# Analysis of polar herbicides and steroids by LC-MS using atmospheric pressure ionisation techniques.

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# Analysis of Polar Herbicides and Steroids by LC-MS Using Atmospheric Pressure Ionisation Techniques

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Bsc. (Hons).

A Thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy in the University of Wales.

Dedicated to my grandmother 'Abuela' Carmen and my

beautiful baby daughter Jannah who made her smile.

DECLARATION

This work has not previously been accepted in substance for any degree and is not being concurrently submitted in candidature for any degree.

Signed.....(candidate)

Date.....30 JANUARY 2003.....

STATEMENT 1

This thesis is the result of my own investigations, except where otherwise stated. Other sources are acknowledged by footnotes giving explicit references. A bibliography is appended.

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I hereby give consent for my thesis, if accepted, to be available for photocopying and for inter-library loan, and for the title and summary to be made available to outside organisations.

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# SUMMARY

Chapter one is a brief introduction to solid phase extraction, chromatography and mass spectrometry. A further explanation of the ionisation processes and the interfaces used to allow for coupling of high performance liquid chromatography (HPLC) to mass spectrometry (MS) is provided.

The basis of chapter two is the separation and analysis of polar herbicides. HPLC-MS was used to perform analysis of triazine, phenylurea and acidic herbicides. Solid phase extraction was investigated for the analysis of these herbicides in river water and analysis of real samples was conducted.

Chapter three involves analysis of free and conjugated estrogen steroids. The analysis of river water extracts spiked with four conjugated estrogen steroids was performed. And solid phase extraction was investigated for the analysis of free and conjugated estrogen steroids in river water and analysis of real river water samples conducted.

The development of a method foe the determination of conjugated steroids in male equine urine with HPLC-MS forms the basis of chapter four. The analysis of urine samples taken before and after administration of an anabolic steroid were investigated.

# ABBREVIATIONS

| | |
|---|---|
| 3-D | 3 dimensional |
| A | ampere |
| ac | alternating current |
| APCI | Atmospheric pressure chemical ionisation |
| API | Atmospheric pressure ionisation |
| °C | degrees centigrade |
| $CH_3CN$ | acetonitrile |
| CI | chemical ionisation |
| CRM | consecutive reaction monitoring |
| DAD | diode array detector |
| dc | direct current |
| DHA | dehydroepiandrosterone |
| EI | electron impact |
| ESI | electrospray ionisation |
| eV | electron volt |
| FAB | fast atom bombardment |
| g | gram |
| GC | gas chromatography |
| GC-MS | gas chromatography-mass spectrometry |
| h | hour |
| HETP | height equivalent to a theoretical plate |
| HPLC | high performance liquid chromatography |
| kg | kilogram |
| kV | kilovolt |
| L | litre |
| LC | liquid chromatography |
| LC-MS | liquid chromatography-mass spectrometry |
| LOD | limit of detection |
| m | meter |
| m | milli |
| M | molar |
| μ | micro |
| μA | micro amps |
| μL | micro litre |

| | |
|---|---|
| mAU | milli absorbance unit |
| MeOH | methanol |
| mg | milligram |
| min | minute |
| mL | millilitre |
| mm | millimetre |
| MS | mass spectrometer |
| MS-MS | tandem mass spectrometry |
| $MS^n$ | mass spectrometry fragmentation n times |
| m/z | mass-to-charge ratio |
| Mwt | molecular weight |
| | |
| n | nano |
| $NH_4Oac$ | ammonium acetate |
| ng | nanogram |
| | |
| p | pico |
| psi | pounds per square inch |
| | |
| Rf | radio frequency |
| RP | reversed phase |
| | |
| s | second |
| SIM | selected ion monitoring |
| SRM | selected reaction monitoring |
| | |
| Th | Thompsons |
| TIC | total ion chromatogram |
| TSP | thermospray |
| | |
| u | atomic mass unit |
| UV | ultraviolet |
| | |
| V | volt |

# Acknowledgements

Firstly I would like to thank my supervisor Prof. Games for giving me the opportunity to be a part of MSRU. Dai thank you for a great experience. I would also like to thank the following people for instigating the various studies contained in this thesis and the advice and help they gave, Gavin Mills (Hyder), Huw James (WRc) Phil Teale and Sue Ormond (HFL). Thanks also go to BMSS for the financial support they gave which allowed for me to present my research at various international conferences.

I would like to take the opportunity to thank all the people who made my time in Swansea so good....bad....challenging....fun....entertaining and particularly thanks to those involved in the memory blanks I acquired after some (?) good nights out, there aren't many people who can say that they've had every secretary in Swansea chemistry department sit on their knee while dressed as Santa.....and I know 4!, I won't mention names because you know who you are!

A big thank you to my family, especially my mum and dad for all the support they have given me, not just while I was a student but always. And my grandmother Carmen, sadly no longer with us, who made this possible.

A special thank you goes to Jash for letting me use his bedroom as an office and keeping an eye on his little sister when everyone else was too tired or busy! Lastly I would like to thank Jade who has given me all the love and support to allow me to get this thesis written. I love you.

# Contents

# Analysis of Polar Herbicides and Steroids by LC-MS Using Atmospheric Pressure Ionisation Techniques

# Chapter 1

# INTRODUCTION

# Chapter 1 Introduction

## Introduction to Solid Phase Extraction

Solid phase extraction (SPE) is a method of sample preparation that concentrates and purifies analytes from solution by sorption onto a disposable solid phase cartridge, followed by elution of the analyte with a solvent appropriate for instrumental analysis. The mechanisms for retention include reversed phase, normal phase and ion exchange. Traditionally, sample preparation consisted of sample dissolution, purification and extraction that was carried out with liquid-liquid extraction. The disadvantages of liquid-liquid extraction include the use of large volumes of organic solvent, cumbersome glassware and cost. Furthermore liquid-liquid extraction often creates emulsions with aqueous samples that are difficult to extract and it is not easily automated. These difficulties are overcome with solid-phase extraction, thus solid phase extraction was invented in the mid-1970s as an alternative approach to liquid-liquid extraction.

