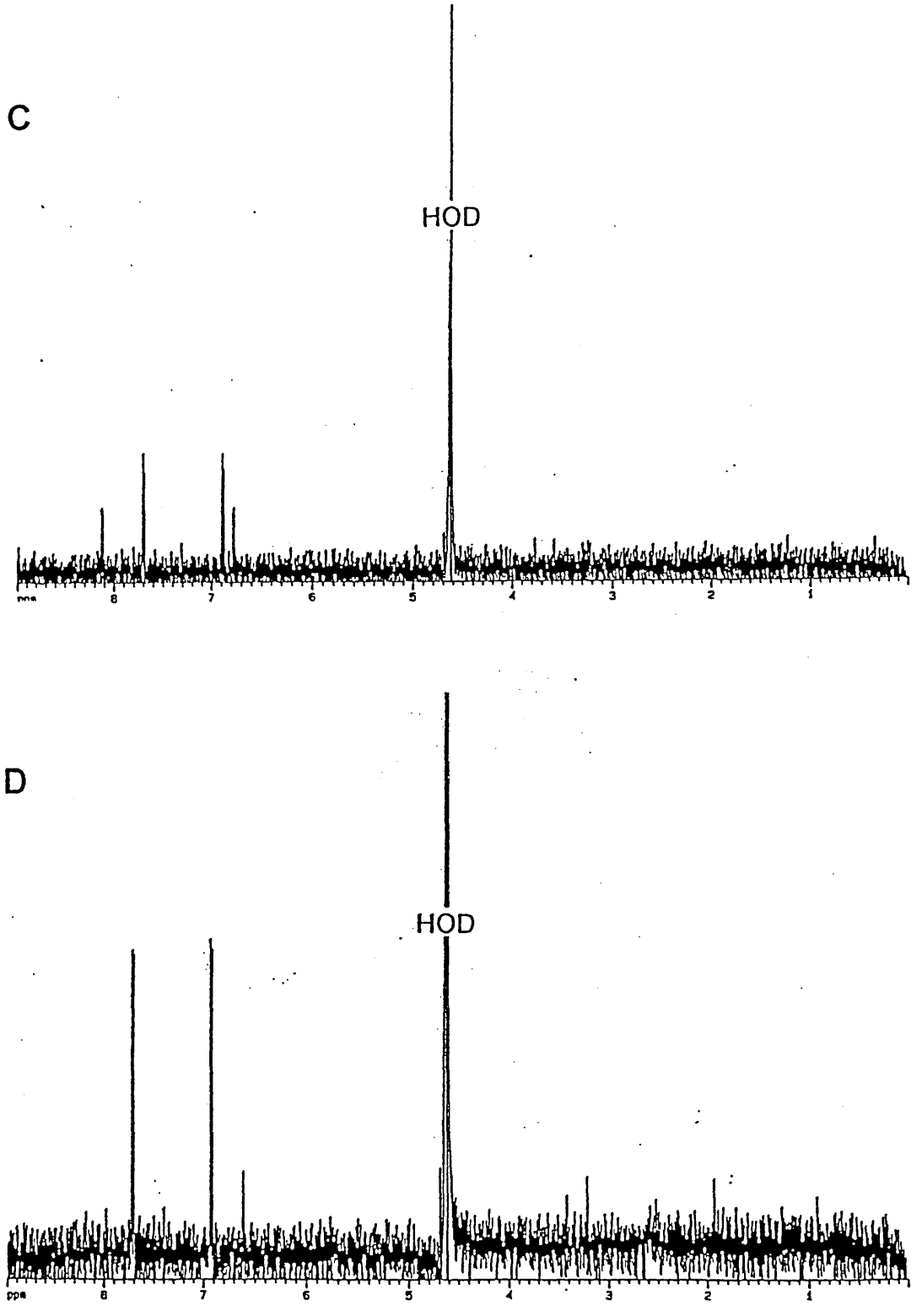



Fig. 3.8 *cont'd stop flow $^{1}H$ NMR spectra of the sulfonamides following separation by superheated water chromatography. C= sulfamerazine and D= sulfamethazine*

positions had undergone a specific and selective deuterium exchange reaction during the elevated temperature conditions of the separation process.

As it appeared that both the 4- and the 4,6-methyl groups on the pyrimidine ring could undergo an exchange, the corresponding 2,4-dimethyl analogue, sulfisomide, was examined under the same conditions. Again the NMR spectrum lacked a significant methyl signal, although small residual peaks were present. The mass spectrum showed that methyl proton exchange had largely occurred. Thus it appears that exchange can occur at both the 2- and the 4- positions of the pyrimidine ring. Previous chemical studies have reported that the alpha carbon of the alkyl group in the 2,4 or 6 positions on the pyrimidine ring is activated[70] presumably via an enamine/imine type tautomerisation. It was also reported that very slow deuteration ($t^1/_2$ = 420 min at pH 0.5 and >1600 min at pH 13.5 in deuterium oxide) occurred on the methyl groups of 2,6-dimethylpyrimidine[71]. This exchange reaction was potentially very interesting as it could represent a facile method for the preparation of deuterated standards for use in mass spectrometry.

Such deuterated standards can at times play a critical role in mass spectrometric analysis, especially in quantitative studies, because they possess almost identical physical characteristics to an un-deuterated analyte but can be readily distinguished by their mass difference. They can therefore be employed as internal standards since they behave in the same way as the analyte of interest on injection, extraction, derivatisation, chromatography and other sample preparation and separation techniques, although some separations have been reported by high

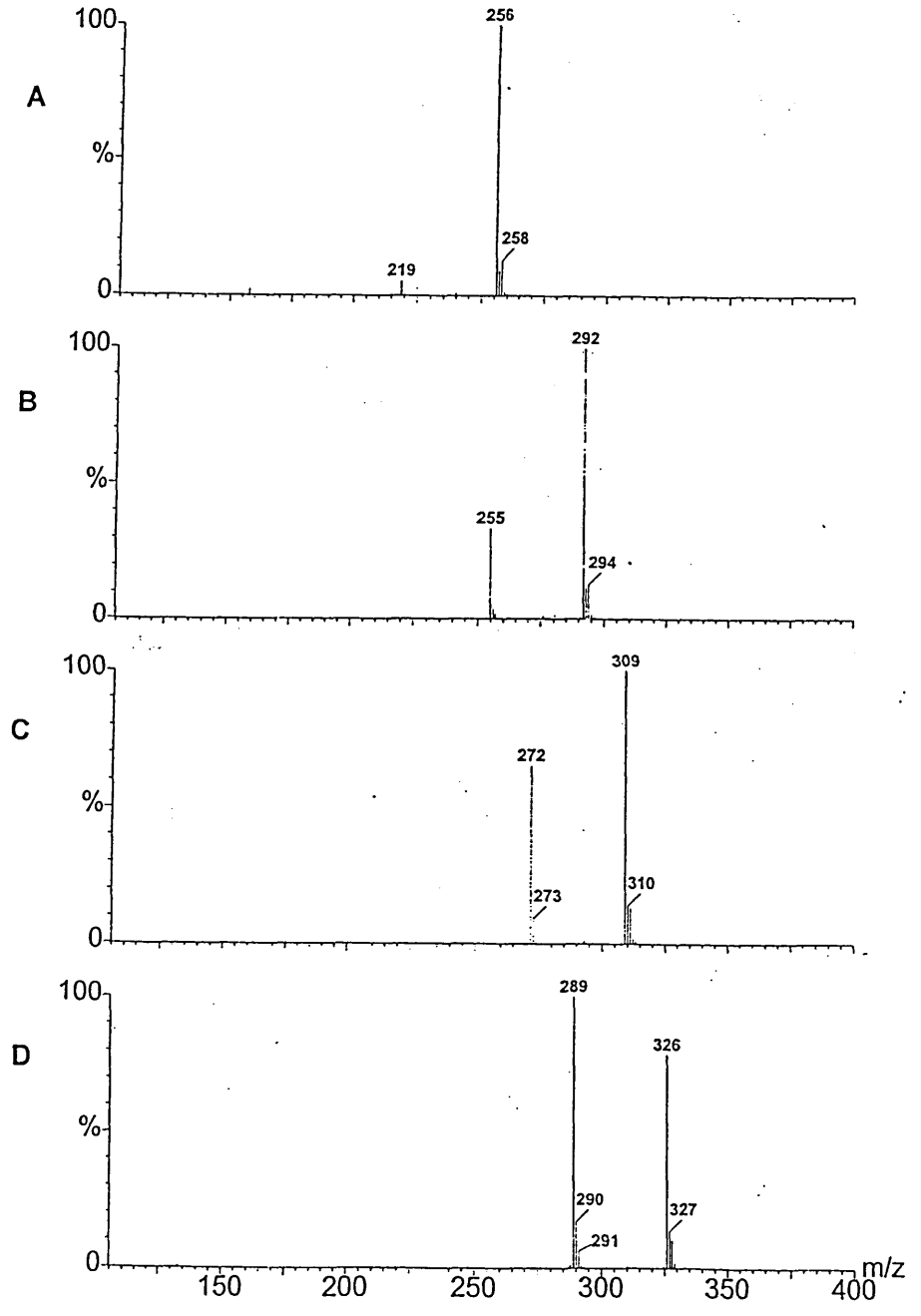



Fig. 3.9 *The mass spectra of the four sulfonamides obtained on flow for the separation shown in Fig. 3.7. A= sulfacetamide, B= sulfadiazine, C= sulfamerazine and D= sulfamethazine*

resolution CE and GC[72]. However, their efficient and selective synthesis in high isotopic purity can be difficult. Previously a number of groups have examined the possibility of using supercritical deuterium oxide at 300-400C to generate isotopic

exchange reactions. Weak acids, including acetophenone, would exchange activated protons in deuterium oxide alone, or per-deuteration (including the exchange of aromatic ring protons) could be obtained efficiently with supercritical deuterated potassium hydroxide[73]. The same method gave per-deuterated compounds in good yields (40-85% with up to 95% deuteration) for unsubstituted aryl compounds but poor yields and frequently complex tars were obtained for substituted aromatic compounds[74]. Pyrazoles and quinoxilines could also be converted into perdeutero analogies[75]. Much of this should not be unexpected since it was indicated earlier in this chapter that supercritical water and hence supercritical deuterium oxide are extremely reactive and aggressive in their nature causing the decomposition of many compounds.

Further preliminary studies were therefore carried out to determine if the reaction could be repeated off-line without using a chromatographic system. Sulfisomide was heated in pD 3.0 $D_2O$ for 10 min at 120C, and the product was examined by NMR spectroscopy, but only a 32% conversion to the deutero compound occurred. However, repeating the experiment this time heating the sample to 160C for the same period of time, gave a 93% exchange of the methyl group protons. Sulfamerazine under the same conditions gave complete exchange of the methyl groups. If these compounds were to be useful as internal standards it was also important to determine that these deuterated analogues would be stable to back exchange of the deuterium to the protonated analogues under normal conditions of use. A sample of the deuterated compound was therefore heated in un-deuterated pH 3.0 water at 80C for 10 min. Examination of the resultant product showed that the deuterated methyl groups were

indeed unaltered, confirming that superheated temperatures were necessary for any exchange to occur.

## 3.5 Summary

- **It was successfully demonstrated that superheated water was indeed a potential alternative for an eluent in LC-NMR-MS studies.**
- **It was specifically demonstrated that superheated deuterated water is an excellent eluent for NMR studies as it provides very minimal interfering peaks while at the same time it is compatible with MS studies.**
- **Gradient temperature elution was achieved with no negative impact on the spectroscopic techniques.**
- **It was shown that superheated water was not as aggressive as supercritical water; however, it was still sufficiently potent to be able to rapidly degrade standard silica based HPLC columns.**
- **Equally some compounds may not be suitable for analysis by superheated water chromatography as they degrade/react in some way with the eluent as was demonstrated in the case of acetylsalicylic acid being hydrolysed to salicylic acid.**
- **It was shown that superheated deuterium oxide has the ability to selectively exchange with certain groups to produce stable deuterated analogues. Furthermore these deuterated analogues are produced in very high yield and are stable under non-superheated conditions.**

# CHAPTER 4

# TOWARDS A TOTAL ORGANIC ANALYSIS DEVICE

## 4.1 Aims and Introduction

**The aims of the work described in this chapter were:**

**1 To determine the initial feasibility of coupling FTIR to LC-NMR in an off-line mode.**

**2 To determine the initial feasibility of coupling FTIR to LC-NMR-MS in an off-line mode.**

Having successfully coupled HPLC with NMR the next logical step was to attempt to couple other spectroscopic techniques with chromatography in order to gain further structural information on the separated components of various mixtures. IR spectroscopy has previously been successfully coupled to a liquid chromatograph in both on-line and off-line modes[76-80]. Initially it was decided to determine whether LC-NMR could be coupled to an IR spectrometer via an off-line collection technique.

### 4.2 The Practicalities of LC-IR

LC-FTIR combines the components of a liquid chromatograph with an FTIR through a specialised interface. In this instance an off line approach was adopted for simplicity. The eluent from the liquid chromatograph passed through a heated

nebuliser and was sprayed onto an aluminium backed germanium disc. A stream of nitrogen gas facilitated nebulisation and transport of the analytes. The rotation speed of the disc was kept constant to allow the re-construction of the chromatogram during off-line collection of the infrared data. The analytes were deposited as solids on the disc (see Fig. 4.1) and at the end of the chromatographic run the disc was removed for subsequent FTIR analysis (see Fig. 4.2 for a schematic of this approach). This whole process took place within a vacuum chamber. This aided the evaporation of the solvents, which were subsequently retained in a cold trap for later disposal.

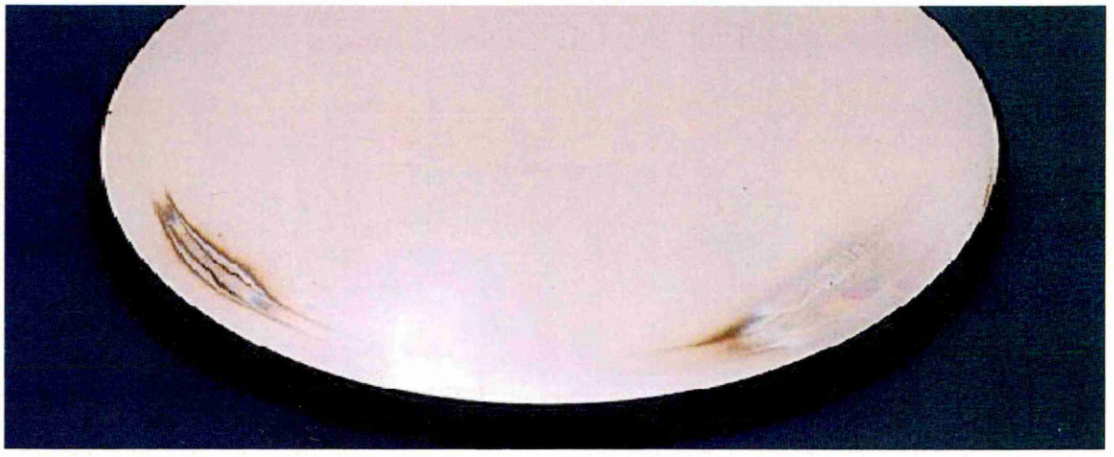


Fig. 4.1 *A typical disc following a chromatographic separation. The dark bands correspond to analytes eluting during the chromatography.*

A Laboratory Connections LC-Transform model 300 (Viscotek, Basingstoke UK) instrument was used for these initial LC-IR experiments. This consisted of the post column interface described above plus an optics module (Fig. 4.4) that fitted directly into the sample compartment of an Nicolet 5DXC FTIR (Nicolet Ltd, Warwick, UK). Infrared energy was focused through the sample deposit on the aluminium backed germanium disc (Fig. 4.3) and then reflected back through the sample to the detector. The disc was rotated at the same speed as during the acquisition to interrogate the

entire chromatogram. Infrared spectra could then be generated for specific positions (peaks) in the sample deposit, or the chromatogram could be reconstructed using total IR absorbance or absorbance along a band associated with a specific functional group, such as a carbonyl. One advantage of this off line technique[81] was that the IR signal to noise could be greatly improved by positioning a segment of the sample deposit under the IR beam and acquiring multiple scans. This signal to noise enhancement would not have been possible with an on line LC-FTIR configuration. Of course both on line and off line FTIR approaches save on costly isolation and sample preparation.

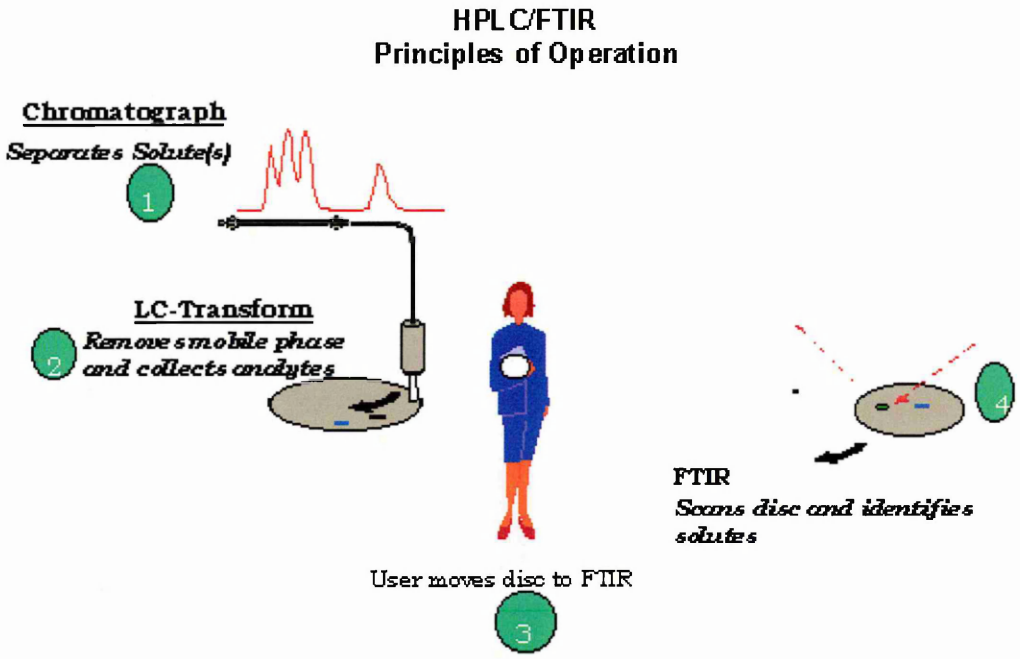



Fig. 4.2 *Schematic of the LC-FTIR process*[82].

Since all traces of solvent were removed the IR spectra generated from the deposits covered the full spectral range. On line systems are only able to operate within spectral windows, which can limit the amount of diagnostic information available. The resultant spectra obtained were high quality and more than adequate for library searching.

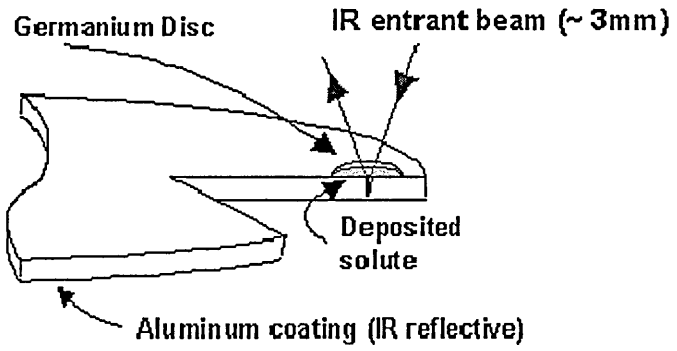



Fig. 4.3 *Diagram of the acquisition of FTIR data from the disc*[82].

### 4.2.1 The Disc Optics Module

In most FTIR spectrophotometers the incident beam would be matched to the size of the sample deposit for optimal sensitivity. To achieve this, the Disc Optics Module used a beam condenser to focus the infrared beam onto the sample deposit.

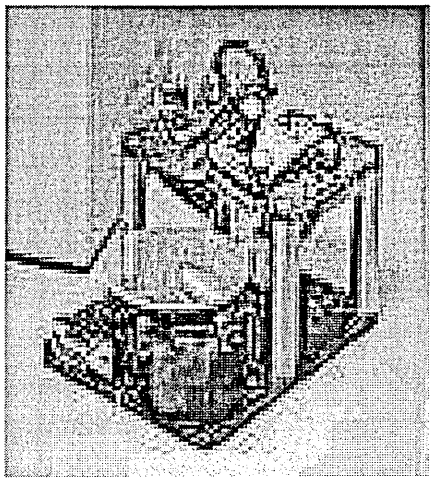


Fig. 4.4 *Picture of the transfer optics module*[82].

Disc rotation could be controlled by either the Automated or Manual Controller (Fig. 4.5) and, in the former case; completely unattended (overnight) spectral collection was possible.

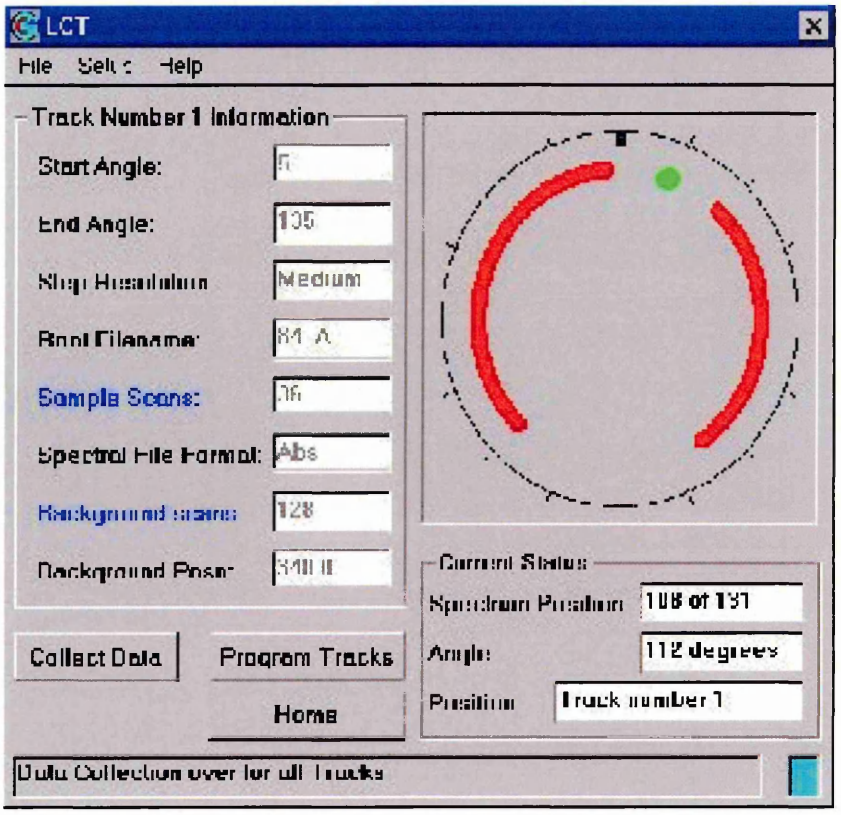



Fig. 4.5 *Control software for the acquisition of FTIR data from the disc*

Disc rotation was either in discrete steps (Automated) or at a selectable, continuous speed (Manual).

In either case the disc was easily positioned at a selected location for extended spectral examination of a particular deposit.

### 4.3 Size Exclusion Chromatography Separation of Polymer Additives[83,84]

In order to minimise the number of unknowns a simple sample mixture, which could be readily characterised by LC-FTIR, was chosen as a starting point. By now many of the difficulties of LC-NMR in the laboratory were known or had been addressed,

but the LC-IR interface was a new addition. Therefore the first experiments described here were tailored to suit the LC-IR first and foremost.

### 4.3.1 Experimental conditions

The NMR conditions were as previously described. The HPLC (Bruker LC22 previously described), was used to deliver 1.0 mL/min of deutero-chloroform ($CDCl_3$) through two Mixed-E columns 3 μm, 30 cm x 7.5 mm i.d. (Polymer Labs) connected in series. The artificial mixture created to test the system consisted of a series of polymer additives; namely 2,6-di-tert-butyl-4-methylyphenol (BHT, Fig. 4.6), octadecyl-3-(3,5-di-tert-butyl-4-hydroxylphenyl)propionate (Irganox 1076, Fig. 4.7) and di-iso-octylphthalate (DIOP, Fig. 4.8) each at an approximate concentration of 100 mg $mL^{-1}$ in $CDCl_3$. 10 μL injections were made through a Rheodyne 7125 valve fitted with a 100 μL loop. After the eluent had passed through both the UV detector and the NMR flow cell it was directed towards the Lab Connections Transform Model 300 LC-FTIR interface. The samples were deposited onto the aluminium backed germanium disc via the pneumatic nebuliser, which was held at 70C. The disc was mounted on a heated stage to aid evaporation; the stage was held at 90C. The disc was driven by a stepping motor at a constant rate of 10 degrees per minute. The whole system was held under vacuum at 8 Torr (6 x $10^{-3}$m bar) again to aid evaporation of the HPLC eluent. Once the chromatographic separation was completed the disc was transferred to the FTIR scanning module and a series of spectra was acquired while rotating the disc under the IR beam. FTIR spectra were obtained using a Nicolet 5DXC spectrometer equipped with a DTGS (deuterated triglycine sulphate) room temperature detector over a spectral width of 4000-650 $cm^{-1}$

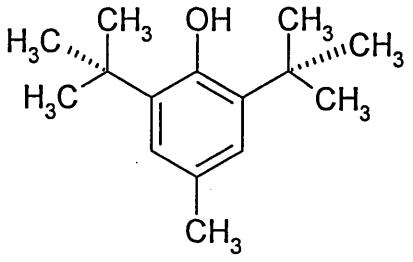



Fig. 4.6 *The structure of 2,6-di-tert-butyl-4-methylyphenol (BHT)*

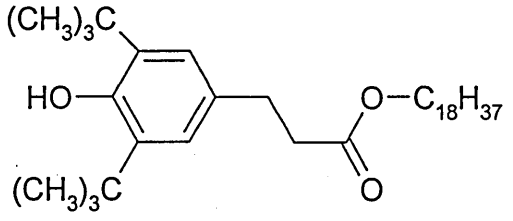



Fig. 4.7 *The structure of octadecyl-3-(3,5-di-tert-butyl-4-hydroxylphenyl) propionate (Irganox 1076)*

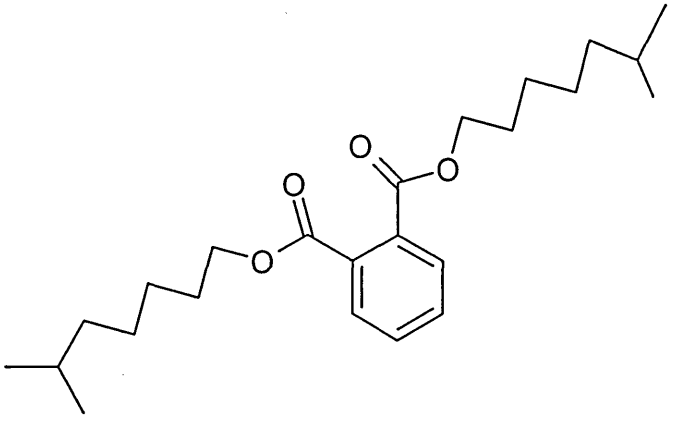



Fig. 4.8 *The structure of di-iso-octylphthalate (DIOP)*

and with a spectral resolution of 8 $cm^{-1}$. During spectral acquisition the disc was rotated at 5 degrees per minute, and 5 scans per spectrum were summed. Rotating at this speed enabled an increase in the signal to noise ratio.

## 4.4 Initial LC-NMR-FTIR results

The UV chromatogram obtained for the separation of the three component mixture is shown in Fig. 4.10 and the on flow $^1$H-NMR spectra for the same separation are shown in Fig. 4.9. The UV chromatogram shows that the use of two low molecular weight range size exclusion chromatography (SEC) columns in series enabled the baseline separation of the three components. The use of $CDCl_3$ as the eluent was particularly beneficial as it enabled $^1$H-NMR spectra to be obtained with very little interference, while the volatility of this solvent ensured that there were no problems with the evaporation step at the LC-FTIR interface. This lack of interference from the solvent is illustrated in the $^1$H-NMR spectra extracted from this on flow experiment shown in Fig. 4.9 (peaks 1-3). There was, however, some interference due to signals from residual acetonitrile ($CH_3CN$) contamination resulting from previous HPLC experiments. These on flow $^1$H-NMR spectra clearly showed the expected signals for the analytes with good signal to noise. Thus the spectrum for Irganox 1076 (peak 1 in Fig. 4.10), shown in Fig. 4.9 (peak 1), was generated from row 50 (marked 1 in Fig. 4.11). This showed a good, clean spectrum in which all the resonances were clearly observed, including the signal due to the phenolic OH group not normally observed in "conventional" reversed phase HPLC-NMR when $D_2O$ is one of the solvents.

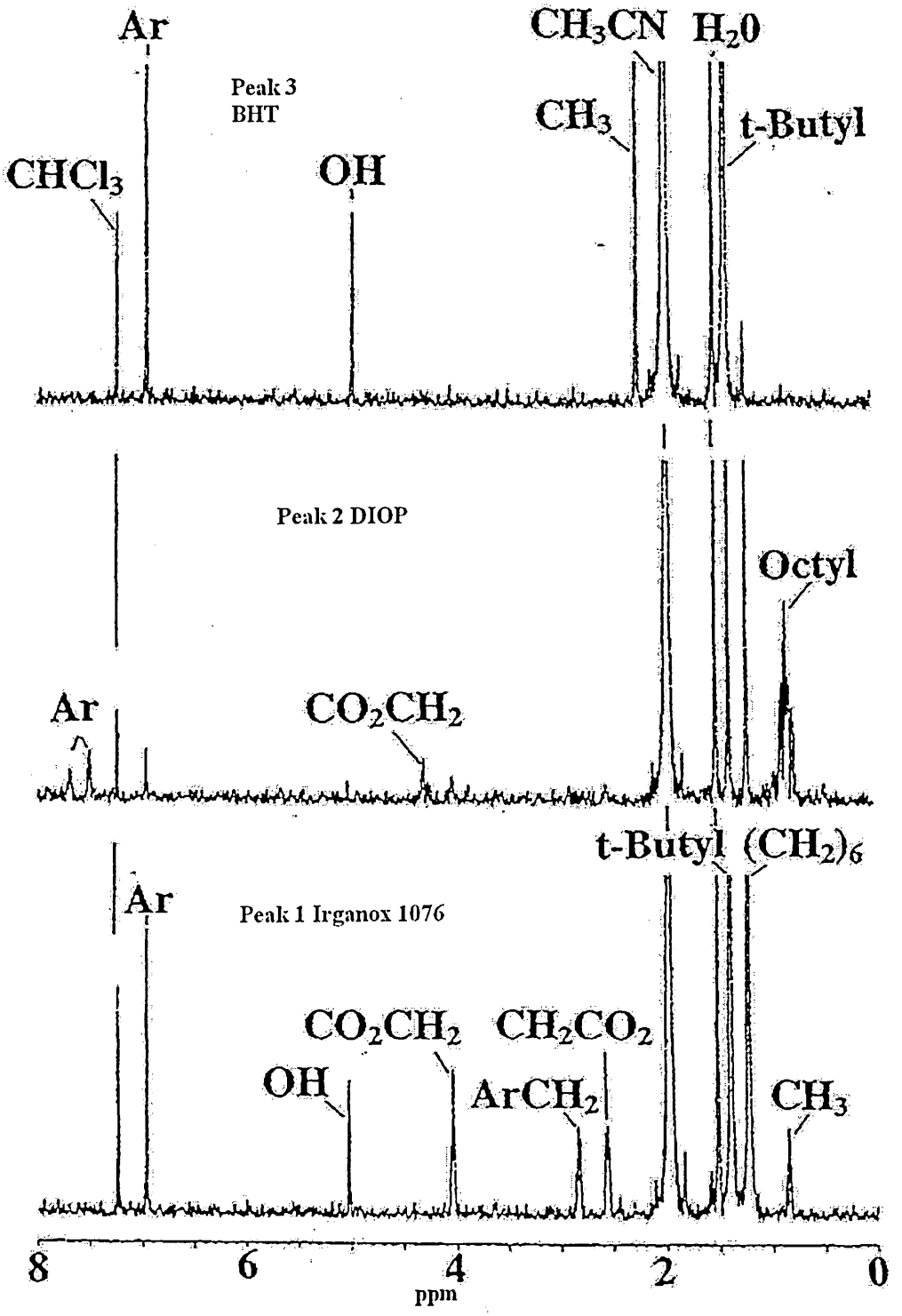



Fig. 4.9 *The $^{1}H$ NMR spectra of peaks 1-3 from Fig. 4.12 with the various chemical shifts labelled for each compound. 1= Irganox 1076 (bottom spectrum), 2= DIOP (middle spectrum) and 3= BHT (top spectrum).*

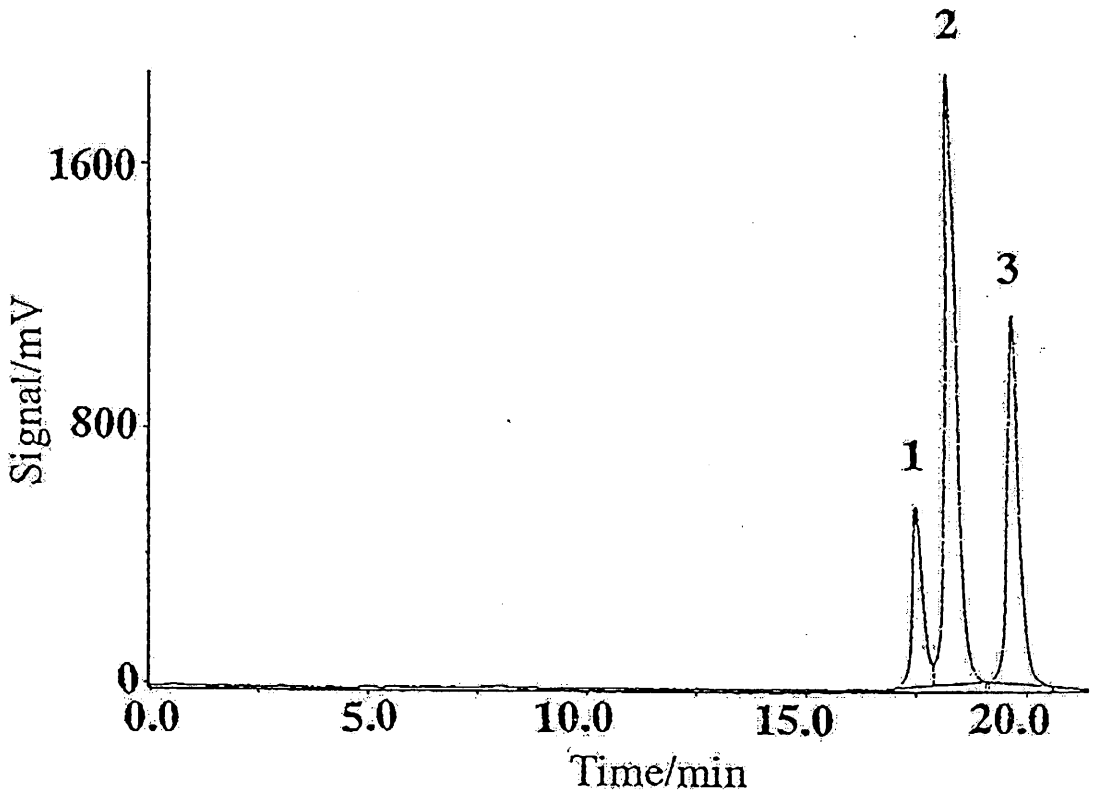



Fig. 4.10 *UV (254nm) chromatogram of the mixture of polymer additives. Irganox 1076 (peak 1), DIOP (peak 2) and BHT (peak 3)*

The second component (peak 2 Figs. 4.9 and 4.10), DIOP, showed the typical phthalate aromatic pattern and a complex long chain hydrocarbon multiplet at 0.8 – 0.9 ppm. This spectrum also showed signals for Irganox 1076 indicating incomplete separation of peaks 1 and 2. The final component (peak 3, Figs. 4.9 and 4.10), BHT, also gave a typical NMR spectrum for this analyte and this was assigned as per Fig. 4.9 peak 3.

FTIR spectra for each of these three eluted peaks were obtained off-line following collection of the eluent from the NMR probe via the LC Transform interface as previously described above. The FTIR spectra for the three test compounds are

shown in Fig. 4.12 (peaks 1-3), and are entirely consistent with those expected for these compounds. There are clearly visible signals for OH in the spectra for Irganox

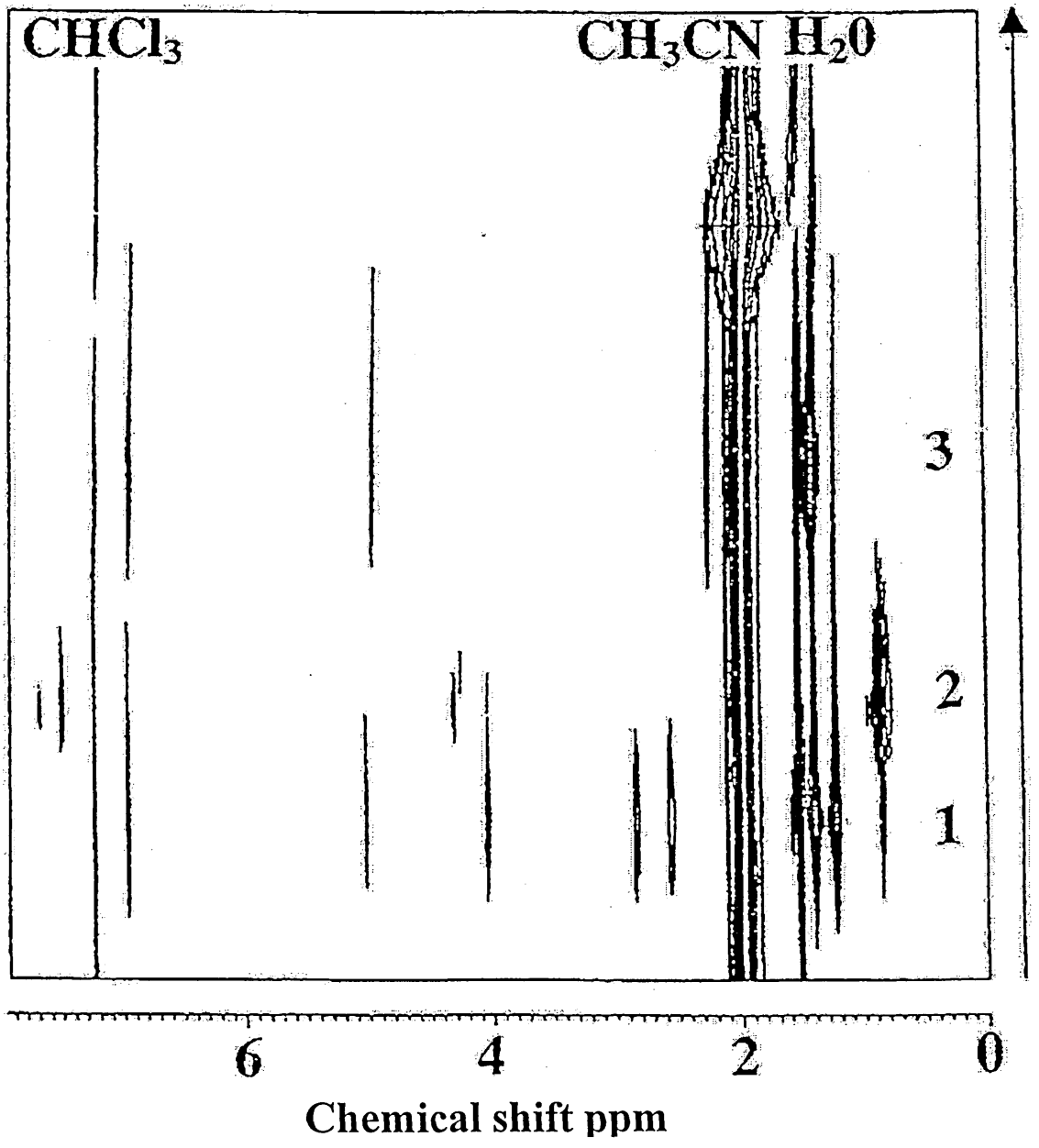



Fig. 4.11 *The portion of the 2D NMR chromatogram on flow trace between approx 17 and 20 minutes, corresponding to the retention times for peaks 1-3. NMR chemical shift is shown along the X axis while the arrow on the right hand side of the vertical axis indicates increasing retention time.*

1076 and BHT at ~3650 $cm^{-1}$ (Fig. 4.12 peaks 1 and 3), aliphatic CH absorptions are visible for all three compounds at ~2900 $cm^{-1}$ while those for carbonyl groups (C=O) are found in the spectra of Irganox 1076 and DIOP (Fig. 4.12 peaks 1 and 2). There was some evidence, in the form of a carbonyl absorption in the spectrum for BHT, of carry-over from the DIOP peak (Fig. 4.12 peak 3), which has been removed by background subtraction.

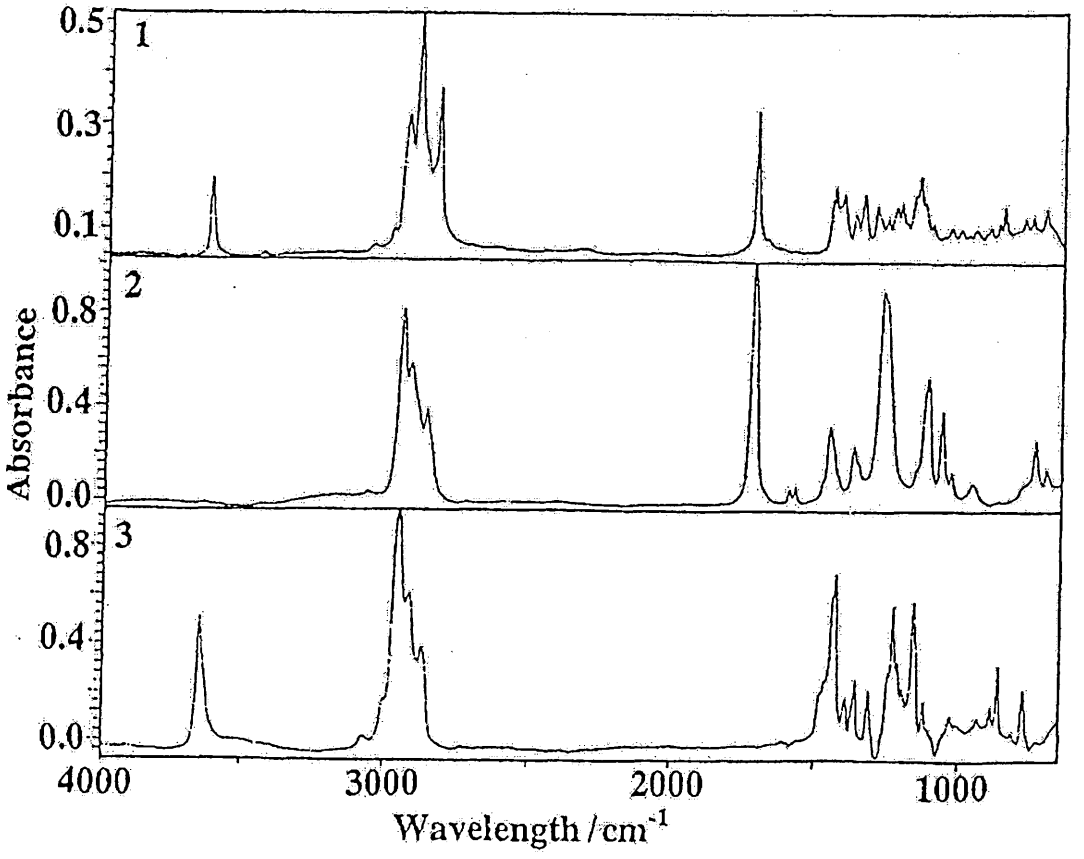



Fig. 4.12 *The FTIR spectra of peaks 1-3 obtained after collection via the LC Transform interface. 1= Irganox 1076 (bottom spectrum), 2= DIOP (middle spectrum) and 3= BHT (top spectrum).*

## 4.5 Conclusions from preliminary experiments

The results above demonstrated the relative ease with which SEC using $CDCl_3$ as the solvent could be coupled to on-line NMR spectroscopy and "in series" for off-line FTIR, thus enabling the generation of both high quality NMR and IR spectra from a single chromatographic run. The complementary nature of NMR and IR is obvious and this experimental set up clearly showed the potential for the simultaneous coupling of these two powerful techniques via chromatographic analysis.

## 4.6 LC-NMR-MS coupled to off-line FTIR

Following the success of the previous experiments the next step was to attempt to add LC-FTIR to the earlier constructed LC-NMR-MS system, thus providing a comprehensive suite of characterisation techniques for sample analysis. The same three component test mixture was again employed to minimise any changes. Unfortunately at this time the FTIR interface used previously was not available and a similar unit had to be substituted although no problems with this substitution were envisaged.

### 4.6.1 Initial Set-up

The first step in constructing a "total solution" type system from the LC-UV-NMR-FTIR described above was to reintroduce the split to the mass spectrometer and to balance the flows for all of the spectroscopic techniques involved. This apparently trivial task proved problematic. The replacement LC-FTIR interface, although an upgraded model, proved to exhibit qualities not previously seen. This later model was designed specifically for use with aqueous eluents and utilised a heated nebuliser.

Most importantly the backpressure exerted on the system and in particular upon the LC-NMR flow probe were sufficiently greater, than with the previous model, to cause the NMR probe to leak. This in turn unbalanced the entire system and altered all of the flows to each of the spectrometers. The only way to prevent the NMR probe from leaking was to introduce a further tee into the system this time just before the NMR spectrometer. Previous experiments had seemed to suggest that the relative sensitivities of the NMR and FTIR spectrometers were approximately equal and so the flow was split at this point 50:50 between the NMR and the FTIR spectrometers. Therefore following separation and having passed through the UV detector flow cell 5% of the eluent was directed to the mass spectrometer and 95% went towards the NMR and IR spectrometers, this being split equally just prior to the NMR spectrometer.

The NMR spectra were once again acquired on the Bruker DRX-500 using a 4mm i.d. flow probe. The portion of the eluent for FTIR analysis was selected with an inline splitter box and was transferred via a 2m length of PEEK tubing. The majority of this portion of the eluent (~70%) was passed through a heated nebuliser nozzle set to 90C, which rapidly evaporated the solvent from the eluent thus depositing a focused track of analytes onto the rotating aluminium backed germanium disc. The remaining 30% of the eluent was directed to waste. As previously the disc was removed after the chromatography was completed for subsequent off line FTIR analysis using the Nicolet 5DXC spectrometer. Mass spectra were acquired with a Micromass LC-Quattro triple quadrupole instrument, although during these experiments data were only acquired with the instrument running as a single quadrupole instrument. Four functions were acquired simultaneously, two in positive ion and two in negative ion,

with cone voltages of 25V and 50V using electrospray ionisation and a Z spray source. The source temperature was set to 150C and the desolvation temperature at 70C. These elevated temperatures were employed to minimise any contamination of the source with the samples as relatively large amounts of sample were being introduced into the mass spectrometer despite the large split in favour of the NMR and FTIR spectrometers. These elevated temperatures did not appear to contribute to any significant degree to any fragmentation of the compounds being investigated. A schematic of the system is shown in Fig. 4.13.

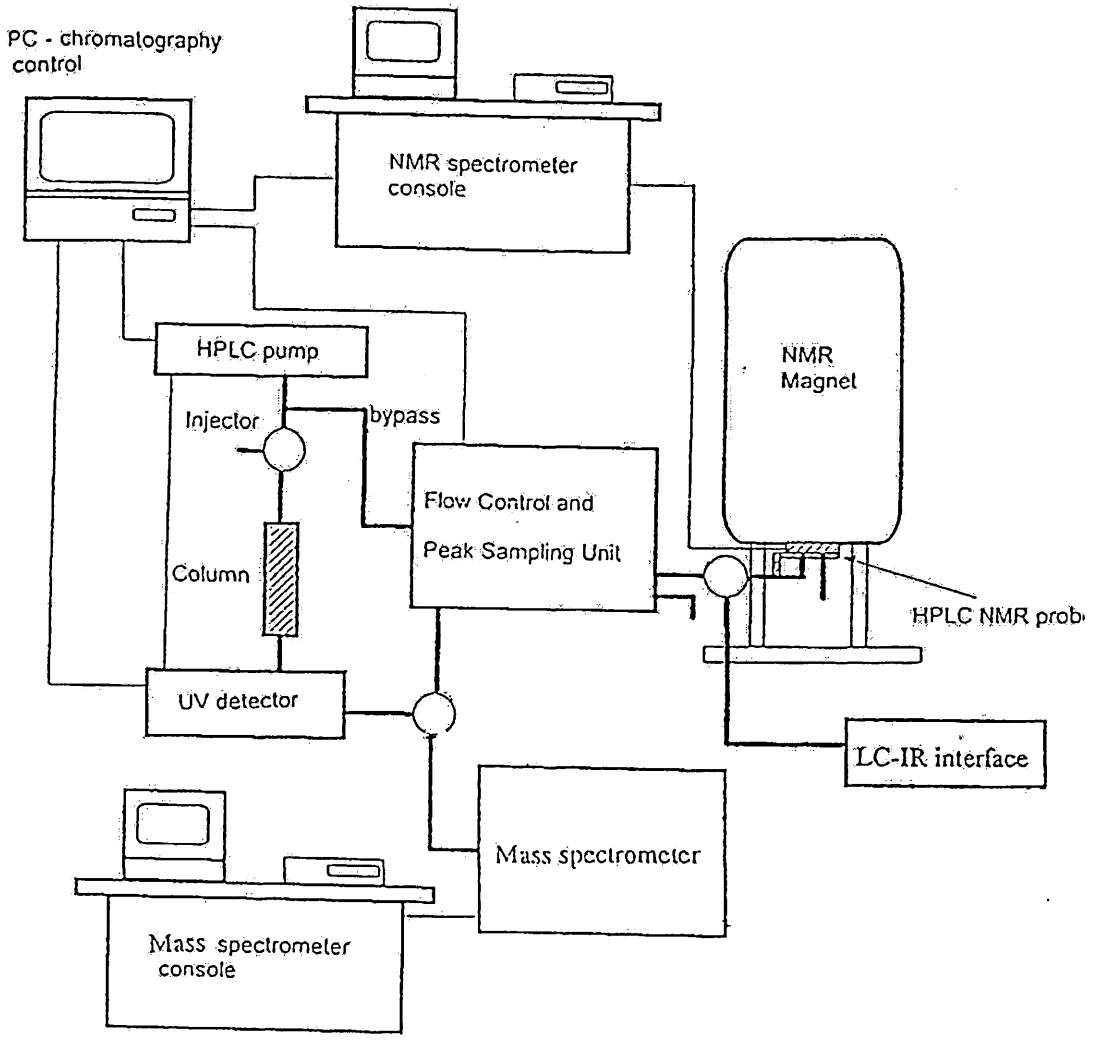



Fig. 4.13 *Schematic diagram of the SEC-UV-MS-NMR-FTIR system used*

## 4.7 SEC-UV-MS-NMR-FTIR results and discussion

The major problem to making this new configuration function was to find a chromatographic eluent that was compatible with the NMR and mass spectrometers as well as the IR interface. The previous experiment had simply used deuterochloroform as the eluent, but while this had several advantages for LC-NMR and for use as a solvent for deposition onto the rotating disc prior to FTIR analysis it was not compatible with MS. It had been hoped that there would be sufficient water present to allow ion formation in the electrospray source but no ions were detected. Performing a shake with $D_2O$, prior to use, as an attempt to dope the deuterochloroform with enough water for successful electrospray also failed to produce any ions in the mass spectrometer. The next approach was to introduce a tee just prior to the mass spectrometer to add in a small amount of methanol/water via a syringe pump. Again this failed as there was sufficient back pressure developed by the syringe pump and the mass spectrometer to cause a back flow of the water and methanol into the main eluent stream directed towards the NMR spectrometer and the IR interface. This caused additional signals to be present in the resulting NMR spectra while at the same time failing to solve the problem of non-ionisation of the peaks entering the mass spectrometer. The only simple available solution remaining was to add a small amount (circa 2%) of ammonium acetate and deutero-methanol to the $CDCl_3$ used as the solvent for the separation. The inclusion of these additives did not significantly alter the appearance of the chromatogram Fig. 4.14 compared with the previous experiment Fig. 4.10, when no methanol or ammonium acetate were used for the separation.

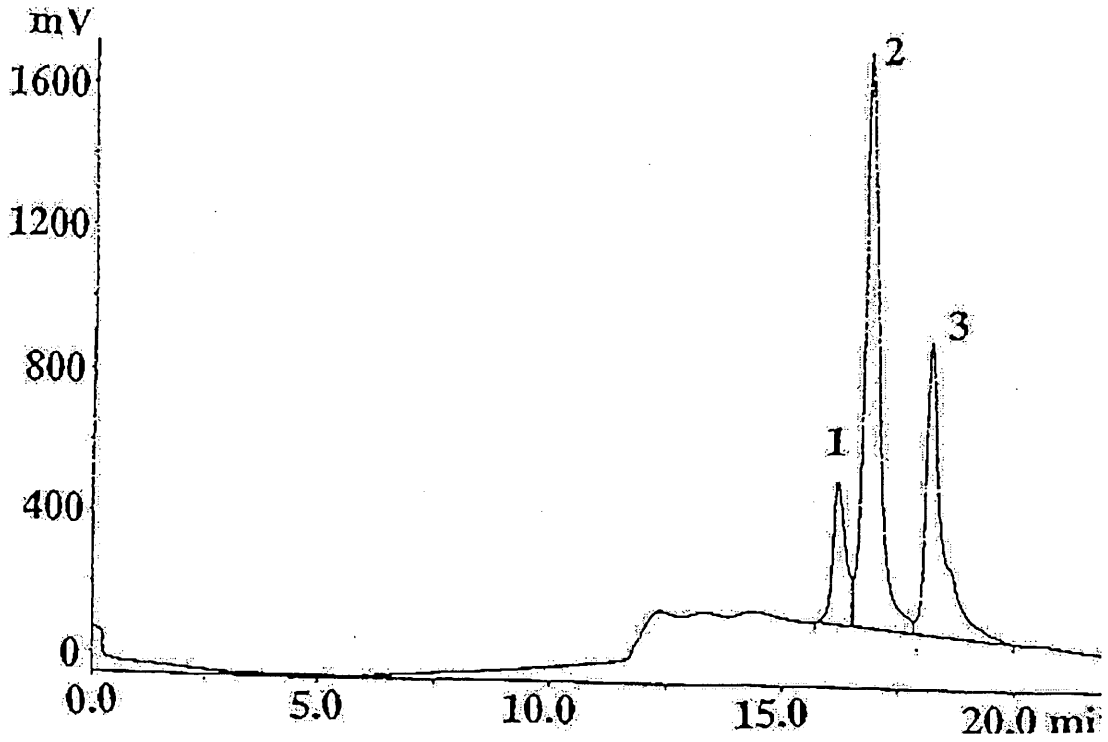



Fig. 4.14 *SEC-UV chromatogram with approx 2% methanol and ammonium acetate for the separation of the mixture of polymer additives, Irganox 1076 (peak 1), DIOP (peak 2) and BHT (peak 3)*

These additives enabled the three components to be ionised and detected by the mass spectrometer. Fig. 4.15 shows the extracted mass chromatograms for each of the three components. Clearly there was some band broadening caused by the transfer to the mass spectrometer. Fig. 4.16 shows the resultant mass spectra obtained using this eluent composition. In each of the spectra A-C all of the exchangeable protons have been replaced with deuterium. An $[M-D]^-$ was observed for both BHT and Irganox 1076 while an $[M+D]^+$ was seen for DIOP. A smaller $[M+ND_4]^+$ ion at m/z 412 was also seen for DIOP due to the presence of the ammonium acetate. Little or no fragmentation was noted in any of the spectra as they were all acquired at a low cone voltage of 25V.

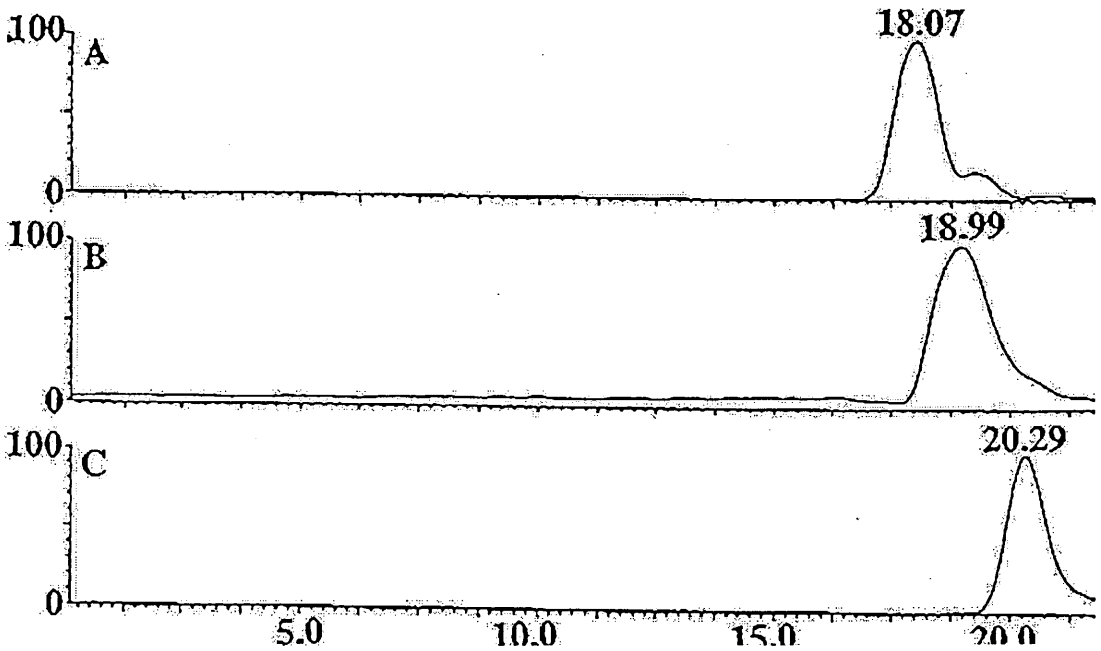



Fig. 4.15 *SEC-MS extracted mass chromatograms for Irganox 1076 (A), DIOP (B) and BHT (C) using the modified eluent containing 2% methanol ammonium acetate to facilitate ionisation. The peak retention times are displayed above each peak.*

While the addition of $CD_3OD$, $D_2O$ and ammonium acetate had allowed for the successful acquisition of mass spectra of the three target compounds without any major affect on the chromatographic separation, there were effects seen in the NMR spectra of the analytes. Although these additives were as NMR friendly as possible they still contributed several additional signals, which, though not preventing the realisation of interpretable NMR spectra in this case, might have caused relevant signals to be masked had other different compounds been chosen. Additionally, the signal for the phenolic OH observed in the previous experiments for both BHT and Irganox 1076 was lost due to deuterium exchange with these new solvents.

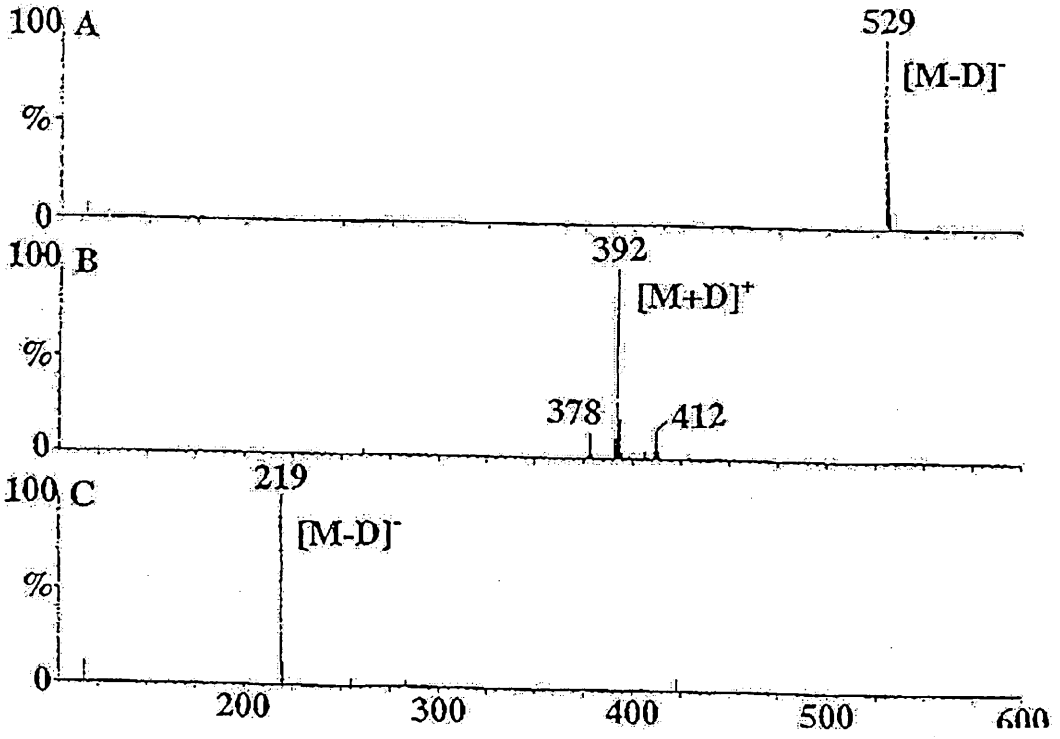



Fig. 4.16 *The mass spectra of the mixture of polymer additives after the eluent was modified with $CD_3OD$ and ammonium acetate to facilitate ionisation. Irganox 1076 (A) gave a $[M-H]^-$ ion at m/z 529, DIOP (B) gave a $[M+D]^+$ at m/z 392 and a $[M+ND_4]^+$ at m/z 412 while BHT (C) gave a $[M-H]^-$ ion at m/z 219*

These effects can be seen in the on flow $^1H$ NMR spectra for BHT shown in Fig. 4.17 A and B. In Fig. 4.17A the spectrum for BHT with just $CDCl_3$ as the eluent is displayed, showing the additional signal due to a residual amount of acetonitrile from another experiment. In Fig. 4.17B the spectrum of BHT is shown this time in the eluent containing $CD_3OD$, $D_2O$ and ammonium acetate. The resonances for all of the protons in BHT are present in both spectra, with the exception of the phenolic OH, which is absent from the latter spectrum due to deuterium exchange. Similar results were obtained for Irganox 1076 and DIOP.

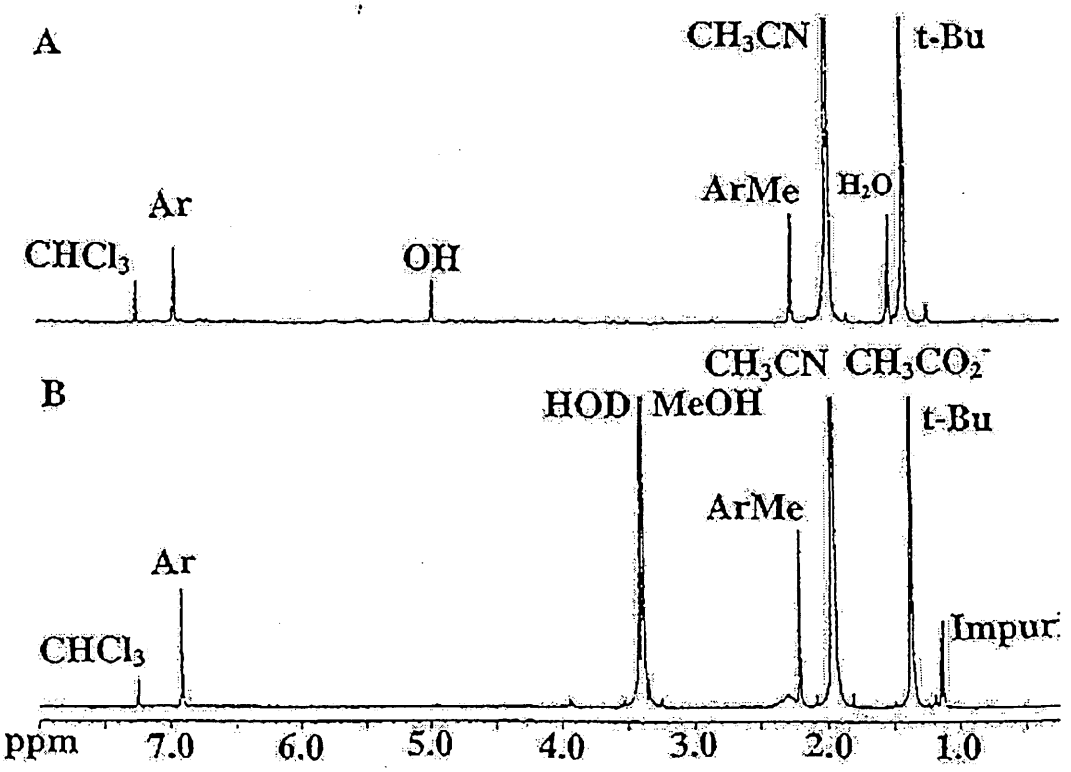



Fig. 4.17 *The on-flow NMR spectra of BHT obtained with $CDCl_3$ (99.9% Fluorochem Glossop, UK) alone as the eluent (A) and with the mobile phase used in this experiment to show the effect on the NMR spectrum of the mobile phase additives required for the MS*

These modifications of the mobile phase did not cause any problems with the evaporation in the LC-Transform interface other than to necessitate a slightly increased nebuliser temperature. As before the spectra were obtained off-line following collection of the peaks of interest using the LC-Transform interface. The resulting spectra were essentially identical to those obtained in the earlier described experiments and clearly showed signals for phenolic OH at approx 3650 $cm^{-1}$ for BHT and Irganox 1076, aliphatic CH absorptions at approx. 2900 $cm^{-1}$ and carbonyl absorptions for Irganox 1076 and DIOP at approx 1750 $cm^{-1}$. An example IR spectrum, for BHT, is given in Fig. 4.18. From this spectrum it can be clearly seen

that the addition of a small amount of ammonium acetate to the solvent did not result in the appearance of any extra signals in the off-line IR spectrum. This is explained by the fact that ammonium acetate under the conditions employed to remove the solvent is sufficiently volatile to also be completely removed. This was to be expected as ammonium acetate is often employed as a buffer salt in mass spectrometric studies because of its relatively high volatility. The excellent quality of the IR spectra obtained opened up the possibility of running library-matching software to compare the spectra obtained with reference spectra and to search for unknowns in other samples.

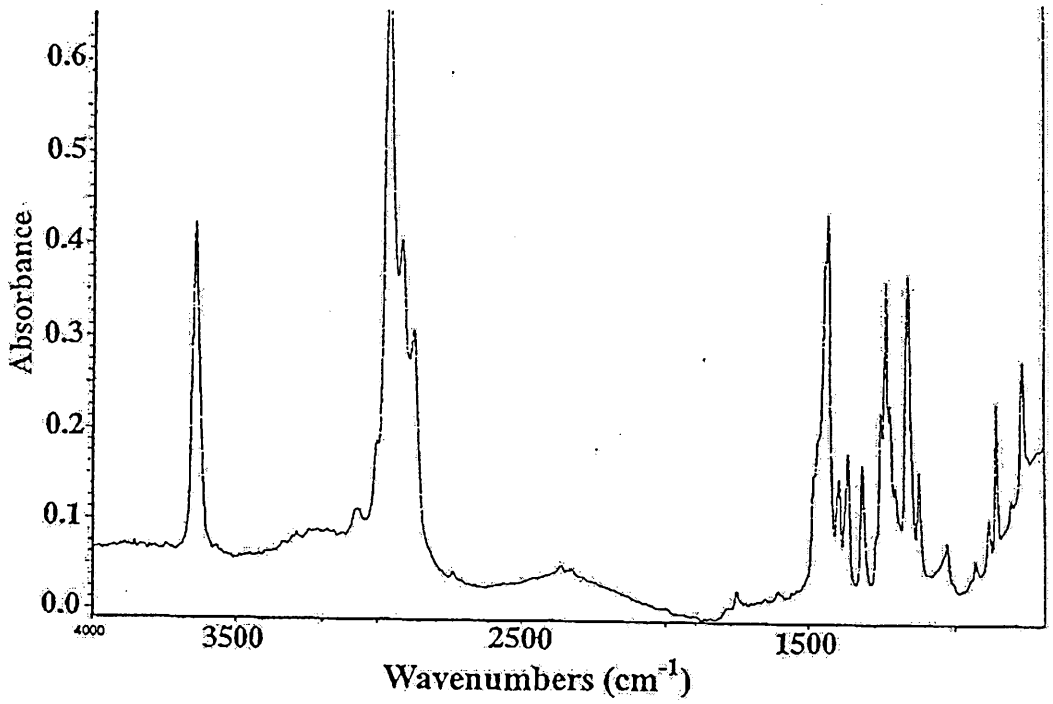



Fig. 4.18 *The off-line FT-IR spectrum of BHT acquired after elution with the modified eluent containing $CD_3OD$ and ammonium acetate to facilitate ionisation in the mass spectrometer. The spectrum is essentially identical to the one acquired previously and shown in Fig. 4.12*

## 4.8 Summary

- **The feasibility of both LC-NMR-IR and LC-NMR-MS-IR were established and working set-ups constructed.**
- **Some of the mobile phase limitations were established showing that a compromise between the ideal requirements for NMR and MS and IR is required.**
- **Library searchable IR spectra were obtained opening up the possibility of using library comparisons to search for unknowns.**
- **Further studies are required to better establish the sensitivity limits of such systems.**
- **The addition of a diode array detector to acquire UV spectra would essentially constitute a total spectroscopic suite of data from a single chromatographic separation.**
- **If used in a flow injection mode i.e. without any chromatographic separation this would in effect become a structure confirmation tool and could possibly open up the ability to introduce an automated structure determination laboratory. This could also reduce some of the problems associated with solvent compatibility, as solvents would no longer be chosen for their ability to achieve a given separation but rather for their ability to solubilise any given sample while producing the minimum number of interfering spectral signals. This will be examined in a later chapter.**

# CHAPTER 5

## Flow Injection Analysis Studies for Structure Confirmation

### 5.1 Aims and Introduction

**The aims of the work described in this chapter were:**

1 **To establish a flow injection analysis system with NMR, MS, IR and diode array UV absorption detection and to analyse a series of known compounds.**

2 **To determine a working minimum quantity of compound required to enable all the spectroscopic techniques employed to produce spectra adequate for structural identification studies.**

The work in this chapter is intended to show how a real problem could be addressed using some of the lessons and techniques covered in the previous chapters. The characterisation of substances by spectroscopy for proof of structure or identity determination is an essential task in all modern pharmaceutical analytical chemistry departments. As the use of libraries continues to grow there is increasing pressure on such departments to process the samples as rapidly and as efficiently as possible. Similarly, as synthetic chemists move away from producing single compounds towards combinatorial chemistry approaches as a means of increasing the number of structures available for testing or high throughput screening, there has been a step

jump in the amount of physical chemical data and in particular spectroscopic data that is required prior to such screens. One of the goals at the outset of this project was to find a means to alleviate this burden on the analysts by the judicious use of multiple hyphenation, to provide a comprehensive suite of spectroscopic information from a single analysis[85-92]. Currently most of these analyses are carried out by different spectroscopists, working on batch sub samples in different laboratories to give individual spectroscopic data such as NMR, mass spectrometry, IR etc. For complex samples there may be some additional separation, usually using HPLC, in combination with one of these spectroscopies[23]. This can introduce uncertainties as the different optimum chromatographic conditions employed in combination with each type of spectroscopy can lead to changes in retention times or even elution orders. As a first step and proof of principle some model compounds were analysed by flow injection analysis to determine some of the limitations of this approach.

## 5.2 The Model Compounds Chosen for these Experiments

The compounds employed in this investigation were, ibuprofen, flurbiprofen, naproxen, indomethacin and atenolol (Sigma, Poole Dorset), antipyrine (Fluka, Gillingham, Dorset), 4-hydroxyantipyrine and salicylic acid (Aldrich, Gillingham Dorset), nor-antipyrine (Janssen Chimica, Geel, Belgium) and propranolol (AstraZeneca Pharmaceuticals, Alderley Park, Cheshire). All the samples were dissolved in deuterium oxide ($D_2O$) at varying concentrations to give samples containing between 0.25 and 100 mg $mL^{-1}$.

### 5.2.1 The Flow Injection System

The Flow Injection Analysis system constructed for these studies was similar to those employed previously including a Bruker LC22 pump, which was set to deliver $D_2O$ at 1mL/min. In the early experiments for FIA typical injections volumes were 200 μL, which were injected via a Rheodyne 7125 fitted with a 200 μL loop. These larger than normal volumes were used to ensure that all of the different systems had ample opportunity to detect the samples as they flowed through the various detectors, especially as multiple splits were being employed. From the injector the entire flow was directed to a Varian 9065 UV diode array detector (Varian UK Ltd, Walton-on-Thames) along a 30cm length of 0.005" i.d. PEEK tubing in order to record the UV spectra of the compounds. The diode array detector (DAD) was placed first as it required no adaptation to accommodate the flows and it is a flow through device so only contributed a small amount to any band broadening prior to the other detectors. UV data were collected over the range 190-360 nm, using the Varian Star Chromatography workstation, version 4.0, and analysed for spectral information using Polyview version 2.0 (Varian). This wavelength range covered the usual area of the spectrum where one would expect pharmaceutical compounds to absorb. By employing $D_2O$ as the eluent spectra could be recorded as low as 190 nm as the normally applied cut-offs for typical HPLC solvents such as methanol or acetonitrile were absent. N.B. the theoretical cut offs for acetonitrile is 190nm and for methanol 205nm for qualitative work, and 180nm and 240nm, respectively, for quantitative work (assuming the solvents are pure). Above 360 nm very few compounds tend to absorb light as this is approaching the visible part of the spectrum and the vast majority of pharmaceutical compounds are colourless, although there are some

exceptions to this. From the diode array detector (DAD) the flow passed along 110cm of 0.005" i.d. PEEK tubing to a Bio-Rad (Cambridge MA, USA) FT-IR model 375C spectrometer fitted with a Spectra Tech (Stamford CT, USA) Macro Circle Cell ATR(attenuated total reflectance) stainless steel flow cell with a volume of 400 μL fitted with a zinc selenide ATR crystal. Spectra were acquired with the Kinetics software, collecting 20 scans per spectrum (5s acquisition time) with a sensitive MCT (mercury cadmium telluride) liquid nitrogen cooled detector. The spectra were acquired at 8 $cm^{-1}$ spectral resolution. The samples were ratioed against a background spectrum of the flowing $D_2O$ eluent as it was passing through the cell just prior to injection of each sample solution. These background spectra were automatically subtracted from the spectra acquired for each sample. This particular FT-IR spectrometer allowed on-line collection of spectra and was also able to cope with the solvent as it flowed so it was placed prior to the mass spectrometer and NMR spectrometer and before any splitting of the solvent flow. Previously it has been shown that FT-IR spectrometer was one of the less responsive of the techniques being attempted and so utilising the total amount of sample would also give an advantage in terms of sensitivity. Following the FT-IR spectrometer the eluent passed through 150 cm of 0.005" i.d. PEEK tubing to the UV detector of the Bruker HPLC system. The reason for this "doubling back" was to allow for a time point to be recorded by the Bruker software, which would allow for stop flow experiments if necessary in the NMR cell. Additionally, using the UV detector here allowed for the determination of band broadening resulting from using the large 400 μL flow cell in the FT-IR spectrometer. From the UV detector the flow was split approximately 95:5 with 5% of the flow being directed towards the mass spectrometer via 250 cm of 0.007" i.d.

PEEK tubing and the remainder to the NMR spectrometer via 280 cm of 0.010" i.d. PEEK tubing. The details of the layout can be found in Fig. 5.1.

Mass spectra were acquired in this instance on a Micromass LCT time of flight (ToF) mass spectrometer using orthogonal acceleration electrospray ionisation with a Z spray source. The nebuliser gas flow was set to 85 L/hour and the desolvation gas to 973 L/h. Spectra were acquired in either positive or negative ion mode as this spectrometer was not capable of switching between positive and negative ion within a useful time. The capillary voltage was set to 3.2 kV and the cone voltage was set at a fairly low value of 25 V to generate as many parent ions and the minimum number of fragment ions as possible. The source temperature was set to 120C and the desolvation temperature to 350C. The pusher cycle time was 50 μs with 0.9 s acquisitions and an inter acquisition delay of 0.1 s over the mass range m/z 100 to 900. Diclofenac, m/z 295.0152, was used to provide a lock mass for the spectrometer in negative ion mode and leucine enkephalin, m/z 556.2771, for positive ion work. These lock masses were introduced via a T-piece at 0.5 mL/min and a concentration of approximately 5 ng/mL; the exact concentration being adjusted to give approximately 300-500 counts signal intensity to allow good accurate mass confirmation.

All the above instrumentation was located outside of the 5 gauss line of the stray magnetic field generated by the 500MHz NMR spectrometer to prevent any interference.

NMR spectra were acquired with the previously described Bruker DRX-500 NMR spectrometer. On-flow $^{1}H$ NMR detection was carried out in the pseudo-2D mode at 500.13 MHz using the 4 mm id, 120μL volume, flow through probe. Typically, 4 FIDs per increment were acquired into 4k data points each with a spectral width

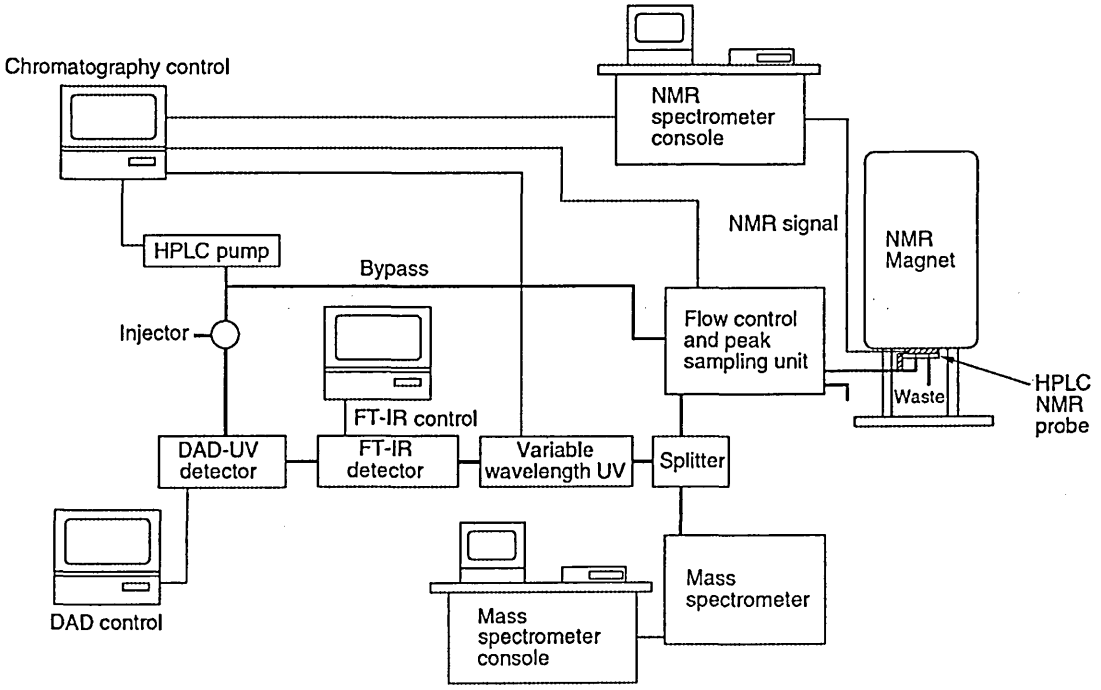



Fig. 5.1 *Schematic of the layout of the various spectrometers used in the FIA system*

of 8278 Hz. Spectra were acquired using the NOESYPRESAT pulse sequence in order to suppress residual acetonitrile signals which were present due to the leaching of acetonitrile from the pump and capillaries from previous experiments conducted using the equipment. 90° pulses were used with an acquisition time of 0.25s, a relaxation delay of 1 s and a mixing time of 100 ms. For those experiments where stopped flow analysis was also conducted, the NOESYPRESAT pulse sequence was used for double solvent suppression. FIDs were then collected into 16k data points over the same spectral width, which resulted in an acquisition time of 0.99 s. A relaxation delay of 2 s and a mixing time of 100 ms were used.

## 5.3 Results

The layout shown in Fig. 5.1 was reached after some trial and error but based on the knowledge acquired from the other systems previously described. The physical size of the equipment and the utilities required placed some constraints on locations within the room available, but the main restriction was to remain outside of the 5 gauss stray field line of the NMR spectrometer. With greater flexibility of utilities and room shape and size this layout could undoubtedly have been improved with potentially improved performance as a result. Nonetheless this layout proved reliable and robust for the purposes of these experiments.

### 5.3.1 Limits of detection

As shown in previous chapters, the wide range of sensitivities of the different techniques required investigation before this set up could ever be considered for use on a routine basis in an analytical laboratory. The ToF mass spectrometer was capable of providing information on substances in the flowing stream at concentrations in the pg range whilst both the NMR and FT-IR instruments used required circa 50 –100 μg (in the on-flow mode) for good quality spectra, with the DAD falling somewhere between the NMR and MS detection limits depending upon the component studied. Given that the sensitivities of the NMR and FT-IR spectrometers were the limiting factors for this device the limits of detection were determined based on the minimum system requirements that would provide complete on-flow spectral characterisation for all techniques i.e. UV, IR, $^{1}$H NMR, and accurate mass MS with atomic composition determination. In order to determine this, 200 μL

aliquots of antipyrine, 4-hydroxyantipyrine and salicylic acid were injected into the FIA system over a wide range of concentrations (see below) at a flow rate of 1 mL/min. For antipyrine the concentrations examined ranged from 250 μg/mL up to 100 mg/mL which corresponded to 50 μg to 20 mg injected. For 4-hydroxyantipyrine and salicylic acid the upper concentration was limited to 25 mg/mL (5mg injected) based on the results obtained with antipyrine. For both ToF MS and UV-DAD even the smallest quantities represented a vast excess over the amount actually necessary to obtain spectra. Therefore in order to obtain meaningful spectral data the spectra had to acquired from the trailing edge of the plug of analyte as it flowed through the system. Ideally the samples would have been diluted after the splitter but there was neither time nor available resources to successfully implement this here. This would have also necessitated the DAD being placed after the split and in line with the mass spectrometer. (Alternatively a smaller UV flow cell than the 1cm one used here could have been employed but this was not an available option in the time available and even had a smaller cell been employed it is likely the detector would still have been overloaded due the large amounts of sample injected to facilitate the acquisition of IR and NMR spectra). However, despite these problems the quality of the UV data obtained was adequate for the purposes of compound identification. Similarly the ToF-MS data obtained from the trailing edge of each peak was suitable for providing accurate molecular mass and atomic composition data once allowances had been made for deuterium exchanges.

The results obtained from these experiments showed that both FT-IR and NMR spectra could be obtained on-flow with as little as 50 μg of material in a 200 μL injection. Thus Fig. 5.2 shows the FT-IR spectra obtained for antipyrine over the

whole range, from 250 μg/mL up to 100 mg/mL. As these spectra show there is little to choose between the FT-IR spectra of the samples containing between 100 and 1 mg/mL (the latter corresponding to 200 μg injected). Even the spectrum obtained with the 250 μg/mL (50 μg injected) sample shows all of the essential features of the

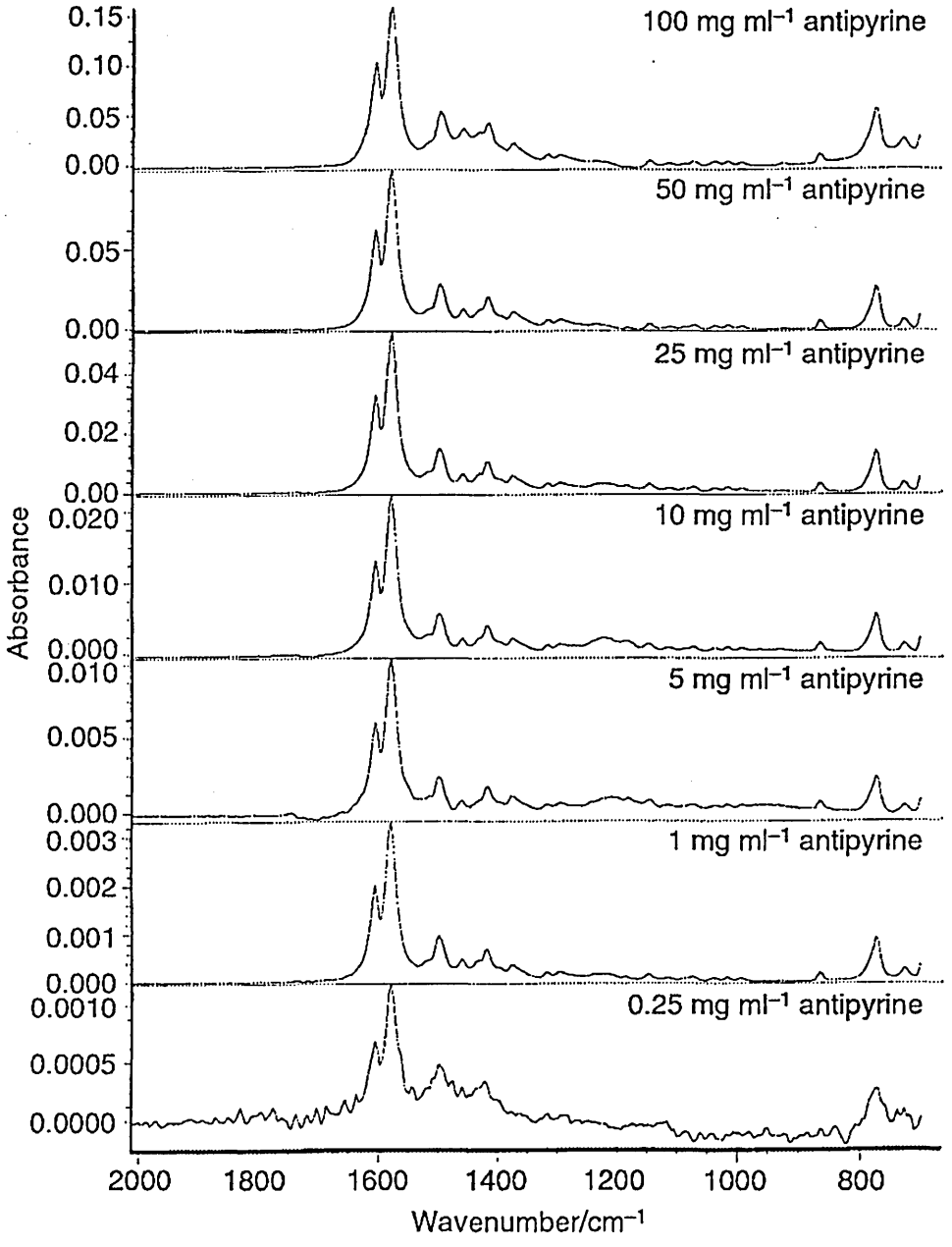



Fig. 5.2 *On-flow FT-IR spectra obtained for antipyrine for 250 μg/mL up to 100 mg/mL*

IR spectrum of antipyrine. With the use of an improved FT-IR flow cell design and a reduced flow rate of say 0.5 or 0.25 mL/min there is little doubt that the sensitivity could have been improved still further.

The equivalent $^{1}H$ NMR and ToF MS spectra are shown for the 250 μg/mL sample of antipyrine in Fig. 5.3 (with the UV spectrum shown inset to the mass spectrum) and, as with the FT-IR spectrum, they are perfectly adequate for the purposes of identification by FIA analysis.

Lowering the flow rates would also enhance the NMR spectra obtained. The configuration of the system allowed for stopped flow NMR analysis due to the positioning of the UV detector of the Bruker LC system prior to the NMR probe, and stopped flow NMR spectroscopy of a sample of 50 μg of antipyrine in this system gave excellent quality spectra after just 64 scans. The use of stopped flow techniques in this manner would also have permitted two dimensional NMR spectra to be obtained when required, giving still further structural information.

These results regarding the effective sensitivity of the combination of techniques were confirmed by data obtained from 4-hydroxyantipyrine and salicylic acid, which gave similar results.

## 5.4 The application of multiple spectroscopic techniques to the assessment of a model library

Having shown that it was possible to obtain on-flow spectra from these three test compounds[93,94] utilising as little as 50 μg of material the next challenge was to attempt

to produce spectra for a library of model compounds. For these experiments 200 μL

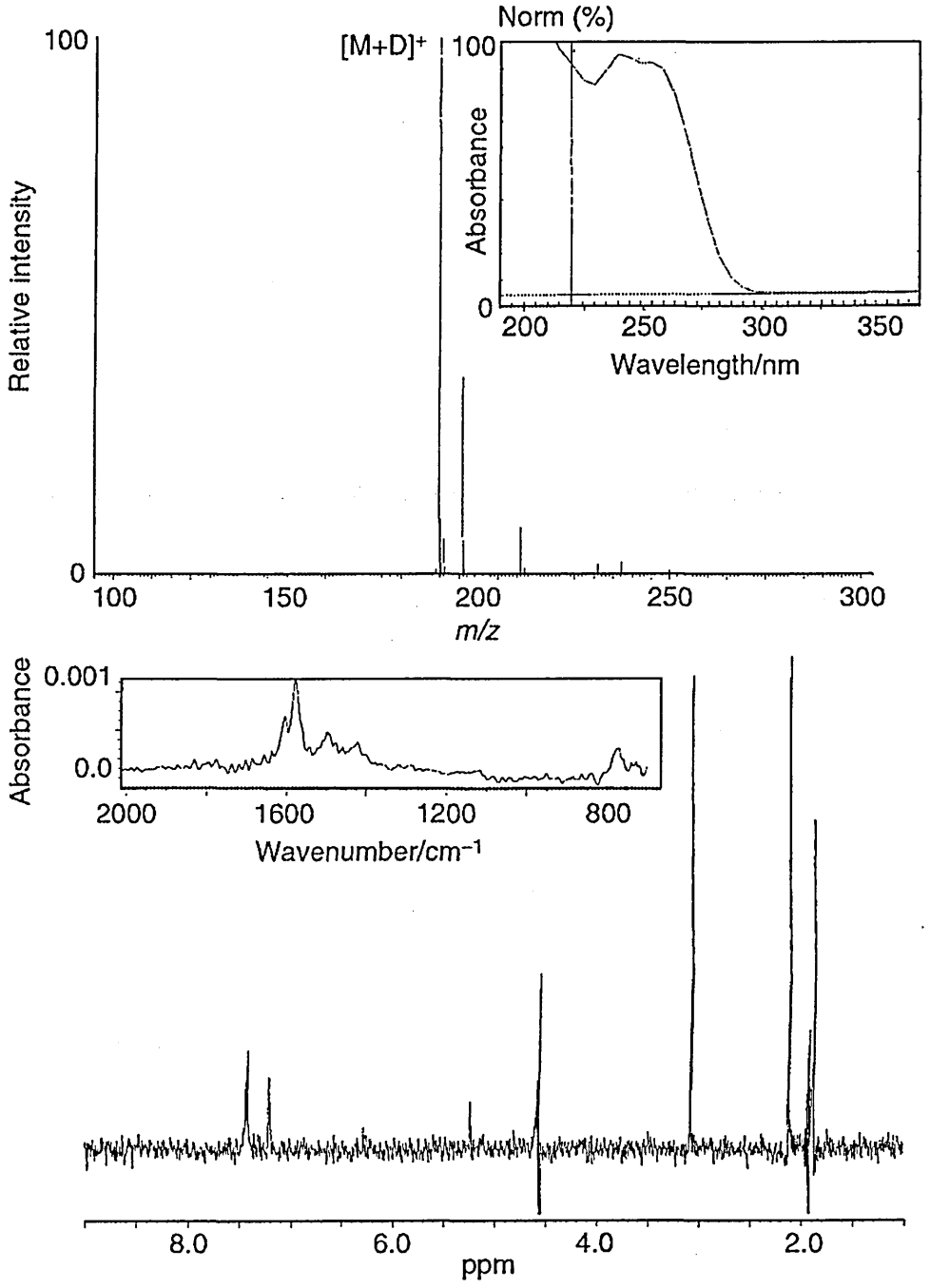



Fig. 5.3. *The ToF mass spectrum (top), the UV spectrum (inset top), the $^{1}H$ NMR spectrum (bottom) and the FTIR spectrum (inset bottom) obtained on-flow for a 250 μg/mL sample of antipyrine. The higher m/z ions in the mass spectrum are contaminants from a previous experiment.*

aliquots of samples containing between 5 and 10 mg/mL (i.e. between 1 and 2 mg injected) were used. The on-flow $^1$H NMR spectra and pseudo chromatogram obtained following the injection of six model compounds of a type similar to those one might expect to find in a typical pharmaceutical company compound collection are shown in Fig. 5.4, together with the on-flow $^1$H NMR spectra extracted from the pseudo-chromatographic experiment for the individual compounds. In all cases this concentration of analyte provided sufficient material to generate $^1$H NMR spectra that

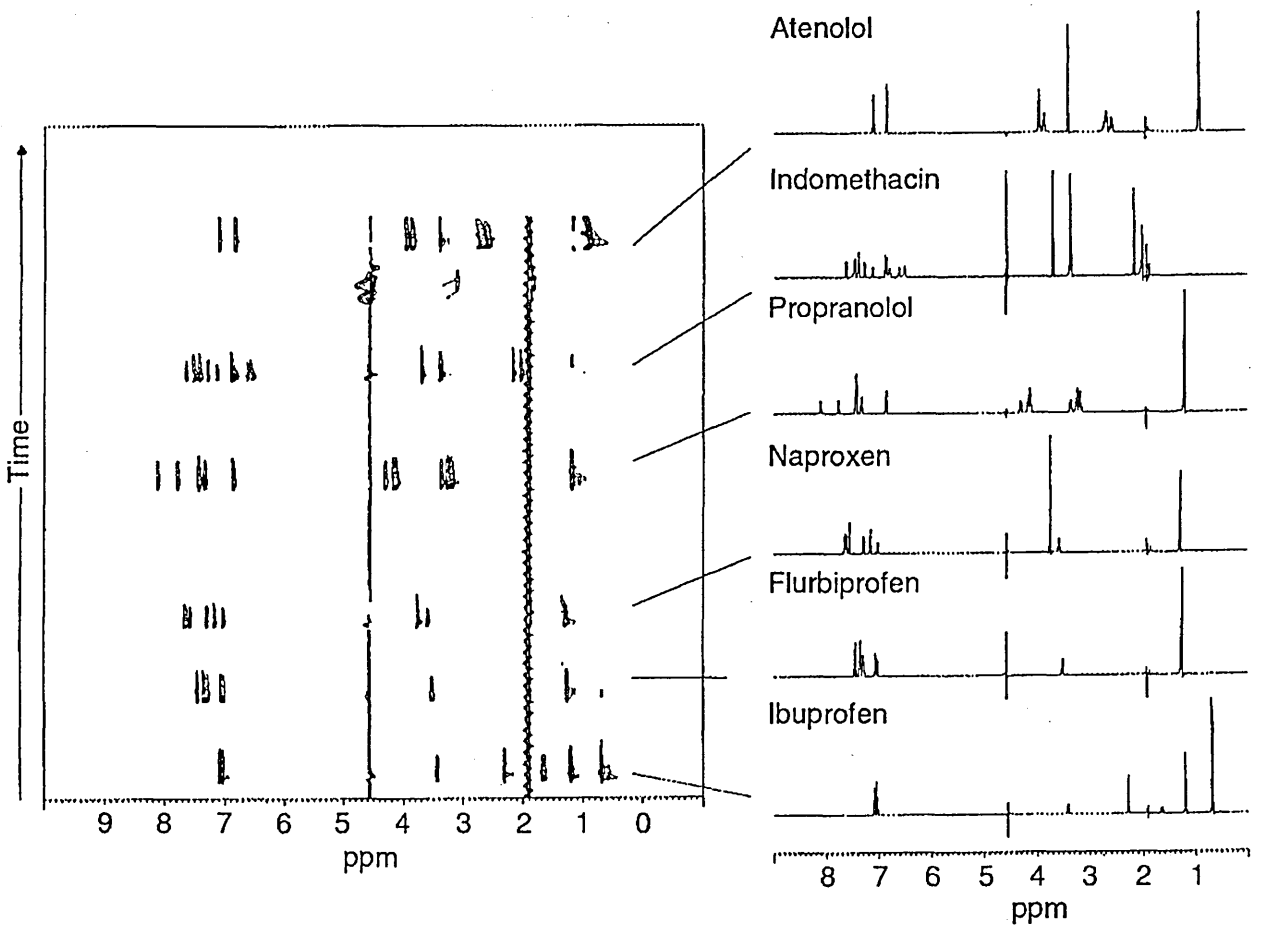



Fig. 5.4 *$^1$H NMR pseudo-chromatogram of a model library (left) showing the spectra extracted (right) from the individual peaks. The time axis is shown on the vertical scale for the pseudo-chromatogram. The chemical shifts are shown on the horizontal axis in both figures.*

showed all of the expected structural features. Similarly, good quality FT-IR and UV data were obtained for all of the components (Fig. 5.5). In the case of the ToF-MS, reasonable results for molecular mass and atomic composition were obtained for the acidic non-steroidal anti-inflammatory drugs used as model analytes by negative ion electrospray. In positive ion electrospray the two basic beta-blockers also gave reasonable results. (see table 5.1)

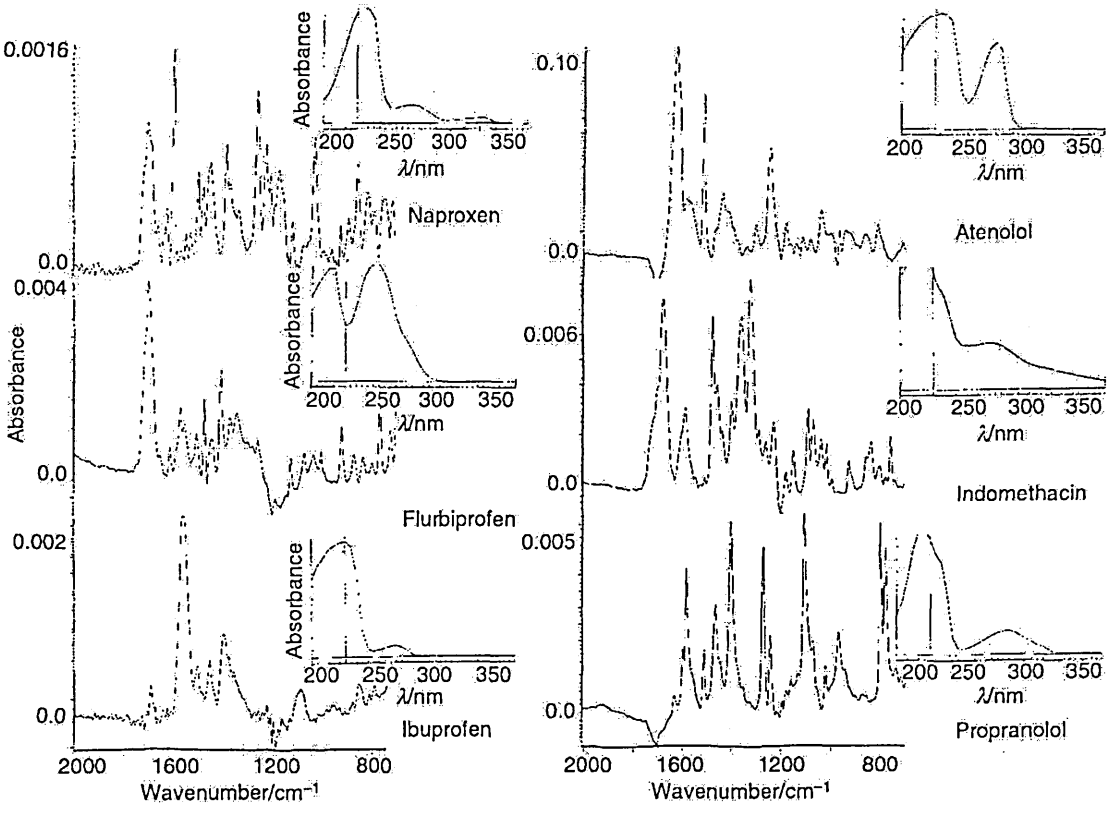



Fig. 5 *FT-IR and UV spectra of the six components extracted following pseudo-chromatography of the model library.*

The inability to switch between positive and negative ion using a time of flight instrument was one of the drawbacks of this series of experiments as it meant that unknowns would need to be run twice to be certain of capturing the parent ion.