SPE columns now are typically constructed of polypropylene or polyethylene and filled with 40μm packing material with different functional groups. A 20μm polypropylene frit is used to contain from 50mg to 10g of packing material. A liquid is passed through the column and analytes are concentrated and purified. The sample volume that can be applied ranges from 1mL to over 1L. The sample may be applied

to the column by positive pressure or by a vacuum manifold. After quantitative sorption of the analyte it is removed with an appropriate elution solvent.

The goal of SPE is to quantitatively remove the analyte from solution and completely recover it in an appropriate solvent. Purification consists of removing the analyte from interfering compounds and concentrating the analyte in a small volume of solvent. As a result of the flexibility that SPE offers, it has found applications in the preparation of environmental, clinical and pharmaceutical samples.

SPE is performed in four steps:

1. First the solid phase is conditioned; typically the conditioning solvent is methanol, which is then followed by water or an aqueous buffer.
2. Next the sample and analyte are applied to the column. Depending on the type of sample it may be applied by either gravity feed, pumping or aspirated by vacuum.
3. This is the rinse step and it will remove the interferences from the column while retaining the sample analytes.
4. Finally the sample is eluted from the sorbent with an appropriate solvent that is chosen to disrupt the analyte-sorbent interaction, resulting in elution of the analyte.

## Introduction to Chromatography

The term chromatography comes from the Greek words 'chroma' meaning colour and 'graphein' meaning to write and was first used by the Russian botanist Mikhail Twsett at the start of the twentieth century. Twestt's studies involved the separation of plant pigments such as chlorophylls and xanthophylls using a glass column packed with finely divided calcium carbonate. The results he obtained appeared as coloured beads along the length of the column corresponding to the separated species, hence his coining of the word chromatography (1). Despite Tswett's early work there was little interest in chromatography until its resurgence in the 1930s and 40s, most notably with the introduction of partition chromatography by Martin and Synge that won them the Nobel Prize in 1952 (2). Since then the practice of chromatography has witnessed a continuous growth in almost every aspect from the number of chromatographers to the variety and complexity of samples being separated. There currently exists many subdivisions within chromatography for the separation of different compounds, the general definition of chromatography as given by IUPAC (3) is:

> 'A method used primarily for the separation of components of a sample, in which the components are distributed between two phases one of which is stationary while the other moves. The stationary phase may be a solid, or a liquid supported on a solid, or gel. The stationary phase may be packed in a column, spread as a layer, or distributed as a film etc. In these definitions, "chromatographic bed" is used as a general term to denote any

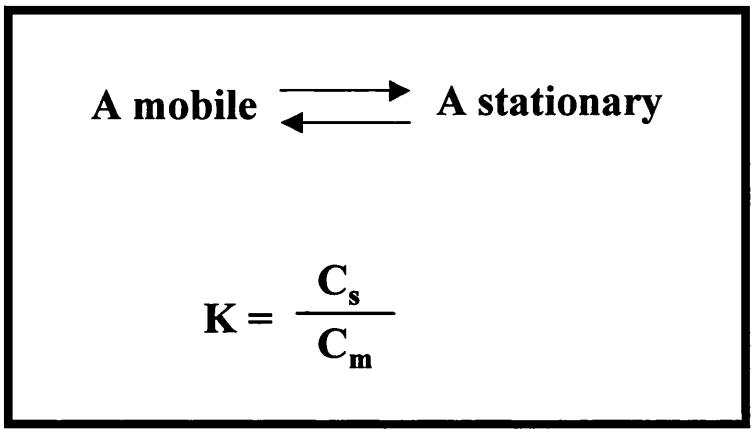
of the different forms in which the stationary phase may be used. The mobile phase may be gaseous or liquid.'

Chromatography is fundamentally a differential migration process where the sample components are distributed between a stationary phase and mobile phase. The sample is dissolved in a mobile phase, which may be a gas, a liquid or a supercritical fluid, and this mobile phase is then forced through an immobile, immiscible stationary phase. The phases are chosen such that the components of the sample have differing affinities in each phase and as a result of this will become separated from each other as they travel through the stationary phase. The table below shows the different combinations of mobile and stationary phase that make up the various classifications of chromatography.

| Mobile Phase | Stationary Phase | Classification |
|---|---|---|
| Gas | Solid | Gas-Solid Chromatography |
| | Liquid | Gas-Liquid Chromatography |
| Liquid | Solid | Liquid-Solid Chromatography |
| | Liquid | Liquid-Liquid Chromatography |
| Supercritical Fluid | Solid | Supercritical Fluid Chromatography |

## Principles of Chromatography

Although the exact mechanisms of retention for the various types of chromatography differ they are all based on the establishment of an equilibrium between the stationary and mobile phases (4). A series of adsorption and desorption processes bring about this equilibrium according to an analytes distribution coefficient K (defined below).




$C_s$ is the concentration of the analyte in the stationary phase.

$C_m$ is the concentration of the analyte in the mobile phase.

Separations therefore occur due to the differing distribution coefficients of individual analytes.

## Chromatographic Retention

The retention time $t_R$ is defined as the time it takes an analyte, after injection, to travel through the column and for the peak maximum to be detected. Figure 1.1 shows the retention parameters for a typical chromatogram.

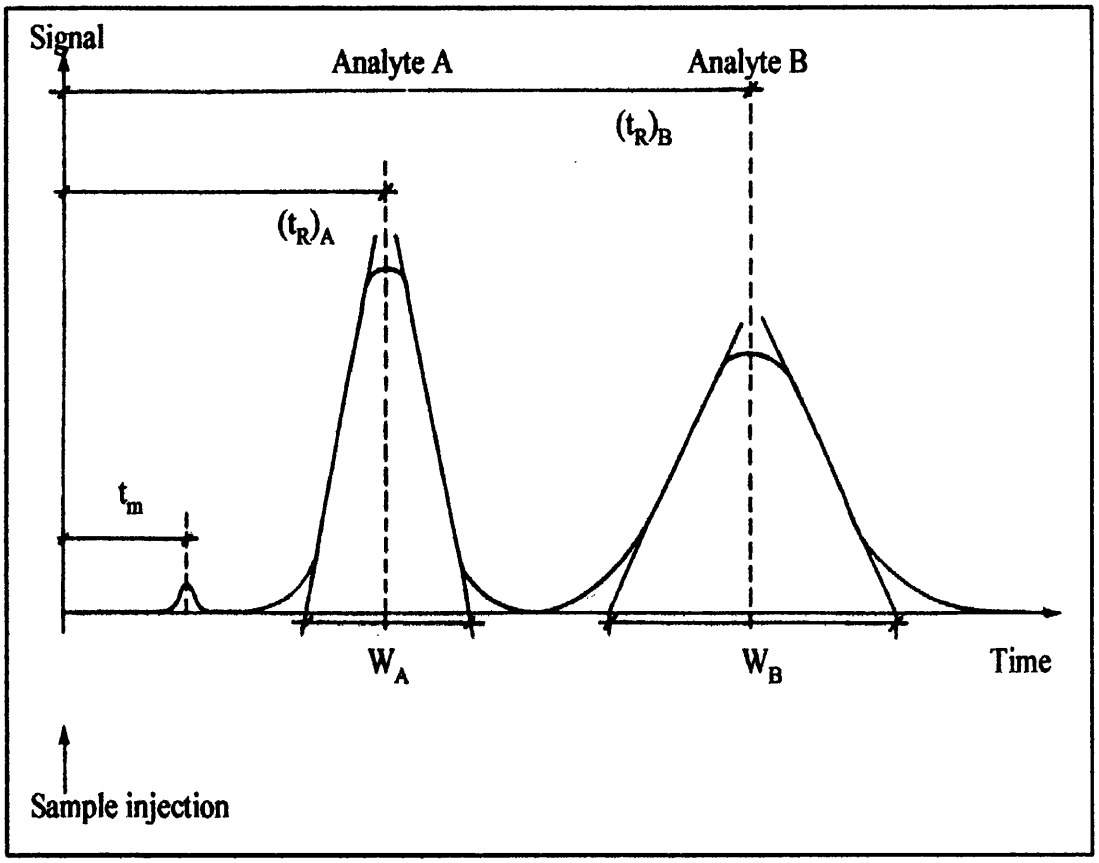



Figure 1.1 Typical chromatogram with retention parameters defined.

$t_m$ is the column dead time and corresponds to the time it takes an unretained analyte to be detected.

$W_A$ and $W_B$ are the respective peak widths of analyte A and B.

Instead of using retention time, a more suitable and preferred method for categorising an analyte is by the respective capacity factor k' (equation 1 below).

$$k' = \frac{t_R - t_m}{t_m}$$

This is more efficient since k' is independent of mobile phase velocity and column length.

**Selectivity**

The selectivity of the column to separate two analytes is defined by the separation factor α, which can be expressed as the ratio of the capacity factors (equation 2).

$$\alpha = \frac{k'_2}{k'_1}$$

Some columns are capable of separating certain analytes where others are not.

**Column Efficiency**

This is expressed as the number of theoretical plates (N) and can be obtained from the chromatogram by using the following equation (equation 3).

$$N = 16\left(\frac{k'_2}{k'_1}\right)^2$$

From this the height equivalent to a theoretical plate (HETP) can be calculated since it refers to the number of theoretical plates per unit length of the column (L) (equation 4).

$$HETP = \frac{L}{N}$$

Each theoretical plate is regarded as an equilibrium step, therefore the column efficiency increases as the number of theoretical plates increases hence the smaller the plate height the more efficient the column. HETP is smaller for small particles of column packing, low mobile phase flow rate, less viscous mobile phases and higher separation temperatures. Therefore, large N values and improved separation are usually favoured by long columns packed with small particles, by non-viscous mobile phases flowing relatively slowly through the column and by higher separation temperatures. N is approximately constant for different bands in a chromatogram for a given set of operating conditions.

**Resolution**

The resolution ($R_s$) is defined as the degree of separation between two analytes. It can be calculated from the chromatogram using the relative retention times and peak widths of the analytes (equation 5).

$$R_s = 2\left[\frac{(t_R)_B - (t_R)_A}{W_B + W_A}\right]$$

To control resolution we must know how $R_s$ varies with experimental factors such as k' and N. From equation 1 we can derive $t_R = t_m (1 + k')$, for two closely spaced peaks the $t_R$ values will be approximately equal we therefore have (assuming N to be

constant for both peaks) $W_A = W_B = W$ the average peak width. Inserting these relationships into equation 5 then gives us equation 6 below:

$$R_s = \frac{t_m(k'_B - k'_A)}{W}$$

Similarly equation 3 gives us $W = 4t_R / \sqrt{N} = 4t_m (1 + k'_R) / \sqrt{N}$. Inserting this expression into equation 6 for W gives (equation 7):

$$R_s = \frac{\sqrt{N}(k'_B - k'_A)}{4(1 + k'_A)}$$

$$R_s = (1/4)\left( [k'_B/k'_A] - 1\right) \sqrt{N}\left( k'_A/[1 + k'_A] \right)$$

If we recognise that $k'_A$ is approximately equal to $k'_B$ and we define the separation factor ($\alpha$) as the ratio of the capacity factors (equation 2) then we get the following expression (equation 8), where k' is the average capacity factor.

$$R_s = (1/4)(\alpha - 1) \sqrt{N}\left( k'/[1 + k'] \right)$$

$$\text{(i)} \qquad \text{(ii)} \qquad \text{(iii)}$$

Equation 8 is a fundamental relationship in liquid chromatography that allows us to control resolution by varying $\alpha$, N or k'. The three terms (i-iii) are roughly independent of each other so we can optimise first one term then another. Separation

selectivity as measured by $\alpha$ (term i) is varied by changing the composition of the mobile phase and/or stationary phase. Separation efficiency as measured by N (term ii) is varied by changing the column length or solvent velocity. Term iii, involving the capacity factor k', is varied by changing solvent strength (the ability of the mobile phase to provide large or small k' values for a given sample) (5).