For good mass assignment and atomic composition determination, an error of less than 10 ppm and preferably less than 5 ppm would have been sought. However, the large samples used to ensure that adequate FT-IR and NMR spectra were obtained quickly and easily made it more difficult to acquire mass spectra where both the

| Name | Error mDa | Error ppm | Hit Number | Formula |
|---|---|---|---|---|
| Ibuprofen | -5.7 | -27.6 | 10 | $C_{13}H_{18}O_2$ |
| Flurbiprofen | 0.5 | 1.9 | 2 | $C_{15}H_{13}FO_2$ |
| Naproxen | -3.2 | -13.8 | 13 | $C_{14}H_{14}O_3$ |
| Indomethacin | -0.7 | -1.9 | 4 | $C_{19}H_{16}ClNO_4$ |
| Propranolol | -0.6 | -2.1 | 3 | $C_{16}H_{21}NO_2$ |
| Atenolol | -3.1 | -11.5 | 6 | $C_{14}H_{22}N_2O_3$ |

Table 5.1 *Relative errors and hit numbers of the six components within the model library after processing within the MassLynx software of the mass spectrometer. For increased certainty of identification an error of less than 10ppm and preferably better than 5ppm is required. The heavy loading of the samples made this type of accuracy unattainable for the components of the model library. As can be seen even when the error was less than 5ppm none of the compounds was identified as the first hit, thus emphasising the importance of good mass accuracy.*

analyte signal and the lock mass signal were also at the correct level. Mostly the data were selected at what appeared to be the very bottom of the peaks so as not to induce errors due to overloading. The application of simple mass spectrometry rules helped

to eliminate many false positives. For instance, in the case of Naproxen four of the hits higher than the real hit all contained chlorine. Chlorine is easily recognised by its very distinct isotope pattern and hence these hits could quickly be ignored. Typical results for flurbiprofen and atenolol are shown in table 5.2. The quality of the mass spectra produced was not an issue, as can be seen from those shown in Fig. 5.6. Each spectrum gave all of the expected ions for each of the samples studied. Some carry

**Atenolol found mass 267.1678**

| Formula | Calc. Mass | mDa | PPM | DBE |
|---|---|---|---|---|
| | | | | |
| $C_{11}H_{25}NO_6$ | 267.1682 | -0.4 | -1.5 | 0 |
| $C_9H_{23}N_4O_5$ | 267.1668 | 1 | 3.6 | 0.5 |
| $C_{12}H_{21}N_5O_2$ | 267.1695 | -1.7 | -6.5 | 5 |
| $C_9H_{24}N_6OCl$ | 267.1700 | -2.2 | -8.3 | 0.5 |
| $C_9H_{22}N_5O_3F$ | 267.1707 | -2.9 | -10.7 | 1 |
| **$C_{14}H_{23}N_2O_3$** | **267.1709** | **-3.1** | **-11.5** | **4.5** |
| $C_{11}H_{26}N_3O_2Cl$ | 267.1714 | -3.6 | -13.3 | 0 |
| $C_{12}H_{25}N_2OFCl$ | 267.1639 | 3.9 | 14.4 | 0.5 |

**Flurbiprofen found mass 243.0826**

| Formula | Calc. Mass | mDa | PPM | DBE |
|---|---|---|---|---|
| | | | | |
| $C_{12}H_{15}NOFCl$ | 243.0826 | 0 | -0.1 | 5 |
| **$C_{15}H_{12}O_2F$** | **243.0821** | **0.5** | **1.9** | **9.5** |
| $C_{15}H_{14}NCl$ | 243.0815 | 1.1 | 4.6 | 9 |
| $C_{15}H_{14}NCl$ | 243.0813 | 1.3 | 5.4 | 5.5 |
| $C_7H_{11}N_6O_4$ | 243.0842 | -1.6 | -6.5 | 5.5 |
| $C_{18}H_{11}O$ | 243.0810 | 1.6 | 6.6 | 13.5 |

Table 2. *Typical elemental composition results for Atenolol and Flurbiprofen (the correct results are shown in bold type). The table shows the nearest exact mass (calc. mass) to the mass found and the formula of that exact mass together with the error of this mass to the found mass in mDa and ppm. DBE is the number of Double Bond Equivalents for that formula and mass combination.*

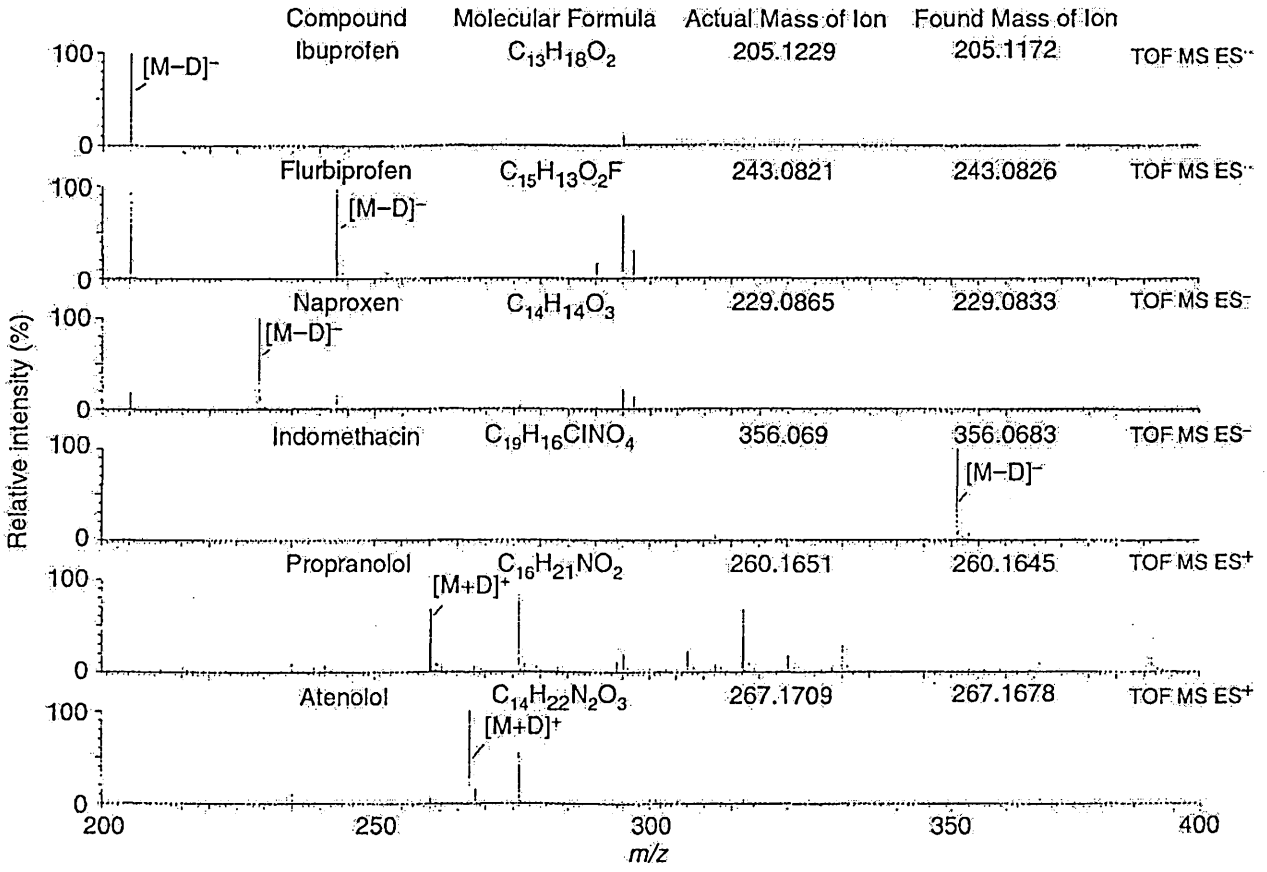



Fig. 5.6 *Mass spectra of the six components showing $(M+D)^+$ or $(M-H)^-$ ions as well as additional ions caused by carry-over from other samples due to the relatively large amounts of sample injected*

over was seen, but at these elevated concentrations this was neither unreasonable nor unexpected.

## 5.5 Further flow injection studies.

These experiments clearly demonstrated the possibility of using this multiply-hyphenated system to characterise samples in a typical analytical laboratory. However, the real aim was to allow some degree of automation and thus to save time

and effort in the acquisition of data sets. Further modifications were made to allow some such automation.

The compounds employed in this new set of experiments were paracetamol (acetaminophen), caffeine, p-aminobenzoic acid, α-hydroxyhippuric acid, propranolol, p-aminohippuric acid, 4-aminoantipyrine, 4-dimethylaminoantipyrine, antipyrine and hippuric acid (Sigma, Fluka and Aldrich UK). The samples were dissolved at concentrations of between 1.4 and 8.4 mg/mL (see below) in deuterium oxide $D_2O$ containing 0.1% (deutero)formic acid.

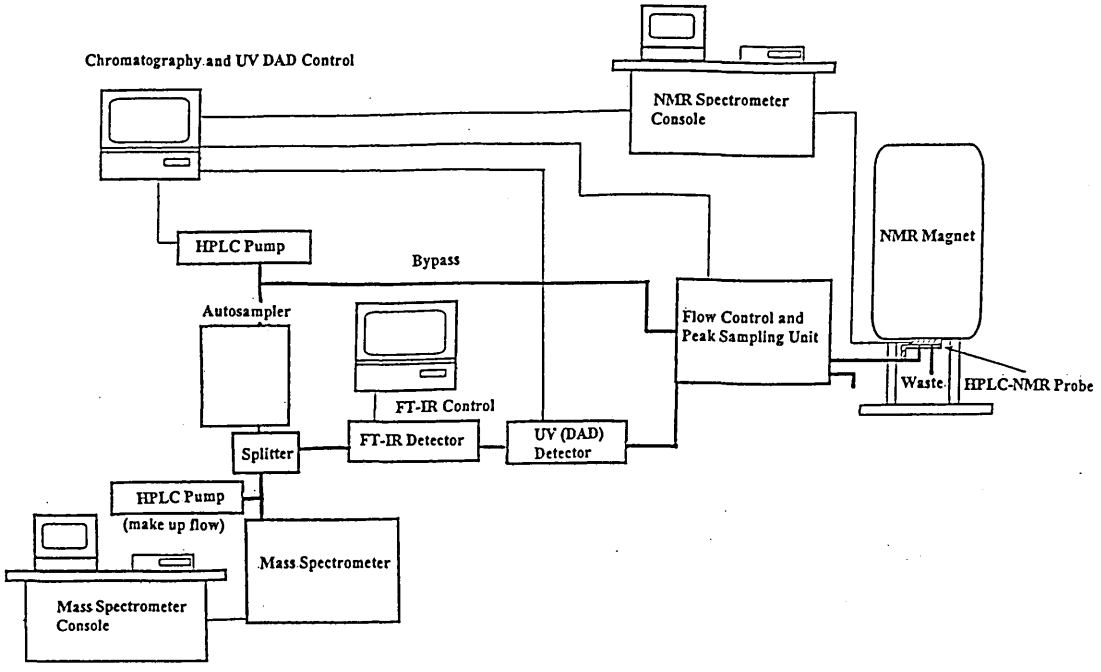



Fig. 5.7 *Layout of the modified flow injection system used to attempt an automated approach. The autosampler, for automated sample introduction, and the make-up flow, introduced prior to the mass spectrometer to create a backpressure to ensure an adequate split, are shown*

This modified flow injection system is shown in Fig. 5.7 and consisted of a Constametric 3200 HPLC pump (LDC, UK) which delivered $D_2O$:0.1% (deutero)formic acid at 1mL/min. Typically, 100 μL of sample was injected into the flow stream at 6 min intervals using a Perkin-Elmer ISS 200 autosampler (Perkin Elmer, UK), which was fitted with a 200 μL sample loop. From the autosampler the flow went via 25 cm of 0.020" i.d. PEEK tubing to a splitter from which 60% of the flow was then directed, via 110 cm of 0.020" i.d. PEEK tubing, to a BioRad model FTS3000 Excalibur series FT-IR spectrometer (Cambridge, MA, USA) fitted with a Spectra Tech (Stamford, CT, USA) Micro Circle ATR high pressure stainless steel flow cell of 25 μL volume zinc selenide ATR crystal. As previously, the spectrometer compartment containing the flow cell was purged with dry nitrogen to minimise any interference from water vapour in the IR beam. Any residual water was subsequently subtracted from the collected data using a water vapour reference spectrum. Spectra were acquired with the kinetics software collecting 57 scans per spectrum (10s acquisition time) with a MCT liquid nitrogen cooled detector. As previously the spectra were acquired at 8 $cm^{-1}$ spectral resolution. Each sample was ratioed against a background spectrum of the solvent flowing through the flow cell prior to injection of the sample solution, thus automatically subtracting out the solvent spectrum from the sample spectra.

Simultaneously, the remaining 40% of the flow from the splitter was directed to a Micromass Platform single quadrupole mass spectrometer (Micromass UK) via a 100 cm length of 0.010" i.d. PEEK tubing. Mass spectra were acquired over the mass range m/z 115 to 650. The lower limit of this range was chosen to cut off the background from a previous eluent containing trifluoroacetic acid which could not be

completely removed within the time available these experiments. Prior to the sample entering the ion source of the mass spectrometer, the flow was mixed with a make up flow of 90:10 methanol:water, introduced via a T-piece at 0.5 mL/min. This was introduced to provide a backpressure to ensure that the bulk of the flow was directed to the IR spectrometer, and then onto the NMR and UV spectrometers. Positive ion spectra were recorded with a cone voltage of 25 V, a scan time of 0.9 s and an inter scan delay of 0.1 s.

The outlet of the FT-IR flow cell was connected to a Bruker DAD (Bruker UK) via 30 cm of 0.010" i.d. PEEK tubing. UV spectra were collected over the wavelength range 188-400nm. From the DAD the solvent stream was connected to the NMR spectrometer via 280 cm of 0.010" i.d. PEEK tubing. As previously all of the instrumentation was kept outside of the 5 Gauss line of the stray magnetic field from the 500MHz NMR spectrometer.

The NMR spectra were acquired using the Bruker DRX-500 NMR spectrometer. On-flow $^{1}$H-NMR detection was carried out in the flow injection mode at 500.13 MHz using a flow probe of 3mm i.d. with a volume of 60 μL. Typically, 16 FIDs per increment were acquired into 8k data points each with a spectral width of 8278 Hz. Spectra were once again acquired with the NOESYPRESAT pulse sequence to suppress residual water resonances. 90° pulses were used with an acquisition time of 0.5 s, a relaxation delay of 0.7 s and a mixing time of 100 ms.

The aim of these changes was to move towards a more automated approach than that described in the earlier part of this chapter, so an auto-sampler was an obvious

addition to provide unattended operation. This system was slightly less elaborate than that described earlier as an upgrade in the software controlling the NMR spectrometer and the Bruker LC system meant that UV data could be obtained directly without the need to pass the solvent through the Bruker UV detector in order to obtain a timing signal or through a separate DAD to acquire the UV spectra. In a further modification the mass spectrometer was positioned directly after the auto-sampler rather than splitting the flow later in the system at the point where the flow was directed to the NMR spectrometer. This ensured that the mass spectra were obtained early in the overall process of spectral acquisitions, allowing mass directed analysis to decide for instance whether to perform a stopped flow NMR study in order to obtain a two dimensional NMR spectrum if such information should be required for complete structural identification. If, as is often the case, a mass list of expected products was to be input at the start of a batch of samples and an unexpected mass was detected it would not be difficult to program the automatic acquisition of a two dimensional NMR spectrum via a stopped flow experiment. Thus a potential impurity could be characterised from a single injection, especially useful for important or precious samples where the use of multiple injections would be less desirable. Alternatively, if stopping the flow was deemed undesirable or too difficult to achieve, the flow could be diverted to the peak sampling unit of the NMR spectrometer. This was a series of sample loops through which the eluent flow could be redirected and the sample peak of interest isolated for subsequent off line NMR analysis after the chromatographic runs had been completed. This approach would also maximise the throughput of the system rather than interrupting the acquisition of a series of samples to focus upon just one. Despite these modifications this system, like previous ones was not fully optimised but time and space constraints prevented further development at the time.

### 5.5.1 System performance

As shown earlier, depending on the chromophore and the ionisation energy of the compound in question under the conditions used for the acquisition of mass spectra, it is generally expected that the UV and MS detection will be more sensitive than either the NMR or IR. Therefore for all practical purposes the minimum amount of sample required was defined as that amount which would permit a compound's characterisation rather than just its detection. Inevitably this amount was compound dependent. As an example, in the case of caffeine (Fig. 5.8), the minimum amount of material required for $^{1}$H-NMR detection under these conditions, based on the three methyl signals seen for caffeine between 3 and 4 ppm, was in the region of 10 μg. However, at this concentration the signal for the single proton at ~ 7.6 ppm was undetectable. In order to obtain a full spectrum approximately 50 μg was required.

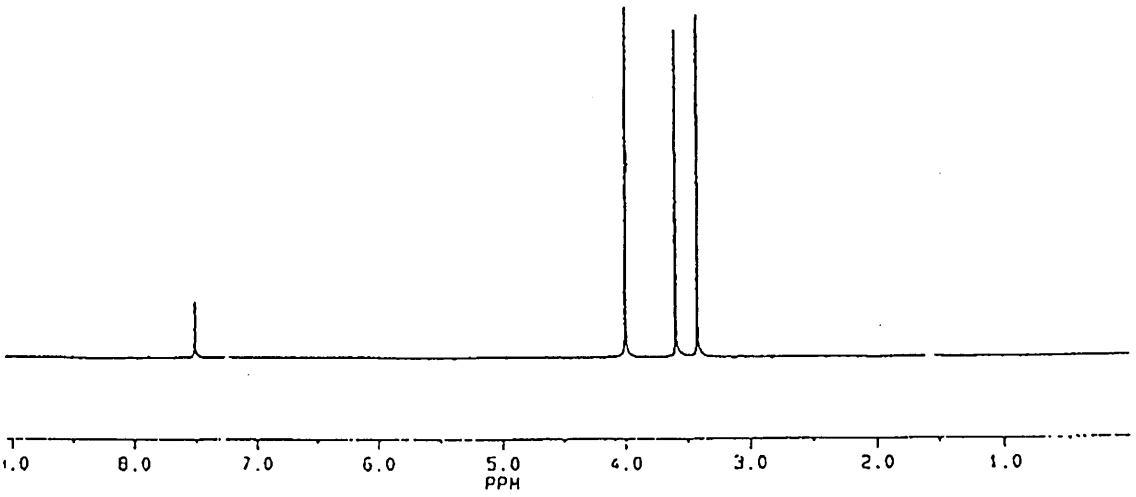



Fig. 5.8 *A typical $^{1}$H-NMR spectrum of caffeine (50 μg) showing the difference in intensities of the four signals and in particular the much weaker signal for the single proton at approx 7.6 ppm. Clearly detection of the three methyl signals could be achieved at a lower concentration but the signal would then not be visible.*

For propranolol, a more realistic model for drugs, the spectrum is complex due to spin-spin coupling and approximately 70 μg of material was required for an adequate $^{1}$H-NMR spectrum. Using this as a guide, twice this amount was chosen as a sensible amount to inject to ensure that adequate spectra would be obtained without the need for stop flow experiments, or to have to re-inject the sample at an elevated concentration. Therefore the range of concentrations used for the 10 compounds studied here in this model library was from 140 μg (paracetamol) up to 840 μg (antipyrine), which was also analysed at 210 μg injected. These figures represented a significant improvement over the earlier experiments and were largely due to the use of the improved NMR cell. The exact amounts injected for each compound are shown in the caption to Fig. 5.13. All samples were injected in 100 μL at 1 mL/min.

### 5.5.2 Automated analysis of a model drug library.

For the analysis of this model drug library, the samples were injected automatically every 6 minutes. This interval was chosen to ensure that there was minimal carry over, which had been a problem in previous studies. Careful examination of the spectra obtained using this approach showed no evidence of cross contamination. Extrapolating this injection rate gives a theoretical throughput of 240 samples per day for this system. This figure might have been improved by further optimisation. The cost of constructing such an array of spectrometers is many hundreds of thousands of pounds and high throughput would be needed to justify the expense.

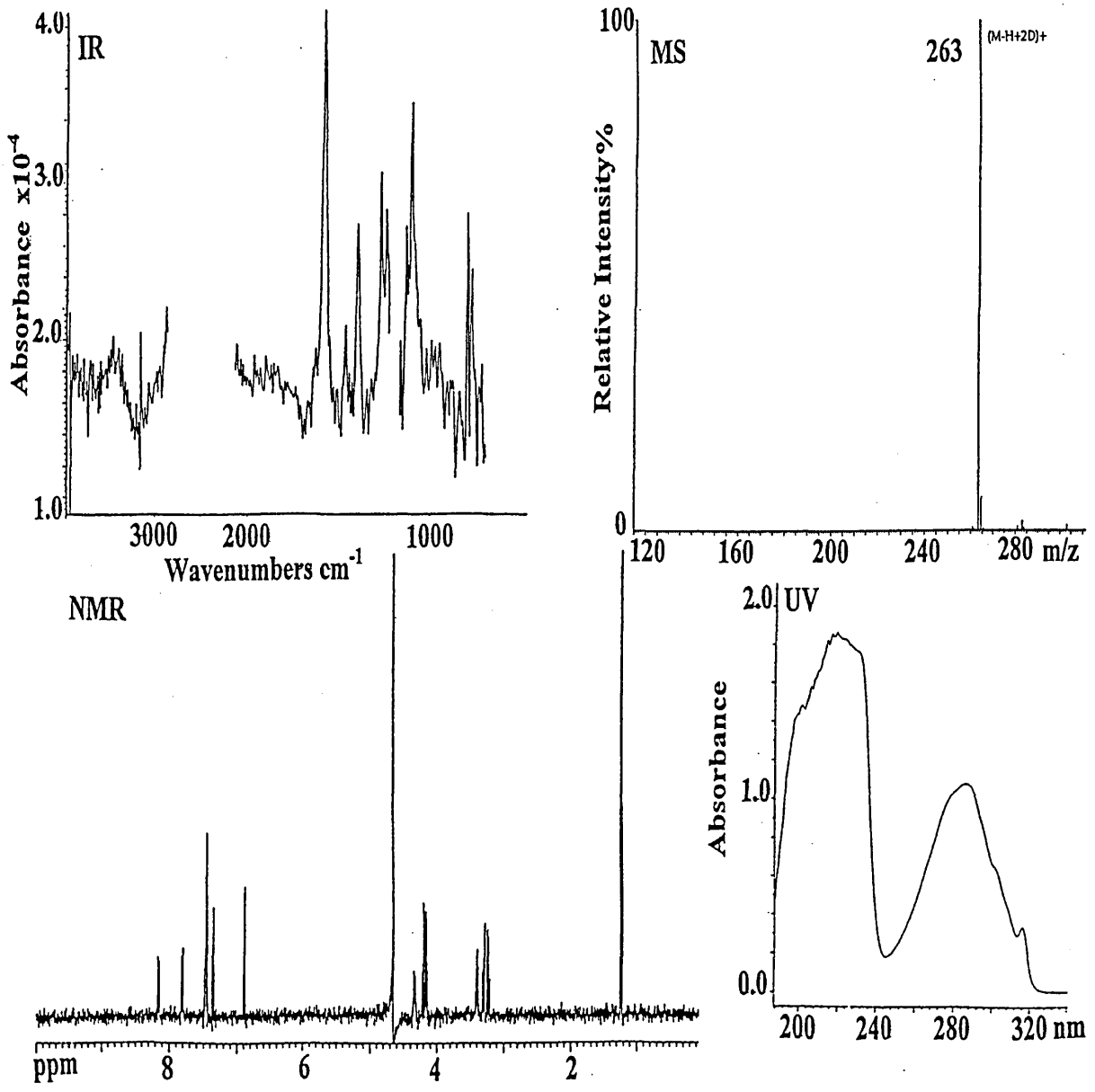



Fig. 5.9 *The full set of spectra (IR, MS, NMR and UV) obtained for propranolol (215μg injected in 100 μL) in flow injection mode.*

As predicted the comparatively high (in MS and UV terms) amounts of analytes used for this study meant that detection of these model compounds was not difficult for either the DAD or the MS. All ten compounds from the library gave the UV spectra expected (e.g. Figs. 5.9 and 5.10). The majority of samples gave ions of the type $(M+D)^+$, and in some cases $(M+Na)^+$ ions were also observed. The exact number of

deuterons associated with each compound was dependent upon the number of exchangeable protons available on each compound. The structures and the number of exchangeable protons for each compound are shown in table 5.3. Typical results (including IR, UV and NMR spectra) for propranolol are shown in Fig. 5.9. The result for p-aminobenzoic acid showed that this sample also

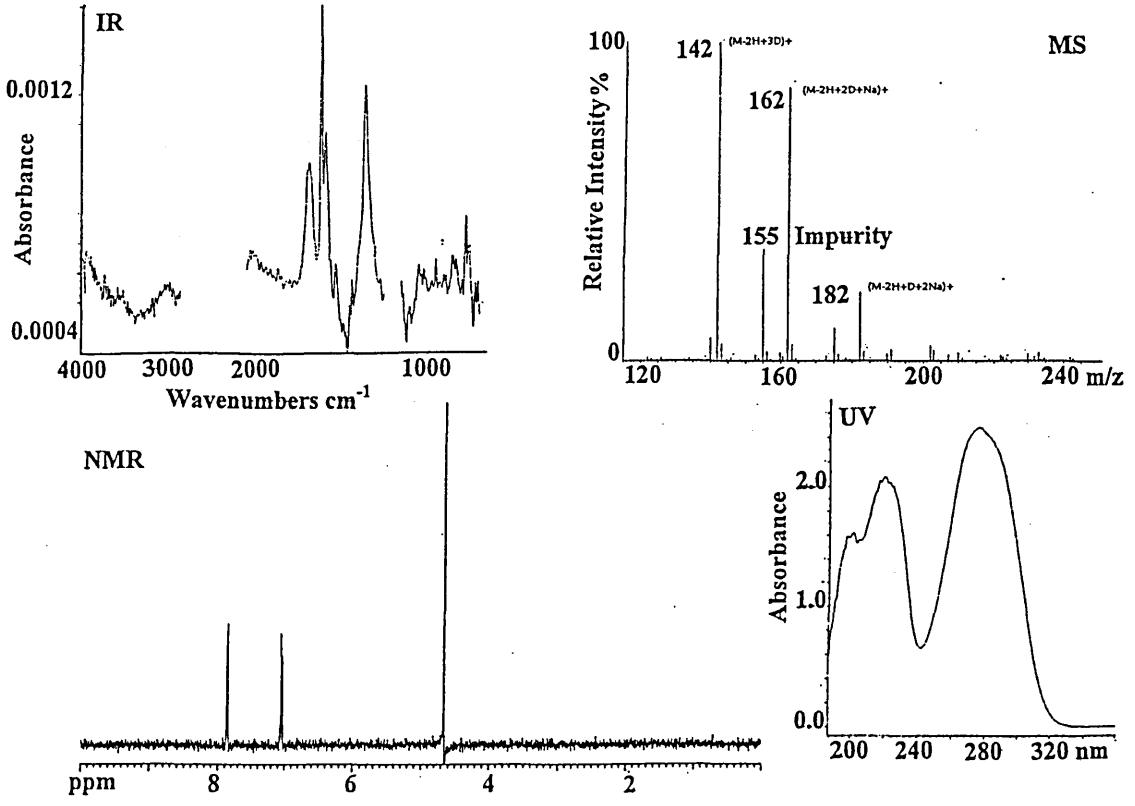



Fig. 5.10 *The IR, MS, NMR and UV spectra obtained for p-aminobenzoic acid (185 μg injected in 100 μL). The MS spectrum shows* $(M\text{-}2H+3D)^+$ *along with sodiated versions as* $(M\text{-}2H+D+Na)^+$ *and* $(M\text{-}2H+D+2Na)^+$*. Additionally a prominent ion at m/z 155 is seen indicating the possible presence of an unknown impurity. The NMR spectrum showed only signals for p-aminobenzoic acid, indicating that the impurity forms only a minor component of the sample.*

appeared to contain an impurity (m/z 155); however, in this on-flow experiment the evidence from other techniques and in particular the NMR spectrum, suggested that this must have been due to the presence of a fairly low concentration component as no additional signals other than those expected were detected. The MS, UV, IR and NMR spectra for p-aminobenzoic acid are shown in Fig. 5.10. Another interesting example was provided by α-hydroxyhippuric acid where the dominant ion was observed at m/z 248 (Fig. 5.12). This ion was not due to the presence of an impurity, but rather represented an adduct of the analyte with the formate present in the $D_2O$ eluent (as with p-aminobenzoic acid the NMR, IR and UV spectra were consistent with those of standards). In some cases the analytes overloaded the MS and adduct

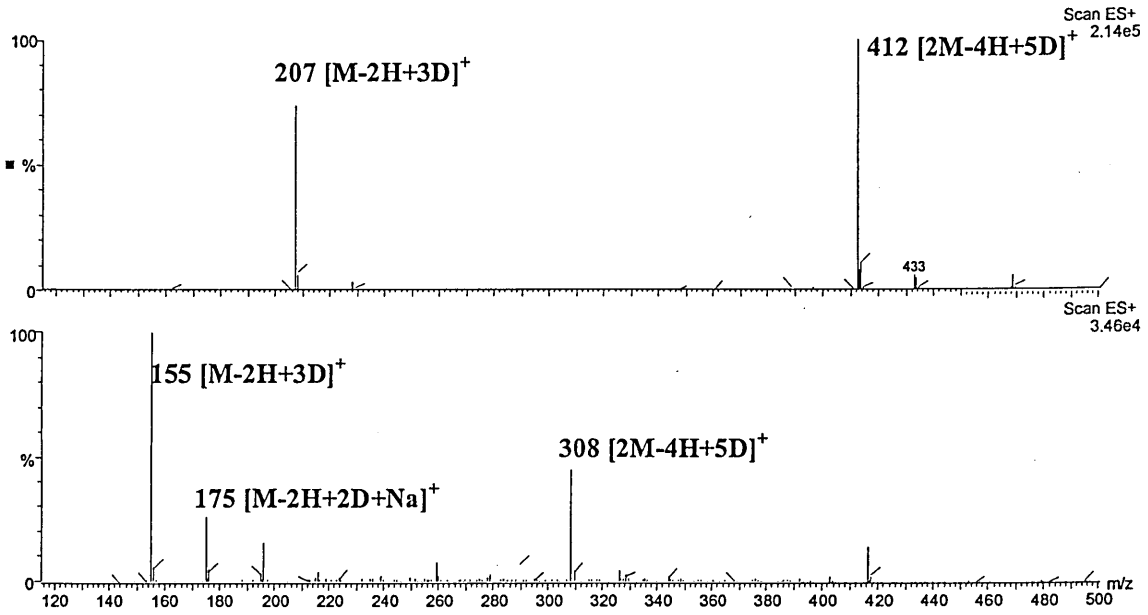



Fig. 5.11 *MS spectra of 4-aminoantipyrine (top) and paracetamol (bottom) obtained from the model library. The top spectrum shows the dimer formed at m/z 412 due to the large amount of material passing to the MS. The lower spectrum for paracetamol shows a similar adduct at m/z 308 along with multiple sodium adducts although only the ion at m/z 175 is labelled.*

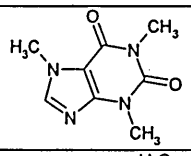
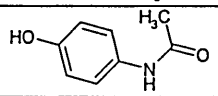
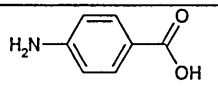
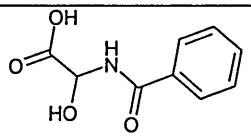
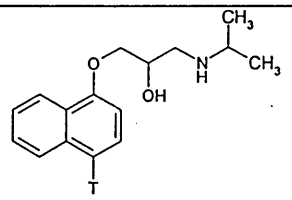
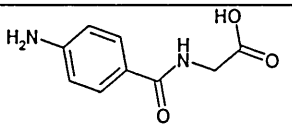
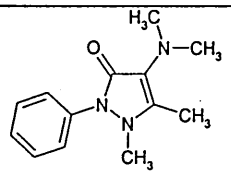
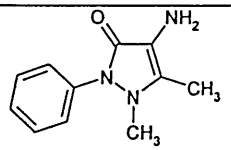
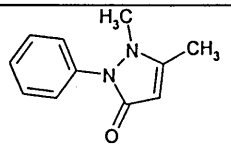
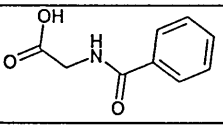
| | | |
|---|---|---|
| caffeine |  | 0 exchangeables |
| acetaminophen |  | 2 exchangeables |
| p-aminobenzoic acid |  | 3 exchangeables |
| α-hydroxyhippuric acid |  | 3 exchangeables |
| propranolol |  | 2 exchangeables |
| 4-aminohippuric acid |  | 4 exchangeables |
| Dimethylamino antipyrine |  | 0 exchangeables |
| aminoantipyrine |  | 2 exchangeables |
| antipyrine |  | 0 exchangeables |
| hippuric acid |  | 2 exchangeables |

Table 5.3 *The structures of the compounds in the model library and the number of exchangeable protons present in each molecule.*

ions of the type $[2M + D]^+$ or $[2M + Na]^+$ were observed. Such results highlight the effect of overloading the MS and show the need for greater care in selecting and setting the split ratio between the MS and the other instruments. The UV, also

showed signs of overloading with the spectra becoming distorted with flat tops as the detector reached saturation point.

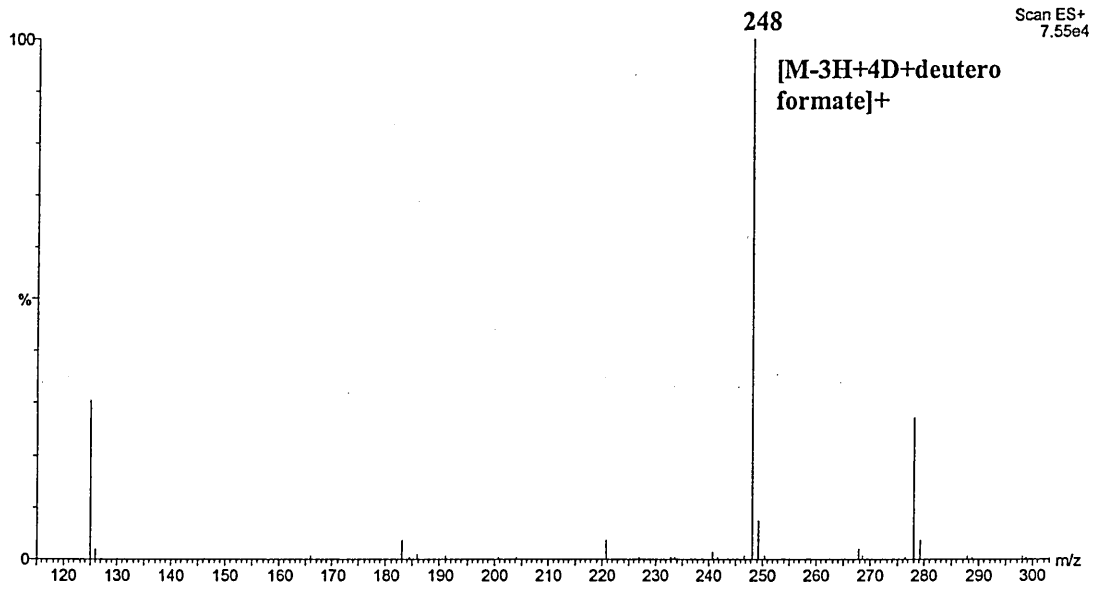



Fig. 5.12 *Mass spectrum of α-hydroxyhippuric acid showing the major ion as a deuterated formate adduct rather than the expected* $(M\text{-}3H+4D)^+$

The FT-IR spectrometer provided good quality, diagnostic spectra for all of the test analytes in the model library. This was reflected in the results of the library searches, where two of the criteria required for confidence in the results are that the top hit has a high percentage match and that there is a large difference between the first and second hits. In these experiments the best spectral match in each case was to the target analyte. In general the top match had a score of greater than 90% for the majority of the compounds examined. For instance in the case of caffeine the match score was 91% with the nearest alternative scoring just 70% by comparison. However, some care must be taken when using library searches because, as might be expected, the FT-IR spectra of antipyrine and its analogues 4-aminoantipyrine and 4-

dimethylamino antipyrine share many common features. The top match score for 4-aminoantipyrine was 80%, which would normally give good confidence in

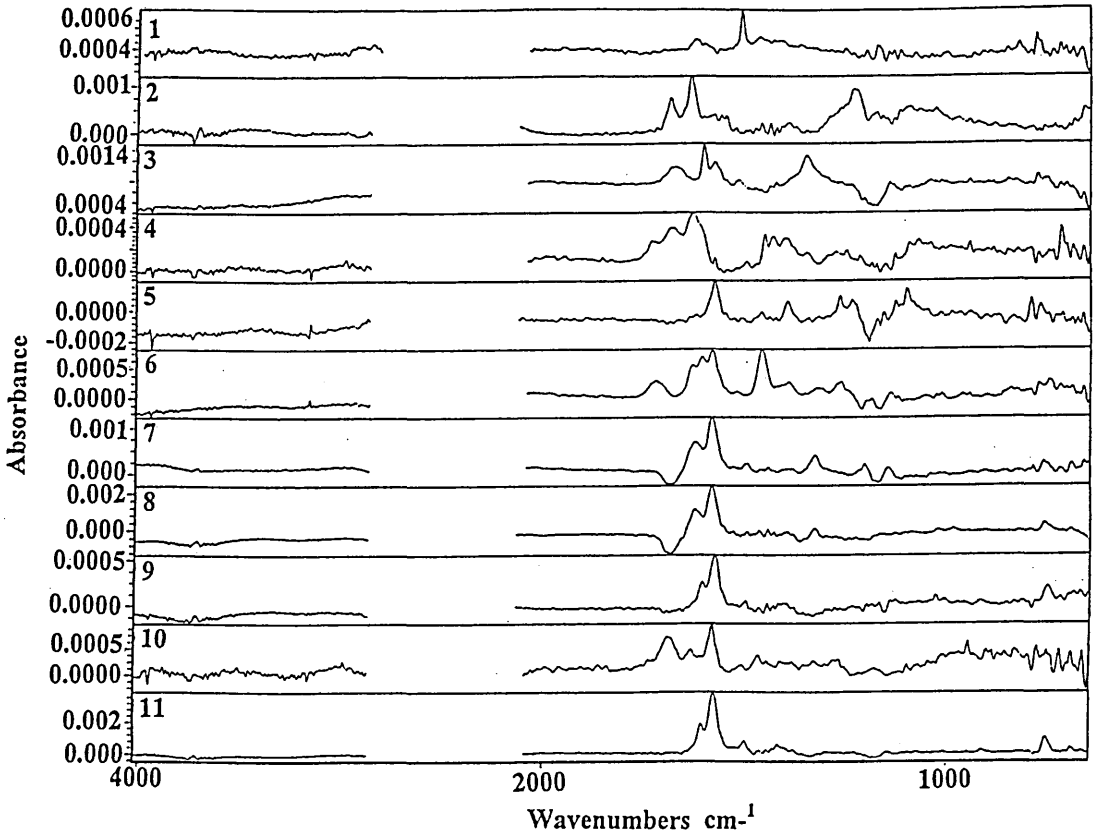



Fig. 5.13 *FT-IR spectra obtained on-line for the model library. 1) paracetamol (140μg ), 2) caffeine (165 μg), 3) p-aminobenzoic acid (185 μg), 4) α-hydroxyhippuric acid (200 μg), 5) propranolol (215 μg ), 6) p-aminohippuric acid (270 μg), 7) 4-aminoantipyrine (295 μg), 8) 4-dimethylamino antipyrine (545μg), 9) antipyrine (840 μg), 10) hippuric acid (365 μg), 11) antipyrine (210 μg)*

the result. However, the second match from the library was quite close at 78% for the structural analogue 4-hydroxyantipyrine, making the result much less clear cut. (Also library search results are heavily dependent on the quality of the spectra in the library as well as the number of spectra they contain). Antipyrine itself, however, was easily

distinguished from these two analogues giving a library search match score of 98%, with dimethylamino antipyrine, 4-aminoantipyrine and a third analogue (present in the spectral library) 4-hydroxyantipyrine scoring 73%, 72% and 70%, respectively, thus providing a fairly unambiguous identification.

The on-flow FT-IR spectra for all of the compounds used for this model library are shown in Fig. 5.13. As with any organic identification, complementary techniques are required for higher certainty of identification (one of the justifications for this multiple hyphenation approach)[95,96]

The on-flow $^{1}$H-NMR spectra were recorded for all of the test compounds as illustrated by the on-flow $^{1}$H-NMR pseudo-chromatogram (Fig. 5.14) and spectra (Fig. 5.15). In each case diagnostic $^{1}$H-NMR spectra were obtained that showed all the expected structural features for each of the compounds.

The amounts of material employed in these investigations were sufficient to obtain a complete set of spectroscopic data for each compound in an automated system without the need for stopped-flow experiments. Whilst a range of sample amounts was employed, the results obtained point towards a practical working range of 150-200 μg for any individual compound utilising this configuration. However, the data suggest that this does not represent the lower limit for a practical system. For instance the Gram Schmidt reconstruction (a pseudo LC-IR trace) of the IR data (not shown) indicated that the peaks were approximately 1 minute wide, implying significant broadening of the peaks due to sample dispersion in the tubing employed before the FTIR. As FTIR spectra were collected every 10 seconds across the peak giving on

average 6-7 IR spectra per compound, clearly less material could have been used if the band broadening were reduced. The UV flow profile (monitored at 254 nm) for the first five injections is shown in Fig. 5.16, and indicates that by the time the peaks had reached the DAD flow cell they had undergone still further band broadening and were approximately 2 minutes wide.

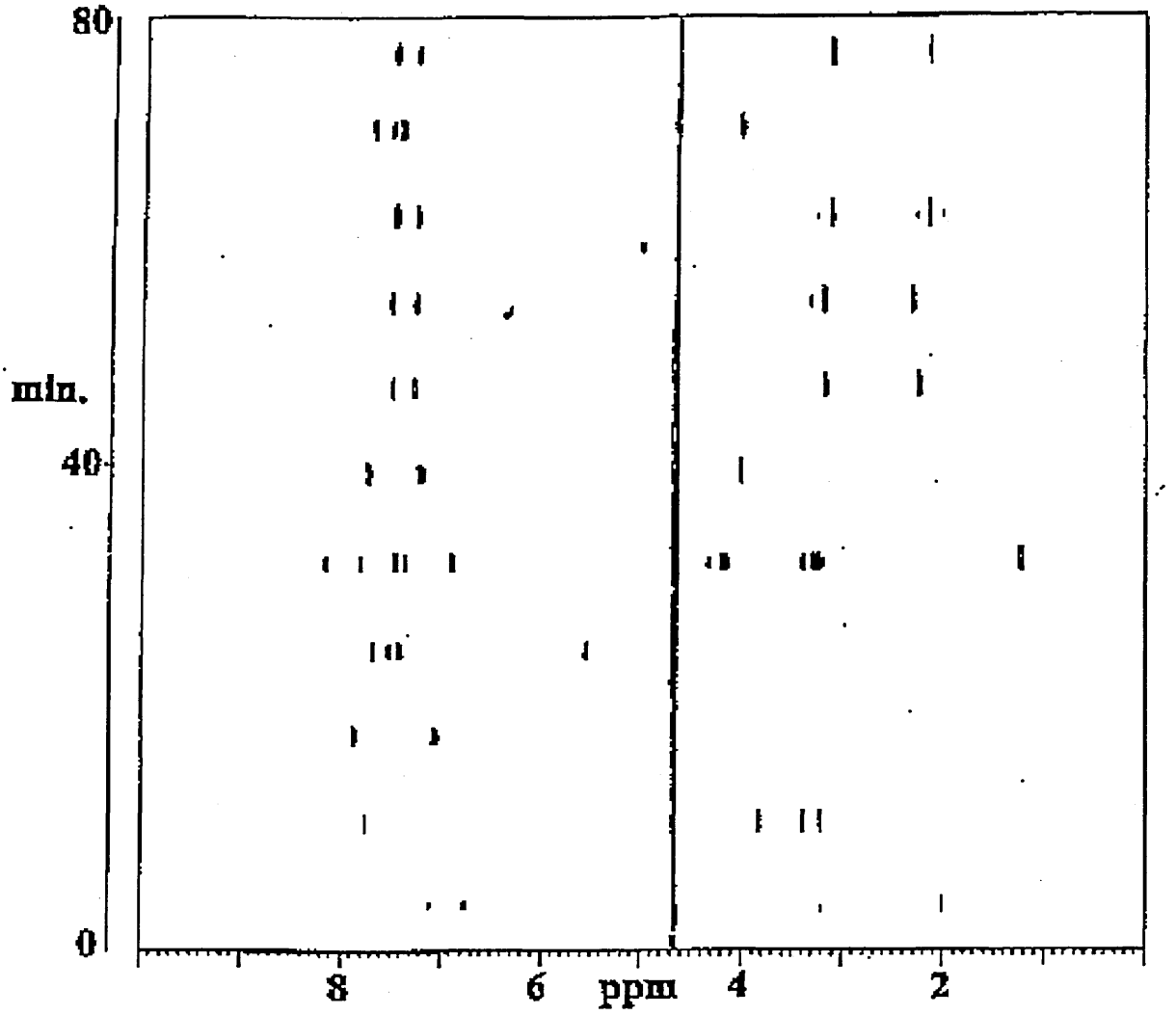



Fig. 5.14 The $^{1}H$ *NMR pseudo-chromatogram for the model library. Key as for Fig. 5. 13 Elution time is indicated on the vertical scale and the horizontal scale shows the chemical shift in ppm.*

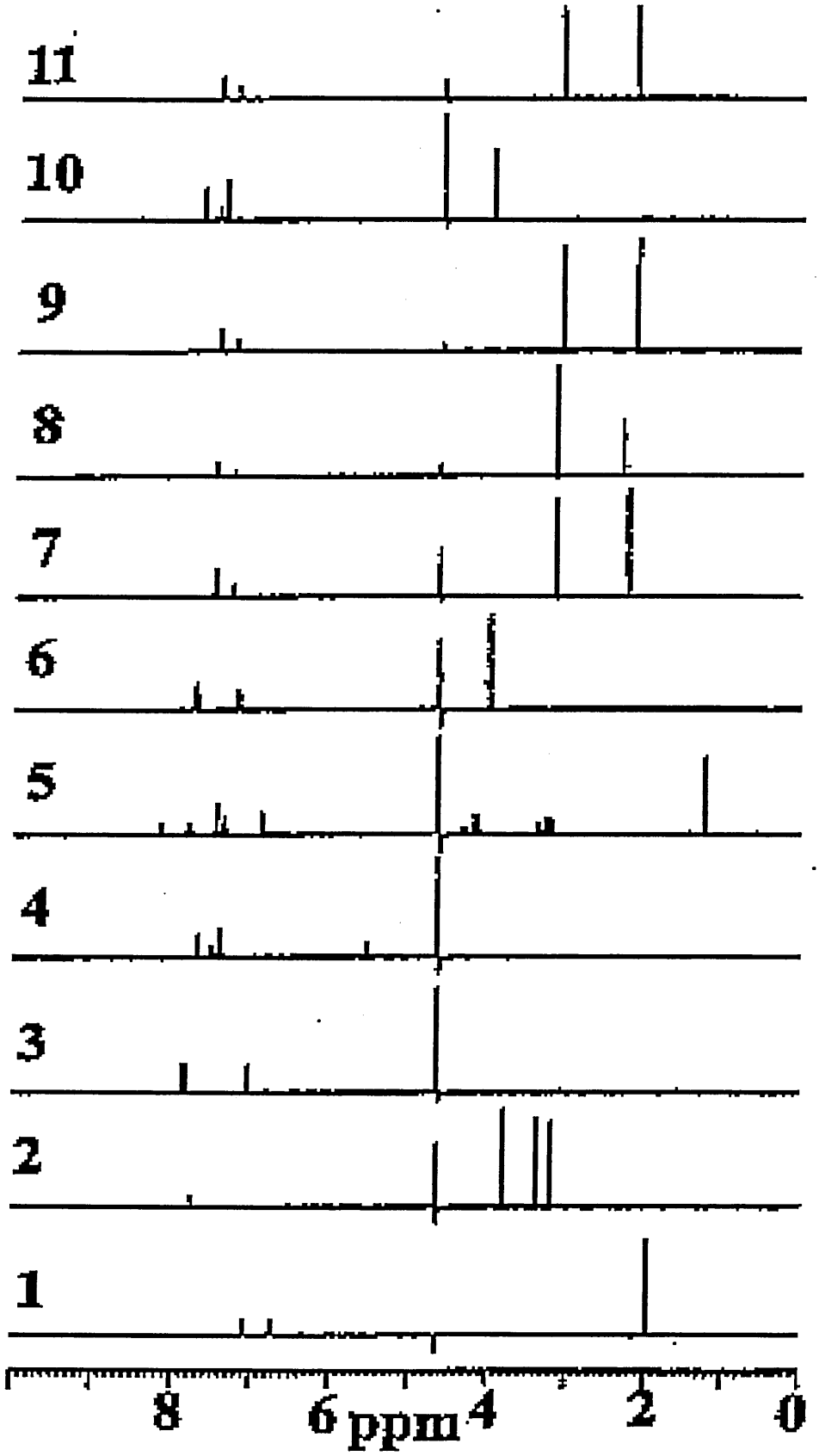



Fig. 5.15 *The extracted $^1$H-NMR spectra from the $^1$H NMR pseudo-chromatogram shown in Fig. 5.14. Key as for Fig. 5.13*

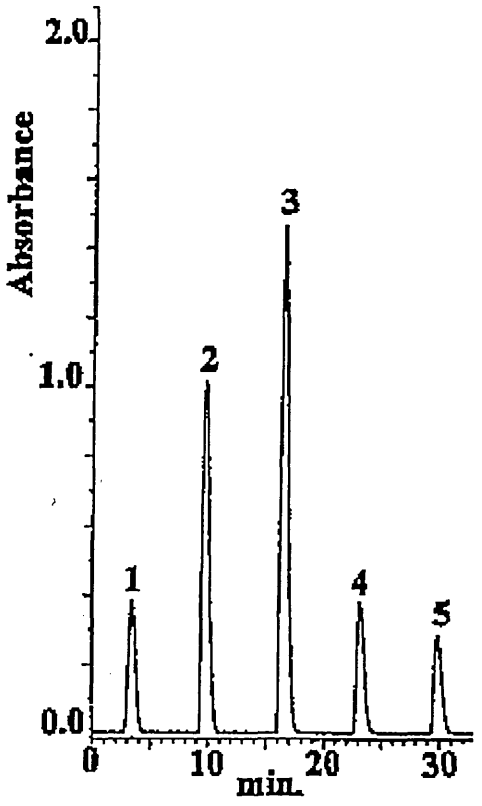



Fig. 5.16 *The UV pseudo-chromatogram for the first five compounds injected from the model library showing the peaks to be approx 2 min wide at the base. 1) paracetamol (140μg ), 2) caffeine (165 μg), 3) p-aminobenzoic acid (185 μg), 4) α-hydroxyhippuric acid (200 μg), 5) propranolol (215 μg )*

Peaks entering the NMR flow cell were approximately 2.1 min wide. When the peak for propranolol entered the NMR flow probe and was analysed incrementally it was clearly possible to discern three spectra, which would have been perfectly acceptable $^{1}$H-NMR spectra for this compound (spectra 2, 3 and 4 in Fig. 5.17). The concentrations of these compounds as they entered each of the spectrometers must therefore have been reduced by dispersion. Further optimisation of the split, to ensure the bulk of the flow was directed to the FTIR spectrometer and the NMR spectrometer rather than only 60%, combined with measures to minimise the amount

of band broadening such as the use of shorter narrower lengths of tubing would provide much greater

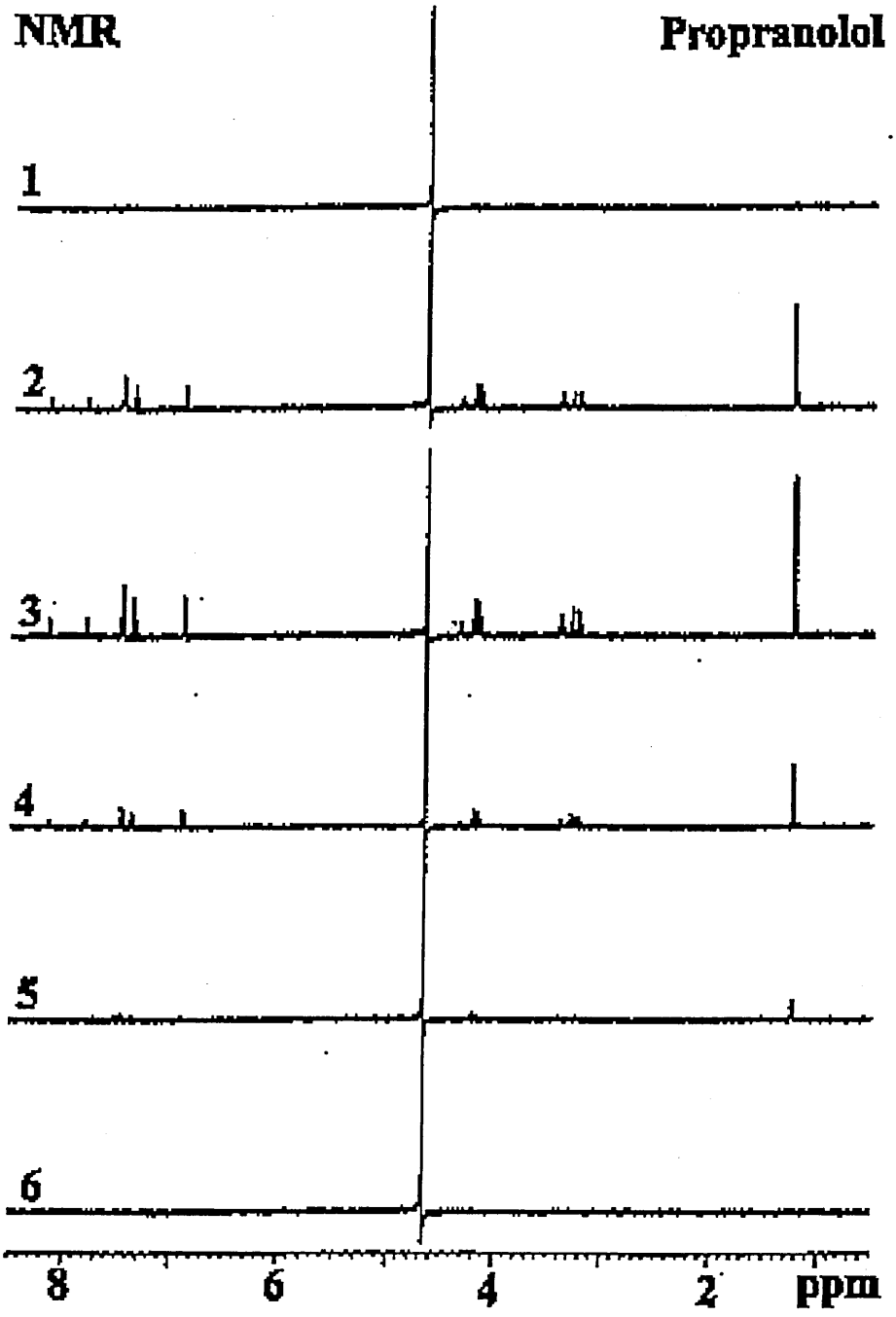



Fig. 5.17 *Sequential $^{1}$H-NMR spectra obtained for propranolol (peak 5 in Fig. 5.16). The spectra are numbered 1-6 from the beginning of the propranolol peak and taken approximately every 15 seconds.*

sensitivity than was observed in these experiments. Another option to improve sensitivity would be to use lower flow rates, as this would increase the residence time in each of the spectrometers and hence improve the signal to noise ratios although, this would lead to some band broadening. A flow rate of 0.5 mL/min enabled the acquisition of on-flow $^{1}$H-NMR spectra for 10 μg of caffeine (results not shown), but the other downside of utilising a lower flow rate would be a reduced throughput.