## Band Broadening

Regardless of how efficient the column there will always be some degree of band broadening. As chromatographers we can reduce this to a certain degree through the use of well-designed equipment. This will minimise effects from injector dead volumes and connector tubing. However, the main causes exist within the column itself. Considerable study into these effects over the last 30 years has taken place, most notably by van Deemter, who derived an equation relating column efficiency and mobile phase velocity (equation 9):

$$\mathbf{HETP} = \mathbf{A} + \frac{\mathbf{B}}{\mu} \mathbf{C_m}\mu + \mathbf{C_s}\mu$$

$\mu$ is the mobile phase velocity.

A corresponds to Eddy diffusion.

B corresponds to molecular diffusion.

C corresponds to the mass transfer effects in both mobile and stationary phases.

The coefficients A, B, $C_m$ and $C_s$ are the main contributing factors to band broadening. The overall effect can be shown by graphically plotting the HETP against the mobile phase velocity as shown below in figure 1.2. The A term is independent of flow rate.

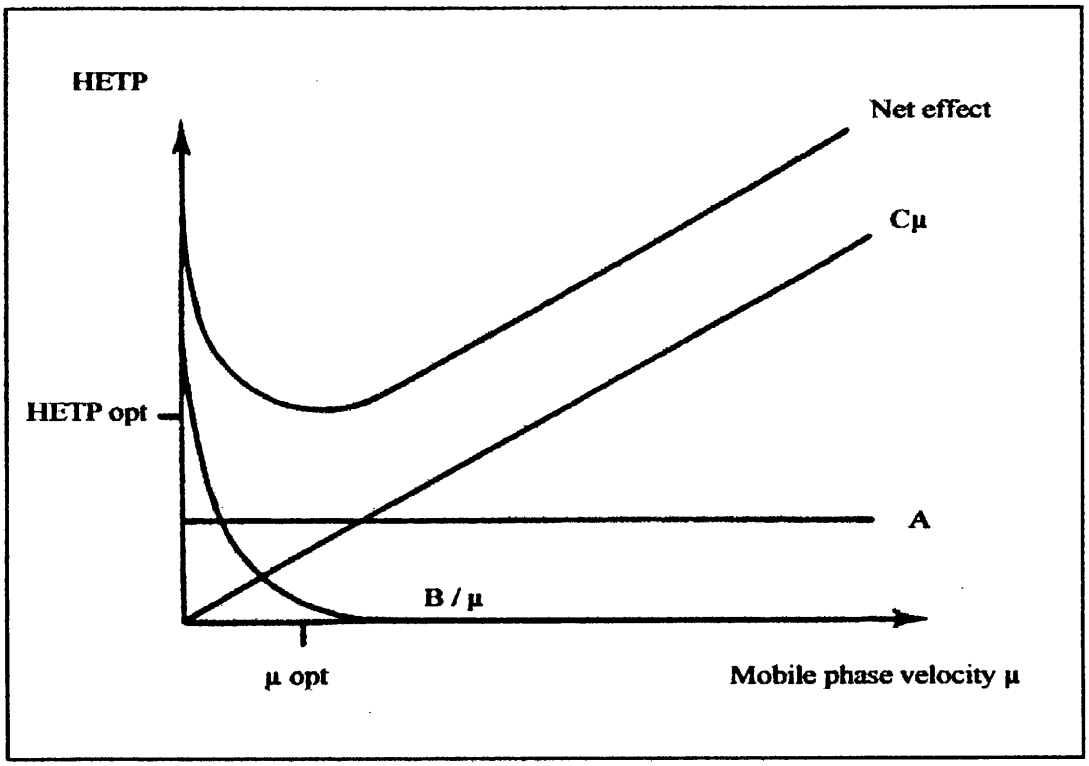



Figure 1.2 Van Deemter plot.

Eddy Diffusion:

As an analyte migrates through a column the molecules find different routes around the stationary phase particles. Band broadening occurs due to the differing lengths travelled by the molecules by these routes or 'multipaths'. This is independent of mobile flow rate but is dependent upon particle size and packing. Thus smaller more uniformly packed columns have better efficiency.

Molecular Diffusion:

Band broadening occurs here because of the tendency of analytes to diffuse in all directions. This movement away from the band centre results in axial spreading of the peak. The effect is much more pronounced in GC than in LC because of the much smaller coefficients in liquids.

Mass Transfer Effects:

This can be divided into two categories, mobile phase and stationary phase transfer. These are dependent upon the adsorption and desorption of analytes to and from the surface of the stationary phase. The initial assumption of equilibrium between the stationary phase and mobile phase is not strictly true since it takes time for these processes to occur due to finite thickness within the two phases. This produces a time lag that offsets the solute concentration profile. The result is that the mobile concentration profile slightly overruns the 'equilibrium portion' and likewise the stationary phase concentration profile lags behind.

## Reversed Phase High Performance Liquid Chromatography

The classification 'liquid chromatography' refers to any chromatographic technique that distributes the analyte between a liquid mobile phase and either a solid or liquid stationary phase. There are many types of high performance liquid chromatography (HPLC), but by far the most commonly used is reversed phase HPLC which accounts for about 80% of all the HPLC separations performed.

Reversed phase (RP) HPLC uses non-polar stationary phases with polar mobile phases. Separations occur due to the differing hydrophobicities of the analytes with hydrophobic non-polar analytes being more strongly retained than hydrophilic polar analytes. By changing the experimental parameters such as the mobile phase and temperature analyte retention can be altered to produce separations.

## Stationary Phases

RP-HPLC is carried out using chemically modified silica-based columns. The surface silanol groups are chemically modified by reaction with different allyl and aryl side chains to produce a range of selective stationary phases. A typical example of one of these reactions is shown on the following page in figure 1.3.

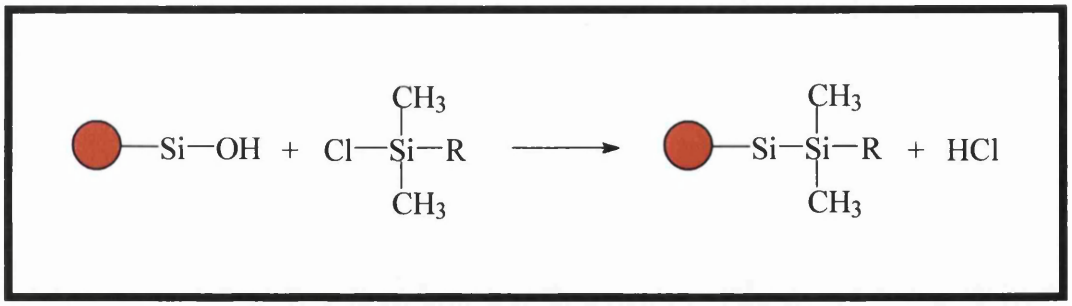



Figure 1.3 Example of typical reaction for producing solid stationary phase for RP-HPLC.

The most widely used stationary phases are based on octadecylsilane (ODS or C18) where R is $(CH_2)_{17}CH_3$, although other shorter alkyl chain columns such as octylsilane, hexyl and phenyl are also commercially available. Due to the steric implications of these groups it is inevitable that there will be a certain amount of free or unreacted silanol groups present; some manufacturers use a process called endcapping to increase coverage of the silanol groups and thereby minimise unwanted interactions and reduce peak tailing.

## Mobile Phases

When selecting a mobile phase certain considerations need to be taken into account. All solvents must be compatible with the detection system, they must be pure and free from particulates that could interfere with analysis, and toxicities and cost must be kept to a minimum where possible.

RP-HPLC mobile phases are usually made up of a mixture of water or an aqueous buffer and an organic solvent such as methanol or acetonitrile. The aqueous part of the mobile phase acts as the weaker component while the organic solvent is the stronger

component. The mobile phase system is either run isocratically whereby the organic content of the mobile phase is kept constant or with a gradient. During gradient elution the proportion of the organic solvent increases over time, hence increasing the elution strength of the mobile phase. Using a gradient can allow for more hydrophilic components to be sufficiently retained on the column while reducing the retention time of more hydrophobic ones.

The use of buffered solutions in RP-HPLC is for two main reasons. Firstly, in the analysis of acids or bases there is need for pH control to stop unwanted ionisation of groups that would cause changes in retention times to occur. Here the pH of the buffer should be kept to about two pH units above or below the pKa of the analytes. Secondly, buffer salts can interact with the stationary phase masking silanol groups and thus minimising potential ion exchange interactions and ultimately reducing peak tailing.

**Detection**

For many HPLC analyses ultraviolet (UV) is used as the mode of detection; it is a relatively sensitive detection method and has a large application area since most organics absorb in the UV region. The wavelength chosen for analysis must provide adequate absorbance for the analytes of interest but also be optically transparent for the mobile phase. Coupling HPLC to mass spectrometry can provide additional specificity and sensitivity of detection; this topic will be discussed latter in this chapter.

The RP-HPLC studies presented in this thesis were performed with a Hewlett Packard HP 1100 liquid chromatography system equipped with a HP diode array detector (DAD), which allows for acquisition of UV spectra for all sample analytes during the method development stage.

## Introduction to Mass Spectrometry

The origins of mass spectrometry can be traced back to the discovery of positive rays by Goldstein in 1886 (6), which were later analysed with the use of magnetic deflection by Wein in 1898 (7). True mass spectrometry was first performed by J. J. Thomson in 1912 when he obtained mass spectra of $O_2$, $N_2$, CO and $COCl_2$ (8). This led to the development, in 1918, of the first mass spectrometer with a sector shape magnet and direction focussing by A. J. Dempster (9) and a year later, in 1919, another type of mass spectrometer with velocity focussing by F. W. Aston (10).

Mass spectrometry took off in the 1930s and advanced technology resulted in the development of double focussing mass spectrometers capable of accurate determination. The 1940s and 50s saw the introduction of time of flight, quadrupole and ion trap mass spectrometers. The eventual coupling of chromatography to mass spectrometry revolutionised the analysis of volatile compounds and much work then centred on the development of different types of sample ionisation techniques. The most recent advances in this field have resulted in the introduction of atmospheric pressure ionisation sources.

## Fundamentals of Mass Spectrometry

Mass spectrometry's underlying principles are to separate, detect and record the mass of an ion. The instrument is made up of a series of components each of which contribute to the final result. Firstly, the sample must be introduced into a source

where the production of ions takes place. Over the years numerous source types have been developed for example electron impact (EI), chemical ionisation (CI), fast atom bombardment (FAB), thermospray (TSP), atmospheric pressure chemical ionisation (APCI) and electrospray ionisation (ESI); the latter two being the source types considered in this thesis. Once ion production has taken place the ions enter the high vacuum region of the mass spectrometer where they pass into the mass analyser, which then separates them according to their mass-to-charge ratio (m/z). In the final stage the ions are focussed into a detector where they are 'counted'; an electrical signal, which is dependent on the number of ions reaching the detector, is produced and a resulting mass spectrum is recorded.

## Atmospheric Pressure ionisation

The greatest problem associated with interfacing chromatographic techniques with mass spectrometry is the high flow rate entering the vacuum region of the mass spectrometer. With atmospheric pressure ionisation (API) the fact that the ionisation source is held at atmospheric pressure alleviates these incompatibilities and simplifies the combination of HPLC to mass spectrometry.

## Atmospheric Pressure Chemical Ionisation

The use of APCI with mass spectrometry was first reported in 1973 by Horning et al. (11) and then as an interface for LC-MS a year later (12). The introduction of the heated nebulizer in the 1980s (13) facilitated and advanced this technique even further.

APCI provides a 'soft' ionisation technique for the analysis of low polarity compounds that have some volatility. Due to the temperatures involved with APCI it is not however a suitable source for thermally labile compounds. The production of ions in this source occurs through gas phase molecular reactions at atmospheric pressure. Figure 1.4 shows the APCI process in the positive ion polarity mode.