## 5.6 Summary

- **A useful working system was established and a series of known compounds was analysed.**
- **The system was automated and a model library analysed**
- **Working levels of 150-200 μg of sample for an automated approach were established**
- **Further modifications to reduce band broadening and increase signal to noise ratios were noted**

# CHAPTER 6

# HOT WATER CHROMATOGRAPHY AND TESTING OF THE MULTIPLY-HYPHENATED SYSTEM WITH A VARIETY OF PROBLEMS

## 6.1 Aims and introduction

**The aims of the work described in this chapter were:**

1) **To thoroughly test the feasibility of the system created with a variety of representative problems**

2) **To compare and contrast the use of hot water systems with conventional chromatography**

3) **Based on the results obtained, to predict the future usefulness of such a multiply hyphenated systems in solving complex problems.**

## 6.2 The application of high temperature separations and their advantages in multiply-hyphenated detection systems.

As described earlier the use of high temperature eluents and in particular the use of superheated water, where the eluent is heated beyond its boiling point at atmospheric pressure, has been investigated by a number of groups[64,68,97]. One of the potential

benefits of such a system, and a particular benefit for NMR studies, is the possibility of greatly reducing or even completely eliminating the organic modifier. The use of temperature gradients can allow for more complex mixture separation as the properties of the eluent change with temperature, while at the same time maintaining the overall composition of the eluent. There are two key mechanisms in play when using high temperature gradients. Standard chromatography often utilises modest increases in temperature up to 40C as a means of reducing viscosity and thus increasing the diffusion rates of compounds with a resultant benefit to speed and separation efficiency. These modest temperatures are also employed to ensure a standard temperature to improve transferability from lab to lab of methodologies. However, when superheated temperatures are employed for reverse phase eluents or for totally aqueous eluents there is a significant alteration in the properties of the water. The elevated temperatures interfere with the strong hydrogen bonding normally associated with water to the point where water has properties very similar to those of methanol for chromatographic purposes. The use of such a totally aqueous eluent for chromatographic separations which include NMR as one of the detection systems is the ability to successfully replace the water with $D_2O$ without affecting the chromatography. Not only are the interferences from organic solvents removed but there is a saving in the cost of expensive deuterated organic modifiers. These modifiers are invariably impure and thus do not completely overcome the problem of interfering signals which can mask areas of interest in the spectra obtained. Similarly the use of a simple eluent greatly facilitates the multiple hyphenation of a liquid chromatograph with various spectroscopic detectors. As has already been demonstrated, one of the most significant problems in trying to hyphenate multiple spectroscopic techniques is the compatibility of the eluent with these various

detectors. For instance the use of inorganic buffers such as sodium phosphate is commonplace in LC-NMR or older LC-UV methodologies as these buffers improve the chromatography obtained and do not interfere with the quality of the spectra obtained. However, the use of these buffers is avoided in LC-MS as their involatility can cause severe problems. Similarly for LC-NMR studies the use of binary systems (usually $D_2O$:acetonitrile due to the cost of deuterated solvent) are preferred so as to minimise the interference of the organic modifier with the signals of the analytes, whilst for LC-MS the use of several organic modifiers presents little or no difficulties as these solvents are relatively easily removed in the LC-MS interface. Thus the desirability of utilising superheated water led to the experiments described here for the separation of a number of model pharmaceutical compounds and their subsequent characterisation by NMR, MS, IR and UV spectroscopies.

### 6.2.1 Experimental conditions

The compounds used for these trials were paracetamol (acetaminophen), antipyrine, 4-aminoantipyrine, norantipyrine, caffeine, phenacetin, p-aminobenzoic acid, propranolol, sulfacetamide and sulphanilamide. They were dissolved at concentrations of 23 to 125 ug/uL in deuterium oxide ($D_2O$). The $D_2O$ was 99 atom % pure for NMR studies, the major impurity being residual $H_2O$. The system employed was broadly similar to that described previously though the details are outlined below. Two different HPLC columns were evaluated during the study, an Xterra C8 150 x 4.6 mm (Waters Ltd, Watford, UK) and an Oasis HLB 150 x 2.1 mm (Waters Ltd) prototype column. Both were packed with 5 μm particles. To raise the temperatures to those necessary for hot water or superheated water applications the

columns were placed inside a Pye 104 GC oven. The temperature was controlled with a Pye oven programmer and was typically maintained at 85C for the Xterra column and 185C for the Oasis HLB. On emergence from the oven the eluent was rapidly cooled using a water bath held at 0-4C using ice. $D_2O$ was maintained in its liquid state at these elevated temperatures by the backpressure generated by the PEEK tubing used to connect the column to the various spectrometers. The liquid chromatograph was the Constametric 3200 described previously. The flow rate was 1.0 ml/min for the Xterra column and 0.8 ml/min for the Oasis HLB. From the column approximately 95 % of the flow was directed via 110cm of 0.020" id PEEK tubing to a Bio-Rad FTS3000 Excaliber FTIR spectrometer. This was fitted with a Spectra Tech ATR Micro Circle high pressure stainless steel flow cell of 25 µl volume, fitted with a zinc selenide ATR crystal. The spectrometer was purged with dry nitrogen to minimise any water vapour interference of the collected spectra. Any residual water vapour signal was subtracted using a water vapour reference spectrum. Spectra were otherwise obtained as previously described.

The remainder of the flow from the column (ca 5%) was directed to a Micromass Platform single quadrupole mass spectrometer via 4.5 m of 75 µm id fused silica capillary tubing. This length of tubing was sufficient to generate the necessary back pressure to direct the bulk of the flow to the FTIR spectrometer. Mass spectra were acquired over the range m/z 115 to 650. Prior to introduction of the sample into the APCI ion source of the mass spectrometer, the eluent was mixed with a make up flow of 90:10 (v/v) methanol:water. This make up flow was necessary to maintain the chromatographic resolution (by minimising the transfer time) and to ensure the APCI interface functioned properly by providing the required flow to obtain efficient

ionisation. This make up flow was provided by a second Constametric pump via a T-piece at 0.5 ml/min. Positive ion spectra were recorded with a cone voltage of 25V, a scan time of 0.9 s and an inter scan delay time of 0.1 s. The multiplier was set at 400V.

From the FT-IR the eluent flowed via 30 cm of 0.005" id PEEK tubing to the Bruker UV-diode array detector previously described, where spectra were acquired over the range 188 to 600nm. The ability to acquire spectra at wavelengths below 210 nm was an additional benefit of a water only eluent. Most organic modifiers have strong UV absorbance at this part of the UV spectrum and so it is usually not possible to acquire UV spectra in this region. From the UV detector the solvent stream was directed to the Bruker 500 MHz NMR spectrometer previously described. On flow NMR detection was carried out using a flow probe of 3mm id and a volume of 60 μL. Typically, 16scans/FID per increment were acquired into 8k data points each with a spectral width of 8278 Hz. Spectra were acquired using the NOESYPRESAT pulse sequence in order to suppress the residual water resonances. 90° pulses were used with an acquisition time of 0.5 s, a relaxation delay of 0.7 s and a mixing time of 100 ms. Water irradiation was carried out during the relaxation delay of 0.7 s and the mixing time of 100 ms.

### 6.2.2 Chromatographic results

Prior to this work superheated water (i.e. water at temperatures in excess of 100C) had been used to achieve separations without the need for organic modifiers. However, it is not necessary to exclusively use such temperatures as even at elevated temperatures it is possible to reduce or eliminate the necessity for organic modifiers; this is

illustrated in the work employing the Xterra column shown below. The chromatographic conditions employed here were based on experience from within the research group.

### 6.2.3 Xterra

The Xterra phase gave good separation of the test compounds with $D_2O$ alone at oven temperatures well below 100C. The chromatography of a test mixture consisting of paracetamol (ca 70 μg), caffeine (ca 23 μg), antipyrine (ca 125 μg) and phenacetin (ca 36 μg) is shown in Fig. 6.1. Under the conditions used baseline resolution, with good peak shape, was achieved in approx 15 min at a temperature of 85C and a flow rate of 1.0 ml/min. It should be noted that the long lengths of tubing necessary to connect all of the spectrometers contributed a significant amount of extra column band broadening, making the separation appear much less efficient than was actually achieved[98].

By repeating the separation several times with different amounts of material it was possible to determine the limits of detection (LODs) and the limits of spectroscopic identification (LOSI ie the minimum amount of material that was necessary to provide a spectrum containing all of the features that should be expected for a molecule) of the system. In this instance it was possible to detect and acquire full UV spectra and mass spectra of all of the test compounds at the smallest quantity injected, as would be expected based on the quantities of material present. In the case of the MS data, as well as the expected $(M+D)^+$ the presence of $(M+Na)^+$ was noted

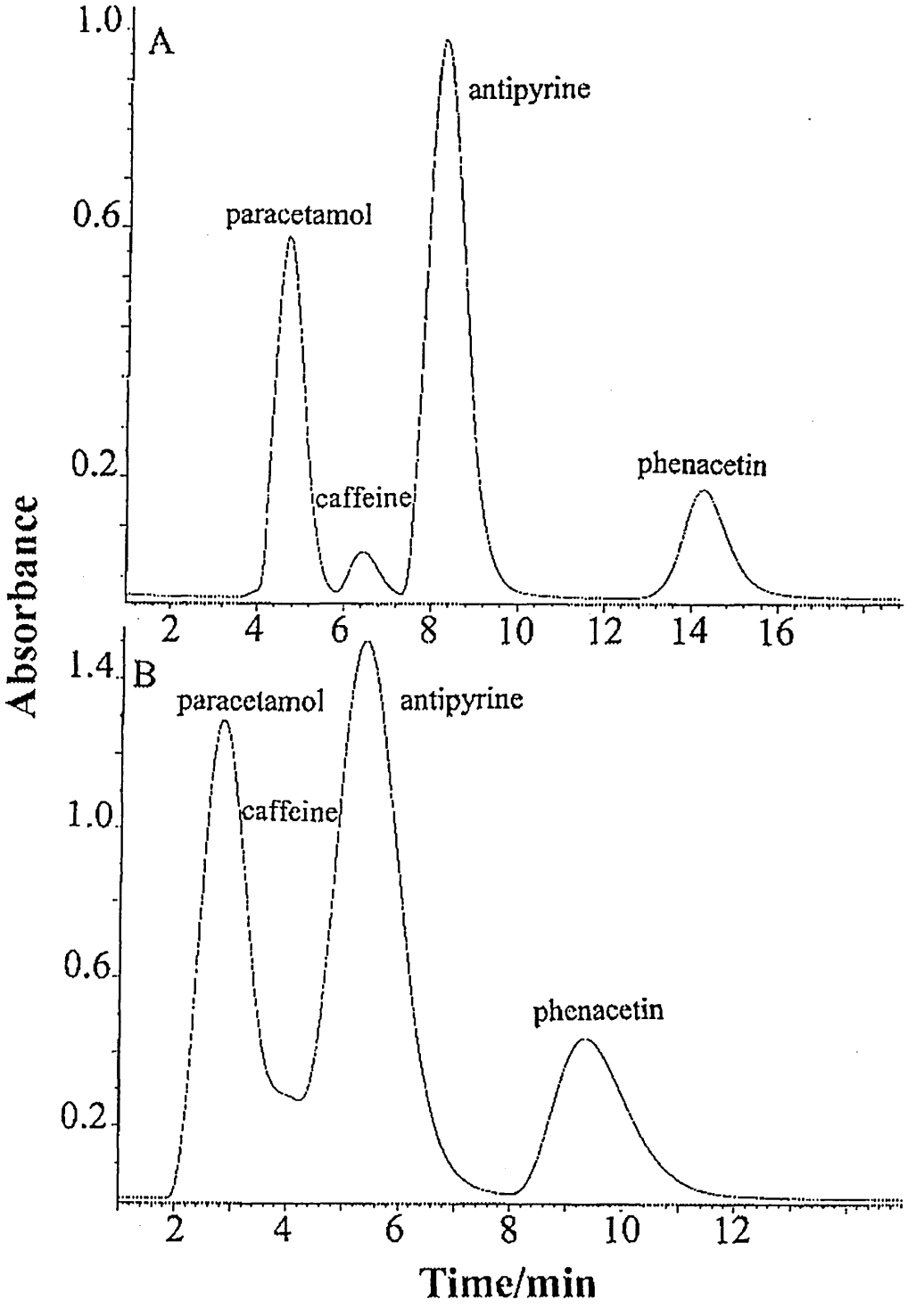



Fig. 6.1 *Representative UV chromatograms at 254 nm for the test mixtures containing (1) paracetamol, (2) caffeine, (3) antipyrine and (4) phenacetin on (A) Xterra at 85C and 1ml/min and (B) Oasis HLB at 185C and ).8ml/min. Clearly under these conditions the Xterra out-performs the Oasis HLB giving sharper peaks and almost baseline resolution of the test components.*

for a number of the analytes and, in addition, in some instances ions of the type $(2M+D)^+$ the latter most probably present due to an access, of analyte. Mixing the eluent with a make-up flow of normal protonated solvents prior to the mass spectrometer had been expected to produce [M+H]+ ions rather than the noted [M+D]+ species. However, this was not the case though the addition of a proton donor may have given the desired back exchange. Such results were seen in other experiments though the data is not available here.

For FT-IR the above test mixture represented the LOD for both phenacetin (36 μg) and caffeine (23 μg) but paracetamol and antipyrine (70 μg and 125 μg) gave diagnostic spectra. Under the same chromatographic conditions repeat injections with a mixture containing 72 μg of phenacetin and 46 μg of caffeine yielded truly representative spectra. For $^1$H NMR, diagnostically useful spectra were obtained for 70 μg of paracetamol, 125 μg of antipyrine and 36 μg of phenacetin although in the case of phenacetin this figure was close to the LOSI. In the case of caffeine, injection of 23 μg of material enabled the ready detection of the three identical protons in each of the three methyl groups present in the molecule, but not the single ring proton. However, the signal for this ring proton was detectable when the amount of caffeine was increased to 46 μg and it was quite prominent at 93 ug, Fig. 2a-c. Similar comments can be applied to the FT-IR spectra (illustrated in Fig. 3a-c with the spectral matches obtained for caffeine being 91.6, 92.9 and 93.5% for 23, 46 and 93 μg of caffeine on column, respectively.

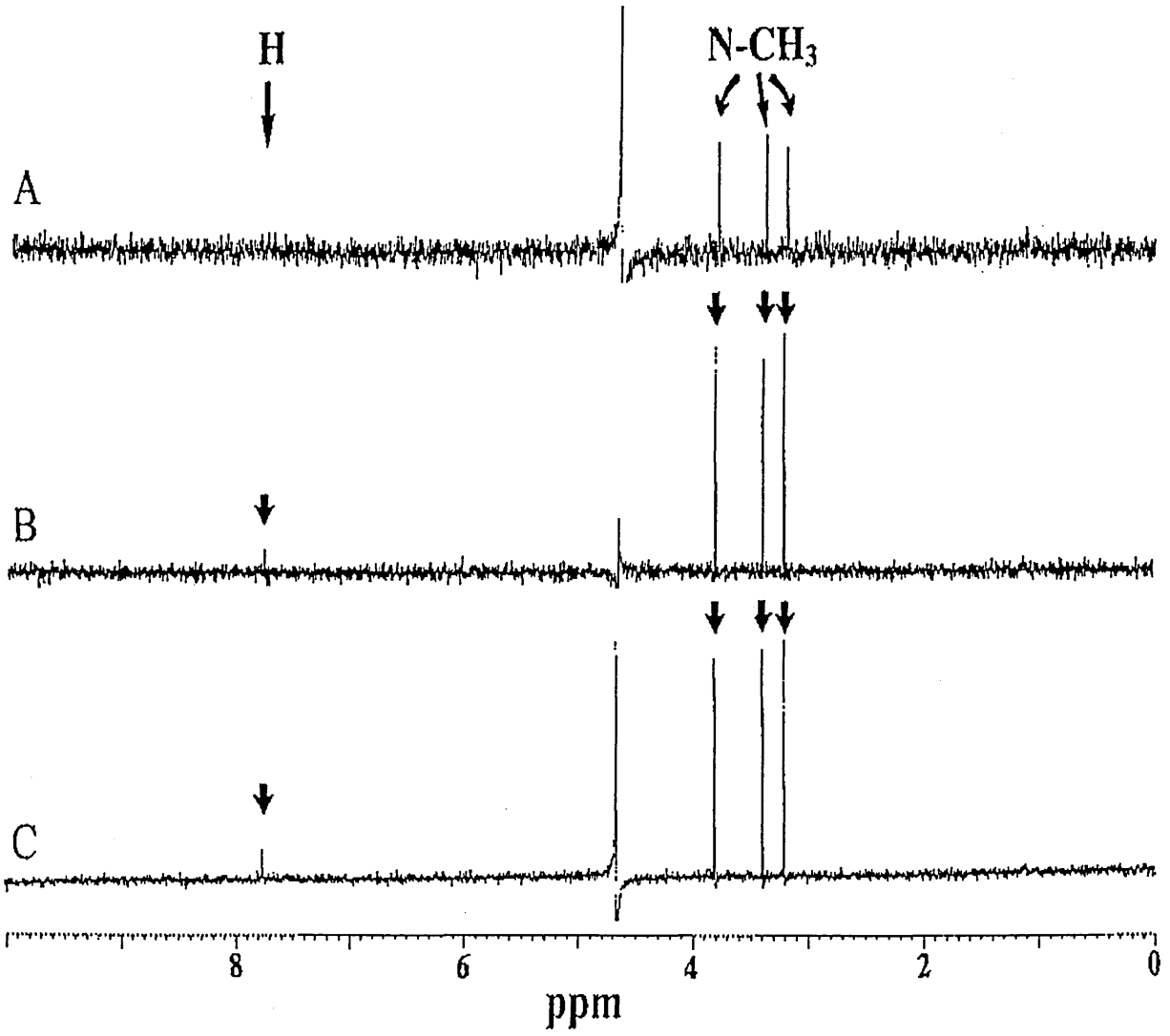



Fig. 6.2 *$^{1}$H-NMR spectra for (A) 23 (B) 46 and (C) 93 μg of caffeine obtained on flow at 1 ml/min following chromatography on Xterra at 85C. The methyl protons are clearly visible in all cases but the ring proton at approx 7.8 ppm is not seen at 23 μg, is just visible at approx twice the background at 46 ug and finally is usefully diagnostic at 93 μg clearly illustrating the difference between LOD and LOSI*

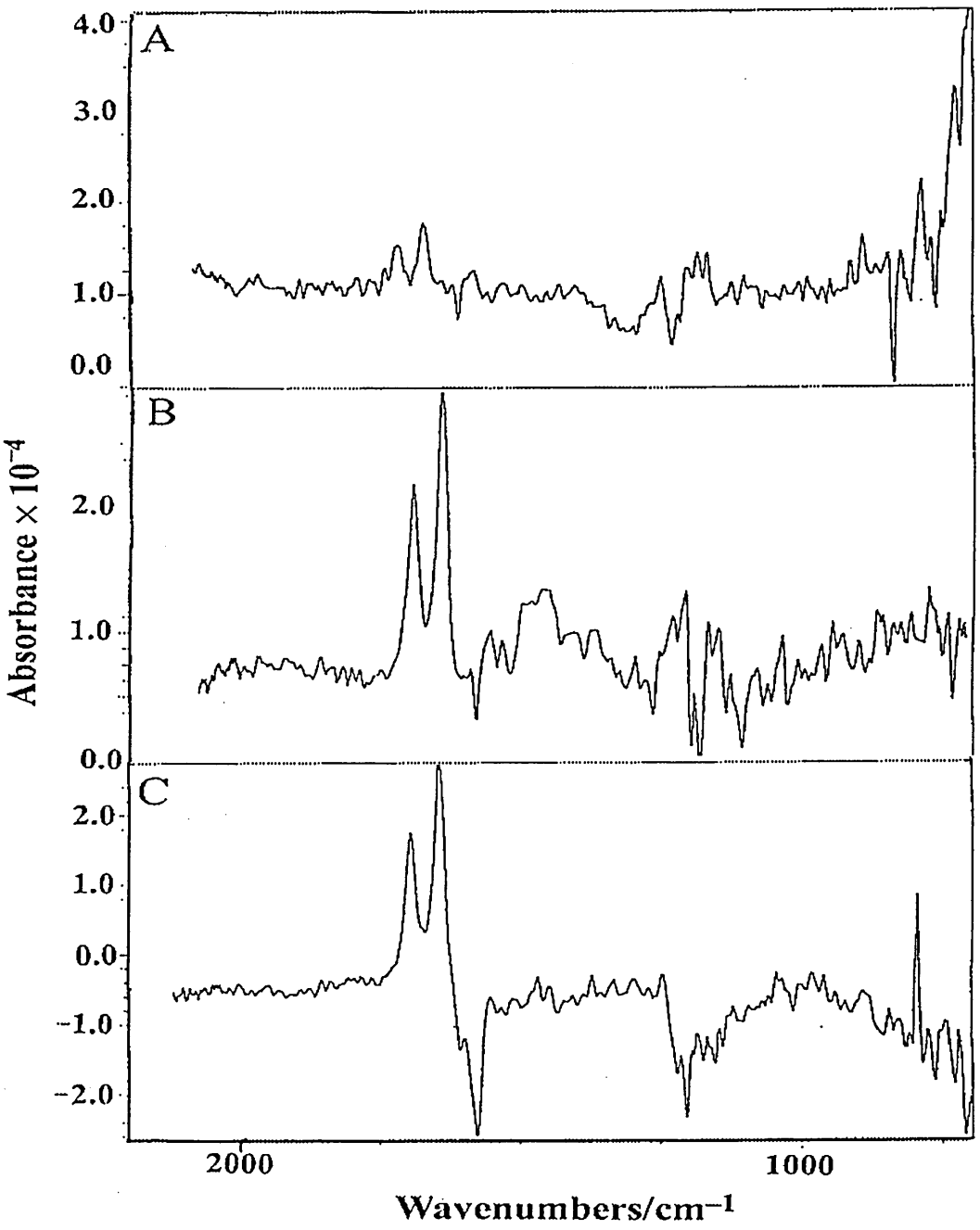



Fig. 6.3 *IR spectra for (A) 23 (B) 46 and (C) 93* μg *of caffeine obtained on flow at 1 ml/min following chromatography on Xterra at 85C. 23ug represents the LOD while more acceptable spectra are obtained at 46 and 93* μg. *The negative peaks were caused by the solvent subtraction routine used.*

The chromatography and spectroscopy of a mixture containing p-aminobenzoic acid (440 μg), 4-aminoantipyrine (425 μg) and propranolol (228 μg) were also

investigated utilising the same conditions. Full spectra were obtained for all three compounds. An illustration of the type of data that can be obtained using this system is provided by the UV, IR, NMR and MS spectra for 72 µg of phenacetin shown in Fig. 6.4a-d.

There was no evidence that the conditions used for chromatography on the Xterra phase resulted in the thermal degradation of any of these compounds.

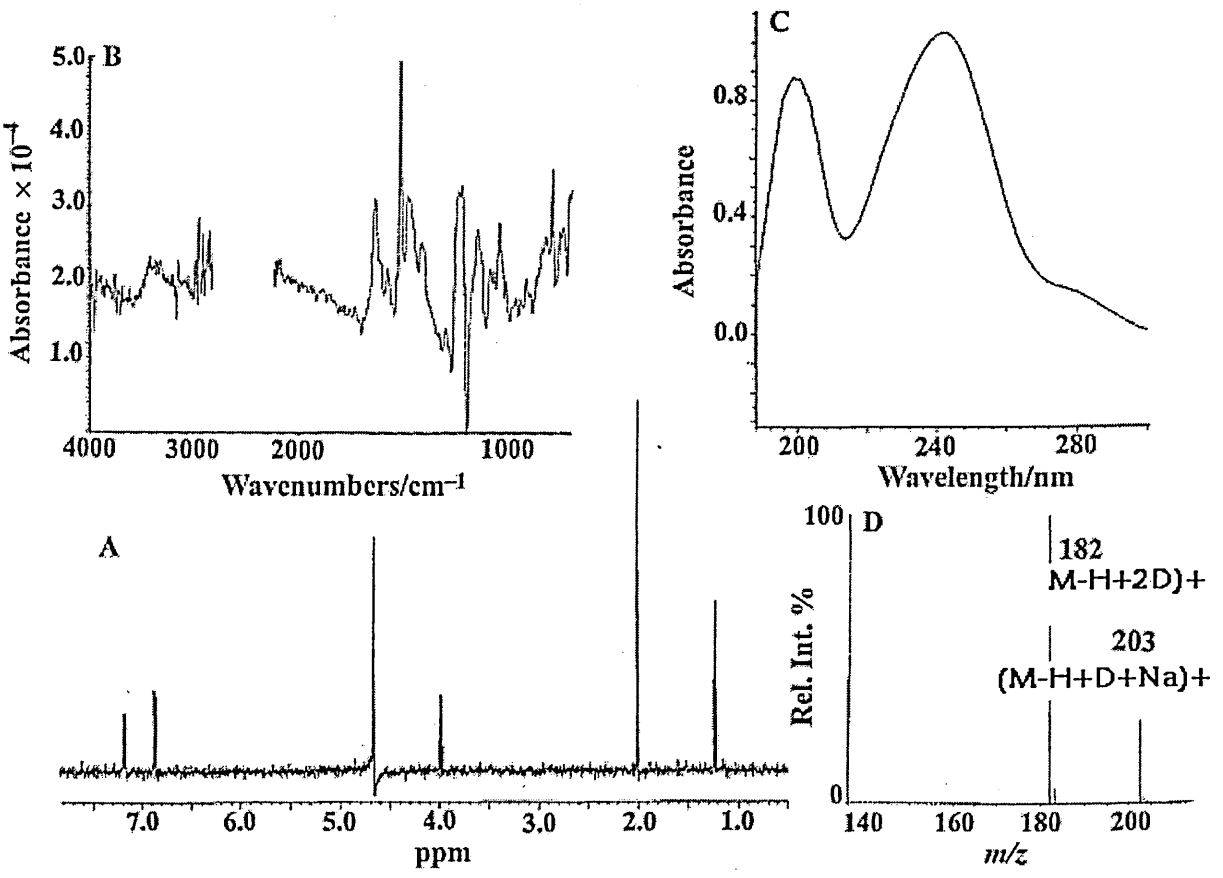



Fig. 6.4a-d *The NMR (A), IR (B), UV (C) and MS (D) spectra obtained for 72µg of phenacetin at 85C on Xterra. All of the spectra show signals which could have usefully been employed to identify this as phenacetin had it been an unknown compound.*

### 6.2.4 Oasis HLB

The Oasis Hydrophilically-Lipophilically Balanced phase is a copolymer based material and has a similar selectivity for paracetamol, caffeine, antipyrine and phenacetin but it is somewhat more retentive than the Xterra phase. Oasis HLB therefore required the use of higher temperatures in order to elute the test analytes and superheated $D_2O$ at 185C and 0.8 ml/min was used to chromatograph the test mixture. In this instance the test mixture contained ca 140, 46 250 and 72 μg of paracetamol, caffeine, antipyrine and phenacetin, respectively. Chromatography of the test mixture on this column was less efficient (Fig. 6.1B) than seen on the Xterra phase, with incomplete resolution of paracetamol, caffeine and antipyrine (probably partly as a result of column overloading). Nevertheless, by carefully selecting the appropriate part of the chromatographic peaks it was still possible to obtain diagnostic spectra for all the components despite the poor resolution of some of the analytes. This is good evidence of the excellently selective nature of some spectroscopic techniques and their ability to extract useful data from less than ideal chromatographic data. In addition to the test mixture, chromatography and spectroscopy were performed on p-amino benzoic acid (220 μg), propranolol (224 μg), 4-aminoantipyrine (113 μg), sulfacetamide (125 μg) and sulphanilamide (131 μg), injected as individual components under the same conditions. The UV, IR, NMR and MS data for 4-aminoantipyrine are shown in Fig. 6.5.

Although the temperature used for the chromatography on the Oasis HLB phase was well in excess of 100C there was no evidence of the thermal decomposition of any of the test analytes.

### 6.2.5 Conclusions on the use of hot water systems

These results provide further evidence that multiple hyphenation does not present insurmountable problems, with diagnostic NMR, IR, UV and MS data being

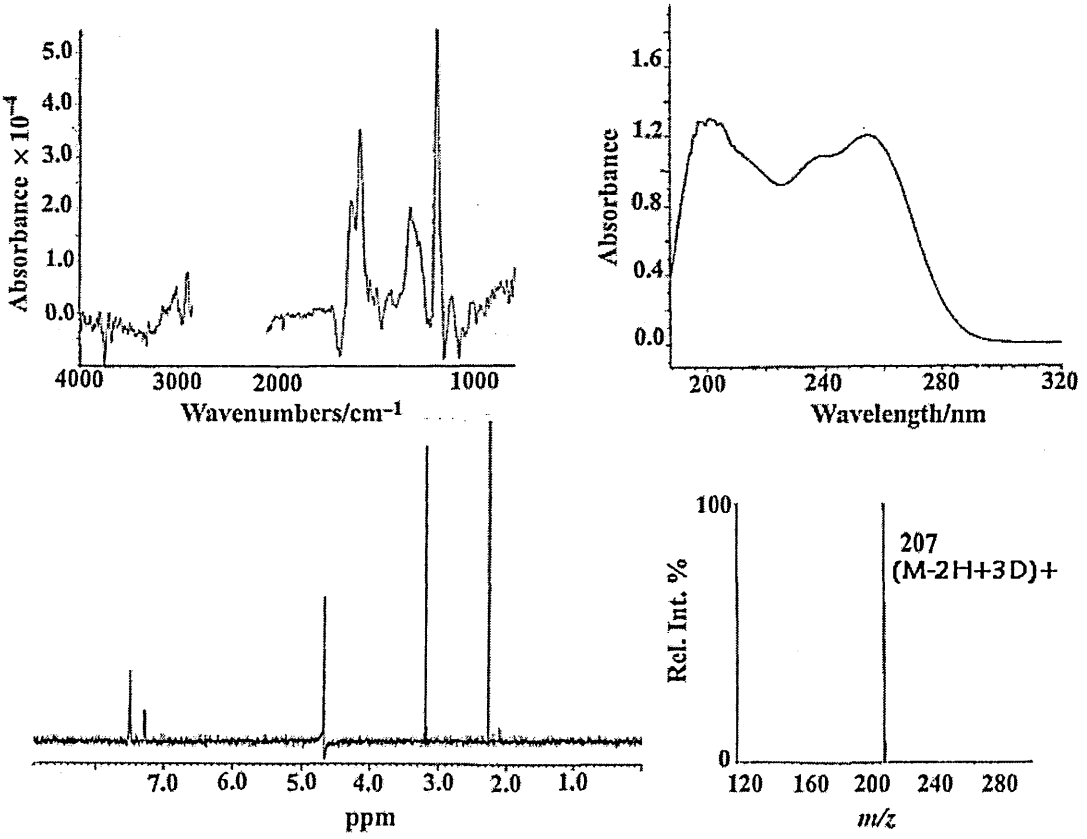



Fig. 6.5 *The spectra obtained for 113 µg of 4-aminoantipyrine on flow at 0.8 ml/min following chromatography on Oasis HLB at 185C. The spectra all clearly show the expected signals and could easily be used to diagnostically identify this as 4-aminoantipyrine.*

obtained on flow with quantities of less than 100µg of each compound. Similarly the use of elevated temperatures posed no practical problems for either the instrumentation or the analystes. The value of this approach is particularly evident in $^{1}$H-NMR spectra where the absence of interferences from organic modifiers greatly simplifies the resulting spectrum. However, the analytes are limited to those which

are thermally stable and this may restrict the use of this particular technique. Likewise current column technology has not been developed to cope with prolonged exposure to such high temperatures and the use of 100 % aqueous eluents may cause "de-wetting" also known as hydrophobic collapse of some stationary phases although neither column degradation nor de-wetting were observed here. Although the sensitivity of the NMR could have been improved by the adoption of stopped flow experiments clearly it would make more commercial sense to maintain on-flow conditions as the array of equipment required necessitates the production of a high throughput of samples to justify the cost.

While the quantities of material required in the present set up preclude trace analysis they do constitute a 20-40 fold increase in sensitivity compared with earlier results using more conventional mobile phases[99,100] and are well within the reach of the amounts encountered in combinatorial chemistry or drug synthesis. Furthermore the system described here does not describe what is possible with state of the art technology. Combining such state of the art spectrometers with steps to reduce the band broadening would yield still further improvements in sensitivity. It is estimated that simply reducing the band broadening would give a 10 fold increase in sensitivity thus allowing analysis of 10 μg of material. This estimate is based on the experiments using individual components introduced as flow injection analysis where 100μl of sample was observed to have broadened to 2.1 min by the time it entered the flow probe of the NMR (ie a 10-20 fold dilution of the peak)[101].

## 6.3 The analysis of a mixture of nonsteroidal anti-inflammatory drugs by multiply-hyphenated spectroscopies.

Although hot water and superheated water chromatography offers much, especially for NMR studies, it is still in its infancy and as such there is still much to develop, particularly stationary phases capable of extended use at elevated temperatures and able to withstand the stresses of high temperature gradients. There are also limits to the hardware, and preheating the eluent to prevent thermal shock of the column and to ensure smooth and reproducible chromatography will require further investigation. A more elegant method of cooling the eluent after the column may be necessary.

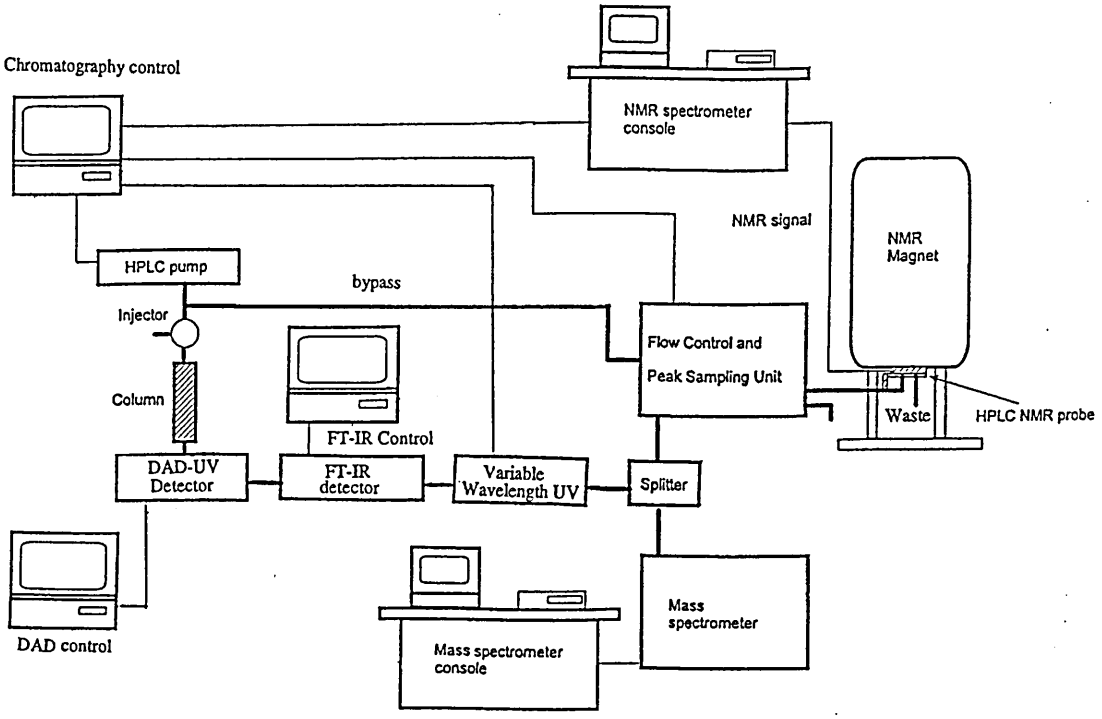



Fig. 6.1 *Schematic of the system used for chromatographic separation and spectroscopic characterisation of a mixture of NSAIDs, showing the addition of an HPLC column from the system shown in chapter 5.*

The remainder of this section therefore focuses on conventional chromatography and on model cases which represent real world problems encountered by research groups.

In chapter 5 the use of accurate mass mass spectrometry was discussed and its use in flow injection analysis studies outlined; here this is extended to include chromatographic separation (Fig. 6.1) in the same system, and analysis of a simple mixture of nonsteroidal anti-inflammatory drugs (NSAIDs) is described. The compounds used were ibuprofen, flurbiprofen, naproxen and indomethacin.

### 6.3.1 Chromatographic conditions.

The injector was fitted with a 200 µl loop for these studies. The eluent was a simple 50:50 mixture of acetonitrile (Pestanal grade, Riedel de Haen) and $D_2O$ (99.8% isotopic purity, Fluorochem) and 1% deuterated formic acid (99.8% isotopic purity, Cambridge Isotope Laboratories) to give a pH of approximately 2, which was necessary as the NSAIDs were all acidic and this pH allowed them to be maintained in their un-ionised form, thus ensuring their retention on the column. The column was a Hichrom 5µm C18 (HiRPB 150 x 4.6 mm id). The flow rate used was 1 ml/min. All other conditions and equipment were as described in chapter 5 except for the NMR spectrometer where a double solvent suppression was achieved via the NOESYSAT pulse sequence with irradiation of the acetonitrile signal and residual water signals during the delay and mixing times. $^1$H NMR spectra were internally referenced to acetonitrile at 1.93 ppm.

The separation obtained with this system, monitored at 254nm using the variable wavelength UV detector placed after the FTIR spectrometer (see Fig. 6.1) is shown in

Fig. 6.2. This UV detector was also used to trigger the acquisition of stopped flow NMR spectra. Even though relatively large amounts of samples were loaded (~2 mg of each compound), good chromatographic peak shapes were obtained with almost baseline separation of all four components achieved in a total analysis time of approximately

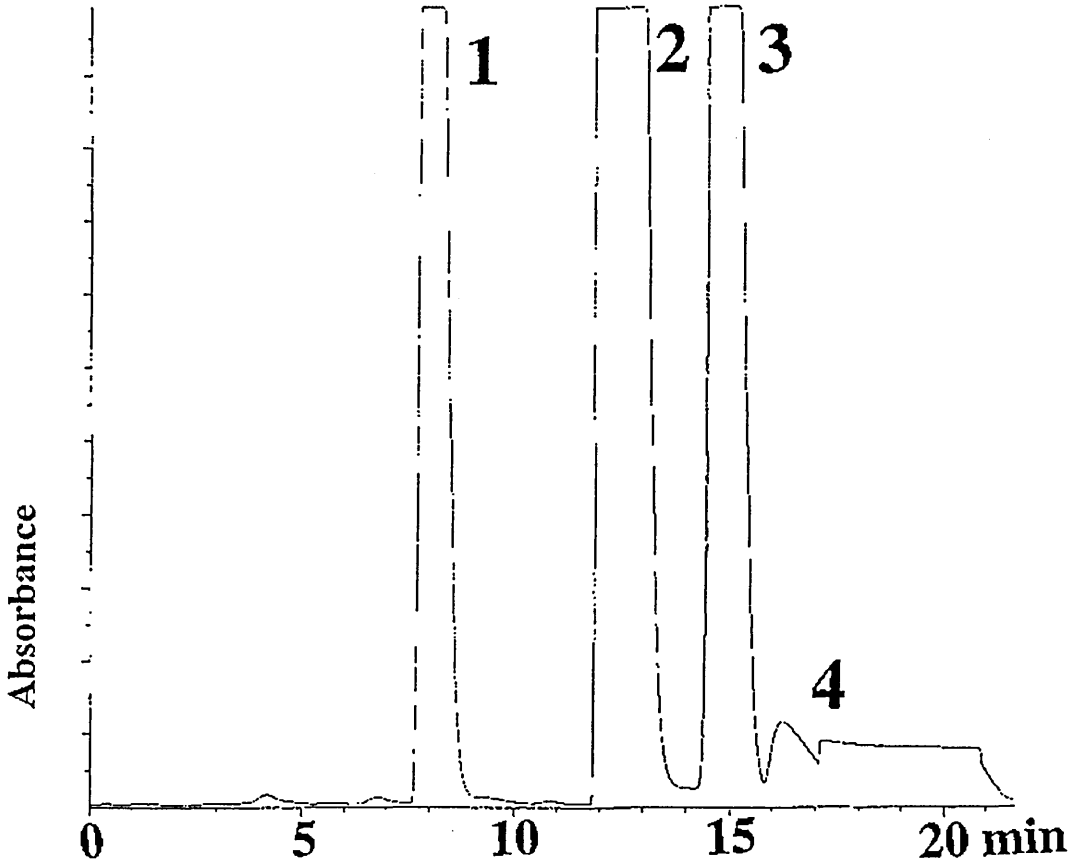



Fig. 6.2 *HPLC-UV (254 nm) chromatogram of the separation of the mixture of nonsteroidal anti-inflammatory drugs. 1. naproxen, 2. flurbiprofen, 3. indomethacin and 4. ibuprofen. The difference in UV absorption is clearly shown by the reduced height of the ibuprofen peak despite there being approximately equal amounts of each component loaded.*

17 minutes. Attempts at reducing this analysis time by increasing the percentage of acetonitrile present in the eluent were unsuccessful as these resulted in the co-elution of indomethacin and ibuprofen (peaks 3 and 4 in Fig. 6.2). One of the disadvantages of UV absorption as a detection method is also highlighted by this separation; as can be seen in Fig. 6.2 the peaks for naproxen, flurbiprofen and indomethacin are all overloaded and off scale. However, the peak for ibuprofen is relatively small. This is not an indication of a reduced sample loading but shows rather the weak absorption of ibuprofen at this wavelength (254 nm).

### 6.3.2 UV Spectra

Although as can be seen from Fig. 6.2 the amount of material loaded on column was sufficient to overload the UV detector for three of the four compounds injected it was still possible to extract characteristic UV spectra at either the leading or trailing edge of the individual peaks. These spectra are shown as insets to Figs. 6.3-6.8, and they compared well with previously obtained spectra of standards (not shown). Although the use of UV data as a means of identification or confirmation of identity is somewhat limited in value, these spectra do nonetheless provide additional evidence, especially when added to the chromatographic retention data, that the peaks correspond to the compounds present in the mixture. Had the UV-DAD been placed on the branch taking the minor portion of the flow to the mass spectrometer and a make up flow been employed so as to dilute the samples to bring the whole peak back on scale, then it would have been possible to retrieve spectra from across the entire peak. This would have enabled the use of peak purity algorithms as commonly found

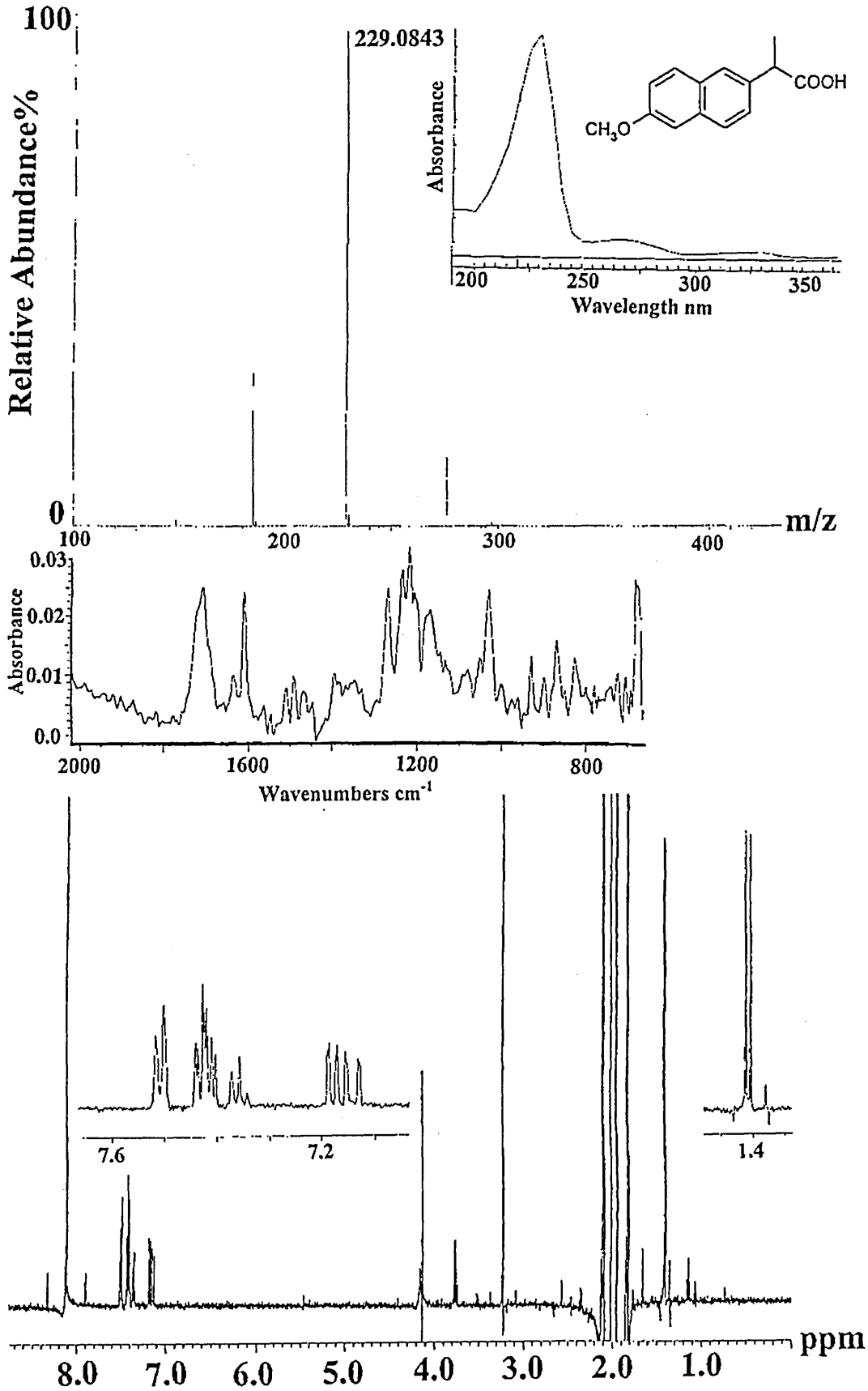



Fig. 6.3 *ToF-MS, UV, IR and $^{1}H$ NMR spectra of naproxen, structure inset to UV spectrum.*

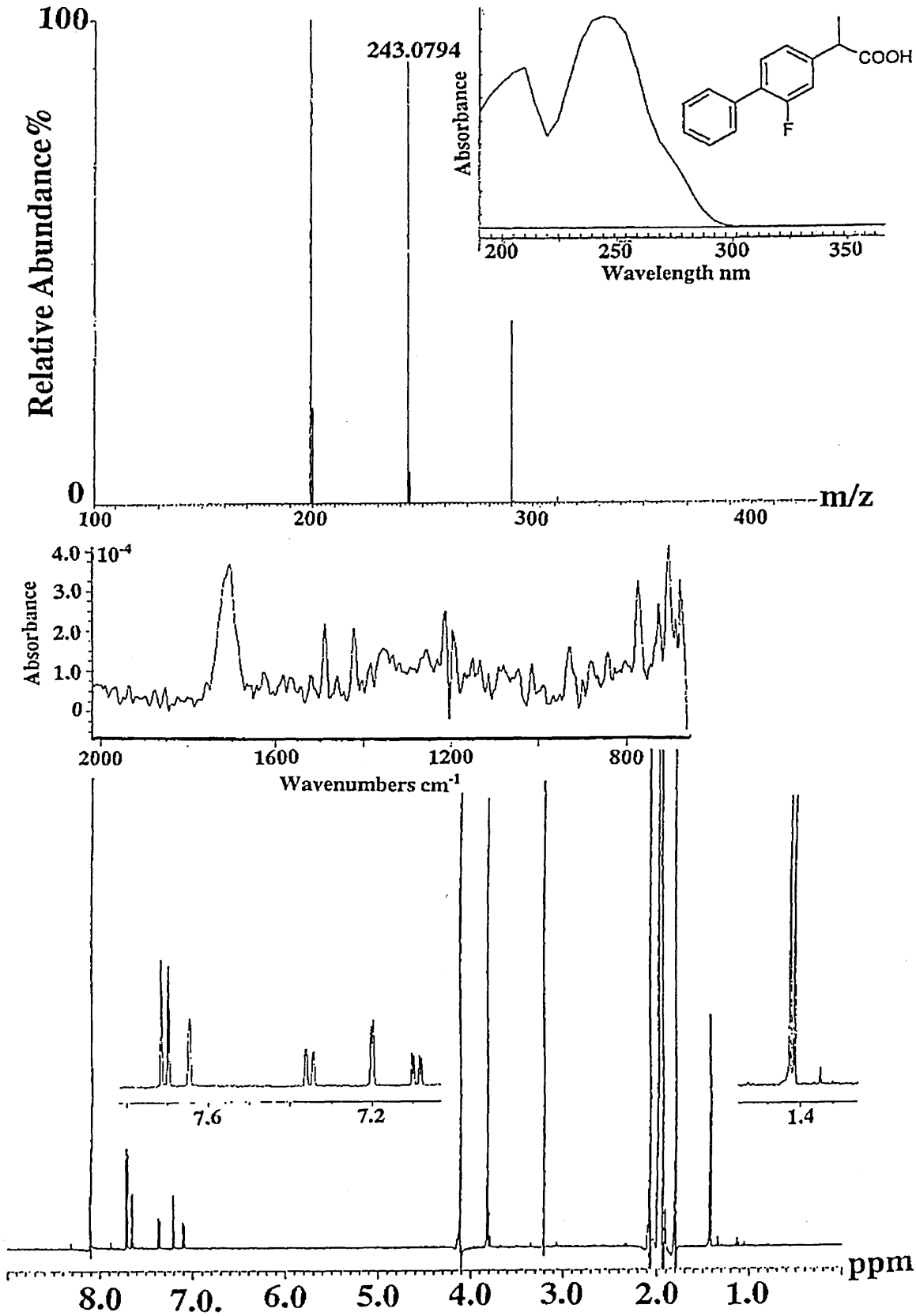



Fig. 6.4 *ToF-MS, UV, IR and $^{1}H$ NMR spectra of flurbiprofen, structure inset to UV spectrum.*