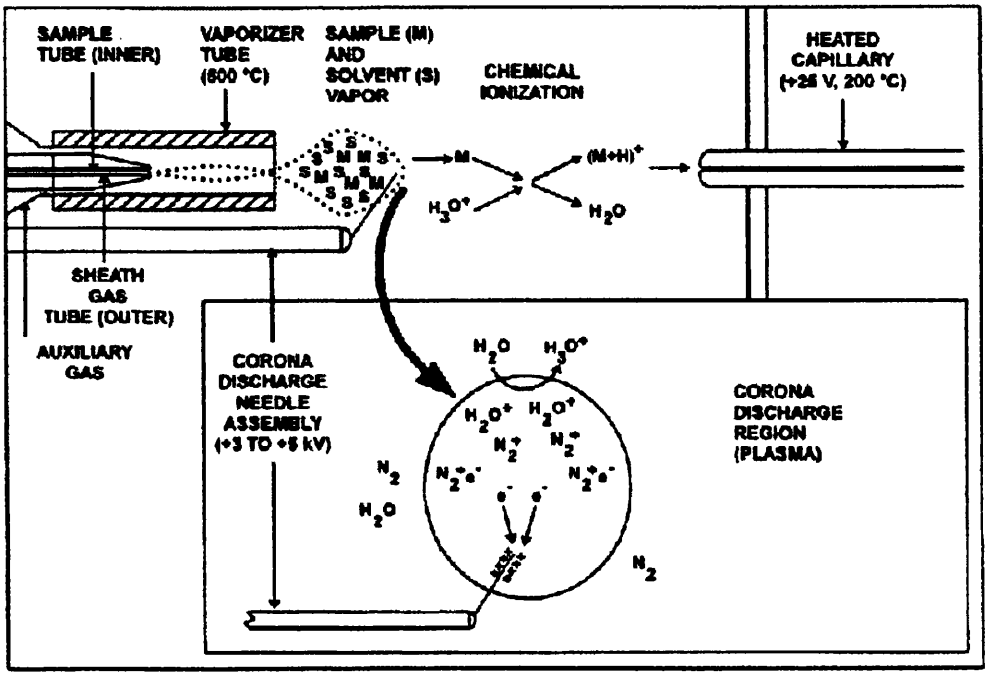



Figure 1.4 Representation of the APCI process in the positive ion polarity mode.

Sample in solution passes into the APCI source through a narrow capillary surrounded by a flow of nitrogen nebulizing gas converting the liquid into a fine spray. The spray along with the nitrogen gas passes into the high temperature vaporiser tube where they are flash vaporised. At the exit of the vaporiser tube there is a corona discharge needle maintained at a voltage of 5-6kV. When the vapour reaches the discharge needle a series of reactions are initiated ultimately resulting in sample ionisation.

Primary ion formation occurs through ionisation of the nitrogen gas. These ions then react with solvent molecules with charge transferral. These in turn react with other solvent molecules and eventually the sample molecules usually resulting in proton transfer. The general reactions are summarised below:

| | |
|---|---|
| **Primary ion formation:** | $N_2 + e^- \longrightarrow N_2^+ + 2e^-$ |
| **Secondary ion formation:** | $N_2^+ + H_2O \longrightarrow N_2 + H_2O^+$ |
| | $H_2O^+ + H_2O \longrightarrow H_3O + OH^-$ |
| **Proton transfer:** | $H_3O + M \longrightarrow [M+H]^+ + H_2O$ |

In APCI the solvents normally used are methanol or acetonitrile with water or buffers such as ammonium acetate. The use of different solvents can give different reactions and sometimes cluster ions can be produced, for example, $[M+NH_4]^+$ instead of the usual $[M+H]^+$. However these cluster ions can usually be minimised by increasing the source temperature.

## Electrospray Ionisation

Zeleny first described the production of a fine mist of charged droplets by the electrospray process in 1917 (14). But it wasn't until the 1960s that Dole et al (15) conducted their pioneering work, generating gas phase ions of macromolecules by spraying a solution through the tip of an electrically charged capillary, that led to the

use of electrospray as an ionisation process for mass spectrometry. In the 1980s, inspired by the work of Dole, Fenn et al demonstrated the techniques potential and developed electrospray as an interface for LC-MS. Since then slight modifications have taken place, for example the introduction of nitrogen drying gas to aid liquid nebulisation in the source, but the underlying principles remain the same.

The production of ions by electrospray has been described extensively in the literature (16, 17, 18, 19,). Figure 1.5 below shows the electrospray ionisation (ESI) process in the positive polarity mode.