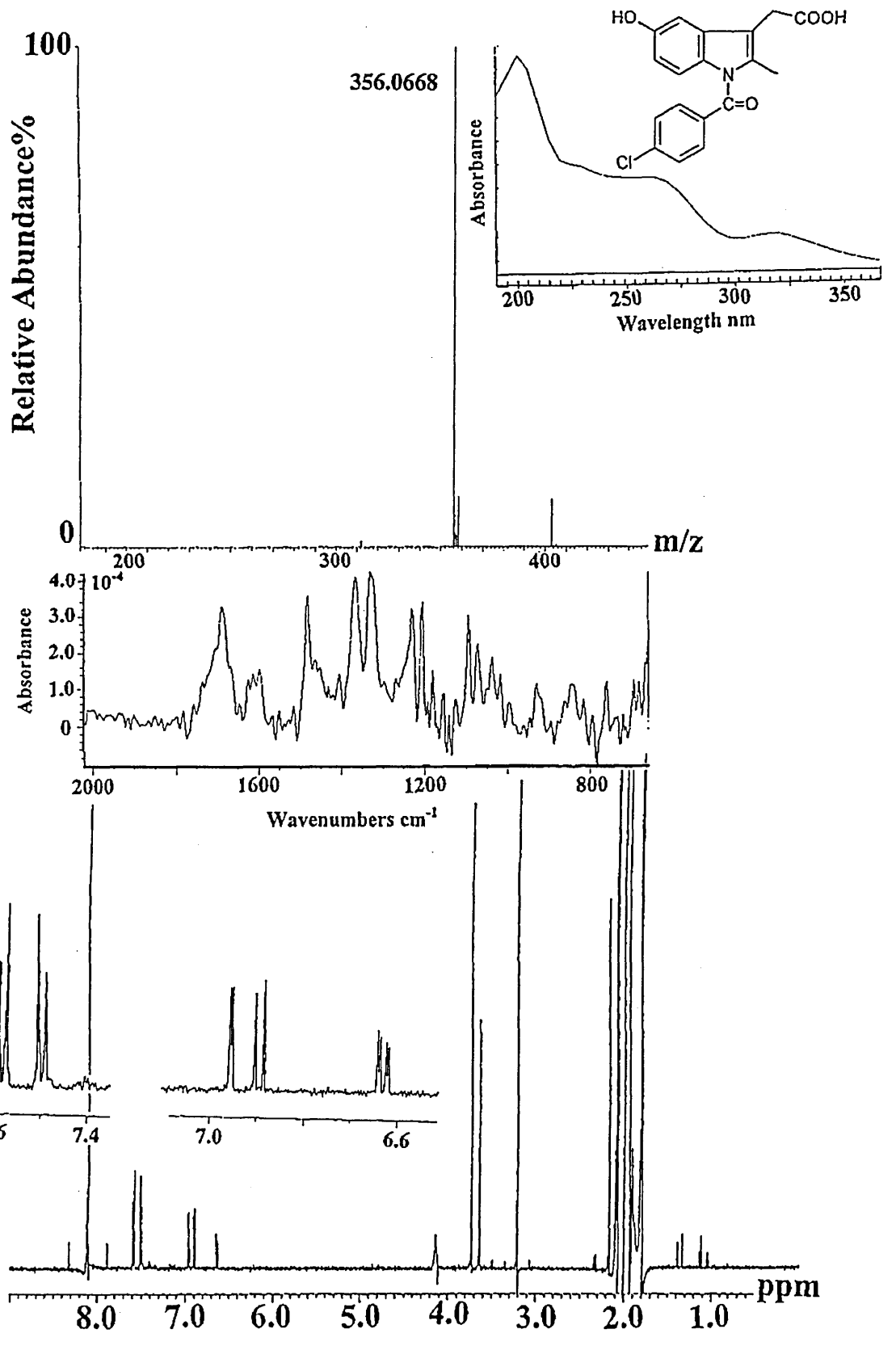



Fig. 6.5 *ToF-MS, UV, IR and $^{1}H$ NMR spectra of indomethacin, structure inset to UV spectrum.*

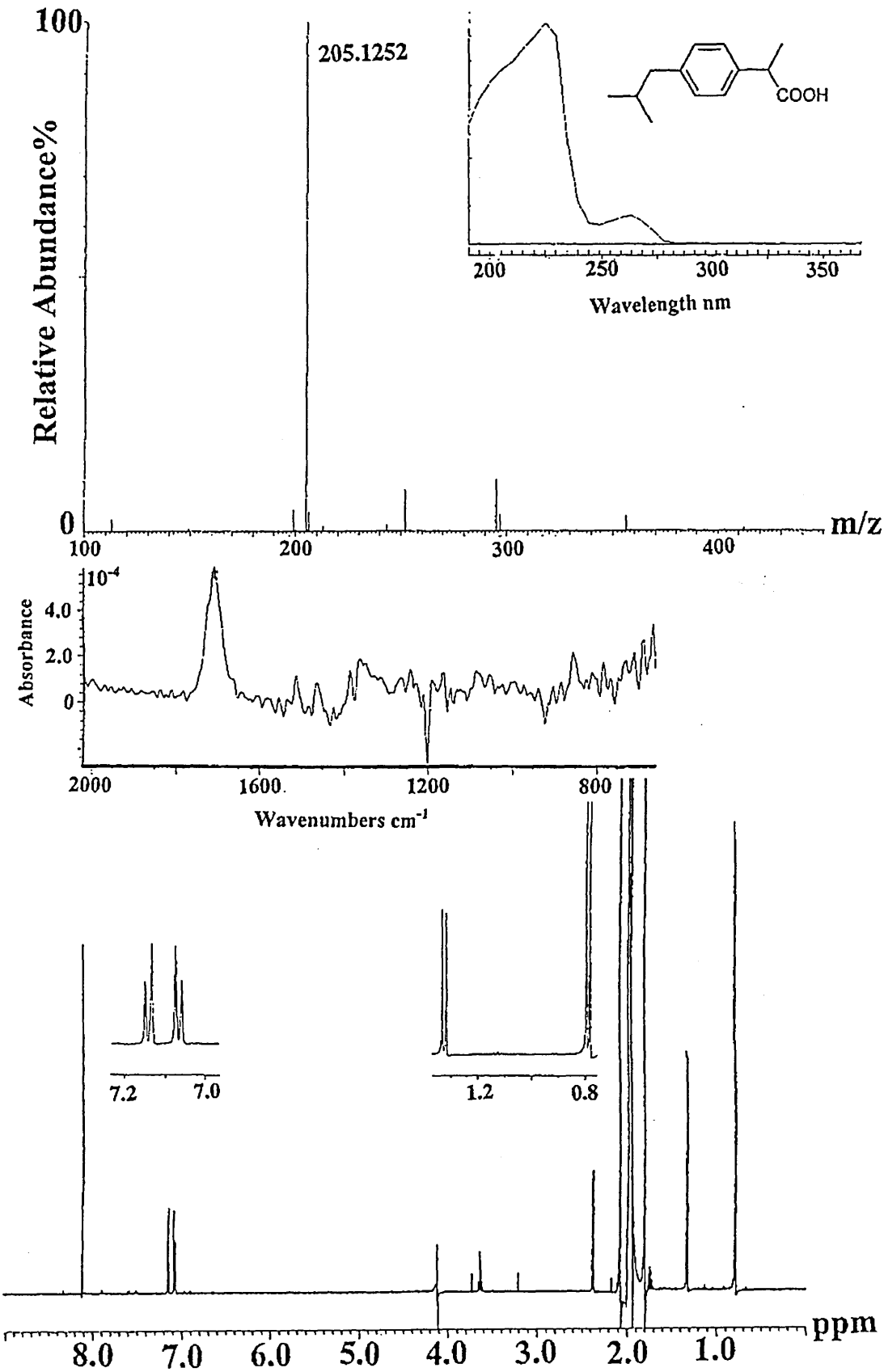



Fig. 6.6 *ToF-MS, UV, IR and $^1$H NMR spectra of ibuprofen, structure inset to UV spectrum.*

in UV-diode array detectors, thereby giving further information on the nature of the samples/peaks.

### 6.3.3 IR Spectra

As shown in Figs. 6.3-6.6, IR spectra were obtained for each of the four NSAIDs, and these were achieved in the on flow mode in each instance. The dominant signal in all four spectra is the carbonyl absorption at ~1700cm$^{-1}$. This is typical for compounds containing a carboxylic acid functionality. The analytes could be distinguished from each other based on the considerable differences in their spectra in the "fingerprint" region of each spectrum. The signal to noise achieved in the on flow mode was sufficient for identification of all of the compound although in the case of ibuprofen it was somewhat borderline.

Infra red spectra are particularly difficult to fully interpret but in this instance automated library searching facilities could be used as the NSAIDs utilised in these experiments were all members of the 6000 compound library available. In each case the top match proved to be the correct solution. In the cases of naproxen, flurbiprofen and indomethacin the spectral matches were 83.7, 84.5 and 92.4%, with the second best fit for these compounds being 53.9, 45.9 and 63.2%, respectively, thus giving a high degree of confidence in the answers. In the case of ibuprofen, the best match from the library was also correct but only with a value of 66.7% and with the second best match being 66.3% the confidence had this been an unknown would have been significantly reduced. This reduced confidence was due to the relatively poor signal to noise obtained for ibuprofen compared with the other compounds. It was possible to boost the signal to noise and hence the confidence in the result by summing the

spectra over the entire chromatographic peak rather than taking spectra at individual time slices. Alternatively employing a lower flow rate would have given the same outcome.

### 6.3.4 NMR Spectra

In these experiments it was decided to use stop flow NMR to ensure that good quality spectra were obtained and to minimise the difficulties of solvent suppression. The $^{1}H$ NMR spectra shown in Figs. 6.3-6.6 for each of the four NSAIDs were acquired using between 32 and 124 scans/spectrum. The spectra contain, in addition to the resonances for analytes themselves, signals for residual water (4.1 ppm), acetonitrile (1.93 ppm), methanol (3.2 ppm) and the formic acid (8.1 ppm) used for suppression of the ionisation of the four NSAIDs. However, none of these additional resonances prevented identification of the compounds present in the mixture.

In greater detail, for the first eluting compound naproxen all the aromatic resonances are clearly visible between 7.0 and 7.8 ppm, together with the singlet for the methyl group at 3.82 ppm and the doublet for the aliphatic methyl at 1.4 ppm (see Fig. 6.3). The quartet for the methine proton coupled to the aliphatic methyl is also visible at ~3.81 ppm though this is partially obscured by the resonance for the methoxy group. Similarly, all of the expected resonances for the two other "profen"-type NSAIDs (flurbiprofen and ibuprofen) were observed in the $^{1}H$ NMR spectra obtained in this experiment (see Figs. 6.4 and 6.6). Indomethacin is structurally dissimilar (structure is inset to Fig. 6.5) but as with the other NSAIDs all the structural features are present in the $^{1}H$ NMR spectrum. Specifically the para-substituted aromatic/phenyl ring

displayed the expected AA'BB' splitting at ~7.55 ppm while the aromatic protons from the indole moiety showed resonances between 6.6 and 7.0 ppm. Additionally, the resonances for the two methyls (3.73 and 2.2 ppm) along with the methylene group (3.64 ppm) can also be clearly noted (see Fig. 6.5).

### 6.3.5 Mass Spectra

Negative ion mass spectra were obtained on all four compounds. The first eluting peak (naproxen) showed an ion at m/z 229.0843 (calculated 229.0865, $C_{14}H_{13}O_3$) corresponding to the $[M-H]^-$. This spectrum also contained ions at m/z 185 and m/z 276, which corresponded to the loss of $CO_2$ from the parent (m/z 229)and the adduction of deuterated formic acid with the parent, respectively. Similarly the second peak to elute showed an ion $[M-H]^-$ for flurbiprofen at m/z 243.0794 (calculated 243.0821, $C_{15}H_{12}O_2F$), together with ions at m/z 199 and m/z 290. As with the naproxen spectrum these ions corresponded to the loss of $CO_2$ from the parent (m/z 243) and the adduction of deuterated formic acid with the parent, respectively. Essentially similar results were also obtained for indomethacin and ibuprofen (peaks 3 and 4) with $[M-D]^-$ ions at m/z 356.0668 (calculated 356.0690, $C_{19}H_{15}NO_4Cl$) and m/z 205.1252 (calculated 205.1229, $C_{13}H_{17}O_2$), respectively. Also present in each spectrum, to varying degrees, was leucine encaphalin, the lock mass (m/z 295).

Due to the high sample loadings used to ensure the simple acquisition of the NMR and IR spectra, there was a large excess of compound reaching the mass spectrometer. Although a large split (~95:5) and a make up flow had been employed, this was done using only a simple T-piece arrangement. Had more time been available these conditions could have been better optimised to allow for improved spectra and mass

accuracy. The concentrations of the compounds, even with the T-piece and make up flow, were still such that only a few scans could be added together and this coupled with the suppression of the lock mass signal meant it was not possible to get within the stated specification for the mass spectrometer of better than 2mDa below m/z 400. Despite this, all of the mass measurements obtained were within 12 ppm of the calculated values for all the compounds present, and when these spectra were processed by the software to assign ionic compositions to the masses found, the correct compound was identified as the best match in every case.

### 6.4 The analysis of plant ecdysteroids using superheated water.

Determination of the structure of active components in plant extracts, and confirmation that the components of interest are either known or novel, is a key task for natural product chemists. The development of routine HPLC/MS and HPLC/NMR has aided this research greatly, but often they must be used in conjunction with one another to provide unambiguous identification. By using multiple hyphenation it was hoped to be able to provide comprehensive spectroscopic information from a single chromatographic separation. In this study a modified version of the system employed to analyse the NSAIDs was used for the detection and identification of ecdysteroids in a number of extracts of plants from the Silene family. The ecdysteroids are a family of polar, polyhydroxylated steroids that function, among other things, as the moulting hormones of insects and crustaceans. They are also found in many species of plants, often in high concentrations, where it is assumed that they have some function as chemical defences against predatory insects. There is considerable structural diversity within the ecdysteroids and some 300 compounds

have previously been identified. The experiments described in this section were aimed at further investigating the multiply hyphenated approach using typical ecdysteroid containing plant extracts.

### 6.4.1 Plant extracts

The extracts used in this study were obtained from *Silene nutans*, *Silene otites* and *Silene frivaldskyana*. The sample of *S. nutans* was collected in the area of Pradelles, (Haute Loire, France) and the *S. otites* sample was collected in the vicinity of Chattillon-en-Dios, Drome (France). *S. frivaldskyana* was obtained as a gift from the Siberian Botanical Garden of Tomsk (Russia) from Dr Larisa Zibareva. All the samples were air dried and then finely milled before being extracted with 96% ethanol (10 or 20 mL/g). After extraction, the samples were filtered and evaporated to dryness under reduced pressure and at room temperature. They were then qualitatively screened for the presence of ecdysteroids by normal phase HPLC with UV detection on silica gel using a solvent system of dichloromethane: 2-propanol: water (125: 40: 3 or 125: 20: 1.5)[102]. Immediately prior to HPLC, the sample was evaporated to dryness and taken up in a small volume of deuterated methanol ($CD_3OD$).

### 6.4.2 Experimental conditions

The ecdysone and 20-hydroxyecdystone used as standards were kindly provided by Rene Lafont from CNRS in Paris. Deuterated solvents were obtained from Fluorochem (Glossop UK)

The system employed (Fig. 6.7) was a modified version of the system used for the analysis of the NSAIDs, consisting of a Constametric 3200 HPLC pump (LDC, Stone UK) which delivered $D_2O$ at 0.8mL/min to a 150 x 4.6 mm i.d. C8 Xterra 5 μm HPLC column (Waters Ltd, Watford UK). Some method development was also performed on an Xterra C18 150 x 4.6 mm i.d. 5 μm column.

Columns were placed in the oven of a Pye 104 gas chromatograph (GC) at elevated temperatures. The temperature was controlled by a Pye oven programmer and was typically set to 160C. The eluent emerging from the column was rapidly cooled using a water bath kept at ~ 4C using ice. The $D_2O$ was maintained in a liquid state above 100C by the backpressure generated by the PEEK tubing connecting the column to the various detectors.

From the column, the eluent entered a splitter from which the minor portion of the flow (approx 5%) was directed to a Micromass Platform LC single quadrupole mass spectrometer (Micromass UK) via 4.5 m of 75 μm i.d. fused silica capillary tubing, the length being determined such that sufficient back pressure was generated to direct the bulk of the flow to the FTIR spectrometer. The Platform LC was operated with an atmospheric pressure chemical ionisation (APCI) interface with mass spectra measured over a range of m/z 115 to 650. Prior to the sample reaching the source of the mass spectrometer, the eluent was mixed with a make-up flow of 90:10 (v:v) methanol:water. The purpose of this make-up flow was to ensure that the chromatographic resolution of the system was maintained by minimising the transfer time and to ensure that the APCI technique functioned correctly by providing sufficient flow of liquid to obtain efficient ionisation. A second Constametric 3200

pump delivered this make-up flow via a T-piece at 0.5mL/min. Positive ion spectra were recorded using a cone voltage of 25V, a scan time of 0.9s and an interscan delay of 0.1s.

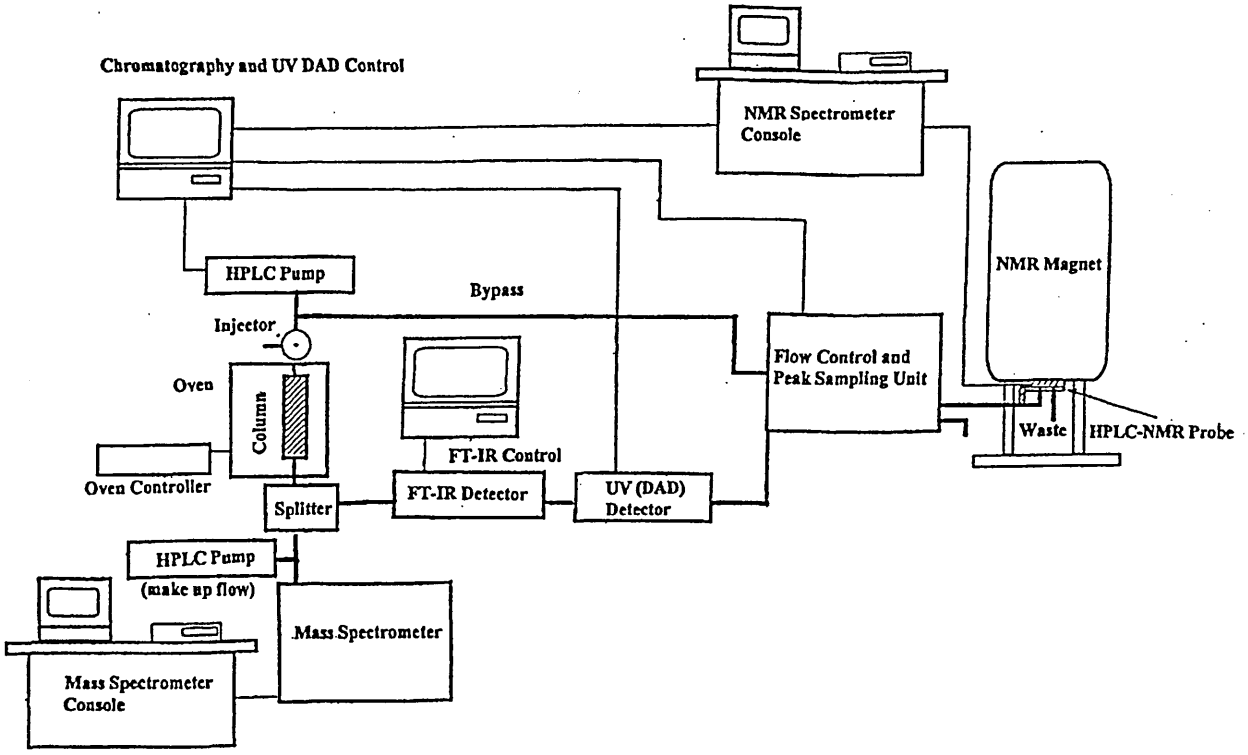



Fig. 6.7 *The modified layout used for the hot water analysis of plant ecdysteroids using multiple detectors to provide a one stop chromatographic and spectroscopic evaluation of the plant extracts. The major changes are the inclusion of a split and make-up flow prior to the mass spectrometer and the use of a single UV detector (DAD) to obtain UV spectra and to monitor the separation rather separate detectors.*

The remainder of the flow (approx 95%) was directed via 110cm of 0.005" i.d. PEEK tubing to a BioRad FT-IR model FTS3000 Excalibur spectrometer (Cambridge MA) fitted with a Micro Circle Cell ATR high pressure stainless steel flow cell of 25 μL volume fitted with a zinc selenide ATR crystal. The spectrometer was purged as previously with dry nitrogen to minimise any water vapour interference of the

collected spectra. Any remaining water vapour in the collected data was subtracted using a water vapour reference spectrum. Spectra were acquired as previously described, collecting 57scans/spectrum (10 second acquisition time) at 8 $cm^{-1}$ spectral resolution. The samples were ratioed against a background spectrum of the solvent flowing through the cell just prior to injection of the sample solution, thus automatically subtracting out the solvent spectrum from the sample spectra.
From the FT-IR the eluent was directed to a Bruker UV-DAD (Bruker UK) via 30 cm of 0.005" i.d. PEEK tubing. UV spectra were acquired over the range 188-1000nm. From the UV-DAD the solvent stream was sent to the NMR flow probe via 280cm of 0.01" i.d. PEEK tubing.

NMR spectra were acquired using a Bruker DRX-500 NMR spectrometer. On flow $^1H$ NMR detection was at 500.13 MHz using a flow through probe of 3 mm i.d. with a cell volume of 60 µL. Typically, 16 scans/FIDs per increment were acquired into 8K data points, each with a spectral width of 8278 Hz. Spectra were acquired with the NOESYPRESAT pulse sequence in order to suppress residual water resonances. Pulses of 90° were used with an acquisition time of 0.5s, a relaxation delay of 0.7s and a mixing time of 100ms.

### 6.4.3 Results and discussion

The use of elevated temperatures always brings concerns over the stability of the compounds being investigated under what are potentially aggressive conditions. In this case in particular the ecdysteroids are known to be temperature sensitive, decomposing by dehydration. It was therefore unclear at the outset whether this approach would be feasible. Some initial method development was conducted, aimed

at reducing the amount of organic modifier used and raising the temperature only moderately, rather than attempting to remove the organic modifier altogether. Preliminary studies using water/methanol mixtures showed that by raising the temperature of the column it was indeed possible to significantly reduce the amount of organic modifier necessary to elute these compounds. At ambient temperature (ca 20C) with 40% methanol as the modifier, ecdysone was eluted at approximately 8 minutes from the C18 bonded Xterra stationary phase. On increasing the column temperature to 120C it was possible to reduce the organic content of the mobile phase to 20% for a similar retention time. By raising the temperature to 140C it was possible to reduce the organic content still further to 10% with ecdysone now eluting at ca 10.5 minutes. For NMR studies, where the cost of deuterated methanol is a major concern, this reduction of 75% in the consumption of deuterated methanol would represent a significant cost saving or if the protonated form were to be used then it would equally represent a useful reduction in the unwanted background signal. It was hoped that by switching to a C8 column there would be a significant reduction in the retention characteristics of the stationary phase but, at 140C and 10% methanol, the retention time for ecdysone was still approx 10.5 minutes. However, by further raising the temperature of the column it was possible to elute ecdysone in 100% deuterated water at approx 25 minutes and although this was a little long the advantages of using 100% $D_2O$ were considered greater than the downside of compounds eluting much later. Hence these conditions were used for the remainder of the work described here.

Utilising these conditions and injecting a standard of 20-hydroxyecdysone (structure shown inset to Fig. 6.8) resulted in a peak with a retention time of approx 17 minutes.

20-hydroxyecdysone was chosen as the results of previous studies had shown this to be the expected major ecdysteroid found in the plant extracts being investigated. Experiments injecting samples of 20-hydroxyecdysone in amounts equivalent to 100 to 400 μg on column showed that good quality UV, IR, MS and NMR spectra could be easily obtained on-flow over this range. The $^{1}H$ NMR spectra showed all of the expected resonances for 20-hydroxyecdysone were present and similarly the UV, MS and IR spectra confirmed that these chromatographic conditions did not cause decomposition. The chromatogram, together with representative spectra of 20-hydroxyecdysone for a sample of 200μg on column, are shown in Fig. 6.8. When the NMR spectrum obtained here is compared with that obtained in experiments using an acetonitrile/water system (20/80) the elimination of the solvent-derived interference with the analyte is most significant (see Figs. 6.16 and 6.17 later in this chapter).

Having established the stability of the compounds under these conditions the crude plant extracts were then analysed. In the case of *S. frivaldskyana* a number of UV absorbing peaks were observed (Fig. 6.9a). The early eluting peaks gave UV and NMR spectra that clearly indicated they were not ecdysteroid related; however, a major UV absorbing compound eluted at the retention time of 20-hydroxyecdysone (peak 3 in Fig. 6.9a). The UV and IR (Figs. 6.9b,e) spectra of this peak were consistent with the presence of an ecdysteroid, showing a maximum at ~250nm in the UV spectrum and a carbonyl absorption at 1643 $cm^{-1}$ in the IR spectrum. Comparison of this IR spectrum with the library gave a match for 20-hydroxyecdysone. By measuring the peak area for this peak and comparing it with those of the standards it was possible to estimate that the sample contained approx 150μg of 20-hydroxyecdysone in the 20μL sample injected. The $^{1}H$ NMR spectrum contained the

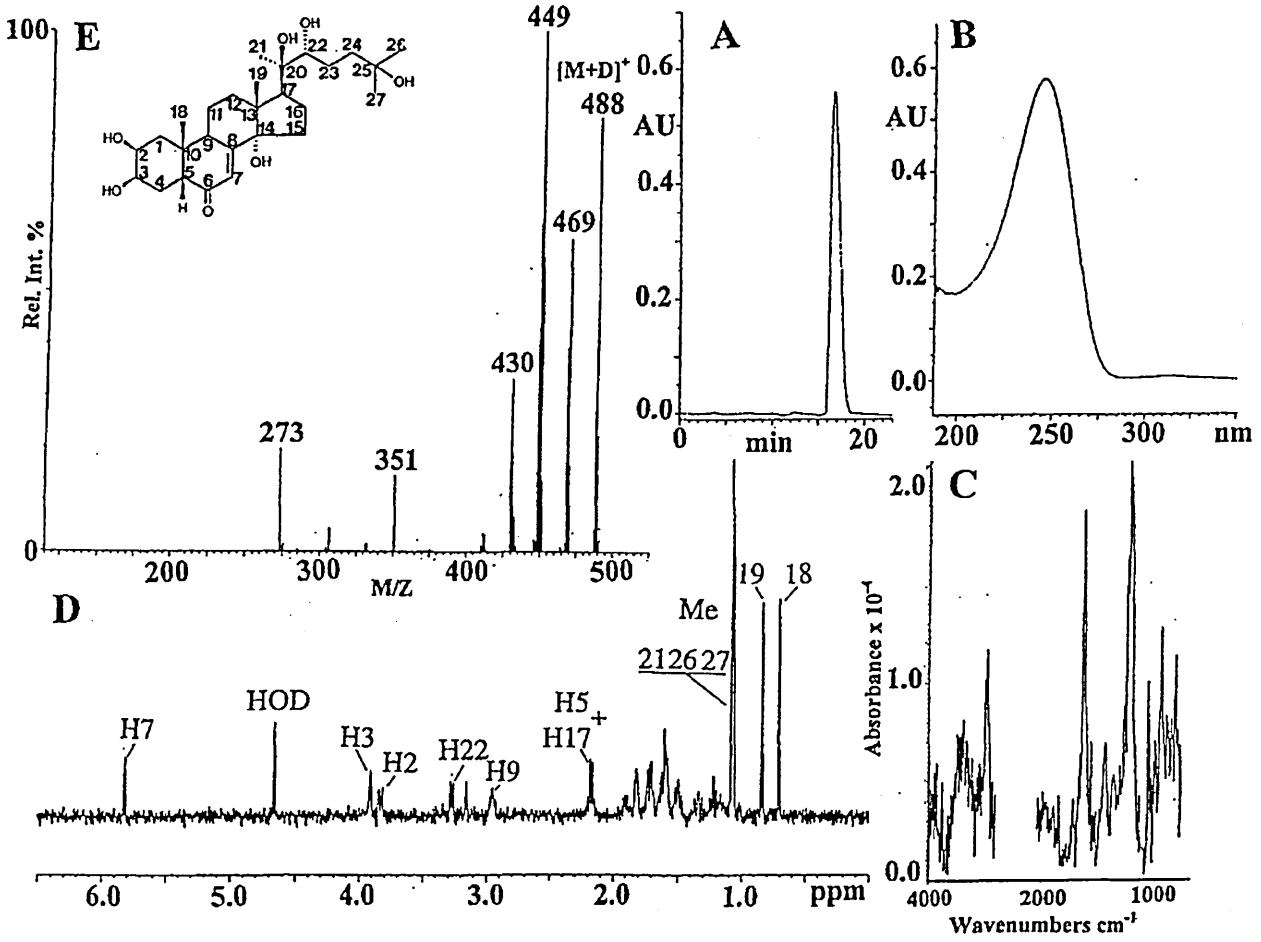



Fig. 6.8 *Chromatographic and spectroscopic data obtained for a 200µg on column sample of 20-hydroxyecdysone standard (structure inset to the MS). Key: A:- the HPLC-UV chromatogram monitored at 254nm, B:- UV spectrum showing a maximum at ~250nm, C:- FT-IR spectrum showing the significant carbonyl absorption band at 1643 cm$^{-1}$, D:- the $^{1}H$ NMR (see text and structure for further explanation),and E:- the mass spectrum showing an $(M+D)^{+}$ ion at m/z 488 and other diagnostic peaks.*

expected methyl resonances for 20-hydroxyecdysone, together with the signal for the diagnostic C-7 proton. These data, together with the $[M+D]^{+}$ for 20-hydroxyecdysone at m/z 488 (Fig. 6.9c) and the associated product ions at m/z 469, 468 and 449 representing the loss of HDO from m/z 488, $D_2O$ from m/z 488 and $D_2O$ from m/z 469, respectively, confirmed the presence of 20-hydroxyecdysone in the sample.

Careful examination of the mass spectrum for this peak revealed the presence of an ion at m/z 505, possibly due to the presence of small amounts of the ecdysteroid polypodine B, (5β 20-hydroxyecdysone), which co-elutes with 20-hydroxyecdysone in reverse phase systems (see data for *S. nutans* below). On the basis of the NMR data, polypodine B could not have constituted a large proportion of the total (less than 10%). Although most of the other UV absorbing peaks did not show ecdysteroid UV characteristics, the small peak eluting just prior to 20-hydroxyecdysone (peak 2 in Fig. 6.9a) gave a UV spectrum consistent with the presence of an ecdysteroid. (N.B. the first ten minutes of the chromatogram is blank as the eluate from the column was diverted to waste). As UV data is not very specific it has been hoped to use the other detectors to unequivocally confirm the presence of an ecdysteroid; however, this component was present in insufficient quantity to provide either an IR or NMR spectrum, but the mass spectrum did give an ion at m/z 505 and a range of characteristic ions at m/z 446, 466 and 486 as the result of the loss of water (see the explanation for 20-hydroxyecdysone above). It would seem probable that this was an ecdysteroid.

The preliminary normal phase HPLC screening of this extract (not shown) carried out prior to the reverse phase chromatographic and spectroscopic analysis suggested that in addition to the major peak for 20-hydroxyecdysone, trace amounts (less than 10%) of polypodine B, 20,26-dihydroxyecdystone, integristerone A (1,20-dihydroxyecdystone) and 26-hydroxypolypodine B were also present. Polypodine B, integristerone A, and 20,26-dihydroxyecdystone would all give a fully deuterated ion at m/z 505, but that for 26-hydroxypolypodine B would be expected to be observed at m/z 522. Assuming that the ion seen in the superheated water separation for the peak

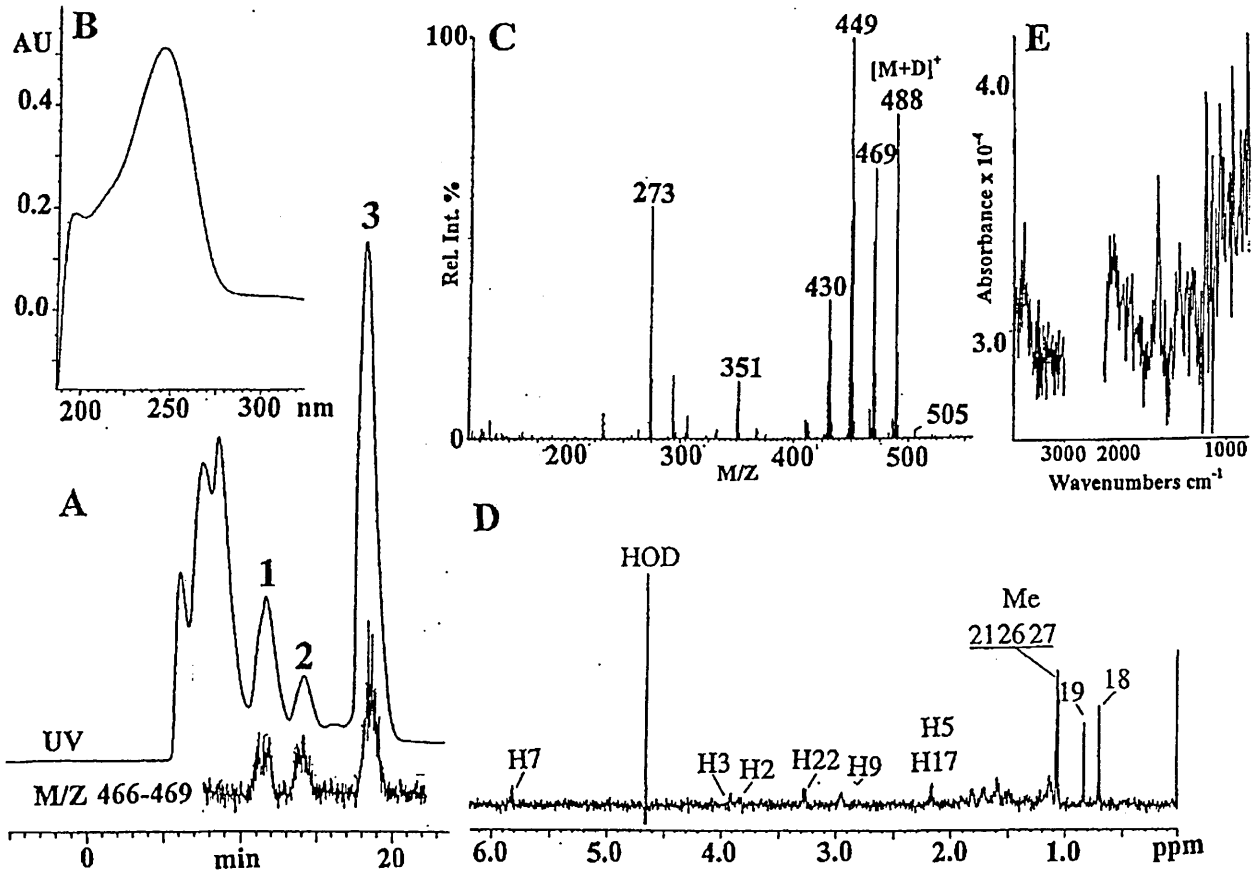



Fig. 6.9 *Chromatographic and spectroscopic data obtained for the extract of S. frivaldskyana. Key: A:- UV and mass (for ions 466-469) chromatograms, peak 1 is thought to be 20,26-dihydroxyecdystone and 26-hydroxypolypodine B; peak 2 is thought to be integristerone A; peak 3 is 20-hydroxyecdysone, B:- UV spectra, C:- mass spectrum, D:- $^{1}H$ NMR spectrum and E:- FT-IR spectra obtained for peak 3. Without supporting evidence from NMR and/or IR it is not possible to confirm the identity of peaks 1 and 2.*

eluting just prior to 20-hydroxyecdysone corresponded to an ecdysteroid then it was probably due to the presence of integristerone A, which is less polar than either 20,26-dihydroxyecdystone or 26-hydroxypolypodine B. Further examination of the MS data for ions characteristic of ecdysteroids (see Fig. 6.9a) detected these ions in two small peaks eluting at approx 11 minutes, each having an ion at m/z 505 which is consistent

with the presence of 20,26-dihydroxyecdystone. In the mass spectrum of the later eluting compound (approx 14 minutes) it was also possible to see an ion at m/z 522 in addition to that at m/z 505, suggesting that this might be 26-hydroxypolypodine B; however, because of the relatively small amounts present and the interference from other components in the extract it was not possible to obtain convincing $^{1}$H NMR, UV, MS or IR spectra for these components. Full characterisation of these minor components would have required much higher loadings and a much greater degree of sample clean up prior to analysis.

For the extract of *S. otites*, the early eluting peaks (up to retention time of approx 11 min) gave spectra that clearly showed them to be unrelated to ecdysteroids. However, there was a major UV absorbing peak eluting at the retention time of 20-hydroxy-ecdysone (peak 2 Fig. 6.10a). This peak contained significantly more material than the corresponding sample from *S. frivaldskyana* (approx 590µg in the 20µL injected aliquot on the basis of peak area using the UV chromatogram). This large amount of sample in a single peak provided ample material to be able to acquire diagnostic UV, IR (97% match), NMR and MS data for 20-hydroxyecdysone. In addition a small peak was observed eluting just prior to the major peak of 20-hydroxyecdysone (peak 1 Fig. 6.10a) that had a typical UV spectrum for an ecdysteroid (see Fig. 6.10b spectrum b). The $^{1}$H NMR obtained for this peak was very weak, and unequivocal identification of this component was not possible; however, signals consistent with the methyl groups of integristerone A could be observed. MS data from this peak gave an ion consistent with the ion expected for integristerone A at m/z 505. The FT-IR gave a weak spectrum containing a carbonyl absorption at 1650 cm$^{-1}$ that gave an

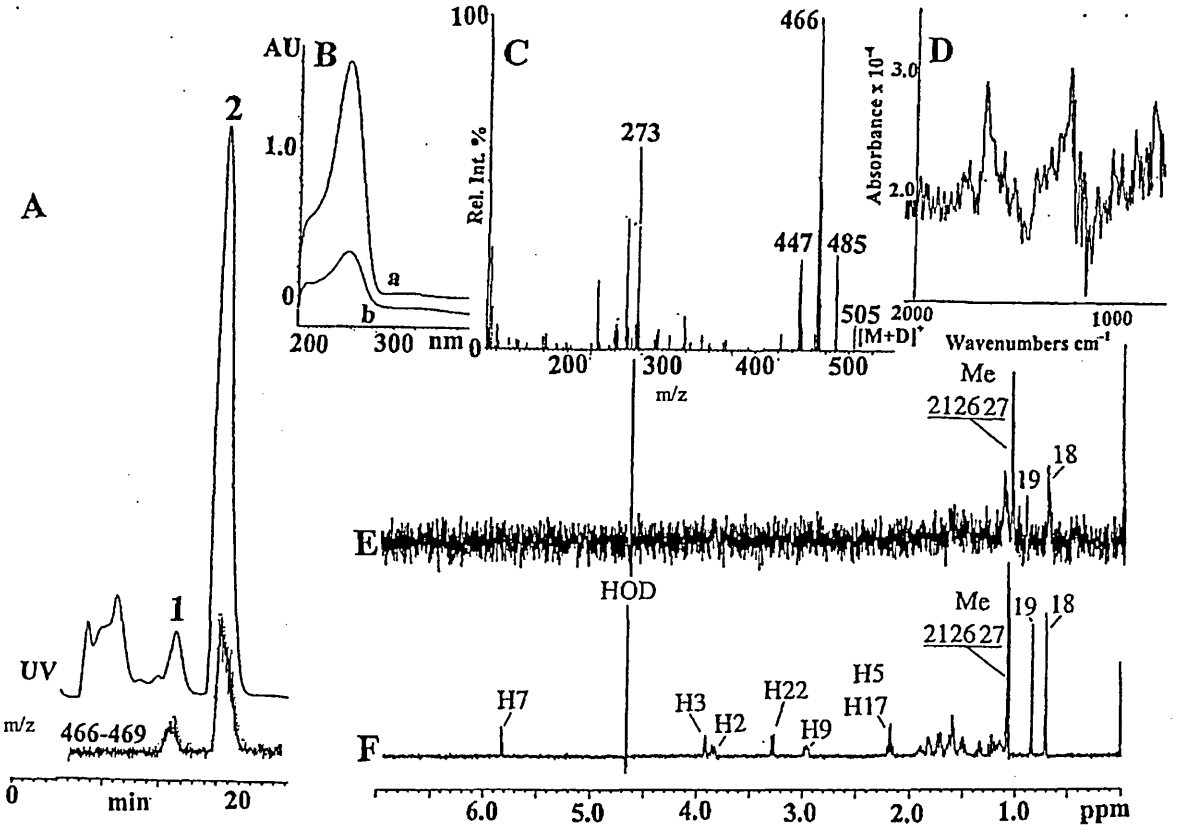



Fig. 6.10 *Chromatographic and spectroscopic data obtained for S. otites. Key: A:- UV (at 254nm) and mass (for ions between m/z 466 and 469) chromatograms, peak identification; peak 1 is integristerone A, peak 2 is 20-hydroxyecdysone, B:- UV spectra for a, peak 2 and b, peak 1, C:- mass spectrum, D:- IR spectrum, E:- $^1$H NMR spectrum for peak 1, and F:- $^1$H NMR spectrum for peak 2. The $^1$H NMR spectrum shown in E for peak 1 is close to the limit of detection and should be compared with that shown in Fig. 6.11f, in which a more comprehensive spectrum for this ecdysteroid was obtained.*

88% match to an ecdysone species. The spectral data for this component are shown in Fig. 6.10b-e with the $^1$H NMR spectrum of the 20-hydroxyecdysone present in the extract given in Fig. 6.10f for comparison.

The preliminary screening of this extract by normal phase HPLC had indicated that as well as 20-hydroxyecdysone, small quantities of integristerone A, 2-deoxyecdysone, and 2-deoxy-20-hydroxyecdysone were also present; however, these latter relatively non-polar ecdysteroids were not observed in the reverse phase separation described here.

The final extract to be examined was of *S. nutans*. Once again this contained a number of components that were unrelated to ecdysteroids eluting in the first 10 minutes of the chromatogram (Fig. 6.11a), together with a major UV absorbing peak at the retention time of 20-hydroxyecdysone (approx 630μg was calculated to be present in the 20μL aliquot injected) (peak 2 Fig. 6.11a); however, closer examination of the $^{1}H$ NMR spectrum for this peak revealed it to be composed of 2 ecdysteroids. The major component as expected was 20-hydroxyecdysone, but polypodine B was also present and comprised approximately 40% of the total as estimated from the relative proportion of the C-7 protons; see Fig. 6.11e). The MS data also confirmed the presence of an ion at m/z 505 for polypodine B. The IR spectra also showed a shift in the carbonyl absorption characteristics with a shift from 1642.3 $cm^{-1}$ on the leading edge of the peak to 1641.8 $cm^{-1}$ on the trailing edge, indicating the possibility of the presence of more than one ecdysteroid co-eluting which was consistent with the presence of polypodine B. Representative spectra are shown in Fig. 6.11b-d.

In addition to these major components a peak with UV characteristics consistent with the presence of an ecdysteroid was also detected (approx 100-200μg present by peak area in the 20μL aliquot). This eluted before the main peak, which contained both the polypodine B and the 20-hydroxyecdysone, with a retention time of approximately 14

minute (peak 1 and spectrum b of Fig. 6.11a,b). This compound was present in sufficient quantity for it to be identified as integristerone A. Screening of the extract by normal phase HPLC had also indicated the presence of integristerone A, polypodine B and 20-hydroxyecdysone, along with very small quantities of 20,26-

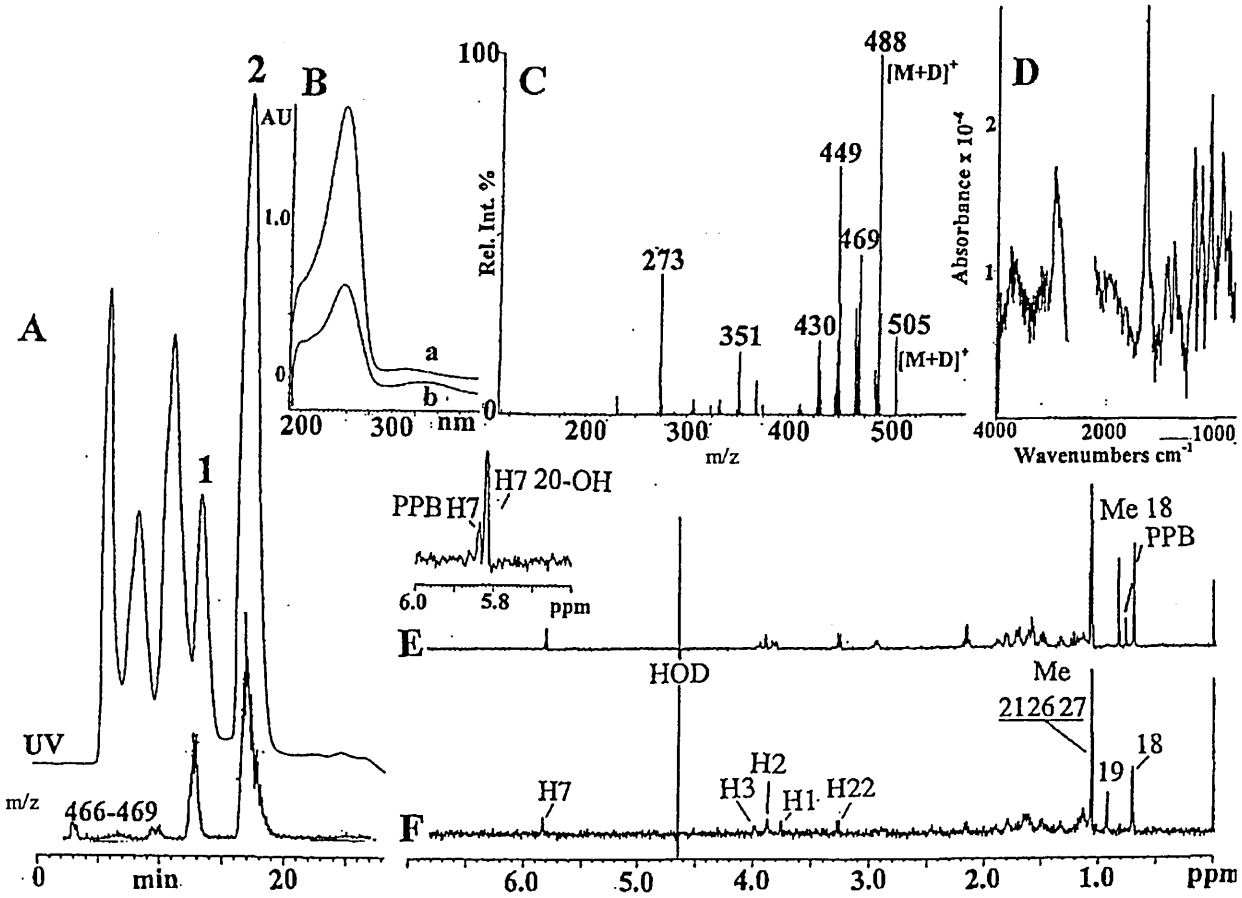



Fig. 6.11 *Chromatographic and spectroscopic data obtained for S. nutans extract. Key: A:- UV (at 254nm) and mass (for ions between m/z 466 and 469) chromatograms, peak identification; peak 1 is integristerone A, peak 2 is 20-hydroxyecdysone and polypodine B, B:- UV spectra for a, peak 2 and b, peak 1, C:- mass spectrum, D:- IR spectrum, E:- $^1$H NMR spectrum for peak 2 (including an expansion of the $^1$H NMR spectrumfor the C-7 protons), and F:- $^1$H NMR spectrum for peak 1.*

dihydroxyecdystone and 26-hydroxypolypodin B. Despite careful examination, no convincing evidence of the presence of these latter components could be obtained from the reverse phase data.

The experimental results obtained from both the standards and the extracts indicated that the ecdysteroids were stable enough to tolerate the conditions used for chromatography without decomposition, thus enabling the use of superheated water or $D_2O$ for separation and analysis. The data obtained for the analysis of the plant extracts also demonstrated that this system could be used to obtain full UV, IR, $^1H$ NMR and MS data on these compounds in the region of 100μg on column. On the basis of these experiments it is suggested that this represents a reasonable limit of spectroscopic identification (LOSI). Clearly, it is possible to obtain a lower limit of detection, as is illustrated by the example of integristerone A present in the *S. otites* sample in which, for example, the methyl resonances were detectable by $^1H$ NMR, but the diagnostic C-7 proton was not.

## 6.5 The analysis of plant ecdysteroids using conventional chromatography

As with the NSAIDs, a comparison was made between superheated water chromatography and conventional chromatography using organic modifiers. The experimental changes and the results are described here.

### 6.5.1 Experimental changes

A number of ecdysteroid standards were introduced and an extract of *Lychnis flos-coculi*, a member of the family of *Caryopyllaceae,* was analysed. The ecdysteroids

ecdysone, makisterone A and 20-hydroxyecdysone were used as pure standards having been received as gifts. Samples of these standards were dissolved in $D_2O$ to give solutions containing 20mg/mL of ecdysone and 20-hydroxyecdysone, and 10mg/mL of makisterone A. The plant extract was obtained from finely ground air-dried *Lychnis flos-coculi*, collected near Rambuoillet in France. An ethanol extract was prepared using 96% ethanol at one litre per 100g dry weight of plant material with continuous stirring for 2-3 days. After extraction the sample was evaporated to dryness, re-dissolved in a small volume of methanol and centrifuged. Just before analysis this was evaporated to dryness and the residue dissolved in $D_2O$. The system was the same as that used to analyse the NSAIDs using conventional chromatography. For the mass spectrometer caffeine was used as a lock mass. Caffeine has a molecular mass of 194.0804 Daltons and was introduced via a T-piece at a concentration of 5ng/mL at a flow rate of 0.5mL/min.

### 6.5.2 Results

The work with the NSAIDs had shown that it was possible to obtain spectra from as little as 50μg of material. However, the extracts provided ample amounts of material, so in order to minimise the time taken to acquire spectra and therefore increase throughput more concentrated samples were employed.