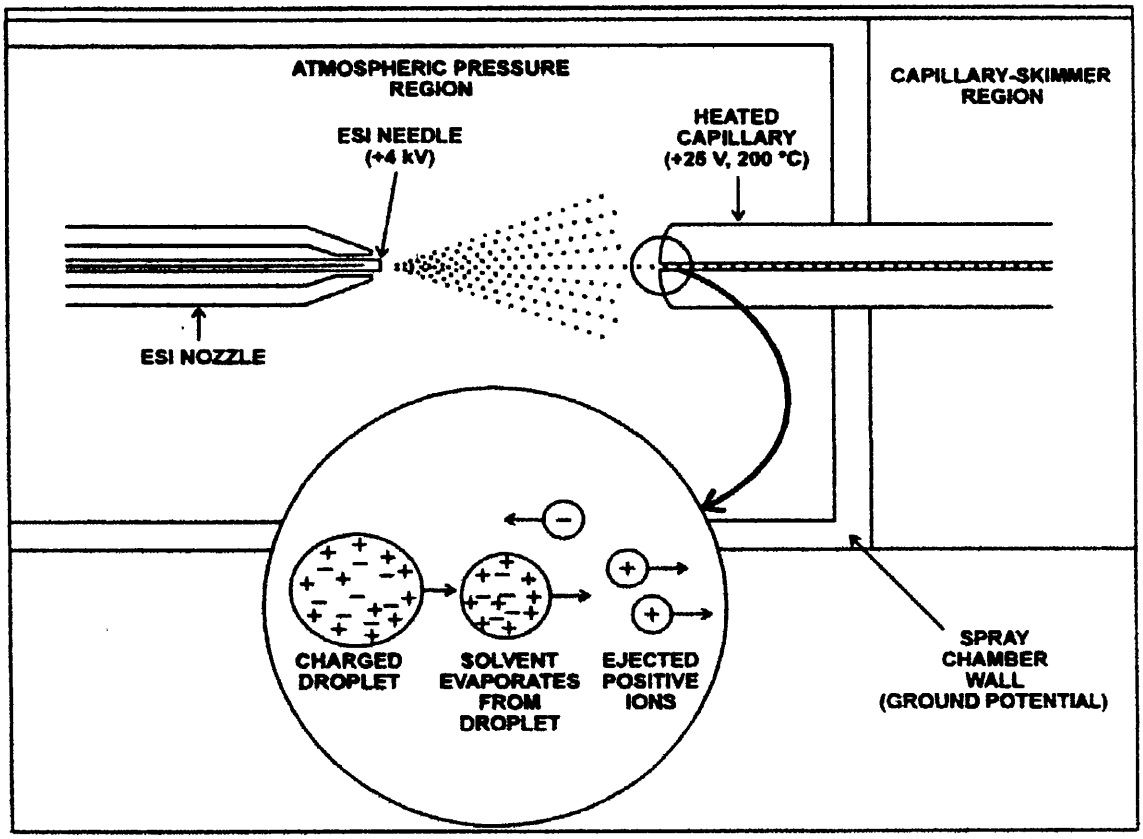



Figure 1.5 Representation of the ESI process in the positive ion polarity mode.

The sample solution is passed into the ESI source via a capillary, the tip of which is held at a high potential relative to the source. The field at the capillary tip charges the surface of the emerging liquid dispersing it as a fine spray of droplets. The use of nitrogen as a nebulizing gas allows higher flow rates to be used as well as directing the spray. During migration towards the heated capillary the highly charged droplets experience solvent evaporation reducing the diameter and increasing the surface charge density. This goes on until the repulsive forces equal the surface tension forces at the Rayleigh limit and extreme instability causes coulombic explosions producing an array of smaller droplets. Repeated solvent evaporation and fission results ultimately in ion ejection. The nitrogen curtain gas carries solvent vapour out of the ion source and the ions enter the vacuum region of the mass spectrometer.

ESI offers a mild form of ionisation and since the ionisation process takes place without the presence of high temperatures it is amenable to the analysis of thermally labile and polar compound.

**Mass Analysers**

There are various types of mass spectrometer commercially available that apply the use of different mass analysers, for example, magnetic sector, ion cyclotron resonance, time of flight, quadrupole and ion trap; the latter of which was used to conduct the studies in this thesis. There are three fundamental parameters to a mass analyser; the upper limit which determines the highest m/z ratio that can be measured; the transmission which is the ratio between the number of ions produced in the source

and the number of ions that reach the detector; and the resolution which is the ability to yield distinct signals for ions with small mass difference.

## Ion Trap Mass Analysers

The design of the ion trap was proposed in 1960 by Paul and Steinwedel (20) but it was not until 1984 that modification by Stafford et al (21) of the Finnigan Corporation resulted in the commercialisation of the ion trap as a mass spectrometer.

The ion trap consists of three hyperbolic stainless steel electrodes, one ring electrode sandwiched between two endcap electrodes. Each electrode posses a small hole in the centre to allow the flow of ions into and out of the trap. The application of an Rf voltage to the ring electrode while the two endcap electrodes are grounded produces a three-dimensional quadrupole field that traps the ions within the mass analyser. This time varying field drives ionic motion in both the axial and radial directions. For ions to remain trapped a stable trajectory in both directions must be maintained. Ions of a wide m/z range are trapped simultaneously with a sharply defined low mass cut-off dependent on the primary Rf voltage applied to the ring electrode. The magnitude of this voltage is such that the ions of water and air do not become stored within the trap. The stored ions are then scanned out of the trap by applying an Rf voltage to the endcap electrodes while at the same time ramping the Rf voltage on the ring electrode. This causes excitation of the ions resulting in unstable trajectories and movement away from the centre of the trap. Ions of increasing m/z ratios are subsequently ejected from the mass analyser. Since the Rf amplitudes and ramping rates are known,

the m/z ratio for each ion being ejected from the trap can be determined (22). Those ions that leave via the exit endcap electrode are focussed into the detector.

Modern ion traps operate with a continuous source of helium damping gas entering the mass analyser cavity. This is done for two main reasons. Firstly, the presence of helium atoms slows the ions through collisions, which reduce their kinetic energies and promote movement to the centre of the trap. Using helium in this way has shown improvements in sensitivity and mass resolution. Secondly, it finds use as a collision gas when performing MS-MS analyses.

When conducting MS-MS analysis the first stage involves the isolation of the precursor ion to be fragmented. Various Rf voltages are applied to both the ring electrode and the endcap electrodes to isolate the selected ion. An excitation Rf voltage is then applied to the endcap electrodes to increase the kinetic energy of the ion. This voltage is below the value necessary for ejection of the ion from the trap. Collisions between the excited ions and the helium damping gas build the internal energy of the ion that ultimately results in fragmentation. The product ions are then scanned out of the trap as discussed previously. Repeated isolations and collisions can occur within the ion trap resulting in $MS^n$ fragmentation steps where n=1-10.

## Finnigan LCQ Ion Trap Instrumentation

The work presented in this thesis involved the use of an LCQ ion trap mass spectrometer. The mass spectrometer consists of an API source capable of performing APCI or ESI, an ion optics system for the transferral of ions, a mass analyser able to

scan between m/z 50-2000 Th, an ion detection system and a vacuum system. A full block diagram of the system is shown in figure 1.6 on the next page.