### 6.5.3 Chromatography

#### *6.5.3.1 The standards*

A simple isocratic method of 80:20 $D_2O$: acetonitrile was used with a Hypersil H5BDS-C18 bonded stationary phase, 100 x 4.6mm, (Hichrom, UK) as this had

previously been used by co-workers to separate these compounds. The separation obtained for a mixture of the three standards on this column is shown in Fig. 6.12. Despite the high sample loading used (approx 3 mg per compound) good chromatographic peak shapes were obtained with almost baseline separation of all three analytes. The compounds eluted in the order 20-hydroxyecdysone (~4.8 min),

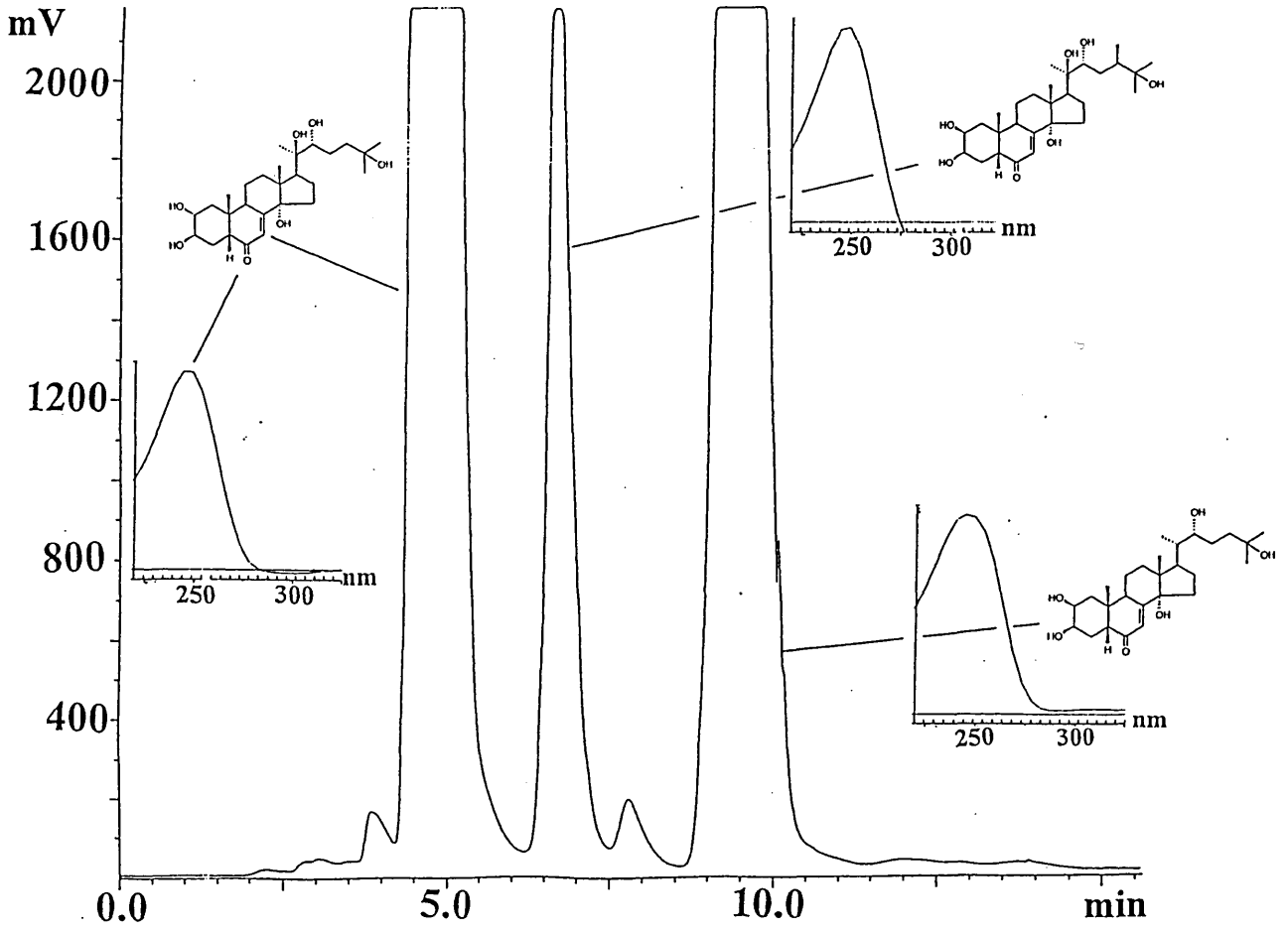



Fig. 6.12 *UV 254nm chromatogram obtained for the HPLC analysis of a mixture of approx 3mg of each of 20-hydroxy-ecdysone (~4.8 min), makisterone A (~6.6 min) and ecdysone (~9.5 min). (Hypersil H5BDS-C18 100x 4.6 mm, 80:20 $D_2O$:acetonitrile 1.0 mL/min). The structures of each compound are shown inset along with the UV spectra obtained during the analysis. The total run time for the analysis was ~12.5 minutes.*

makisterone A (~6.6 min) and finally ecdysone (~9.5 min). The UV spectra shown inset to Fig. 6.12 together with the structures of the three standards were of reasonable quality with no significant signs of the detector reaching overload.

### *6.5.3.2 Lychnis flos-coculi*

*Lychnis flos-coculi* represents a rich source of phytoecdysteroids (plant ecdysteroids) and has been shown to contain approx 0.17% of 20-hydroxyecdysone by dry weight. This and polypodine B, are two of the main ecdysteroids present in this species. Numerous minor ecdysteroids including 2-deoxy-20-hydroxyecdysone, dihydrorubrosterone, 20,26- hydroxyecdysone, 20- hydroxyecdysone 2 and 3 acetates, 26-hydroxypolopodine B, integristerone A, makisterone A, poststerone, rubrosterone, taxisterone and viticosterone have also been detected[103]. In order to improve the resolution of the system and thus enable better identification of the ecdysterones from the endogenous materials also present in the extract the column was increased in length to 25 cm. The UV chromatogram for the separation of the extract is shown in Fig. 6.13. This shows a number of peaks eluting at or close to the solvent front, with a major UV absorbing peak at 34 minutes which corresponded to the retention time of 20-hydroxyecdysone. Two other smaller peaks were also detected at approximately 36 and 39 minutes.

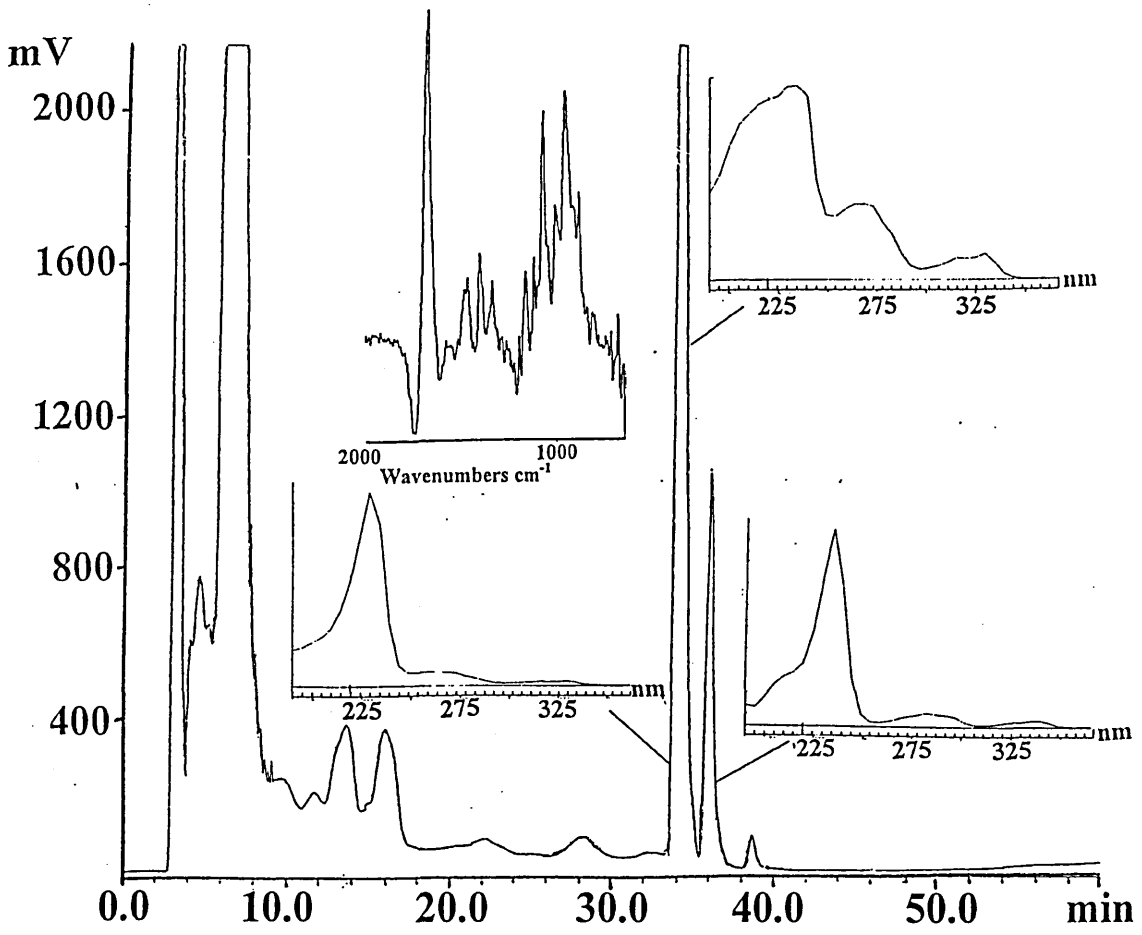



Fig. 6.13 *The UV chromatogram obtained following the analysis of the extract of Lychnis flos-coculi. The UV spectra obtained at the leading and trailing edges of the main peak together with that of the close eluting peak at approx 36 minutes are shown inset to the chromatogram along with the FT-IR spectrum for the main peak.*

### 6.5.4 UV Spectra

As was shown earlier the ecdysteroids typically show a UV absorbance maximum between 240 and 250 nm. As in previous experiments, the high sample loadings used to facilitate the collection of IR and NMR spectra meant that often the DAD overloaded other than at the leading or trailing edge of the chromatographic peaks. As would be expected the spectra obtained from the trailing edge of each of the standards (inset Fig. 6.12) are essentially identical, because the compounds are

structurally very similar and the differences being distant from chromophore. However, despite the presence of large amounts of ecdysteroids known to be in the plant the UV spectra obtained from the main chromatographic peak present in the crude *Lychnis* extract were not typical of those expected for an ecdysteroid. The spectra inset to Fig. 6.13 show a UV maximum at ~230nm early in the peak with a gradual increase to higher wavelengths (260-280nm) as the peak continues to be eluted. This suggests that the peak is not due to ecdysteroid-like substances. Similarly the smaller later eluting peaks at approximately 36 and 39 minutes failed to give ecdysteroid like spectra.

### 6.5.5 IR spectra

On-flow FT-IR spectra were obtained for all the analytes present in the mixed standards sample (Fig. 6.14A-C). All of these spectra were dominated by the presence of a strong absorption at approximately 1645 $cm^{-1}$ due to the carbonyl group in the ecdysteroids. The fingerprint regions of each spectrum showed differences that allowed for the compounds to be distinguished from one another. The FT-IR spectrum of the main peak in the UV chromatogram of the plant extract (inset to Fig. 6.13) showed a strong absorption at 1645 $cm^{-1}$, with similarities in the fingerprint region to 20-hydroxyecdysone. Because large amounts of material had been loaded the resulting spectra were of sufficiently good quality to be able to identify the spectrum as that of 20-hydroxyecdysone when searched against the reference library. It was possible to collect spectra in 5-second time slices through the peak and spectra for the beginning, middle and the trailing edge of the peak are shown in Figs. 6.14A-C. The carbonyl peak maximum shifted from approximately 1645 $cm^{-1}$ at the beginning and middle of the peak to 1665 $cm^{-1}$ at the end of the peak, and the

spectrum at the trailing edge of the peak shows a distinct shoulder at approximately 1645 $cm^{-1}$. This shows that the peak is impure and that at least two components are contributing to the resulting spectrum. Figs. 6.14D-F show the second derivative spectra from the beginning, middle and trailing edge of this ecdysteroid-containing peak. These sharpened second derivative spectra clearly show that the spectra obtained from the beginning and middle of the peak were obtained from an entity with a carbonyl absorption at 1645 $cm^{-1}$, while the spectrum acquired from the trailing edge of the peak is from a distinct compound which shows two carbonyl absorption bands at 1667 and 1642 $cm^{-1}$, thus suggesting the presence of at least two ecdysteroids. The similarity of the IR spectra in terms of the carbonyl absorption of the three ecdysteroid standards used in the original model mixture suggests that if the second carbonyl signal was also due to an ecdysteroid then there must be some structural difference close to the 7-en-6-one group. This would be consistent with the presence of polypodine B which contains a 5-β hydroxyl (structure inset to Fig. 6.19A). Polypodine B is known to co-elute with 20-hydroxyecdysone in many reverse phase chromatography systems.

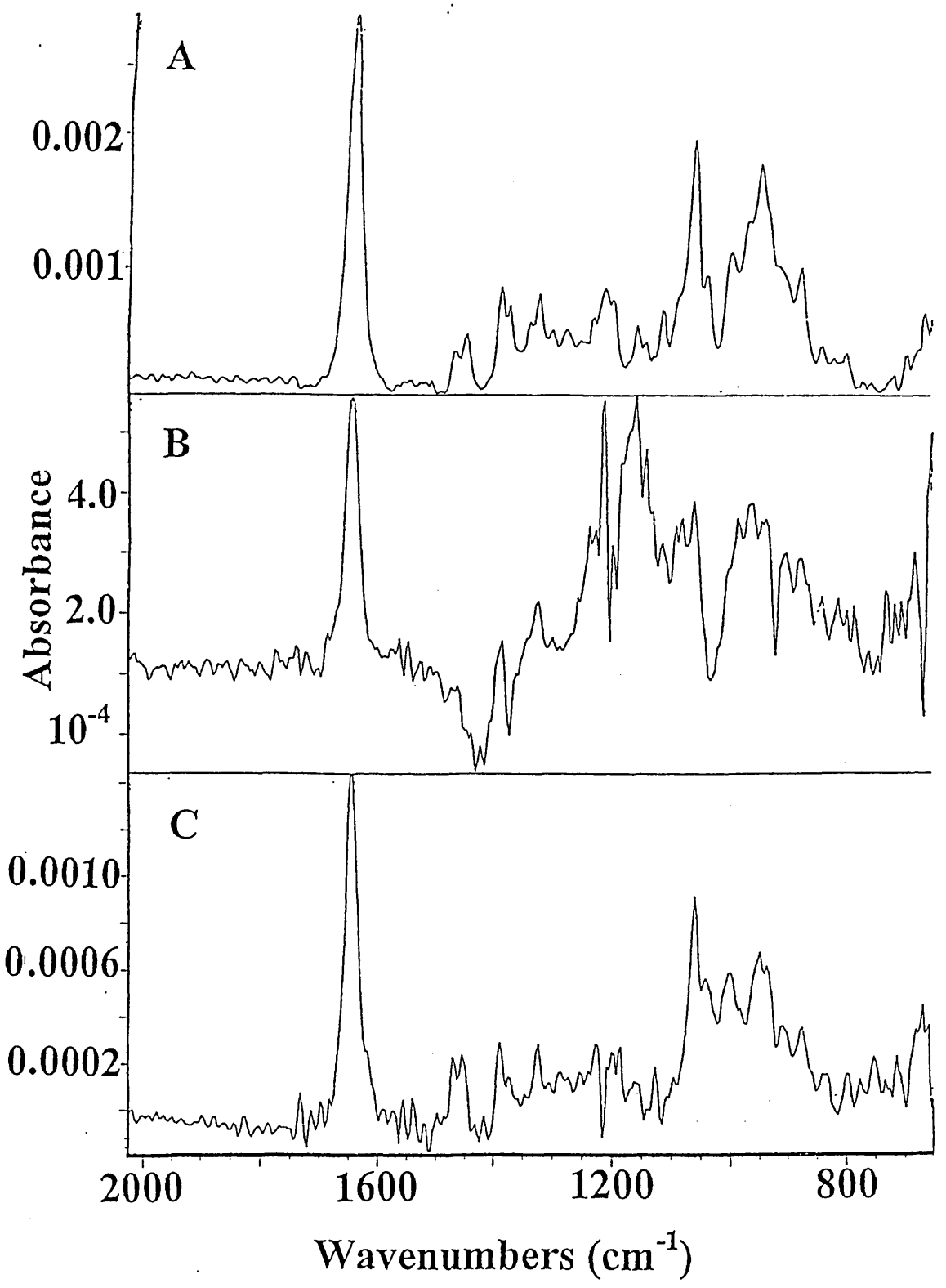



Fig. 6.14 *FT-IR spectra obtained for the standards A:- 20-hydroxyecdysone, B:- makisterone A and C:- ecdysone from the standard mixture injected.*

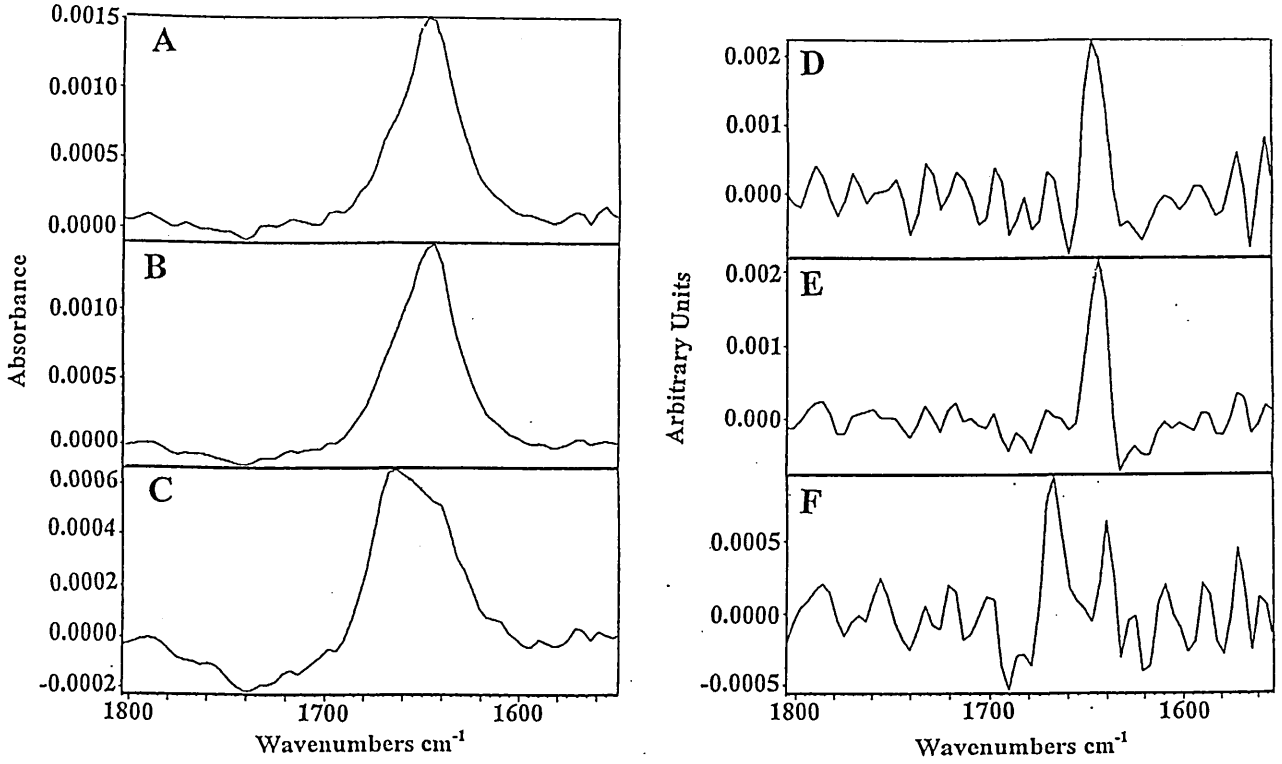



Fig. 6.15 *FT-IR spectra of the ecdysteroid containing peak of the Lychnis flos-coculi extract shown in Fig. 6.13, showing the carbonyl absorption at A:- the beginning, B:- the middle and C:- the trailing edge of the chromatographic peak. The second derivative spectra of the carbonyl absorptions corresponding to D:- the beginning E:- the middle and F:- the trailing edge clearly show the shift in the position and the splitting of the carbonyl absorption band.*

### 6.5.6 NMR Spectra

The NMR spectra were acquired in the stop flow mode to allow better solvent suppression. The $^{1}H$ NMR spectra shown in Fig. 6.16A-C acquired for each of the ecdysteroids in the standard mixture were acquired with between 24 and 128 scans per spectrum. The spectra contained signals for residual HOD and acetonitrile, in addition to the resonances for the ecdysteroids. These additional signals did not result in any significant problems for the spectroscopy of the ecdysteroids used in the

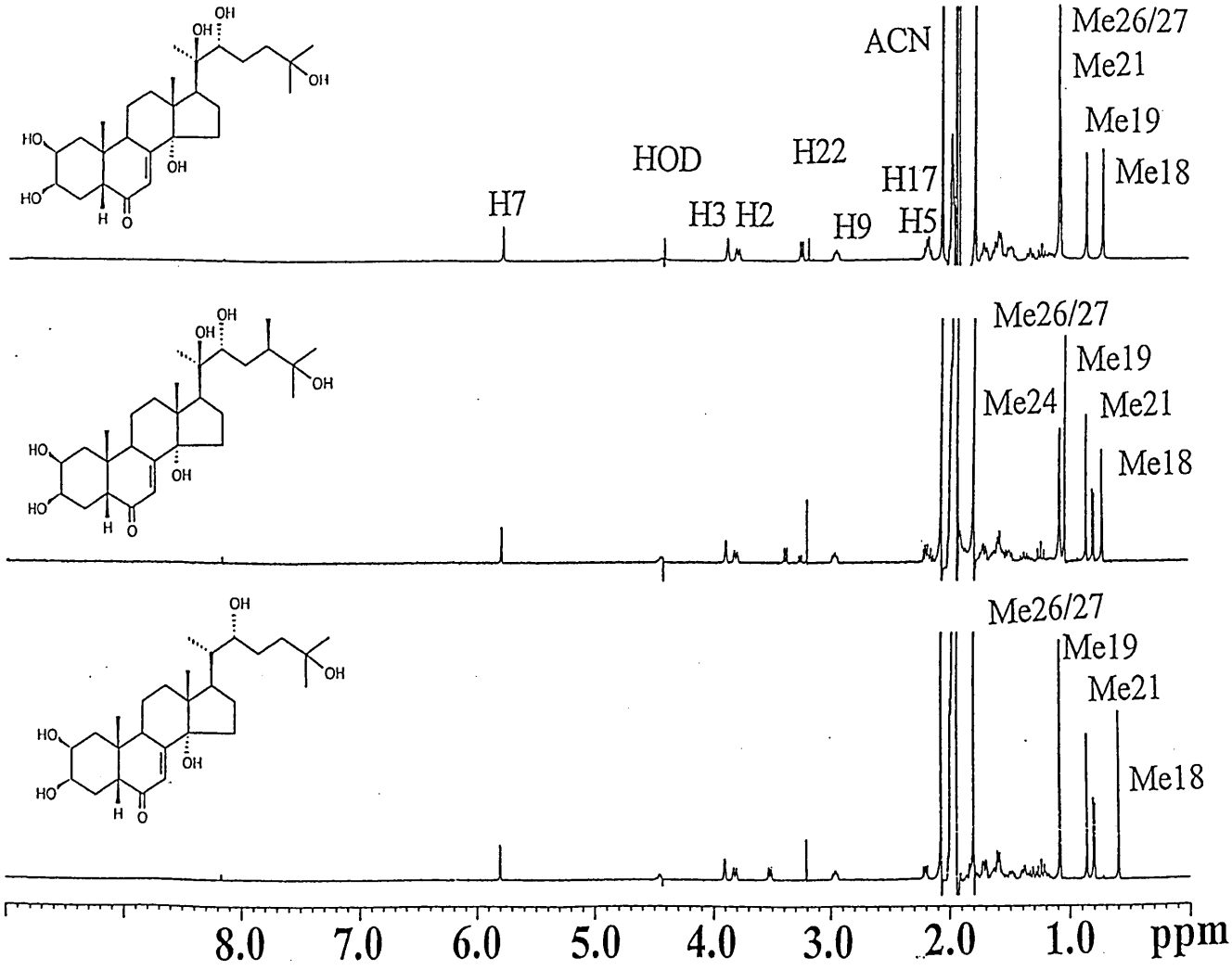



Fig. 6.16 *Stop flow HPLC $^1$H NMR spectra of A:- 20-hydroxyecdysone, B:- makisterone A and C:- ecdysone*

standard mixture. For the first eluting peak which corresponded to 20-hydroxyecdysone, all of the methyl resonances were clearly visible between 0.6 and 1.2 ppm, together with the diagnostic 7-en proton at approximately 5.8 ppm (see Fig. 6.16A). Similarly all of the methyl resonances for makisterone A and ecdysone were also clearly visible in the same region (see Figs. 6.16B and 6.16C). For the *Lychnis flos-coculi* extract it was apparent from the $^1$H NMR spectra that at the front, early eluting part, of the main chromatographic peak there were signals consistent with the

presence of ecdysteroids and in particular the methyl protons in the region 0.6-1.2 ppm and the 7-en proton at approximately 5.8 ppm. However, the chromatographic peak also contained significant other NMR signals, in particular a number of anomeric protons consistent with the presence of sugars. In order to better characterise the peak 20 second time slices were taken and the resulting $^1$H NMR spectra obtained. Typical spectra for the beginning, middle and end of the chromatographic peak are shown in Figs. 6.17 A-C. These spectra clearly show that the impurity profile of the peak changed with time and as the time slices moved forwards through the peak the region of the $^1$H NMR spectrum containing the resonance for the 7-ene proton gradually acquired a second signal. The NMR spectrum for the first part of this chromatographic peak corresponded to 20-hydroxyecdysone, but the chemical shift of the C7 proton in the latter part of the peak was consistent with the presence of polypodine B, supporting the FT-IR results suggesting that the structural differences between the two co-eluting ecdysteroids was close to the en-one group. This result was in accordance with the known chromatographic properties of 20-hydroxyecdysone and polypodine B in reverse phase HPLC separations. The $^1$H NMR spectra obtained for this peak were therefore clearly able to indicate the presence and the proportions of the two major ecdysteroids co-eluting in this chromatographic peak.

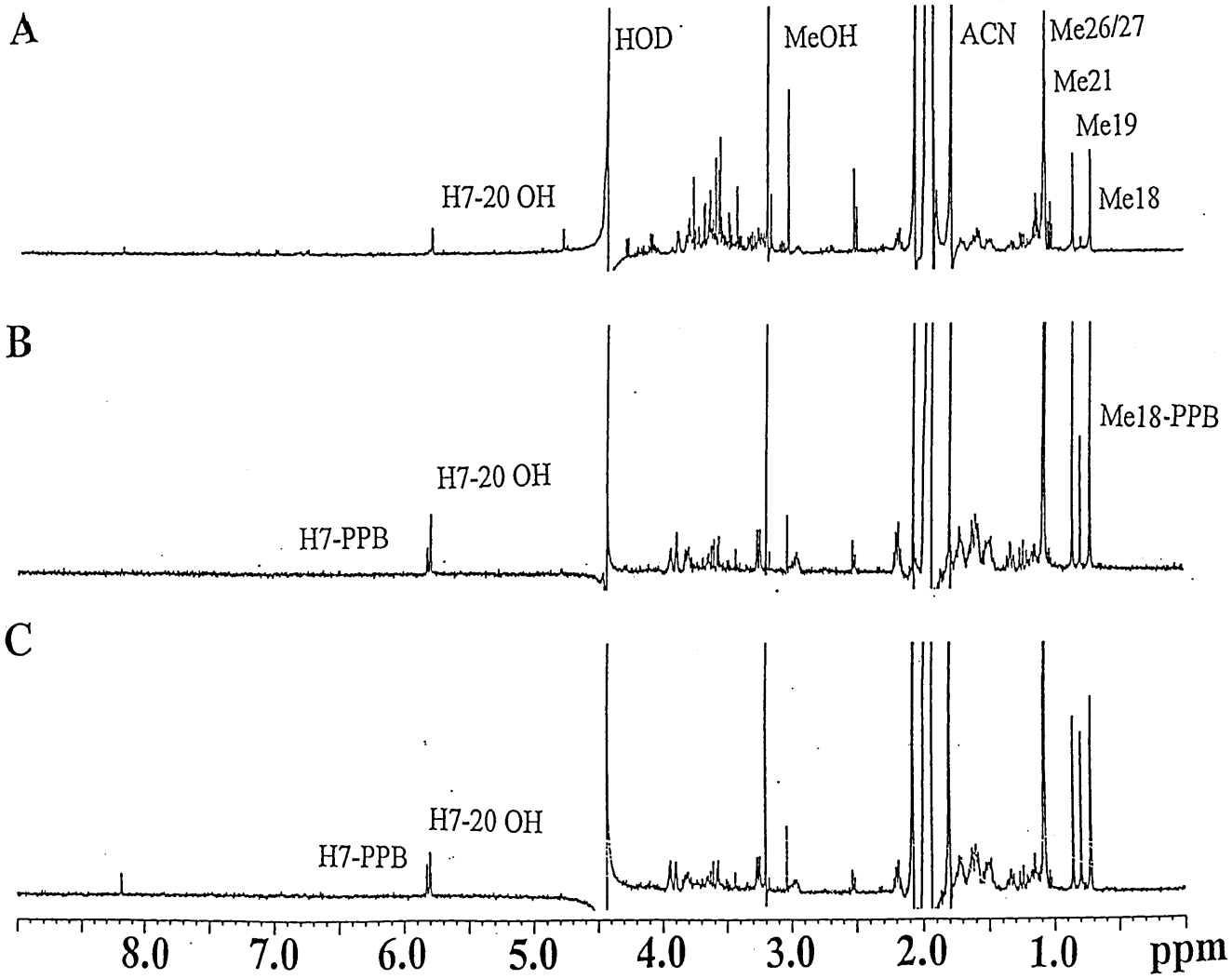



Fig. 6.17 *The $^{1}H$ NMR spectra obtained for A:- the beginning, B:- the middle and C:- the trailing edge of the ecdysteroid-containing peak of the Lychnis flos-coculi extract shown in Fig. 6.13. The emergence of the second signal at approx 5.8 ppm indicating the presence of a second component can be clearly seen in B and C.*

### 6.5.7 Mass Spectra

The ecdysterone mixed standards sample showed ions for both 20-hydroxyecdysone and makisterone A at m/z 488 and 502, respectively. These two standards also

showed base peak ions at m/z 469 and 483 respectively (Figs. 6.18A and 6.18B). In the case of ecdysone only a weak $(M+D)^+$ ion was detected at m/z 471 with the base peak ion at m/z 452, (Fig. 6.18C). In each instance the spectrum was dominated by an ion 19 m/z units down from the deuterated molecule, indicative of the loss of HOD from the $(M+D)^+$ ion. It was shown earlier in this chapter that the loss of "water" from these ecdysteroids was very easily achieved and so these results are consistent with those previously recorded. The reduced height of the $(M+D)^+$ ions compared with the ions for the loss of water were probably indicative of the cone voltage being slightly too high. Other ions noted in these spectra were related to sodium and acetonitrile adduction. It was not possible to attain any satisfactory accurate mass data for the three standards due to the large amounts of sample loaded to facilitate FT-IR and NMR acquisitions. These high loadings caused the ion source and the detector of the mass spectrometer to become overloaded while simultaneously suppressing the lock mass spectrum.

In the case of the *Lychnis flos-coculi* extract it was possible to obtain spectra consistent with the presence of 20-hydroxyecdysone and polypodine B co-eluting in the major UV absorbing chromatographic peak (Figs. 6.19A and 6.19B). For 20-hydroxyecdysone at the front of the chromatographic peak a $(M+D)^+$ ion was observed at m/z 488 together with ions at m/z 469 (corresponding to the loss of HOD from the $(M+D)^+$ ion) and m/z 529 (corresponding to an acetonitrile adduct with the $(M+D)^+$ ion) shown in Fig. 6.19B. These are similar to those observed for the standard and reported above. The spectrum for polypodine B proved to be rather more complex, with instead of a single ion corresponding to the $(M+D)^+$ ion, a cluster of ions was observed around the expected ion at m/z 505 (see Fig. 6.19A). These

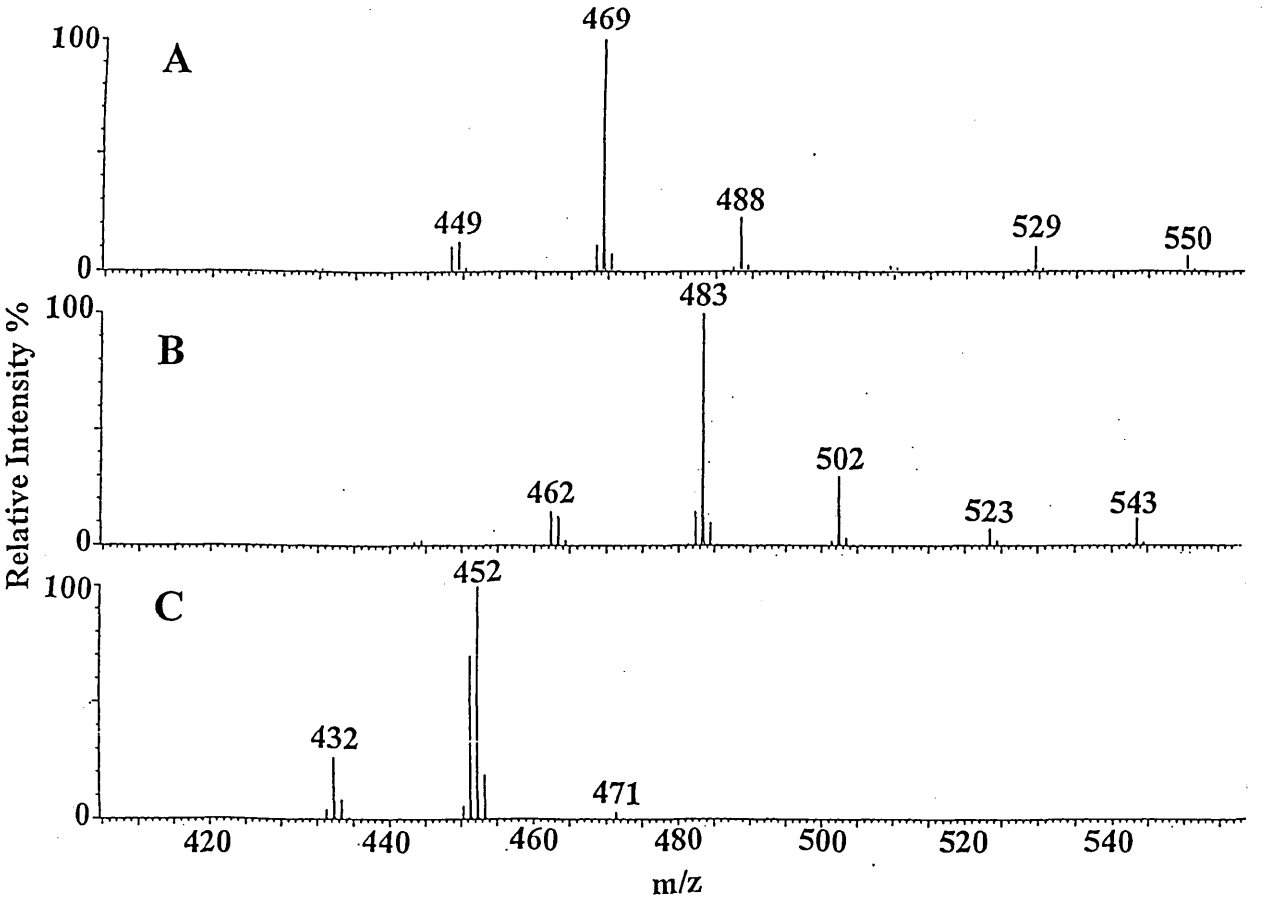



Fig. 6.18 *Mass spectra obtained for A:- 20-hydroxyecdysone, B:- makisterone A and C:- ecdysone from the standard mixture of ecdysteroids. In each case the spectrum is dominated by the loss of HOD.*

results could only rationally be explained in terms of varying degrees of deuteration of the exchangeable protons of polypodine B, which logically must be related to the presence of the 5-β-hydroxyl, given the rapid and complete deuteration of the 20-hydroxyecdysone in the same sample. It is interesting to note that this partial deuteration was not observed in the superheated water system described earlier, possibly because the elevated temperatures employed facilitated the complete deuteration of the polypodine B present in those samples. The two most significant ions in the spectrum of polypodine B were observed at m/z 485 and 486,

corresponding to the loss of HOD from the fully deuterated (7 hydroxyl protons replaced) and the hexadeuterated steroid respectively. The proportions of the ions at m/z 486 and 505 suggest that the hydroxyl in the 6 position is less easily deuterated than the other hydroxyl groups and this has indeed been reported previously[104,105]. In order to confirm the identification of polypodine B the mass spectrometry was repeated using protonated solvents, which gave the expected ions for both 20-hydroxyecdysone (m/z 481) and polypodine B (m/z 497) together with ions

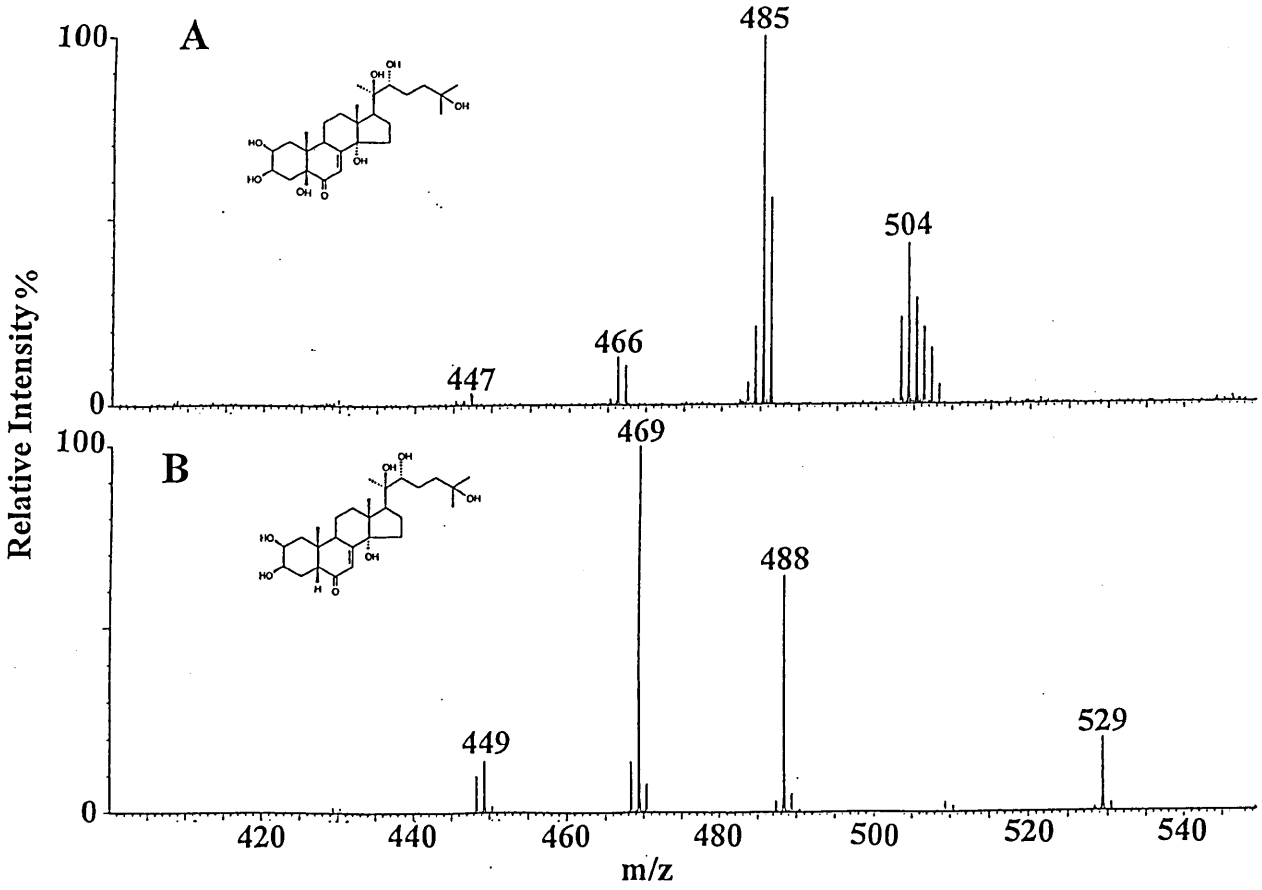



Fig. 6.19 *Mass spectra of A:- polypodine B and B:- 20-hydroxyecdysone obtained from the trailing edge and the front respectively of the ecdysteroid containing chromatographic peak at approximately 5 minutes.*

corresponding to the loss of water. This slow exchange could potentially be used diagnostically when attempting to verify the sites of hydroxylation of novel ecdysteroids.

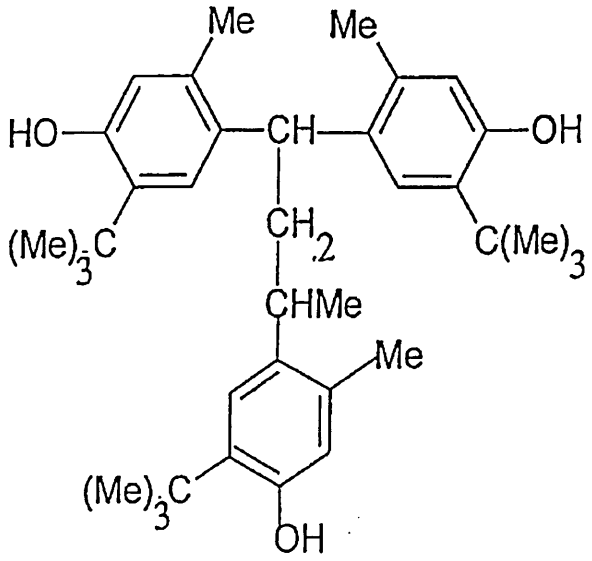
The mass spectra of this chromatographic peak support the data from both FT-IR and NMR in indicating that it consisted of at least two components and that the front (i.e. earlier eluting portion) of the peak was predominantly 20-hydroxy-ecdysone with the latter part of the peak being predominantly polypodine B.

### 6.6 The analysis of polymer additives

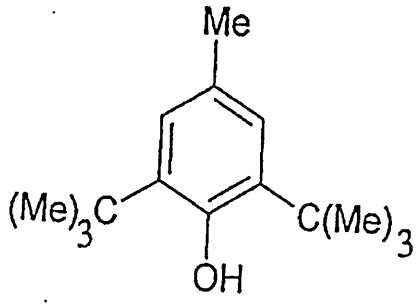
The previous studies in this chapter have focused on the analysis of polar compounds using reverse phase partition chromatography. In chapter 4 the analysis of some polymer additives was briefly considered in a normal phase chromatographic system. This proved partially successful but the results were less than ideal. In the experiments described here, this series of compounds was extended and their analysis investigated further. In order to facilitate primarily the mass spectrometry a reverse phase system was employed for these non-polar compounds. The compounds studied were Irganox 245 (triethyleneglycol bis-3(3-tertiarybutyl-4-hydroxy-5-methylphenylpropiionate)), BHA (butylated hydroxyanisole), BHT (butylated hydroxytoluene), Bisphenol A and Topanol CA (1,1,3-tris-(2-methyl-4-hydroxy-tertiarybutylphenyl)butane). The structures of these compounds are given in Fig. 6.20.

### 6.6.1 Experimental conditions

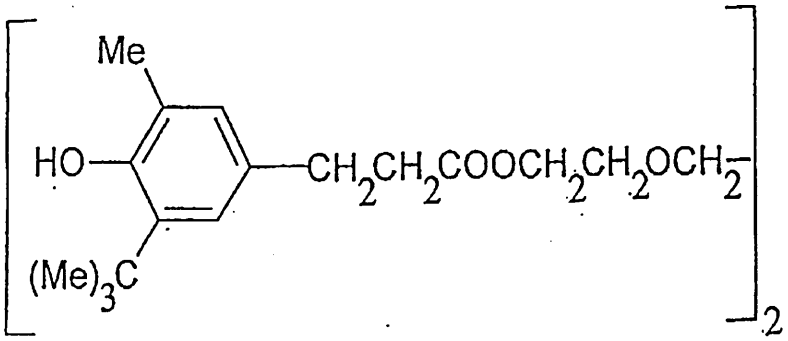
The system used was the same as described earlier for the analysis of ecdysteroids using super heated water and is shown schematically in Fig. 6.7. The previous experiments on these compound types used a polymer based size exclusion column with deuterated chloroform as the eluent. This enabled the acquisition of good quality NMR and IR data with minimal interference, and allowed NMR experiments to observe exchangeable protons such as those on the phenolic hydroxyls. The volatility of $CDCl_3$ also simplified the conditions required for solvent evaporation when the eluent was collected off-line for the FT-IR although this solvent was unsuitable for MS analysis due to its "dryness" (which prevented the ionisation of the analytes due to the absence of a suitable proton donor). Suitable MS data were only obtained when deuterated methanol and ammonium acetate were added, but this resulted in unwanted signals in the NMR spectra and also the loss of signals for the exchangeable protons. However, in this set of experiments where solvent volatility was not an issue, the use of reverse phase chromatography was possible with standard C18 bonded phases, enabling acquisition of MS data. The non-polar nature of the compounds studied necessitated the use of high proportions of acetonitrile in the mobile phase to ensure elution of the samples in a reasonable time. This resulted in large signals in the NMR for the protons present in the acetonitrile, so deuterated acetonitrile was substituted ($CD_3CN$). Suitable chromatography was then attained using 80:20 $CD_3CN$: $D_2O$ (v/v) and a flow rate of 1.0 mL/min. The resultant chromatogram using this eluent and a Hypersil H5ODS 100 x 4.6 mm column for a mixture containing Bisphenol A (371 μg), BHA (338 μg), Irganox 254 (332 μg), BHT (992 μg) and Topanol CA (338 μg) is shown in Fig. 6.21. As can be seen almost baseline separation was achieved for Irganox 254, BHT and Topanol CA, but the earlier eluting components Bisphenol A



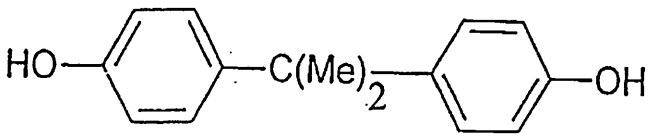

**TOPANOL CA**



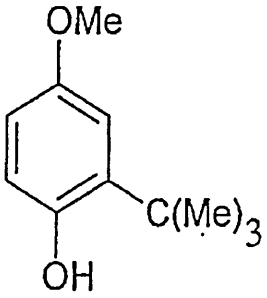

**BHT**




**IRGANOX 245**




**BISPHENOL A**




**BHA**

Fig. 6.20 *The structures of the polymer additives used in these experiments.*

and BHA co-eluted. However, as will be seen below, Bisphenol A eluted in the leading edge of this peak while BHA eluted in the trailing edge, enabling spectra to be obtained for each component. In addition to chromatographing these components as a mixture the individual compounds were also injected separately in order to obtain clean spectra to be used in the library as reference spectra.

### 6.6.2 Infra Red Spectra

As with most experiments the quantities of compounds injected ensured that good spectra were obtained on-flow. Examination of the leading and trailing edges of the combined peak of Bisphenol A and BHA revealed clear spectra for the individual components (Figs. 6.22 a and b). Similarly, clear spectra were obtained for both Irganox 245 and BHT (Figs. 6.22c and 6.22d). The only difficulty encountered in obtaining IR spectra from the test mixture under these conditions was with Topanol CA. After treatment to remove the solvent signals the resultant spectrum was too weak to be useful. For the other four compounds library comparisons gave very good matches (Bisphenol A : 99.1%, BHA : 91.6%, Irganox 245 : 97.4% and BHT : 97.2%)

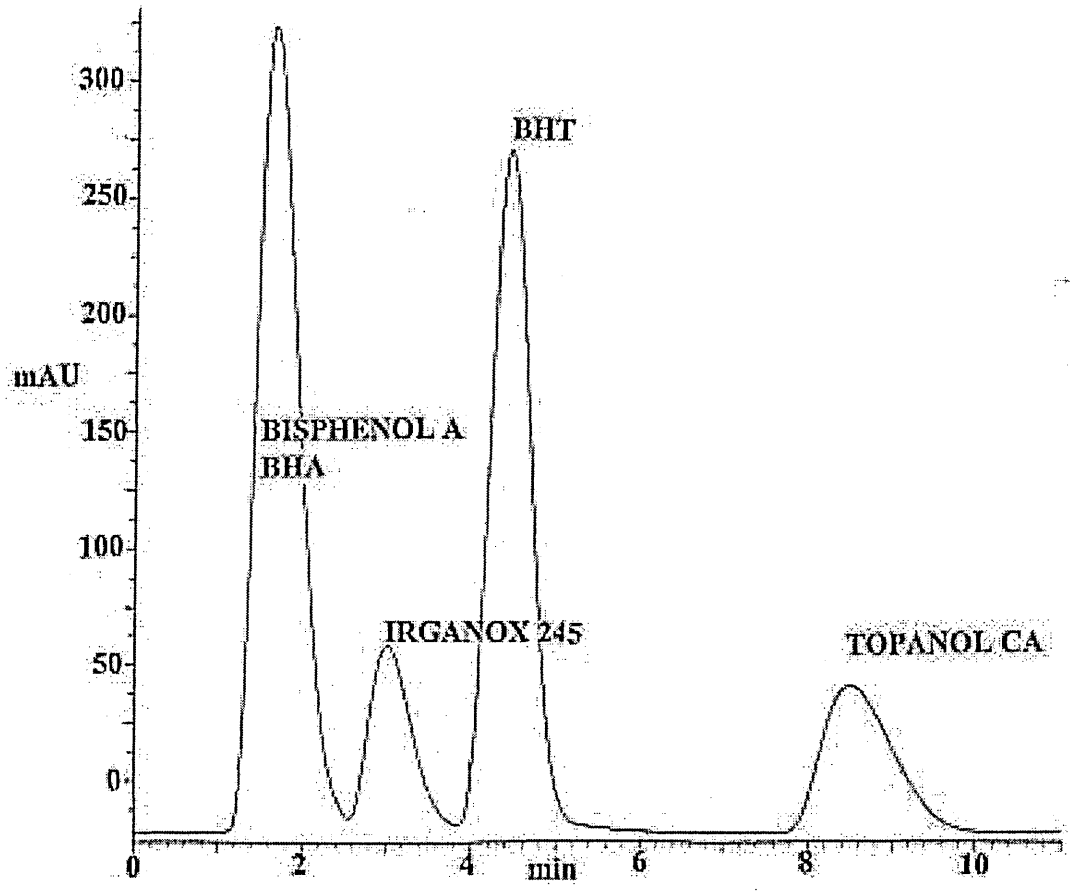



Fig. 6.21 *HPLC UV chromatogram at 254nm for Bisphenol A (371 μg), BHA (338 μg), Irganox 254 (332 μg), BHT (992 μg) and Topanol CA (338 μg). Although the Bisphenol A and BHA coeluted good spectra could be obtained as the Bisphenol A eluted early in the peak and the BHA in the tail of the peak. Topanol CA eluted last and was therefore more diffuse, which may have contributed to the inability to obtain a useful IR spectrum.*

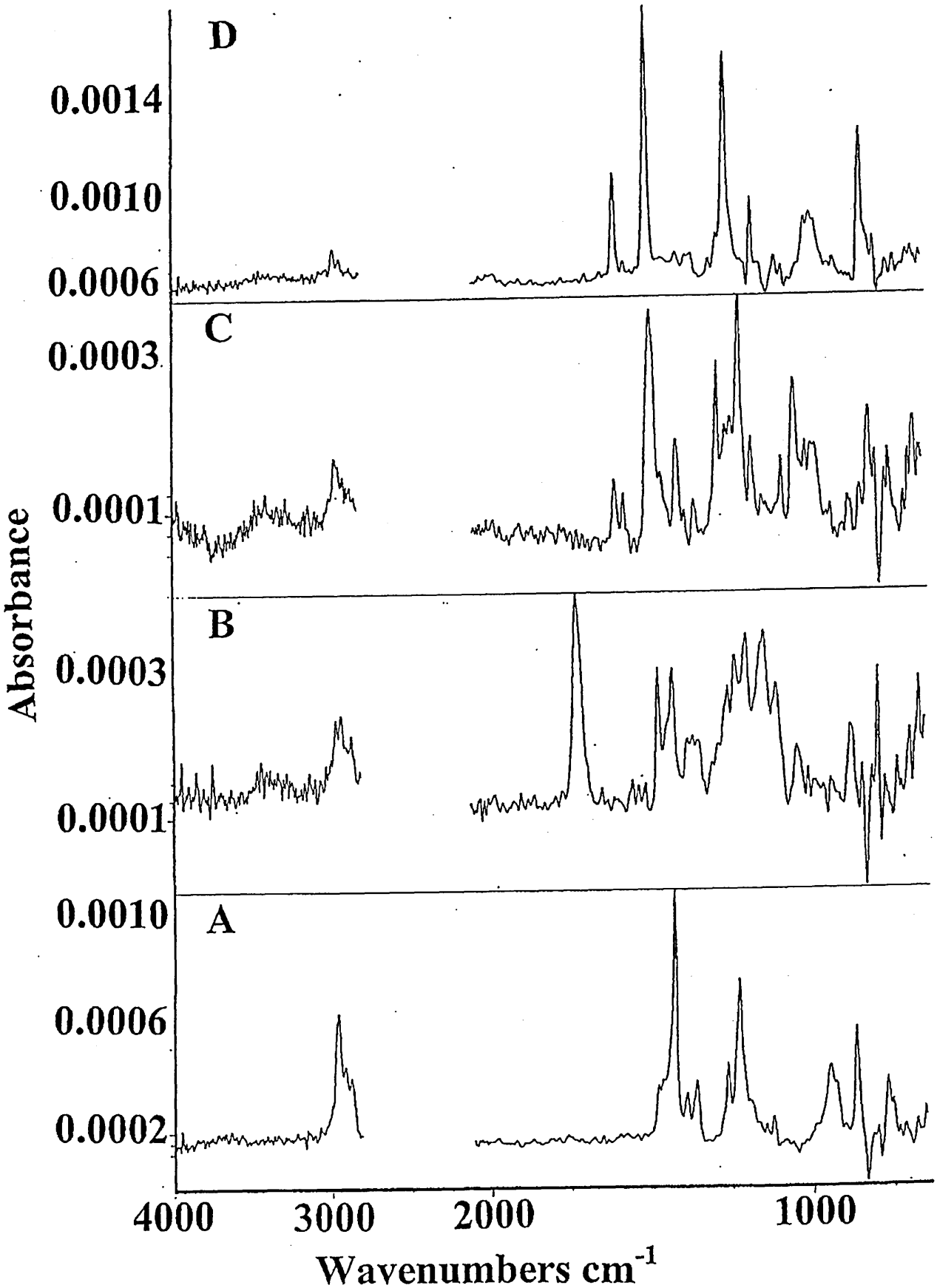



Fig. 6.22 *IR spectra for A: Bisphenol A, B: BHA, C: Irganox 245 and D: BHT obtained from the injected mixture shown in Fig. 6.21.*

### 6.6.3 UV Spectra

The large quantities of the polymer additives used provided no challenge with respect to sensitivity for the UV-DAD spectrometer.

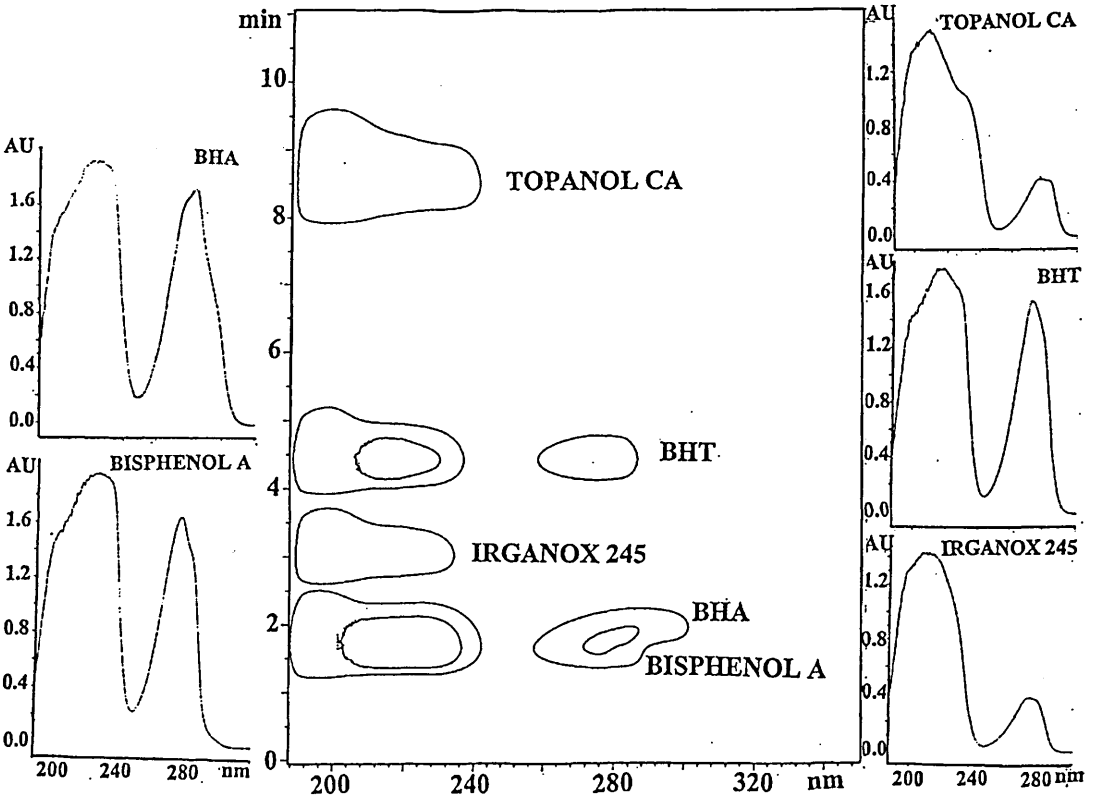



Fig. 6.23 *2D-UV chromatogram for the mixture shown in Fig. 6.21. The spectra of the individual components are shown inset and individually labelled. The distortion of the Bisphenol A /BHA peak can be better seen and the shift in the UV maxima of the two compounds is also evident.*

The resulting 2D-UV chromatogram for the 5 compound mixture is shown in Fig. 6.23. The first eluting peak was composed of Bisphenol A and BHA and although the peak appears symmetrical (see Fig. 6.21), spectroscopic examination showed the components to be partially resolved, with Bisphenol A eluting in the leading edge and

BHA in the trailing edge. Evidence for the non-homogeneous nature of this peak can be seen in the distorted shape of the contour plot for the region 260-300nm (Fig. 6.23). The individual UV spectra obtained from this two-component peak (inset to Fig. 6.23) reflect this in the different UV maxima of the two analytes. Bisphenol A gave a secondary UV maximum at 275 nm, whereas the secondary maximum for BHA was at 285 nm. These values agreed well with those obtained from injecting the compounds individually and chromatographing them under the same conditions. Similarly, the UV spectra for the remaining compounds in the test mixture were comparable with those of the individual standards. Spectra for each of the compounds obtained from the chromatograph of the mixture are shown inset to Fig. 6.23.

### 6.6.4 NMR Spectroscopy

Deuterated acetonitrile was used for the experiments described here. Normal protonated solvents had been tried initially but while it was possible to obtain spectra it was not possible to obtain adequate suppression of the acetonitrile signals. The use of deuterated acetonitrile was limited only by its very high cost in comparison with standard solvents; hence in other prior experiments the normal protonated solvent was used. The use of $CD_3CN$ enabled the acquisition of diagnostic $^1H$ NMR spectra for all 5 of the compounds in the mixture. In the instance of Bisphenol A and BHA which co-eluted as a single peak, it was still possible to obtain the appropriate spectra from the leading and tailing edges of the chromatographic peak as had been the case of the UV and IR spectra. The NMR chromatogram obtained for the mixture is shown in Fig. 6.24 together with the spectra extracted for each individual component (A-E). The spectra were of good quality and showed all of the expected signals for the analytes. For Irganox 245, the spectrum (Fig. 6.24C) consisted of two sets of

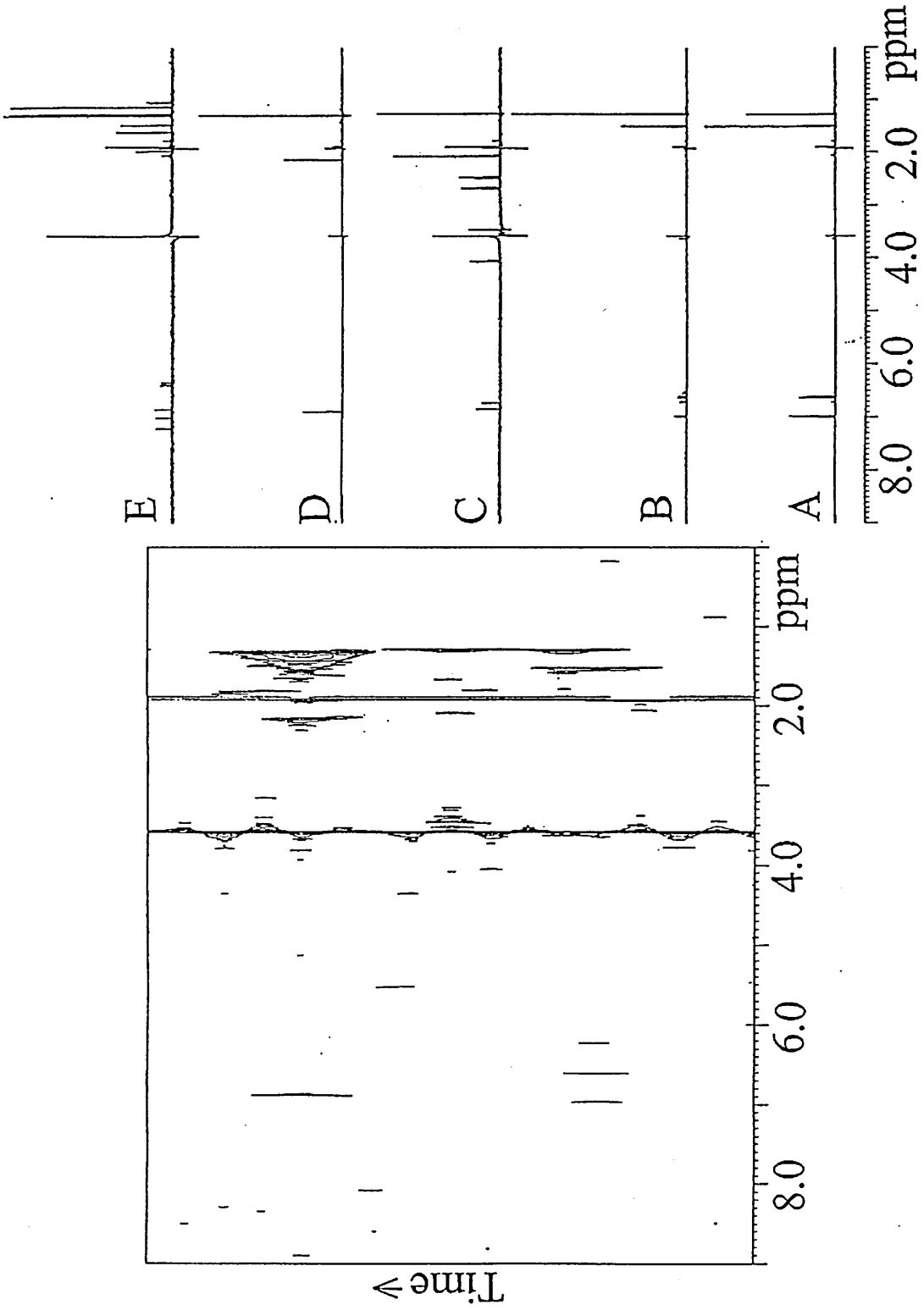



Fig. 6.24 *$^{1}H$ NMR chromatogram of the mixture of polymer additives together with extracted spectra for the individual components. A: Bisphenol A, B: BHA, C: Irganox 245, D: BHT and E: Topanol CA*

triplets at 2.49 and 2.69 ppm (due to the proprionate group) and two sets of doublets-of-doublets at 3.55 and 4.08 ppm (from the ethylene glycol moiety next to the carboxy function). The latter appear significantly smaller as a result of the suppression of residual water signals at 3.58 ppm. A single line at 3.44 ppm represented the central ethylene glycol group. The aromatic protons were visible at 6.73 and 6.8 ppm, while the tertiary butyl group and methyl group gave signals at 1.27 and 2.09 ppm, respectively. Suppression of the residual acetonitrile signal at 1.9 ppm resulted in the slight distortion of the singlet at 2.09 ppm. Overall the quantities of compound present were adequate to obtain on-flow $^{1}H$ NMR spectra of good signal quality.

### 6.6.5 Mass Spectrometry

The quantities of sample present in the peaks were more than adequate for spectra to be obtained on all five of the compounds in the mixture. The total ion current (TIC) chromatogram is shown in Fig. 6.25A. Ions were observed for Bisphenol A, BHA and BHT (extracted mass chromatograms are shown in Figs. 6.25 C, E and F). The spectrum of Bisphenol A was dominated by the product ion of m/z 136 which represented the loss of one of the phenol groups from the precursor ion. In the case of Topanol CA, an ion related to the molecular mass was not observed as the molecule fragmented in the ion source of the mass spectrometer to give an ion at m/z 192 (see Fig. 6.25B). Irganox 245 was observed as a poly-deuterated ion at m/z 592 (see Fig. 6.25D). The greater degree of deuteration than would have been predicted based on the number of available exchangeable protons in the structure was unexpected. The resultant mass spectrum showed an unusual isotope ratios and this region is shown in Fig. 6.26.

Further evidence of this over deuteration is provided by the cluster of ions at m/z 535 – 540 which represent the facile loss of the tertiary butyl group from the cluster at m/z 591-596. Given that the NMR data obtained concomitantly showed no evidence of additional deuterium exchange of the Irganox 245 it is postulated that this extra deuteration occurred in the ion source of the mass spectrometer rather than in the mobile phase of the HPLC. Further off-line investigation suggested that the additional deuteration involved the replacement of the protons adjacent to the carbonyl group (data not shown).

Some of the mass chromatograms in Fig. 6.25 and in particular 6.25 A and B exhibit a periodicity to the baseline noise. These oscillations appear to have a period of approximately one minute. The peak for Topanol A appeared to be split into two peaks in phase with this cyclic effect. This periodicity is illustrated further in the two-dimensional mass chromatogram shown in Fig. 6.27 where the appearance of regular mass bands at approximately one minute intervals are especially apparent just after the elution of Irganox 245 (about 3 minutes). The peaks for all of the analytes are also irregular over a much shorter time period. An example of this is shown in Fig. 6.27B where the trace for m/z 484 (one of the fragment ions associated with Irganox 245) clearly shows three distinct peaks over what was in reality a single peak. As none of these phenomena were observed in either the UV chromatogram or the NMR chromatogram it seems likely that this periodicity was an artefact generated from the use of two distinct pumping systems to deliver the flow to the mass spectrometer for the make-up flow and from the HPLC. The measured piston cycle times for the pumps were 10 and 20 seconds at flow rates of 1.0 and 0.5 mL/min, respectively. An

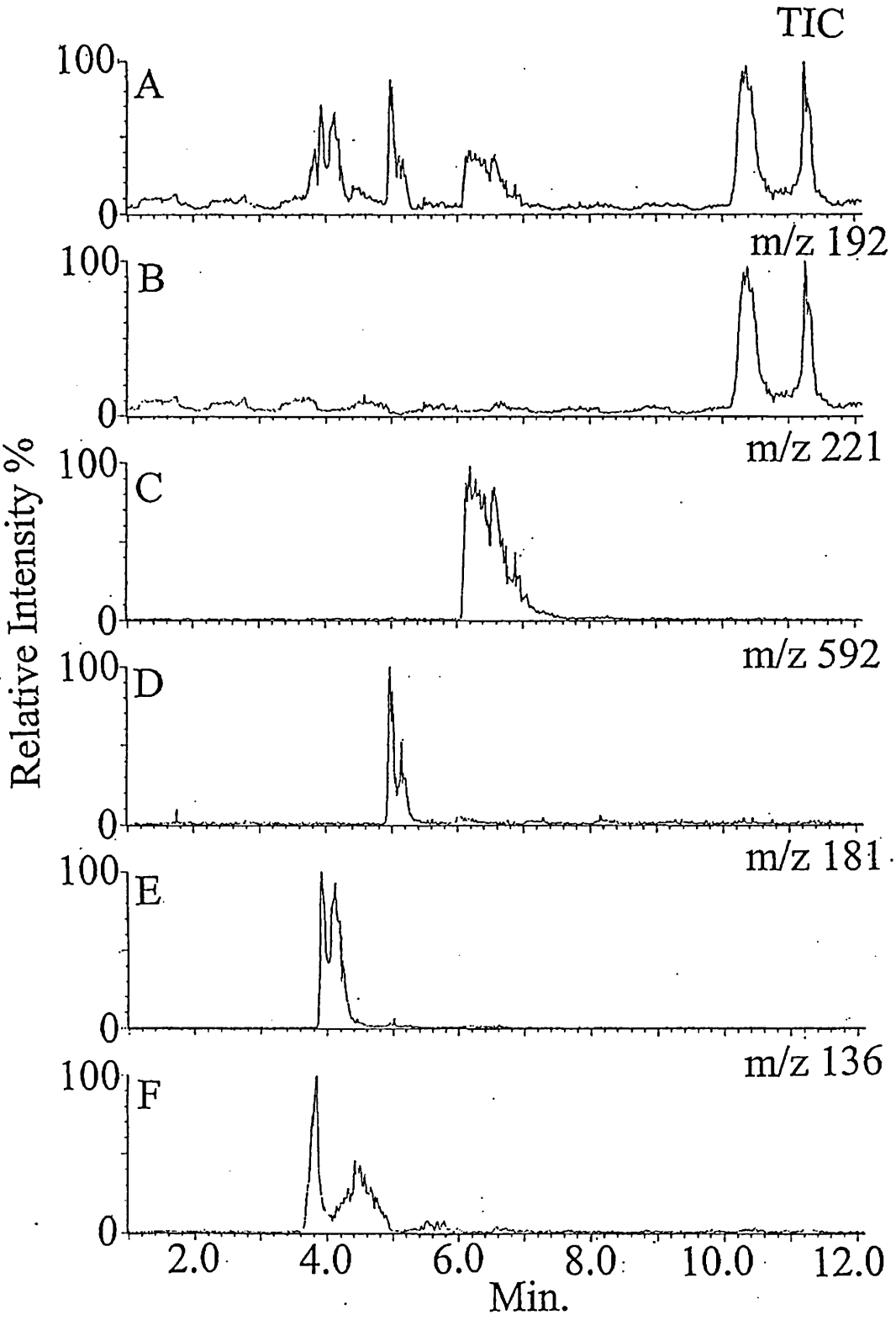



Fig. 6.25 *Mass chromatograms for the separation of the mixture of polymers. A:- TIC, B:- m/z 192 the major fragment ion in the spectrum of Topanol CA, C:- m/z 221 BHT, D:- m/z 592 Irganox 245, E:- m/z 181 BHA and F:- m/z 136 the base peak in the spectrum of Bisphenol A*

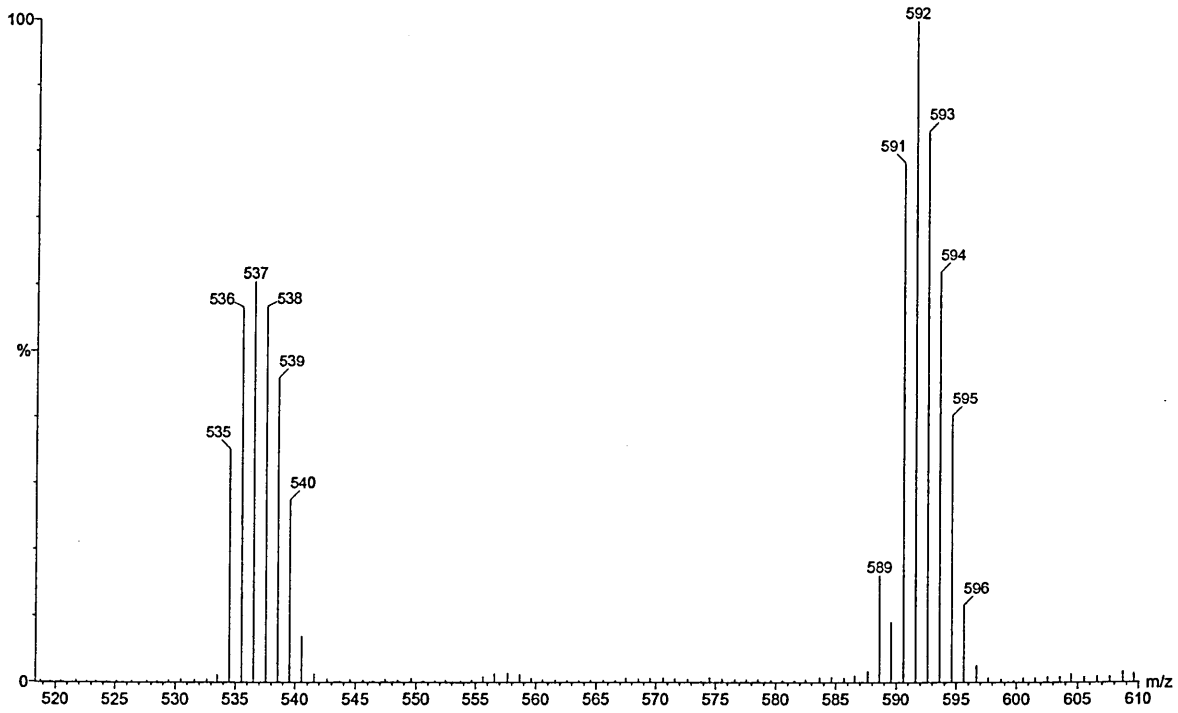



Fig. 6.26 *The detailed view of the mass spectrum of Irganox 245 showing the unexpectedly high degree of deuteration. The loss of 56 Da representing the loss of the tertiary butyl group gives rise to the cluster of ions from m/z 535 to 540.*

irregular flow of solvent into the mass spectrometer was probably the cause of these effects.These effects had not been noted previously when the NSAIDs had been analysed with $D_2O$ as the eluent and methanol/water (90/10) had been used as the make-up flow. The use of dynamic mixing and/or pulse dampeners might have reduced or eliminated these effects, but these were not available at the time of the experiments. The use of matched eluents would also (with hindsight) have been a better option which might have at least reduced the observed phenomenon if the problem was one of miscibility.

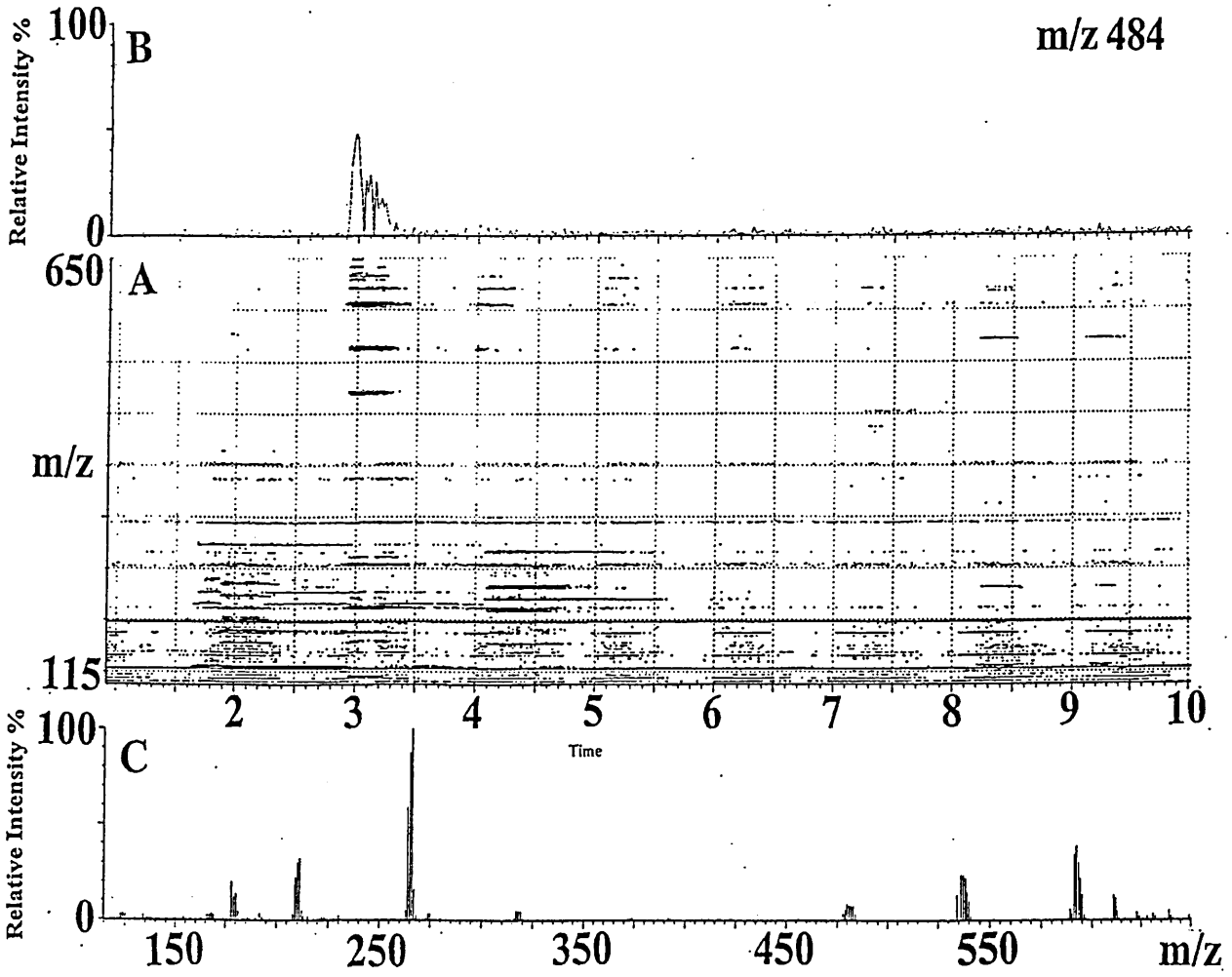



Fig. 6.27A *The two-dimensional mass spectrum for the mixture of polymer additives*

Fig. 6.27B *The mass chromatogram for m/z 484 one of the higher mass product ions of Irganox 245 showing the peak clearly being split into three "peaks" due to the underlying cyclic effects.*

Fig.6.27C *The mass spectrum of Irganox 245 taken at 3.01 min.*

Despite these drawbacks the system was used to acquire spectroscopic data on a sample of a suspected polymer additive which was conclusively identified as being BHT (Fig. 6.28).

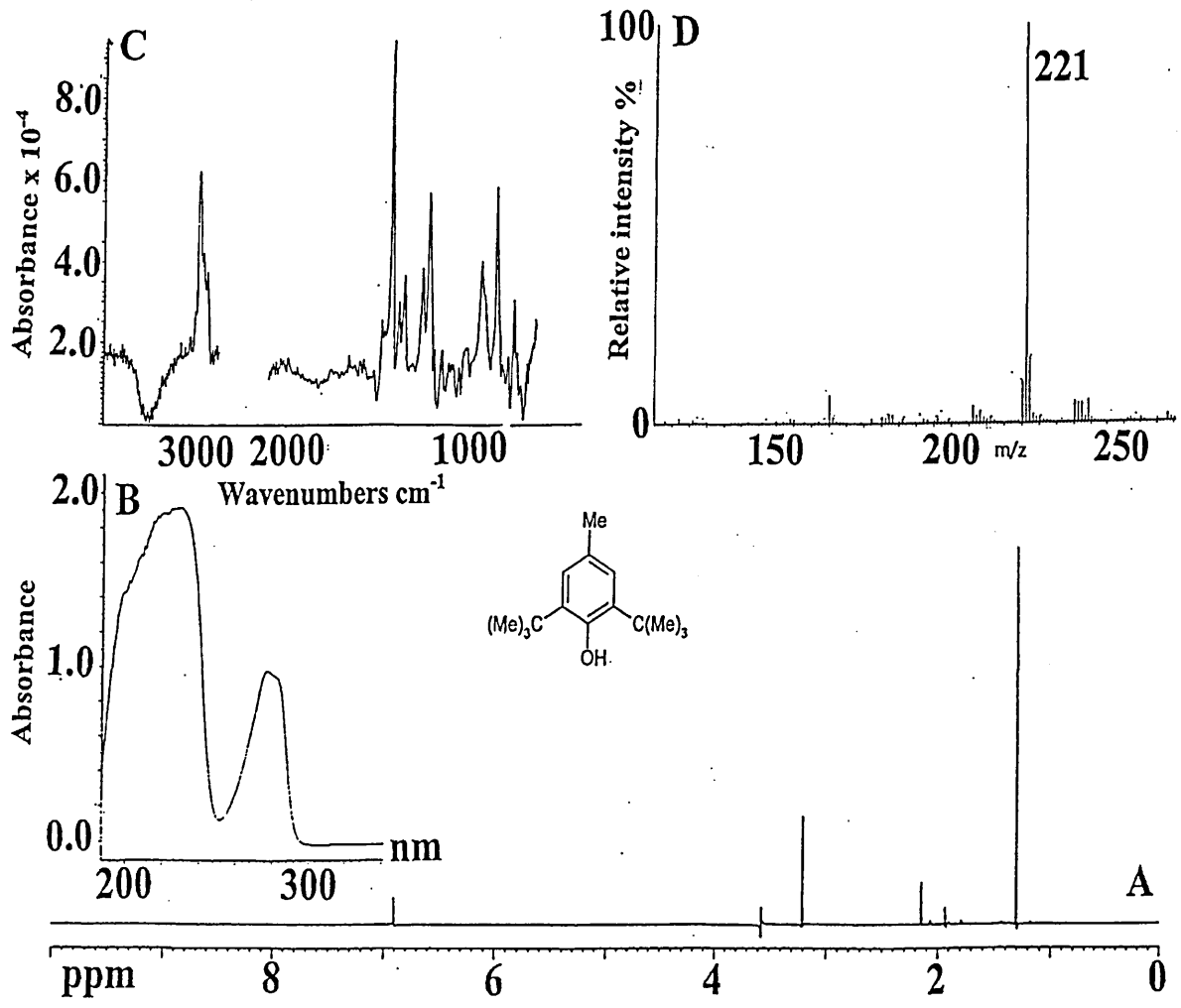



Fig. 6.28 *Spectroscopic data acquired for the suspected polymer additive subsequently identified as BHT.*

## 6.7 Summary

- **The system described was capable of producing good quality spectroscopic data from various chromatographic techniques.**
- **Hot water systems show promise for both multiple-hyphenation and as an additional tool for the production of deuterated compounds or the monitoring of deuterated sites.**
- **Multiple-hyphenation will only become more widely used with the input of manufacturers as the costs involved are often prohibitive for individual laboratories to explore.**

# CHAPTER 7

## OVERALL CONCLUSIONS

All the work described in this thesis provided excellent evidence that a working multiply hyphenated system could be established and utilised for the analysis of complex mixtures. The major difficulties have been highlighted at different points throughout, but centre on the mutual compatibility of all of the techniques. The pressures generated by linking the various spectrometers and the knock-on effect of these pressures, for instance in causing unwanted flow during stop-flow experiments or more seriously causing the NMR probe to leak, are not insurmountable given sufficient time and the necessary equipment. Solvent compatibility is somewhat more difficult to overcome but the use of high quality deuterated solvents would help greatly. The removal of residual proton signals could be achieved through prolonged utilisation of these deuterated solvents, thus removing other additional signals from the NMR spectra. Deuterated solvents had no discernible detrimental effects on either the UV or the IR spectra acquired and in the case of the mass spectrometry could be used to identify the number of deuterated sites on a molecule. However, the work described in chapter 6 on the polymer additives, and some of the hot water data, should act as a warning that assumptions about the number of deuteration sites should be treated with care. The IR off-line data suffered less from interfering solvent signals than those spectra acquired in an on-line mode.

The choice between on-line and off-line acquisition is rather more difficult to judge. In on-line systems all of the data is acquired simultaneously and the possibility of using one technique to direct another exists. Conversely, in the off-line mode better quality spectra could generally be achieved through the reduction of solvent signals and with greater time available to improve signal to noise ratios. Ultimately the choice will depend on the application.

The relative sensitivities of each of the techniques also needed careful consideration. Generally the mass spectrometer and the UV-DAD were orders of magnitude more sensitive than the IR or the NMR spectrometers, with the latter generally being the least sensitive. By loading the columns with higher than usual amounts of sample it was a relatively straightforward matter to acquire good quality IR and NMR data. However, this often resulted in the overloading of the mass spectrometer and UV-DAD resulting in poorer spectra than could otherwise have been achieved. The NMR sensitivity could have been improved and good results obtained in the low μg or even ng range had a miniaturised flow system been available[106,107].

The stray field from the NMR caused little problem in terms of quality of the spectra observed for the other instruments; however, the physical distances required to place the other spectrometers beyond the 10 or 5 gauss line meant long connecting lengths of tubing were required. Although no major problems were encountered due to this it could easily be envisaged that a situation would arise where these excessive lengths of tubing could cause sufficient band broadening to effect the co-elution of two previously separated components. The placement of physically moving motors in close proximity to the NMR spectrometer did in at least one early experiment have

some detrimental effect on the NMR data. The advent of shielded magnet technology towards the end of this work will no doubt enable the placement of instruments in close proximity to the NMR spectrometer and therefore help to reduce transfer times from the liquid chromatograph to the NMR spectrometer and any other spectroscopic technique.

Data interpretation was one of the most demanding tasks resulting from the multiple hyphenation experiments described here, but coordination was also difficult. Multiple operators were often required to just start experiments concurrently, as there was no simple way to automatically start multiple spectrometer data acquisition systems simultaneously. Although there are a limited number of data systems which are capable of displaying the data from multiple vendors, and to a certain extent multiple spectroscopic techniques, this will need to be addressed in the future if such multiple hyphenation systems are to become widespread and useable. Generally, spectroscopic interpretation was carried out by specialists from each technique; thus, NMR spectra were interpreted by a NMR specialist and IR spectra were interpreted by an IR specialist etc. For multiple hyphenation to work successfully, either a greater degree of cross training would be necessary or computer controlled interpretation software will need to be employed. The manufacturers of all of the spectroscopic techniques employed in the work described are striving to provide greater computer control and data interpretation, and as computing power continues to grow this will provide significant assistance for multiple hyphenation data interpretation.

## 7.1 Summary

- **Multiple hyphenation has been shown to be feasible and desirable**

- **Improvements in instrument design are a continuous process and many of these improvements will make multiple-hyphenation easier to achieve.**
- **Along with instrument improvements new software and increased computing power will help with data management.**
- **Multiple-hyphenation will become more widespread as the requirement for greater certainty in the identification of compounds in mixtures is driven forward by regulatory bodies.**
- **Despite the significant costs involved in bringing these various techniques together the potential savings in reduced staff hours or even reduced staff numbers will ultimately overcome the instrumentation costs. This has been shown already with the widespread introduction of "open access" systems in many establishments.**
- **Shielded magnet technology offers the solution to the physical constraints placed on the work described here and this technology will become commonplace.**
- **Better NMR probe design and increased magnetic field strengths will lead to better NMR sensitivity.**
- **The introduction, shortly after the work here was completed, of complete LC-NMR-MS systems by instrument manufacturers is a testament to the usefulness of multiple-hyphenation and to the work described in this thesis.**

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# Appendix 1

## Published Papers on

## Multiple-Hyphenation

RAPID COMMUNICATIONS IN MASS SPECTROMETRY
*Rapid Commun. Mass Spectrom.* **12**, 1732±1736 (1998)

# Practical Aspects of the Use of High Performance Liquid Chromatography Combined with Simultaneous Nuclear Magnetic Resonance and Mass Spectrometry

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**Applications of liquid Chromatography/nuclear magnetic resonance/mass spectrometry have recently been described which have shown the bene®ts of this double hyphenation for identi®cation and structure determination. However, the combination of HPLC/NMR and HPLC/MS is not without its dif®culties and, as the number of applications of this technology has increased in our laboratory, we have gained considerable insight into the pitfalls that can be encountered when attempting to perform HPLC/NMR/MS. Guidelines are presented to facilitate the effective combination of HPLC with NMR and MS in tandem together with some suggestions as to how the technique may develop. # 1998 John Wiley & Sons, Ltd.**

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LC/MS has been employed for many years but only since advent of electrospray ionization has it become a truly ust and routine method for the analysis of mixtures. LC/NMR is, by comparison, a relatively recent introduc- ı; however, technological advances in this area have ulted in HPLC/NMR being extensively employed in the rmaceutical industry.[1,2] Now that both of these spectro- pic techniques have been coupled to HPLC, it has for the t time become possible to acquire both NMR and MS a simultaneously from a single chromatographic analy-

A number of groups have begun to investigate the ınique of HPLC/NMR/MS (e.g see Refs. 3±9) for the lysis of mixtures, especially those of pharmaceuticals drug metabolites. However, because of the novelty of approach, published work has tended to concentrate on results of these studies without detailed discussion of the :tical problems associated with this technique. Here we :ribe our own experiences of HPLC/NMR/MS and offer suggestions as to the direction of future developments.

## PERIMENTAL

### LC conditions

omatography was performed using a variety of C18 _ 4.6mm columns, attached to a standard Bruker LC em comprising Bruker LC22 pump, autosampler, UV ctor and BPSU-12 collector (Bruker Spectrospin, entry, UK). For the majority of experiments the outlet he UV detector was connected immediately to a splitter :h directed the flow to the NMR and MS in the ratio . Both linear and gradient elution were used with 0.1% ic or trifluoroacetic (TFA) acids in $^2H_2O$ (99.9 atom %, Fluorochem, Glossop, UK) and acetonitrile (Riedel de Haen, Seelze, Germany) at a flow rate of 1.0 mL/min. UV detection was performed at 254nm. The splitter was a simple Valco stainless steel T-piece and all transfer lines were in PEEK. A small length of silica capillary was used to connect the PEEK tubing to the mass spectrometer and to adjust the split ratio. The use of an all silica capillary transfer line to the mass spectrometer was not investigated as this has been known to act as a chromatographic stationary phase and alter the elution times and orders of the peaks.

The samples used in this study were from various sources and are described, where appropriate, in the text.

### NMR spectroscopy

HPLC/NMR/MS was performed using a Bruker DRX500 spectrometer equipped with a dedicated $^1H/^{19}F$ -flow probe with cell volume 120 mL. Stopped-flow experiments were carried out using a double solvent suppression pulse sequence (1D-NOESY) to remove the residual protonated peaks from acetonitrile and water. Data were acquired with between 32 and 160 transients into 16K data points with pulse repetition time *ca.* 3 s. When the flow was split in order to perform NMR and MS it was necessary to recalculate the transfer time of the peaks from the UV detector to the NMR probe (52 s instead of 30 s in the absence of flow splitting, at a nominal flow rate of 1 mL/min.) because of pressure changes within the system when connected to the mass spectrometer. Chemical shifts were referenced to acetonitrile at _1.93.

### Mass spectrometry

The mass spectrometer was a Platform LC (Micromass, Altrincham, UK) fitted with an electrospray probe and a standard source. Data were acquired over the range 200±600 u with a scan time of 1 second and an interscan delay of 0.1

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# The Application of High Performance Liquid Chromatography, Coupled to Nuclear Magnetic Resonance Spectroscopy and Mass Spectrometry (HPLC-NMR-MS), to the Characterisation of Ibuprofen Metabolites from Human Urine

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## Key Words

Column liquid chromatography
NMR and MS detection
Ibuprofen metabolites
Urine extracts

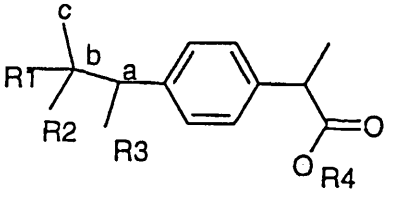
## Summary

The use of HPLC-NMR-MS for the detection and identification of the metabolites of ibuprofen present in a solid phase extract of human urine is described. Gradient reversed-phase HPLC was used to separate the components present in the extract, which were then characterised by a combination of stopped-flow $^1H$ NMR and on line electrospray-MS. This approach led to the rapid identification of the known phase 1 human metabolites of ibuprofen, including hydroxy- and carboxy- metabolites, together with their respective glucuronide conjugates. In addition a probable artefact resulting from the dehydration of one of the side chain-hydroxylated glucuronides was also identified.

## Introduction

The identification of the individual components present in a complex mixture frequently requires the use of an efficient separation system in order to isolate the analytes in a relatively pure state followed by spectroscopy for structure elucidation. Increasingly such mixtures are being routinely analysed using HPLC linked to either mass spectrometry or, more recently, high field NMR [1–3]. Often however, both MS and NMR data are required to enable a sample component to be unequivocally identified and, as a result, a number of groups have begun to evaluate the use uf combined HPLC-NMR-MS systems [4–9]. To date such systems have been applied to the analysis of simple pharmaceutical [4, 6] or peptide mixtures [8], and to drug or xenobiotic metabolites in urine samples [5–7, 9]. These HPLC-NMR-MS systems provide a very powerful tool for structure elucidation and enable the rapid determination of the structure of chromatographic peaks. As such the approach may offer advantages in terms of efficiency when compared to either HPLC-MS or HPLC-NMR alone.

Here we describe the results of LC-NMR-MS studies undertaken on an extract of human urine obtained following the oral administration of the non-steroidal anti-inflammatory drug ibuprofen (structure below).




For ibuprofen R1, R3 and R4 = H and R2 = Methyl.

## Experimental

### HPLC Conditions

HPLC was performed using a Hypersil BDS C-18 column (5 µ, 250 × 4.6 mm i.d.), attached to a standard Bruker LC system comprising Bruker LC22 pump, autosampler, UV detector and BPSU-12 collector (Bruker Spectrospin, Coventry, UK). The outlet of the UV detector was connected immediately to a splitter which directed the flow to the NMR and MS in the ratio 95:5. Gradient chromatography was used with 0.2 % formic acid in $^2H_2O$ (99.9 atom %, Fluorochem) and acetonitrile (Riedel de Haën). A linear gradient from 20 % to 60 % acetonitrile was employed over 45 min. at a flow rate of 1.0 mL $min^{-1}$ with UV detection at 254 nm. Under these conditions the transfer time for peaks from the UV detector to the NMR probe was 52 s and to the MS was 36 s.



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# High-Performance Liquid Chromatography On-Line Coupled to High-Field NMR and Mass Spectrometry for Structure Elucidation of Constituents of Hypericum perforatum L.

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**The on-line separation and structure elucidation of naphthodianthrones, flavonoids, and other constituents of an extract from *Hypericum perforatum* L. using high performance liquid chromatography (HPLC) coupled on-line with ultraviolet-visible, nuclear magnetic resonance (NMR), and mass spectrometry (MS) is described. A conventional reversed-phase HPLC system using ammonium acetate as the buffer substance in the eluent was used, and proton NMR spectra were obtained on a 500 MHz NMR instrument. The MS and MS/MS analyses were performed using negative electrospray ionization. In the present study, all of the major known constituents in extracts from *Hypericum perforatum* L. were identified, and two new substances which had not previously been reported as constituents of extracts of *Hypericum perforatum* L. were identified and their structures elucidated.**

Over the last 10 years there has been an increasing interest in the investigation of natural products in order to unveil new pharmacologically active substances, either for direct investigation or as lead structures for further synthetic research. This interest has primarily been concentrated upon plants or plant extracts[1-3] but marine organisms[4] have also been investigated.

The isolation and structure elucidation of unknown substances in biological materials, e.g. metabolites in biofluids or constituents in plant extracts, has, in the past, been lengthy and technically demanding. The traditional way to investigate natural products is to prepare extracts, test their pharmacological activity, isolate the individual components of the extract using liquid chromatography, and then perform structure elucidation by nuclear magnetic resonance (NMR) and mass spectrometry (MS) off-line. The isolation procedure is often a very tedious and time-consuming process. Thus, it would be advantageous to be able to speed up this part of the work, and the most obvious way to do this is to perform the separation and structure elucidation on-line.

High-performance liquid chromatography (HPLC) coupled to UV detectors is a routine technique at most laboratories for separation and detection of chemical substances. HPLC is also the separation technique of choice for the isolation of the often polar, nonvolatile, and unstable constituents found in natural products. However, the UV detectorsseven diode array detectorss do not provide enough data for full structure elucidation. During the last five years HPLC-MS has become a routine technique, but most of the new ionization techniques in MS provide only a molecular ion and eventually a few fragments, and thus, MS and MS/MS combined with UV-Vis are still often insufficient for the full structure elucidation. However, within the past few years NMR technology has improved considerably, and it is now possible, in certain circumstances, to obtain good proton spectra in the nanogramme range[5] when coupled on-line to HPLC. If all these techniques are coupled together at the same time in an on-line system an optimal system, for fast separation and structure elucidation of chemical constituents of natural products or biological fluids is constructed. A few examples of this are given in the literature,[6-11] including a recent example of the use of HPLC-UV-NMR-MS with a complex plant extract.

Extracts of *Hypericum perforatum* L. are used in drugs as an antidepressant. The major constituents of this extract have been known for many years, and structures have been elucidated by

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# Application of Directly Coupled HPLC-NMR-MS/MS to the Identification of Metabolites of 5-Trifluoromethylpyridone (2-Hydroxy-5-trifluoromethylpyridine) in Hydroponically Grown Plants

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Directly coupled HPLC-NMR-MS was used to characterize two major metabolites of 5-trifluoromethylpyridone (2-hydroxy-5-trifluoromethylpyridine), a model compound for herbicides, after it had been dosed into hydroponically grown maize plants. The combination of NMR and MS data allowed the identification of both of these metabolites, namely, the *N*-glucoside and *O*-malonylglucoside conjugates of the parent pyridone. This work demonstrates the efficiency and the potential application of HPLC-NMR-MS to the investigation of the metabolism of agrochemicals. The work also indicates that combination of the use of hydroponically grown plants and directly coupled HPLC-NMR-MS allows rapid identification of metabolites with little sample preparation.

**Keywords:** *HPLC; NMR; MS; 2-pyridone; hydroponics; metabolism; xenobiotic*

## INTRODUCTION

The hyphenated technique of HPLC-NMR is now well established as a powerful technique for the structure elucidation of unknown species in a variety of applications (Lindon et al., 1996). Recently, the hyphenation has been extended with the direct connection of HPLC-NMR-MS (Pullen et al., 1995; Scarfe et al., 1997; Shockcor et al., 1996). HPLC-NMR-MS has several advantages over both "stand alone" HPLC-NMR and HPLC-MS systems. A potential problem overcome by HPLC-NMR-MS is that of correlating spectroscopic data if the chromatography alters between HPLC-NMR and HPLC-MS separations. It is also possible to use either MS or NMR as a direct detection trigger to implement data collection by the other technique. By using a triple-quadrupole mass spectrometer, it is possible to obtain additional information from secondary fragmentation of selected ions (MS/MS).

HPLC-NMR-MS/MS has been used to study the metabolism of a trifluoromethylpyridone in hydroponically grown maize plants. Hydroponically grown plants offer several advantages over either whole plant systems or tissue culture (Miller et al., 1989). The conditions are more easily controllable than plants grown in soil and allow the introduction of the xenobiotic directly into the nutrient solution. Unlike tissue culture, the hydroponically grown plant is more akin to the whole plants and may give results that are more easily translated into what is likely to occur in whole plant systems.

5-Trifluoromethylpyridone (5-TFMP) was chosen for study as it serves as a simple substrate to examine the possible occurrence of *N*- versus *O*-glycosylation in plants.

## EXPERIMENTAL PROCEDURES

**Chemicals.** 5-TFMP (2-hydroxy-5-trifluoromethylpyridine) was obtained from Fluorochem (U.K.).

**Growth of Maize Plants.** The plants (maize, seeds obtained from Zeneca Agrochemicals, U.K.) were germinated for 2 days at nightime temperatures of 20-25 °C, daytime temperatures of 23-30 °C, with 16 h day length.

Germinated seeds were then placed into 3 in. pots with washed grit and silver sand (at a ratio of 1:1), _2 cm under the surface. The plants were then placed in a closed frame and grown at nightime temperatures of 19-23 °C, daytime temperatures of 22-27 °C, and a 16 h day length. The plants were watered with a solution of half-strength Hoagland's solution (standard nutrient solution) once a day. After 2 weeks, the plants were removed from the pots, and the roots were washed to remove sand and grit. The plants were then placed in hydroponic units (supplied by Zeneca Agrochemicals, U.K.) and secured with sponge bungs (three plants in each pot). The units contained full-strength Hoagland's solution, the level of which was maintained throughout the experiment. The plants were then left at ambient temperature, with a day length of 16 h. The plants were left in the pots to acclimatize for 1 week before dosing.

**Dosing of 5-TFMP.** A single dose was given to each plant, by direct addition into the nutrient (Hoagland's solution). 5-TFMP was prepared as a fine powder in a pestle and mortar to aid solution. Approximately 100 mg (in water) of 5-TFMP was added to each hydroponic pot (400 mL total volume).

**Sample Preparation.** At time periods of 1, 3, 7, 14, 28, and 38 days after dosing, plants were harvested. Roots and

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# Superheated Heavy Water as the Eluent for HPLC-NMR and HPLC-NMR-MS of Model Drugs

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**Superheated heavy water can be used as the eluent for reversed-phase chromatography with on-line HPLC-NMR and HPLC-NMR-MS detection. The method has been demonstrated for the separation of model drugs (analgesics and caffeine) on a poly(styrene-divinylbenzene) stationary phase in isothermal and temperature-programmed modes. One- and two-dimensional spectra could be obtained with less interference from the mobile phase than with conventional HPLC-NMR eluents. Unlike supercritical fluid chromatography-NMR, the spectra could be measured at room temperature and atmospheric pressure. The combination of superheated water HPLC-NMR-MS chromatography enables both NMR and MS spectra to be obtained simultaneously for the same sample.**

Liquid chromatography coupled with nuclear magnetic resonance (NMR) spectroscopy[1-7] and/or mass spectrometry (MS)[8] is an important technique for the identification and structural elucidation of analytes from complex mixtures obtained from drug metabolism,[1] environmental samples,[7] natural products, and combinatorial synthetic methods. Many of the practical problems associated with coupling these techniques have been resolved in recent years.[8,9] Although on-line HPLC-NMR spectroscopy has become practical for routine applications in recent years, there are still complications arising from strong background signals for protons in mobile-phase constituents, which can overlap with resonances from the analyte. In addition, even the purest conventional "HPLC-grade" mobile-phase constituents can also contain impurities, which contribute additional interfering signals. The interfering signals can be suppressed by using a suitable pulse sequence but this can result in loss of signals from a region of the spectrum. NMR-quality deuterated solvents with minimal interfering proton signals, such as $D_3$-acetonitrile, can be used, but these are expensive. Alternatively proton-free solvents, such as supercritical carbon dioxide,[10-13] can be used. However, in supercritical carbon dioxide the spin-lattice relaxation time is increased resulting in a longer sample acquisition time.[11] The NMR flow cell must also be capable of operating at high pressures up to 400 bar, and these must be maintained under stop-flow conditions. It has also been reported that problems can be encountered when pressure gradients are employed due to changes in the chemical shift with eluent density.[13] More importantly, SFC-NMR is essentially a normal-phase separation technique and is more restricted in its application than reversed-phase HPLC-NMR. Consequently, unmodified $CO_2$ is only really suitable for relatively nonpolar analytes, such as the phthalate esters[10] and vitamin A.[11]

Recently, superheated water at temperatures from 100 to 220 °C and at pressures up to 50 bar was found to be an effective low-polarity solvent and was initially employed for extraction.[14,15] Subsequently it has been used as an eluent for reversed-phase chromatography[16] and has been employed for the analysis of a range of analytes, including phenols, amides, esters, and barbiturates.[16-18] These conditions cause little hydrolysis or oxidation, unlike supercritical water at 400 °C and 350 bar, which is chemically aggressive. Superheated water chromatography has also been shown to be compatible with typical LC detectors (e.g.,

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Short communication

# Selective deuterium exchange during superheated heavy water chromatography ±nuclear magnetic resonance spectroscopy ±mass spectrometry of sulfonamides

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**Abstract**

Superheated deuterium oxide has been investigated as an eluent for reversed-phase HPLC on a polystyrene ± divinylbenzene column with UV, H NMR and MS detection using a series of sulfonamides as model compounds. In the [1] course of these studies, a selective, speci®c and ef®cient deuteration of the methyl groups on a pyrimidine ring was observed during chromatography of certain of the sulfonamides. The potential of this methodology for producing deuterium-labelled compounds from substances bearing suitable substituents is considered. The utility of HPLC ±NMR ±MS as a means for studying on-column reactions is discussed. © 2000 Elsevier Science B.V. All rights reserved.

*Keywords*: Superheated water chromatography; Deuterium exchange; Nuclear magnetic resonance spectroscopy; Mass spectrometry; Sulfonamides

## 1. Introduction

Recently, superheated water at temperatures from 100 to 2208C and up to 50 bar has been found to provide an effective eluent for reversed-phase chromatography [1 ± 3] and has been employed for the analysis of a range of analytes, including phenols, amides, esters and barbiturates. During these studies, superheated water chromatography has been shown to be compatible with typical LC spectroscopic detectors. In addition the absence of an organic eluent modi®er means that a ¯ame ionization detector can also be used [4 ± 6]. The eluent strength can be increased by increasing the temperature enabling gradient elution to be carried out and recently the separation of sulfonamides at a range of pH values with buffered aqueous eluents has been reported [7]. We have also demonstrated the application of deuterium oxide as a mobile phase for the HPLC ± NMR and HPLC±NMR±MS separation of a number of analytes including barbiturates, analgesics and related analytes [8,9]. The absence of large signals, for the protons of the organic modi®er in the HPLC solvents, considerably simpli®ed the NMR spectroscopic determination. The use of coupled NMR and MS enabled us to con®rm that the analytes were stable under the chromatographic conditions. In the

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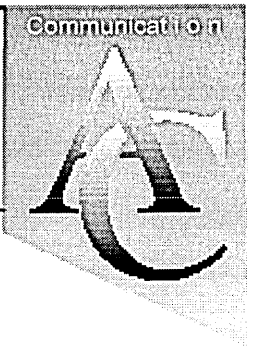
# Size exclusion chromatography with UV detection coupled to on-line $^1$H-NMR and on-line collection *via* a dedicated interface for subsequent off-line FT-IR

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The coupling of size exclusion chromatography (SEC) to on-line NMR spectroscopy and a dedicated interface for the collection of the chromatographic eluent for subsequent FT-IR has been investigated using a number of polymer additives as model compounds. SEC was performed using euterated chloroform as eluent with the separation mon-tored on-line by UV detection at 254 nm and on-flow $^1$H-MR. The effluent from the NMR probe was directed to a edicated HPLC/FT-IR interface where it was deposited on germanium plate. FT-NMR and FT-IR spectra were uccessfully obtained for 2,6-di-*tert*-butyl-4-methylphenol BHT), octadecyl-3-(3,5-di-*tert*-butyl-4-hydroxyphenyl)pro-ionate (Irganox 1076) and di-iso-octylphthalate.

## ntroduction

he successful coupling of high performance liquid chromatog-phy (HPLC) to nuclear magnetic resonance (NMR) spectros-opy to enable spectra to be obtained directly on chromato-raphic peaks, using either on-flow or stopped-flow methods eviewed in refs. 1-4) has been amply demonstrated. More cently a number of examples of the use of HPLC-NMR-MS ave been shown which have enabled the more complete aracterisation of samples by providing both NMR and MS ta for the same separation.[5-9] The success of this combination helping speed structure determination has encouraged us to ok at other multiple hyphenations of HPLC-NMR with other ectroscopic detectors in order to acquire additional structural formation on the separated sample components. The coupling IR spectroscopy to liquid chromatographic separations has en demonstrated using both on-line and off-line method-ogies with some success.[10-13] We have therefore examined e coupling of HPLC-NMR on-line with an interface which th removes the solvent present and deposits the solutes in a ndition that is compatible for subsequent FT-IR of relevant aks. This type of system has certain advantages over on-line w FT-IR systems in that the contribution of strong absorption nds inherent to HPLC solvents, which can restrict the ailable 'windows' in the resulting IR spectra, are removed, d in addition the solute bands can be focused to provide proved detection.[14]

Here we describe preliminary results for the separation and ectroscopic characterisation of a number of polymer additives llowing GPC with deuterochloroform as the eluent.

## xperimental section

### agents

-di-*tert*-butyl-4-methylphenol (BHT), octatadecyl-3-(3,5-di-t-butyl-4-hydroxyphenyl)propionate (Irganox 1076) and di-iso-octylphthalate (DIOP) were obtained from EVC Ltd (Runcorn, Cheshire, UK). Deuterochloroform ($CDCl_3$) 99.9 atom% was from Fluorochem Ltd (Glossop, Derbyshire, UK).

Solutions of the test analytes of approximately 100 mg ml$^{-1}$ in $CDCl_3$ were used.

### Chromatographic conditions

The HPLC-NMR system consisted of a Bruker LC22 pump (Bruker, Coventry, UK) which delivered $CDCl_3$ at 1.0 ml min$^{-1}$ to two Mixed-E columns (3 mm, 30 cm 3 7.5 mm id, Polymer Laboratories, Shropshire, UK) connected in series. Typically 10 ml of sample were introduced onto the column *via* a model 7125 Rheodyne injector (Rheodyne, USA) fitted with a 100 ml sample loop.

The eluent from the columns was monitored at 254 nm *via* a Bischoff Lambda 1000 UV detector and then delivered to the flow probe of the Bruker DRX-500 NMR spectrometer *via* 3 m of PEEK tubing. On-flow $^1$H-NMR detection was carried out in the pseudo-2D mode at 500.13 MHz using a flow-through probe of 4 mm id with a cell volume of 120 ml. FIDs were collected into 4 K data points with a spectral width of 8278 Hz, 90° pulses were used with an acquisition time of 0.25 s and each row in the pseudo-2D plots was acquired from 24 scans.

The eluent from the NMR probe was then taken to a commercial HPLC/FT-IR interface (Lab Connections-LC Transform Model 300 supplied by Viscotek, Basingstoke, Hampshire, UK) where it was deposited *via* the ultrasonic nebuliser onto a slowly rotating germanium composite disc (placed on a heated stage at 90 °C) and driven by a stepping motor at a constant rate of 10° min$^{-1}$.

The ultrasonic nozzle of the interface was held at 70 °C under vacuum (8 Torr) in order to evaporate the eluent. Following chromatographic separation the disc was transferred to the FT-IR scanning module (Lab Connections) and a series of spectra collected whilst rotating the disk under the IR beam. FT-IR spectra were obtained using a Nicolet 5DXC spectrometer (Nicolet Instruments Ltd, Warwick, UK) equipped with a DTGS room temperature detector over a spectral width of 4000-650 cm$^{-1}$ and a spectral resolution of 8 cm$^{-1}$. The disc was rotated at 5° min$^{-1}$ with 5 scans per spectrum co-added.

## Results and discussion

A typical GPC-UV trace obtained for the separation of the 3 test analytes is shown in Fig. 1 whilst the on-flow $^1$H-NMR pseudo 2D spectrum obtained for the same separation is shown in Fig. 2. As shown in the chromatogram (Fig. 1), the use of two low molecular weight range size exclusive chromatography (SEC) columns connected in-line, enabled essentially base line separations to be made on the test analytes. The use of $CDCl_3$ as eluent was of particular benefit as it enabled $^1$H-NMR to be

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# Size-exclusion chromatography with on-line ultraviolet, proton nuclear magnetic resonance and mass spectrometric detection and on-line collection for off-line Fourier transform infrared spectroscopy

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**Abstract**

The coupling of HPLC with UV detection and on-line NMR spectroscopy and mass spectrometry combined with a dedicated interface for the collection of the chromatographic eluent for subsequent Fourier transform (FT) IR has been investigated using a number of polymer additives as model compounds. Size-exclusion chromatography was performed using deuterated chloroform as eluent with the separation monitored on-line by UV detection at 254 nm and on-flow H-NMR and MS. The effluent from the NMR probe was directed to a dedicated HPLC interface where it was deposited on a germanium plate for subsequent FT-IR. NMR and MS spectra were successfully obtained for 2,6-di-*tert.*-butyl-4-methylphenol, octadecyl-3-(3,5-di-*tert.*-butyl-4-hydroxyphenyl) propionate (Irganox 1076) and diisooctyl phthalate on-line and FT-IR spectra for all three compounds were obtained off-line. Practical problems encountered with this multiple hyphenation are described. © 1999 Elsevier Science B.V. All rights reserved.

*Keywords*: Hyphenated techniques; Interfaces, LC ±NMR ±MS ±IR; Detection, LC; Dibutylmethylphenol; Octadecyl(dibutylhydroxyphenyl) propionate; Diisooctyl phthalate

## 1. Introduction

High-performance liquid chromatography (HPLC) is now routinely coupled to both nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS) enabling spectra of organic molecules to be obtained directly on chromatographic peaks (reviewed in Refs. [1 ± 5]). Normally HPLC ±NMR and HPLC ±MS have been performed separately but more recently examples where both NMR and MS coupled to the same separation (HPLC ±NMR ±MS) have been shown [6 ± 10]. Clearly such systems enable a much more complete characterisation of samples by providing both types of spectroscopic data on the same separation. The success of this combination has led us to examine the multiple hyphenation of HPLC ±NMR with other spectro-

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# Flow injection analysis with multiple on-line spectroscopic analysis (UV, IR, $^1$H-NMR and MS)

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**Abstract**

Studies on the capabilities of flow injection analysis (FIA) combined with on-line characterisation of model compounds via a combination of diode array UV, $^1$H-NMR, FT-IR spectroscopy and mass spectrometry are described. Using this combination of spectrometers enabled the on-flow collection of UV, $^1$H-NMR, IR and MS for a range of model compounds. Samples were introduced into the system as solutions in deuterium oxide in concentrations ranging from 1.4 to 8.4 mg ml$^{-1}$. A sample volume of 100 l was used for FIA at a flow rate of 1 ml min$^{-1}$. From these studies a practical working quantity of ca. 140 g/sample of analyte was determined which provided characteristic spectra. 

*Keywords*: Flow injection analysis; Multiple hyphenation; Spectroscopic characterisation

## 1. Introduction

The determination of the structure of unknowns, or the confirmation of structure of compounds thought to be present in a sample, is an important and often time consuming, task for analytical chemists in the pharmaceutical and other industries. The effect of combinatorial synthesis and the use of chemical libraries for high throughput screening, etc. has greatly added to this burden. We, together with a number of other groups, have been attracted by the potential of multiple hyphenation ('hypernation') of several spectroscopies in a single system as a means of speeding up this type of analysis, enabling comprehensive spectroscopic information to be obtained in a single analysis. This has led to the use of systems in which chromatography has been linked to nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS) to provide HPLC -NMR -MS analysis of complex mixtures (e.g. see Refs. [1 - 9], reviewed in Ref. [10]). More recently we have investigated systems

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# Reversed-Phase High-Performance Liquid Chromatography Combined with On-Line UV Diode Array, FT Infrared, and $^1$H Nuclear Magnetic Resonance Spectroscopy and Time-of-Flight Mass Spectrometry: Application to a Mixture of Nonsteroidal Antiinflammatory Drugs

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**A prototype multiply hyphenated system has been applied to the analysis of a mixture of nonsteroidal antiinflammatory drugs separated by reversed-phase HPLC. Characterization of the model NSAIDs was achieved via a combination of diode array UV, $^1$H NMR, FT-IR spectroscopy, and time-of-flight mass spectrometry. This combination of spectrometers allowed the collection of UV, $^1$H NMR, IR, and mass spectra together with atomic composition data enabling almost complete structural characterization to be performed.**

We have been interested for some time in the possibilities of characterizing compounds present in complex mixtures via the multiple hyphenation of the required spectroscopies in order to provide comprehensive spectroscopic information in a single analysis. In particular, together with a number of other groups, we have actively investigated the utility of chromatography doubly hyphenated with nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS) for this purpose (e.g., see refs 1-9, reviewed in ref 10). Such systems provide a very powerful means for structure determination, and indeed, the combination of retention time with NMR and MS spectral data is often enough to enable the unequivocal identification of an analyte. However, other spectroscopic techniques can also be linked to chromatographic separations that can be used to provide additional structural information. Thus, ultraviolet (UV) spectra can also provide supportive data for confirmation of identity and infrared (IR) spectroscopy can aid in the identification of structural features such as carbonyl or nitrile groups. In previous work, we have examined the practicality of coupling of size exclusion chromatography (SEC) with on-flow NMR and on-line collection of peaks via a dedicated interface for subsequent off-line FT-IR spectroscopy.[11] A further refinement of this system was provided by the subsequent addition of on-line MS.[12] However, while this system did enable NMR, MS, and IR spectra to be obtained for components of simple mixtures separated in a single chromatographic experiment, the need to obtain the FT-IR spectra off-line did make the methodology somewhat cumbersome.

The experience gained with this system did however show the potential for multiple spectroscopic characterization, and more recently, we constructed a flow injection analysis system (FIA) for the characterization of compounds from, for example, chemical libraries.[13] This prototype system consisted of a UV diode array

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The Analyst
FULL PAPER

# Flow injection spectroscopic analysis of model drugs using on-line UV-diode array, FT-infrared and $^1$H-nuclear magnetic resonance spectroscopy and time-of-flight mass spectrometry

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A prototype flow injection analysis (FIA) system for the characterisation of compounds *via* a combination of diode array UV, $^1$H NMR, FT-IR spectroscopy and time-of-flight (TOF) mass spectrometry has been investigated using a number of pharmaceuticals and related compounds as model compounds. This combination of spectrometers allowed the on-flow collection of UV, $^1$H NMR, IR and mass spectra together with atomic composition data, enabling almost complete structural characterisation to be performed. Practical detection limits with the current system were in the region of 50 mg, however, the use of state of the art spectrometers would result in a significant reduction in the amount of material required.

## ntroduction

he characterisation of substances by spectroscopy to prove dentity or determine structure remains an essential feature of ıodern pharmaceutical chemistry. There is an urgent need to be ble to perform such work rapidly and efficiently as a result of he vastly increased use of large 'libraries' of compounds for igh throughput screening. Correspondingly the increased umbers of compounds synthesised as a result of the wide-pread adoption of combinatorial chemistry as a means of ıcreasing the number of structures available for testing has laced a similar burden on the production of structure purity ata. We have therefore been interested in the possibilities for ccomplishing such characterisation *via* the multiple hyphen-tion of the required spectroscopies in order to provide omprehensive spectroscopic information in a single analysis. hus we, together with a number of other groups, have been ctively investigating the potential of chromatography linked ›intly to nuclear magnetic resonance (NMR) spectroscopy and ıass spectrometry (MS) for complex mixture analysis [*e.g.*, see ›fs. 1-9, reviewed in ref. 10]. Indeed this technology has now ıatured to the extent that it is now possible to purchase an ıtegrated HPLC-NMR-MS system from one manufacturer Bruker). However, whilst NMR and MS data are often ıfficient to confirm identity, or to determine the structure of ıknowns, other spectroscopies are available that also provide seful structural information. In particular, infrared (IR) ›ectroscopy can provide direct evidence for structural features ch as, *e.g.*, carbonyl or nitrile groups. We have, therefore, also vestigated size exclusion chromatography-NMR combined ith on-line collection of peaks *via* a dedicated interface for bsequent FT-IR spectroscopy.[11] More recently this system as extended by the addition of MS[12] enabling the collection of MR and mass spectra on-line with off-line FT-IR of the peaks interest.

The experience gained with these preliminary experiments, ıereby chromatography has been linked to several spectro-›pic detectors in a single run, has encouraged us to construct low injection analysis system for the characterisation of compounds from, *e.g.*, chemical libraries. Here results obtained when this prototype system, which consisted of a UV-diode array (DAD) detector, an FT-IR spectrometer, an NMR spectrometer and a time-of-flight (TOF) MS, was applied to a selection of model compounds are described.

## Experimental section

### Reagents

The compounds employed in this investigation were, ibuprofen, flurbiprofen, naproxen, indomethacin and atenolol (Sigma, Poole, Dorset, UK), antipyrine (Fluka, Gillingham, Dorset, UK), 4-hydroxyantipyrine and salicylic acid (Aldrich, Gillingham, Dorset, UK), nor-antipyrine (Janssen Chimica, Geel, Belgium) and propranolol (AstraZeneca Pharmaceuticals, Alderley Park, Cheshire, UK). Samples were dissolved in deuterium oxide ($D_2O$) at varying concentrations to give samples containing between 0.25 and 100 mg ml$^{-1}$.

The FIA system (Fig. 1) consisted of a Bruker LC22 pump (Bruker, Coventry, UK) which delivered $D_2O$ at 1 ml min$^{-1}$. Typically 200 ml of sample were introduced into the flowing steam *via* a model 7125 Rheodyne injector (Rheodyne, Cotati, CA, USA) fitted with a 200 ml sample loop.

From the injector the flow was directed to a Varian 9065 UV-diode array detector (Varian UK Ltd., Walton-on-Thames, Surrey, UK) *via* 30 cm of 0.005" id PEEK (polyethyl ether ketone) tubing in order to obtain UV spectra. UV data was collected over the wavelength range 190-360 nm, using the Star Chromatography Workstation, Version 4.0 (Varian), and analysed for spectral information using Polyview Version 2.0 (Varian).

Following DAD the flow went *via* 110 cm of 0.005" id PEEK tubing to a Bio-Rad (Cambridge, MA, USA) FT-IR model 375C spectrometer fitted with a Spectra Tech (Stamford, CT, USA) Macro Circle Cell ATR (attenuated total reflectance) stainless-steel flow cell of 400 ml volume fitted with a zinc selenide ATR

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# HPLC Analysis of Ecdysteroids in Plant Extracts Using Superheated Deuterium Oxide with Multiple On-Line Spectroscopic Analysis (UV, IR, $^1$H NMR, and MS)

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**HPLC, using superheated $D_2O$ as the mobile phase, combined with on-line characterization via a combination of diode array UV, $^1$H NMR, FT-IR spectroscopy, and mass spectrometry has been used for the analysis of a standard of 20-hydroxyecdysone- and ecdysteroid-containing plant extracts. This combination of spectrometers enabled the on-flow collection of UV, $^1$H NMR, IR, and mass spectra not only for pure 20-hydroxyecdysone (100-400 ig on column) but also the major ecdysteroids present in crude extracts of *Silene otites*, *Silene nutans*, and *Silene frivaldiskyana*. The ecdysteroids unequivocally identified in these extracts included 20-hydroxyecdysone, polypodine B, and integristerone A.**

The determination of the structure of active components in plant extracts, and the confirmation that the components of interest are either known or novel, is a key task for natural product chemists. The development of routine HPLC/MS and HPLC/NMR instrumentation has greatly eased this burden, but often both methods must be employed for unambiguous identification. We, together with a number of other groups, have been attracted by the potential of multiple hyphenation ("hypernation") of several spectroscopies in a single system in order to be able to perform this type of work more efficiently, by providing comprehensive spectroscopic information from a single separation. Although the bulk of this work has involved the use of HPLC/NMR-MS (e.g., see refs 1-9, reviewed in ref 10), we have also begun to investigate systems that, in addition, provide infrared (IR) spectra. In these studies, we have examined systems that enabled spectra to be obtained either off-line (with on-line collection of peaks)[11,12] or directly on-line.[13,14] The most recent of these studies, in which all spectra were obtained on-line, enabled the collection of UV-diode array (DAD), FT-IR, $^1$H NMR, and time-of-flight (TOF) MS data. This system was used in conjunction with reversed-phase HPLC and was applied to the characterization of a model mixture of nonsteroidal anti-inflammatory drugs[13] and the major ecdysteroids present in a plant extract.[14] Here we describe studies using a modified version of this system and employing eluents at elevated temperatures applied to the detection and identification of ecdysteroids in a number of extracts of plants from the *Silene* family. The ecdysteroids are a family of polar, polyhydroxylated, steroids that function, among other things, as the moulting hormones of insects and crustaceans. They are also found in many species of plants, often in high concentration, where it is assumed that they have some function as chemical defenses against predacious insects. There is considerable structural diversity within the ecdysteroids and some 300 compounds have been identified.[15] The

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# High temperature reversed-phase HPLC using deuterium oxide as a mobile phase for the separation of model pharmaceuticals with multiple on-line spectroscopic analysis (UV, IR, $^1$H-NMR and MS)

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The reversed-phase chromatography of a number of model pharmaceuticals using deuterium oxide ($D_2O$) as the mobile phase at elevated temperatures, including superheated conditions (greater than 100 °C), is described. Following elution the analytes were characterised on-line *via* a combination of diode array UV, $^1$H-NMR, FT-IR spectroscopy and mass spectrometry. This combination of spectrometers enabled the on-flow collection of full UV, $^1$H-NMR, IR and mass spectra for a range of compounds in amounts anging from 46 to 500 mg on-column. The advantages of the use of $D_2O$ alone as mobile phase for chromatography with nultiple spectroscopic characterisation of analytes is dissussed.

## ntroduction

he use of high temperatures (greater than 100 °C) for reversed-hase HPLC has recently begun to receive attention.[1-3] One of he advantages of the use of elevated temperatures is the otential to greatly reduce, or even eliminate, the organic nodifier. There is also the possibility of employing temperature rogramming to effect gradient elution whilst keeping the verall composition of the mobile phase constant. A particular dvantage of entirely aqueous mobile phases is found in HPLC-MR where the use of $D_2O$ alone results in the elimination of nterferences from the organic modifier which, even when euterated solvents are employed, can result in areas of interest n the spectra of analytes being obscured. Examples of the use f "superheated" $D_2O$, to good effect, as the mobile phase for IPLC-NMR for a range of analytes have been described.[4-6] imilarly, there is no doubt that a simple mobile phase greatly acilitates multiple hyphenation ("hypernation") where a range f spectroscopic detectors (*e.g.* combinations of HPLC-NMR-IS, HPLC-UV(DAD)-NMR-MS and HPLC-UV(DAD)-IR-MR-MS) are used in a single system for compound identification. Whilst not yet routine the application of such hypernated ystems is being investigated by a number of groups (*e.g.*, refs. -13 reviewed in ref. 14). In our view, a significant problem ncountered with hypernated systems is the difficulty in finding obile phases that are compatible with the various spectroopic detectors employed. Thus, for example, in the case of PLC-NMR the use of inorganic buffers is quite acceptable as ese additives do not result in interferences in the resulting ectra. However, the same buffers used for HPLC-MS can be ite problematic because of their involatility. For HPLC-NMR mple binary solvent systems (generally $D_2O$-acetonitrile) are eferred, so as to minimise the interference of the organic modifier with the signals of the analyte, whilst for HPLC-MS the use of several organic modifiers presents little or no difficulties. As a result of the problems of matching chromatographic solvent and spectrometers we have begun to investigate the use of entirely aqueous mobile phases. This approach has been applied in studies using superheated $D_2O$ for the HPLC-NMR and HPLC-NMR-MS of a variety of model pharmaceuticals[4-6] and most recently in experiments using superheated $D_2O$ in combination with a HPLC-UV(DAD)-IR-NMR-MS system for the separation and analysis of ecdysteroids in plant extracts[13]. Here we describe further studies to evaluate this system using a number of model pharmaceuticals with two stationary phases that we have previously found to be suitable for this type of chromatography[15].

## Experimental section

### Reagents

The compounds employed in this investigation were paracetamol (acetaminophen), antipyrine, 4-aminoantipyrine, norantipyrine, caffeine, phenacetin, *p*-aminobenzoic acid, propranolol, sulfacetamide, sulfanilamide (Sigma, Poole UK, Fluka, Gillingham, UK and Aldrich, Gillingham, UK). Samples were dissolved, at concentrations of between 23 to 125 mg ml$^{-1}$ (see text) in deuterium oxide ($D_2O$) (99 atom %, Aldrich, UK).

The HPLC system consisted of a Constametric 3200 HPLC pump (Laboratory Data Control, Stone, Staffs, UK) which delivered $D_2O$ at 1.0 ml min$^{-1}$ to a 150 3 4.6 mm C8 XTerra (5mm particle size), HPLC column (Waters Ltd, Watford, UK, part no 186000493 ser. No. W01031N007) or at 0.8 ml min$^{-1}$ to a prototype 2.1 3 150 mm Oasis HLB column (5 mm particle size, lot no., M90762D01) (Waters Ltd). The column was placed in the oven of a Pye 104 gas chromatograph (Pye Unicam, Cambridge, UK) in order to perform chromatography at elevated temperatures. The temperature of the oven was controlled using a Pye oven programmer and typically was maintained at 85 and 185 °C for the XTerra and Oasis columns respectively. On emergence from the oven the eluent was rapidly cooled using a water bath kept at *ca.* 0-4 °C using ice. $D_2O$ was maintained in a liquid state at these temperatures by the backpressure generated by the PEEK tubing connecting the column to the various detectors described below.

From the column the eluent entered a splitter from which *ca* 95% of the flow was directed, *via* 110 cm of 0.020B id PEEK tubing, to a Bio-Rad FT-IR model FTS3000 Excaliber spectrometer (Cambridge, MA, USA). The spectrometer was fitted with a Spectra Tech (Stamford, CT, USA) Micro Circle Cell ATR (attenuated total reflectance) high pressure stainless steel flow cell of 25 ml volume, fitted with a zinc selenide ATR crystal. The spectrometer was purged with dry nitrogen to

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ORIGINAL PAPER

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# Reversed-phase HPLC of polymer additives with multiple on-line spectroscopic analysis (UV, IR, $^1$H NMR and MS)

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**Abstract** The reversed-phase chromatography of a number of polymer additives has been undertaken with on-line characterisation via a combination of diode array UV, $^1$H NMR, IR spectroscopy and mass spectrometry. This combination of spectrometers enabled the on-flow collection of full UV, $^1$H NMR, IR and mass spectra for a range of common polymer additives in amounts ranging from ca. 230 to 900 ∝g on-column. The practical difficulties associated with multiple hyphenation and potential future developments are discussed.

**Keywords** HPLC · Polymer additives · Multiple hyphenation · Spectroscopic characterisation

## Introduction

The use of techniques such as HPLC-MS and HPLC-NMR is now routine in many laboratories for the analysis of mixtures. This is possible as a result of the development of robust chromatographic interfaces that have evolved to the extent that relatively few compromises need to be made by either separation system or spectrometer. The successful development of this type of hyphenation has led to the construction of more sophisticated systems combining more than one spectrometer. Such multiple hyphenation ("hypernation") is perhaps most widely developed for HPLC-NMR-MS (reviewed in [1]). Here, the complementary nature of the NMR and MS data provide a powerful and efficient method for the characterisation and identification of chromatographic peaks. However, even more elaborate concatenations of instruments are possible and systems capable of providing NMR, IR, MS [2] and IR, UV, NMR and mass spectra [3, 4,5] have been reported. In a recent series of experiments, we have investigated the potential of such an HPLC-IR-UV(DAD)-NMR-MS system in combination with $D_2O$ at high temperatures for the separation and analysis of ecdysteroids in plant extracts [6] and model pharmaceuticals [7] to obtain on-line spectra. Here, we describe further studies to evaluate this system, using a number of common polymer additives as models, with a more conventional reversed-phase chromatographic system. These results are compared with those from earlier studies on the separation and spectroscopic characterisation of polymer additives with normal phase eluents and off-line FT-IR following on-line collection using various commercial ("LC-Transform") interfaces [2,3].

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## Experimental section

### Reagents

The model polymer additives (structures in Fig. 1) used in this investigation were Irganox 245 (triethyleneglycol bis-3(3-tertiary-butyl-4-hydroxy-5-methylphenylpropionate), BHA (butylated hydroxyanisole, 2-tertiary-butyl-4-methoxyphenol), BHT (butylated hydroxytoluene, 2,6-di-tertiary-butyl-4-methylphenol), Bisphenol A and Topanol CA (1,1,3-tris-(2-methyl-4-hydroxy-5-tertiary-butylphenyl)butane) (purchased from Sigma, Poole UK, Fluka, Gillingham, UK and Aldrich, Gillingham, UK). Samples were dissolved, at concentrations of 46-99 ∝g ∝L$^{-1}$ (see text) in ($CD_3CN$) (99 atom%, Aldrich, UK). For this investigation, a test mixture comprising Bisphenol A, BHA, Irganox 245, BHT and Topanol CA was prepared and analysed as described below. In addition, the various model analytes were chromatographed individually.

The HPLC system consisted of a Constametric 3200 HPLC pump (Laboratory Data Control, Stone, Staffs UK) which delivered $CD_3CN$-$D_2O$ (80:20 v/v) at 1.0 mL min$^{-1}$ to a 100· 4.6 mm Hypersil H5ODS (5 ∝m particle size), HPLC column (Hichrom, Reading, UK, Batch No. 4421, SER. NO. H5ODS-5886).

From the column, the eluent entered a splitter from which ca. 95% of the flow was directed, via 110 cm of 0.020"-i.d. PEEK tubing, to a Bio-Rad FT-IR model FTS3000 Excaliber spectrometer (Cambridge, MA USA). The spectrometer was fitted with a Spectra Tech (Stamford, CT USA) Micro Circle Cell ATR (attenuated total reflectance) high-pressure stainless steel flow cell of 25 ∝L volume. The flow cell was fitted with a zinc selenide ATR crystal. The spectrometer was purged with dry nitrogen to minimise any

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# Spectroscopic characterisation and identi®cation of ecdysteroids using high-performance liquid chromatography combined with on-line UV±diode array, FT-infrared and $^{1}$H-nuclear magnetic resonance spectroscopy and time of ¯ight mass spectrometry

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**Abstract**

A prototype multiply hyphenated reversed-phase HPLC system has been applied to the analysis of a mixture of pure ecdysteroids and an ecdysteroid-containing plant extract. Characterisation was achieved via a combination of diode array UV, $^{1}$H NMR, FT-IR spectroscopy and time of ¯ight (TOF) mass spectrometry. This combination of spectrometers allowed the collection of UV, $^{1}$H NMR, IR and mass spectra for a mixture of pure standards enabling almost complete structural characterisation to be performed. The technique was then applied to a partially puri®ed plant extract in which 20hydroxyecdysone and polypodine B were identi®ed despite incomplete chromatographic resolution and the presence of co-chromatographing interferents. The experimental dif®culties in the use of such a systems for these analytes are described. 

*Keywords*: Multiple hyphenation; Mixture analysis; Detection, LC; Spectroscopic characterization; Plant extracts; Ecdysteroids

## 1. Introduction

The identi®cation of analytes in complex mixtures via multiple hyphenation of chromatography with a range of spectroscopic detectors such as, e.g. HPLC ± NMR±MS, etc. is becoming an established means for obtaining unambiguous characterisation (e.g., see [1 ± 9], reviewed in Ref. [10]). Recently, we have begun to look at systems that enable ultraviolet (UV) and infrared (IR) spectra to be obtained in addition to NMR and MS [11 ±13]. The most recent version of these prototype systems combined a UV±diode array (DAD) detector, an FT-IR spectrometer, an NMR spectrometer and a time of ¯ight (TOF) MS (providing the ability to determine molecular formulae via accurate mass determination) and was

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# $^{19}$F -NMR and directly coupled HPLC -NMR -MS investigations into the metabolism of 2 -bromo -4 -tri¯ uorom ethylaniline in rat : a urinary excretion balance study without the use of radiolabelling

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1. The metabolic fate and urinary excretion of 2 -bromo-4-tri¯ uoromethylaniline has been studied in rat using " * F -NMR spectroscopic and directly coupled HPLC -NMR MS methods. The compound was dosed to Sprague-Dawley rats (5 0 mg kg' " , i.p.) and urine collected over 0 ± 8 , 8± 24 and 24 ± 4 8 h post-dosing.

2. A total urinary recovery of 53± 5 7 ± 0 % of the dose was achieved up to 4 8 h after dosing. The major metabolite in the urine was identi® ed as 2-amino-3-bromo -5 -tri¯ uoromethylphenylsulphate accounting for a total of 35± 7 6 ± 2 % of the dose.

3. Further metabolites detected were 2-bromo -4 -tri¯ uoromethylphenylhydroxyl-amine-*N* -glucuronide (9±7 0± 2 % of the dose ), 2-bromo-4-tri¯ uoromethylaniline-*N* -glucuronide (3±0 0± 3 % ) and 2-amino-3 -bromo-5-tri¯ uoromethylphenylglucuronide (2±8 0± 4). Minor metabolites, including 2-bromo -4 -tri¯ uoromethylphenylhydroxyl-amine-*O*-glucuronide , 2-amino -3-bromo-5-tri¯ uoromethylphenol and 2-bromo -4 -tri¯ uoromethylphenylsulphamate , in total accounted for 2± 3 0± 9 % of the dose.

4. Directly coupled HPLC -NMR -MS and " * F -NMR spectroscopy proved to be eœ cient techniques for the unequivocal and rapid determination of the urinary metabolic fate and excretion balance of ¯ uorinated xenobiotics without the need for radiolabelling.

## Introduction

The metabolism of primary aromatic amines and acetanilides is of widespread interest because of their relevance to drug design and diverse toxicological eå ects including hepatoxicity , nephrotoxicity, carcinogenesis and methaemoglobinaemia (Radomski 1979) . In addition certain aromatic amines , such as sulphanilamide (Fries *et al* . 1 9 71 ), 4 -chloroaniline (Kiese and Lenk 1 9 71 , Ehlhardt *et al* . 1 9 9 1) and 4-tri¯ uoromethylaniline (Wilson *et al.* 1 98 5 , Wade *et al.* 1 98 8 ), are of interest because they undergo *N* -acetylation and subsequent oxidation to produce unusual oxanilic acid metabolites which account for a signi® cant proportion of the dose . In the case of tri¯ uoromethylanilines (TFMAs ) the presence of the CF $group also provides a useful ` handle ' for the detection and quanti® cation of metabolites using

F -NMR spectroscopy without the need for radiolabelling (Wade *et al.* 19 8 8) . One of the aims of the current work is to gain a better understanding of the molecular physicochemical properties of TFMAs that may predispose them towards the formation of oxanilic acids . We are , therefore , investigating the metabolism of various substituted 4 -TFMAs to determine structure ± metabolism relationships of

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# Investigation of the metabolism of $^{14}C/^{13}C$-practolol in rat using directly coupled radio-HPLC-NMR-MS

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1. The metabolic fate of "%C}"$C-practolol was investigated using on-line HPLCNMR-MS following oral administration to rat. The major route of elimination for the radiolabel was via the urine with the principal biotransformation products con® rmed as the 2-hydroxy- and 2-hydroxyglucronide metabolites.

2. In addition, futile deacetylation, determined by the replacement of "$C-labelled acetyl groups with endogenous "#C-acetyls accounted for C 7± 10% of the urinary metabolites, corresponding to C 5% of the dose undergoing N-deacetylation.

3. Evidence for chiral metabolism was sought via NMR of isolated metabolites using bcyclodextrin as a chiral shift agent. Practolol was excreted as a racemate. However, some enantioselective metabolism}excretion had occurred as the hydroxy- and hydroxyglucuronide were not excreted as racemic mixtures.

4. Directly coupled radio-HPLC-NMR-MS is extremely eå ective for the identication of the metabolites of radiolabelled xenobiotics in urine samples.

## Introduction

Practolol (Eraldin4) was developed as a b-adrenoceptor antagonist for the treatment of angina pectoris, systemic hypertension and cardiac arrhythmias, but was subsequently withdrawn due to unwanted side-eå ects observed in a comparatively small proportion of patients receiving the drug. The metabolic fate of the compound has been extensively studied in a number of species. Thus, following administration of practolol to rat (20± 500 mg kg ") and dog (16 mg kg "), urinary excretion was the major route of elimination (Scales and Cosgrove 1970) with

85% of the dose excreted as unchanged Practolol. The major metabolite (9 % of total) was identi® ed as the 2-hydroxy-4-(2-hydroxy-3-isopropylaminopropoxy)-acetanilide, which was excreted in the ratio 1 : 2 as the free and glucuronide conjugated metabolite. Practolol metabolism has also been studied in healthy human volunteers and patients (Reeves *et al.* 1978). Similar metabolism was seen in man and other species including mouse, guinea pig, hamster, rabbit and marmoset (Reeves *et al.* 1979). The only species diå erence that was noted was the extent of N-deacetylation (followed by re-acetylation). While this futile deacetylation was low in most species (! 5%), in the marmoset C 57% of the dose was N-deacetylated (Reeves *et al.* 1979). The extent of this deacetylation was monitored by measuring the evolution of "%CO

# after the incorporation of a "%C-acetyl group into the drug.

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# Quantitative studies on the urinary m etabolic fate of 2-chloro -4 -tri¯ uoromethylaniline in the rat using $^{19}$F -NMR spectroscopy and directly coupled HPLC - NMR -MS

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1. The metabolism and urinary excretion of 2 -chloro-4-tri¯ uoromethylaniline has been studied in the rat using "* F -NMR spectroscopy and directly coupled HPLC -NMR - MS methods. The compound was dosed to three male Sprague-Dawley rats (5 0 mg kg' * i.p . ) and urine collected over 0± 8,8± 24 and 2 4± 48 h post-dosing.

2. A total urinary recovery of 5 6.3 ‰2.2 % of the dose was achieved up to 48 h after dosing. The major metabolite in the urine was identi® ed as 2-amino-3-chloro -5 tri¯ uoromethylphenylsulphate accounting for a total of 33.5 ‰2.2 % of the dose.

3. Further metabolites detected and characterized included 2-chloro-4-tri¯ uoro - methylphenylhydroxylamine glucuronide (13.2 ‰0.5 % of the dose ), 2-amino-3 - chloro -5-tri ¯ uoromethylphenylglucuronide (3.8 ‰0. 4 % of the dose) and 2-chloro -4 - tri¯ uoromethylaniline-*N*-glucuronide (3.6 ‰0.1 % of the dose ). Several minor metabolites were also found and identi® ed, including 2-chloro-4 -tri¯ uoromethylphenylsulphamate , which together accounted for 2.1 ‰0.4 % of the dose.

4. Directly coupled HPLC -NMR -MS and "* F -NMR spectroscopy is shown to provide an eæ cient approach for the unequivocal and rapid determination of the quantitative urinary metabolic fate and excretion balance of a uorinated xenobiotic without the necessity for speci® c radiolabelling .

## Introduction

Understanding the metabolic fate of primary aromatic amines is important because of the toxicological insights that such information may provide on a class of compound that has widespread uses as intermediates in the production of drugs , pesticides and bulk chemicals . Aromatic amines can also be generated *in vivo* via the metabolism of a variety of *N* -containing drugs . The well-known toxicological properties of aromatic amines include hepatotoxicity , nephrotoxicity, carcinogenesis and methaemoglobinaemia (Radomski 1979). As part of a series of studies aimed at building metabolic databases , from which it is planned to develop predictive *in vivo* models of drug metabolism (Ghauri *et al.* 1 99 2 a , b , Holmes *et al.* 1 9 95 , Cupid *et al.* 1996), we have used NMR -based methods to generate data on the metabolic fate of a number of anilines (e .g . Scarfe *et al* . 1 99 8 ).

For xenobiotics containing uorine , we have found "* F nuclear magnetic resonance (NMR ) spectroscopy to provide a useful alternative to radiolabelling in metabolism studies . Thus the presence of one or more ¯ uorine atoms provides a

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# Appendix 2

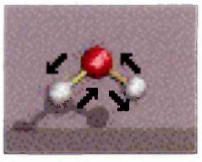
# FT-IR and NMR

# Interpretation Aids

## Basic IR Background and theory

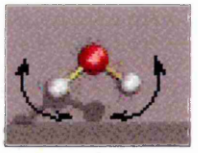
The region of the infrared spectrum that is of greatest interest to organic chemists is the wavelength range from approximately 2.5 to about 15 micrometers (μ). In practice, units proportional to frequency, (wave number in units of $cm^{-1}$) rather than wavelength, are commonly used and the region 2.5 to 15 μ corresponds to approximately 4000 to 600 $cm^{-1}$.

Absorption of radiation in this region by a typical organic molecule results in the excitation of vibrational, rotational and bending modes, while the molecule itself remains in its electronic ground state. These vibrational and bending modes for water ($H_2O$) are illustrated below:



**Symmetric Stretch**

**Asymmetric Stretch**



**Symmetric Bend**

N.B. Molecular asymmetry is a requirement for excitation by infrared radiation and fully symmetric molecules do not display absorbances in this region unless asymmetric stretching or bending transitions are possible.

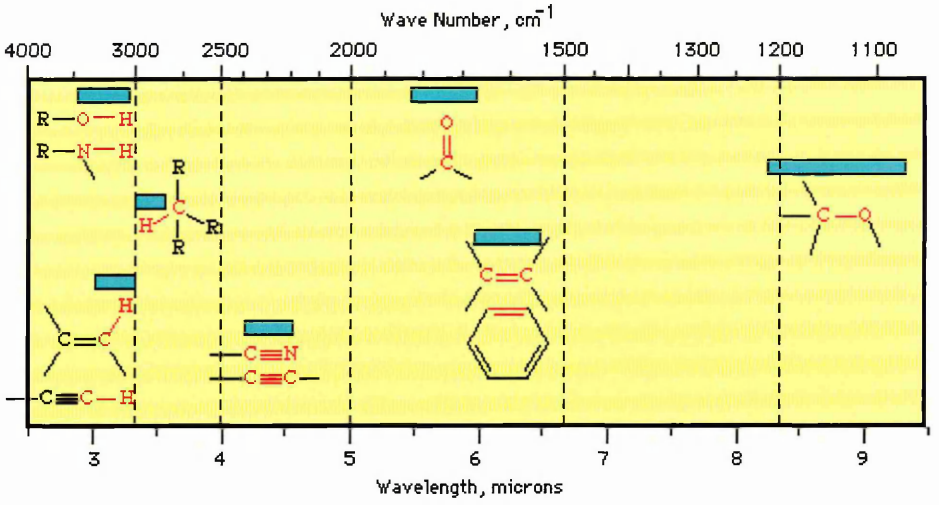
For the purpose of routine organic structure determination, the most important absorptions in the infrared region are the simple stretching vibrations. The stretching

For the purpose of routine organic structure determination, the most important absorptions in the infrared region are the simple stretching vibrations. The stretching vibrations of typical organic molecules tend to fall within distinct regions of the infrared spectrum, as shown below:

- 3700 - 2500 $cm^{-1}$: X-H stretching (X = C, N, O, S)
- 2300 - 2000 $cm^{-1}$: C≡X stretching (X = C or N)
- 1900 - 1500 $cm^{-1}$: C=X stretching (X = C, N, O)
- 1300 - 800 $cm^{-1}$: C-X stretching (X = C, N, O)

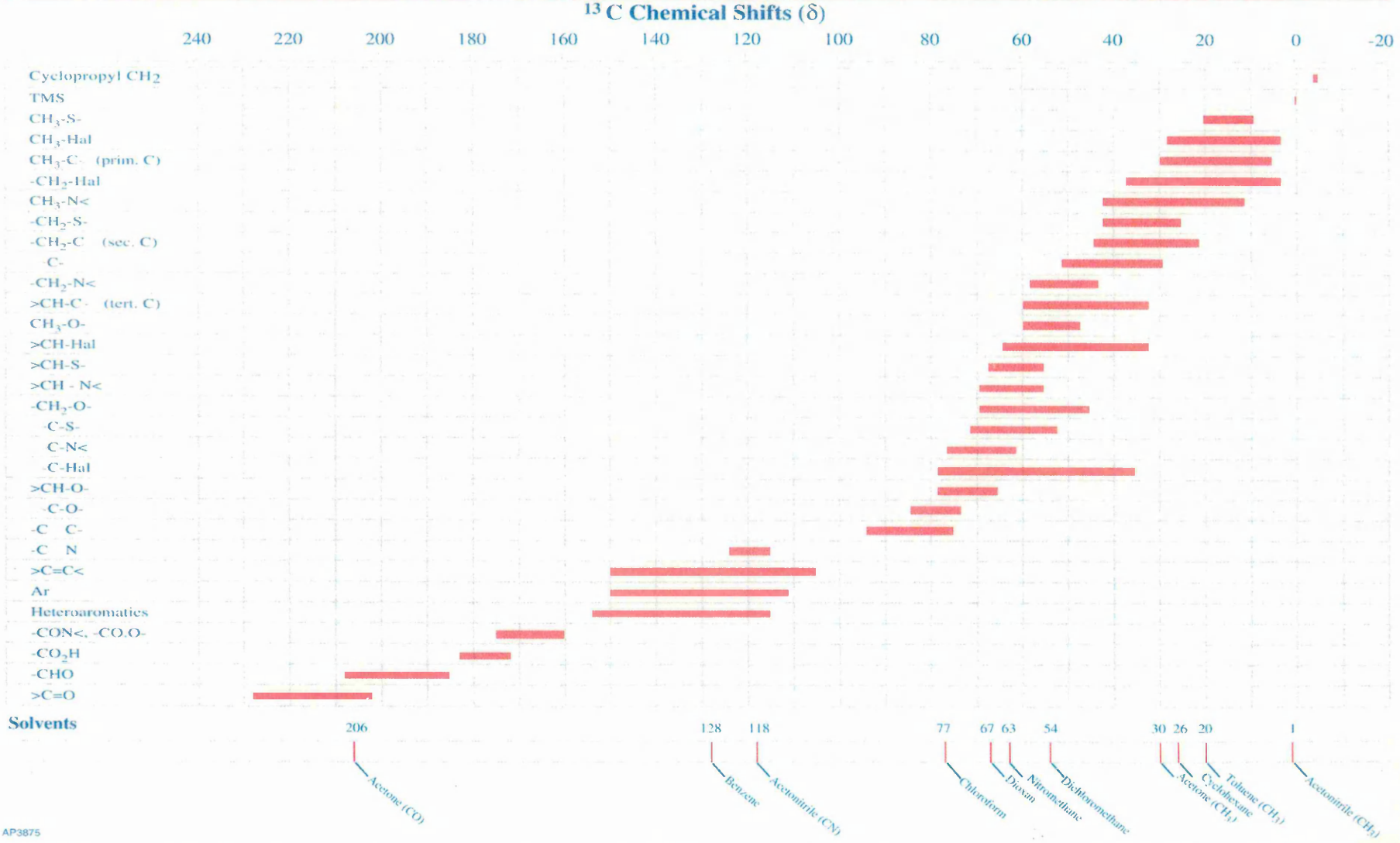
Since most organic molecules have single bonds, the region below 1500 $cm^{-1}$ can become quite complex and is often referred to as the 'fingerprint region'. Therefore if you are comparing an unknown molecule which has the same 'fingerprint' in this region as that of a library compound, it is considered strong evidence that the two molecules may be identical.

Because of the complexity of the region below 1500 $cm^{-1}$, the assignment of this part of the spectrum is difficult and so the focus here will be on functional group stretching bands in the higher frequency region. Many of these bands overlap in the IR spectrum and therefore may give equivocal structural information. However, often the absence of a band is as informative as the presence of a particular band. A correlation table showing many of the common IR absorbance bands is given below.




- Alcohols and amines display strong broad O-H and N-H stretching bands in the region 3400-3100 $cm^{-1}$. The bands are broadened due to hydrogen bonding and a sharp 'non-bonded' peak can often be seen at around 3400 $cm^{-1}$.
- Alkene and alkyne C-H bonds display sharp stretching absorptions in the region 3100-3000 $cm^{-1}$. The bands are of medium intensity and may be obscured by other absorbances in the region (i.e., OH).
- Triple bond stretching absorptions occur in the region 2400-2200 $cm^{-1}$. Absorptions from nitriles are generally of medium intensity and are clearly defined. Alkynes absorb weakly in this region unless they are highly asymmetric; symmetrical alkynes do not show absorption bands.
- Carbonyl stretching bands occur in the region 1800-1700 $cm^{-1}$. The bands are generally very strong and broad. Carbonyl compounds which are more reactive in nucleophilic addition reactions (acyl halides, esters) are generally at higher wave number than simple ketones and aldehydes, and amides are the lowest, absorbing in the region 1700-1650 $cm^{-1}$.
- Carbon-carbon double bond stretching occurs in the region around 1650-1600 $cm^{-1}$. The bands are generally sharp and of medium intensity. Aromatic compounds will typically display a series of sharp bands in this region.

- Carbon-oxygen single bonds display stretching bands in the region 1200-1100 $cm^{-1}$. The bands are generally strong and broad. However, many other functional groups have bands in this region and therefore they may appear similar.

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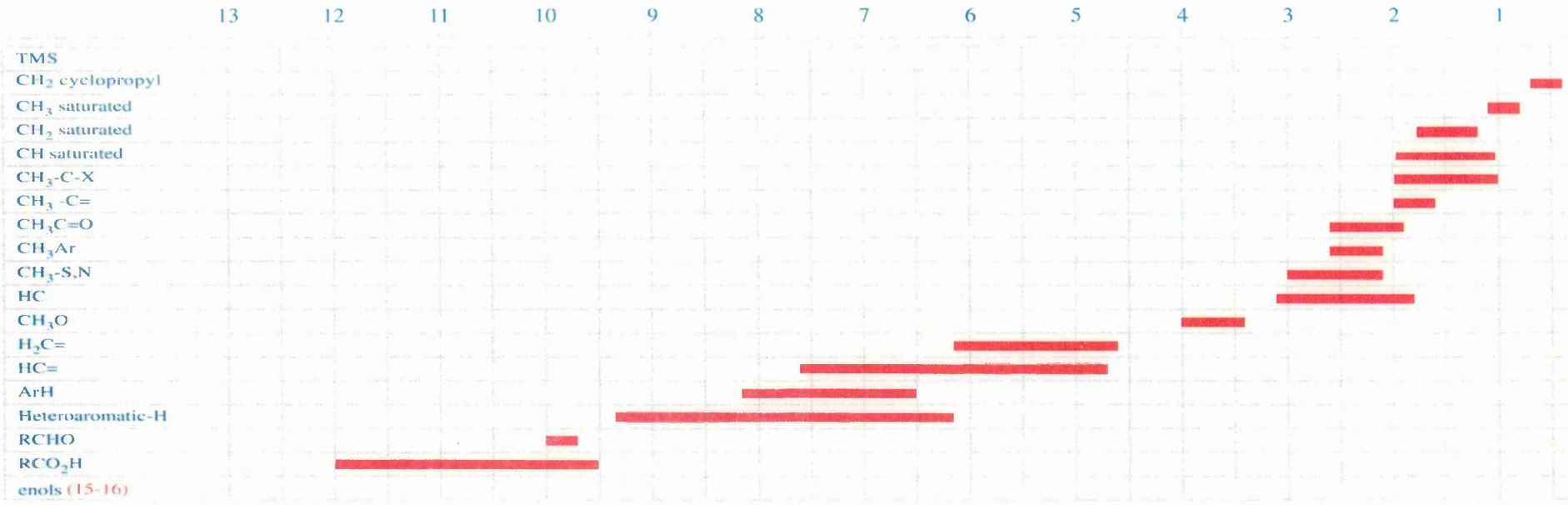
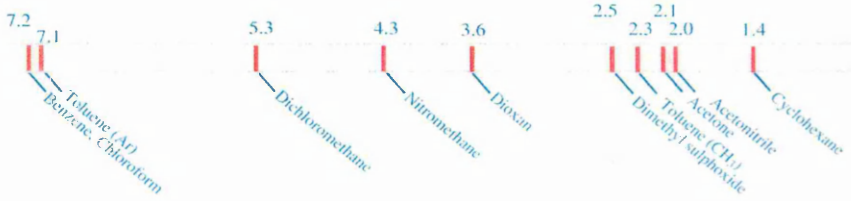
# Proton Chemical Shifts (δ)




## Solvents




*The chemical shifts of alcohols, phenols, thiols and amines are concentration dependent, being shifted to lower δ values as dilution increases*

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