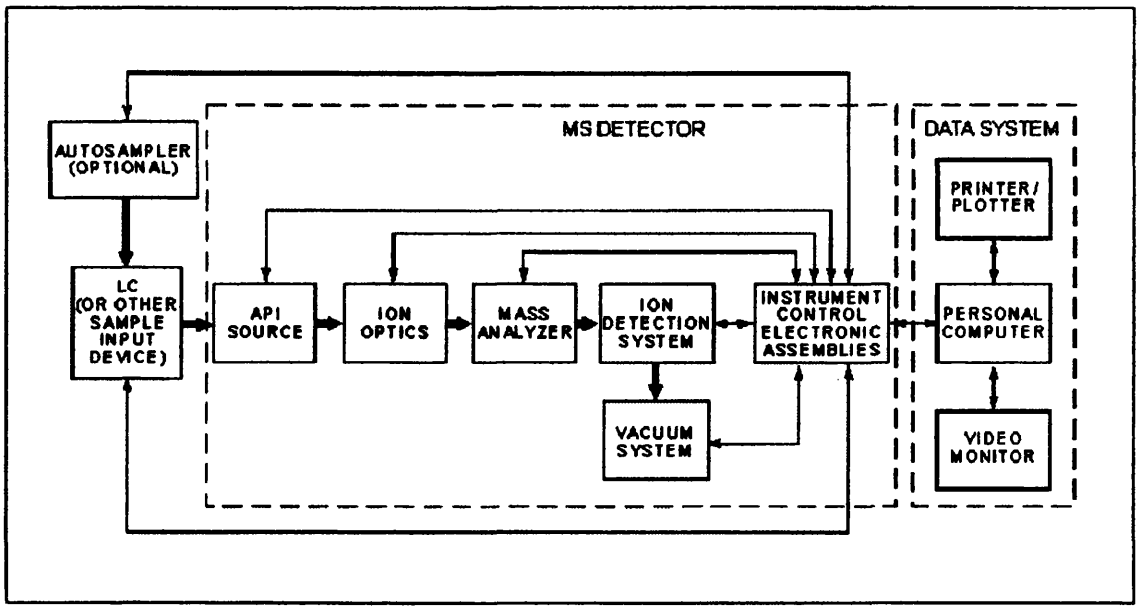



Figure 1.6 Schematic diagram of the LCQ system.

Operation can be performed in both the positive and negative ion polarity modes and the instrument has the ability to perform $MS^n$ analysis n = 1-10.

## API Source

A cross sectional view of the APCI source can be seen in figure 1.7 on the following page. Typical flow rates used for APCI are typically higher than for ESI, in most instances the entire flow from the HPLC can be directed into the APCI source. The HPLC flow enters the APCI source through a short piece of fused silica capillary held by an APCI nozzle. As the sample and solvent enter the vaporiser tube they are flash vaporised and swept along the tube by the sheath and auxiliary gas flows. As the

sample and vapour exits the vaporiser tube they are ionised by a corona discharge needle held at voltages of 3-5kV, as described earlier in this chapter.

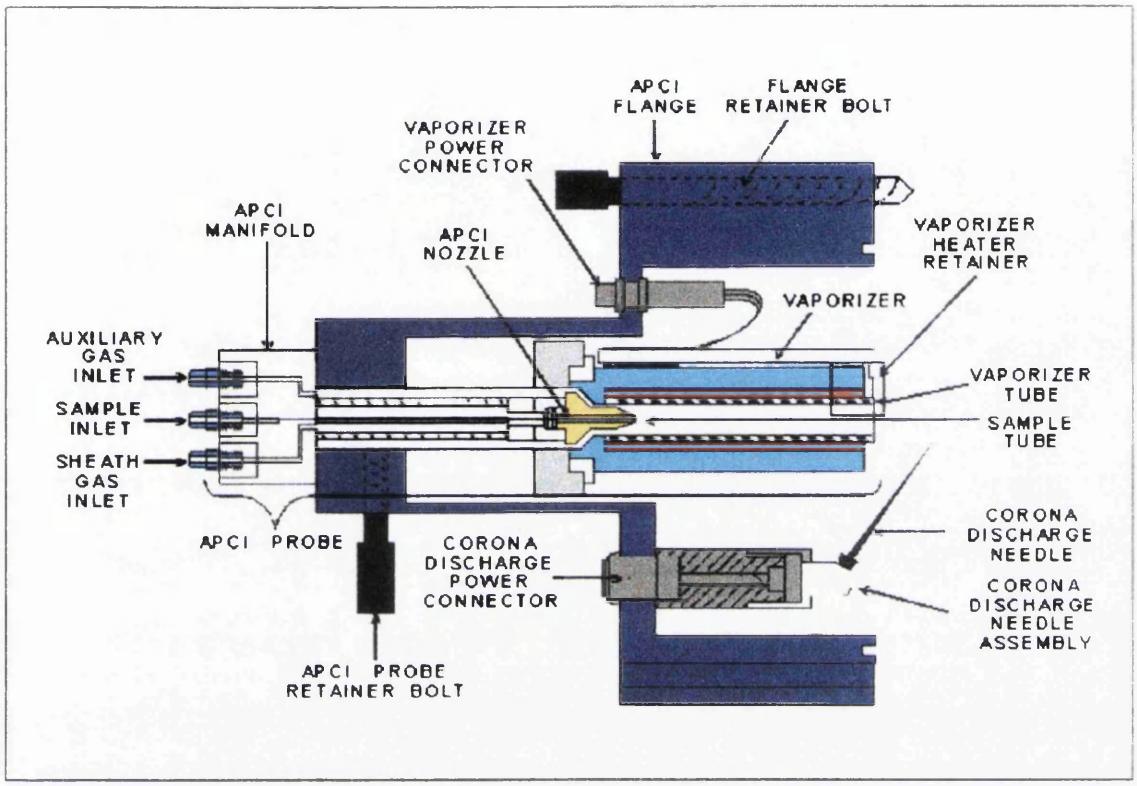



Figure 1.7 Cross sectional view of the LCQ APCI source.

With ESI the usual flow rates entering the ion source from the HPLC are in the region of 200μL/min, this is achieved with conventional HPLC via the use of a split. The flow enters a short length of fused silica capillary held in place by a grounded fitting. The capillary is fed into the source and through the needle so that it protrudes by approximately 1mm. The ESI nozzle surrounding the needle has a high voltage applied to it, typically 3-5kV, which causes charging and the solvent dispersion of the emerging liquid. The ESI probe allows the addition of nitrogen gas that aids in the sample nebulisation and provides a curtain gas within the source. A sheath liquid line is also accommodated to enable the addition of solvent to enhance the ESI process.

This is usually necessary when high aqueous solvent contents are used. Figure 1.8 on the following page shows a cross sectional view of the ESI source.

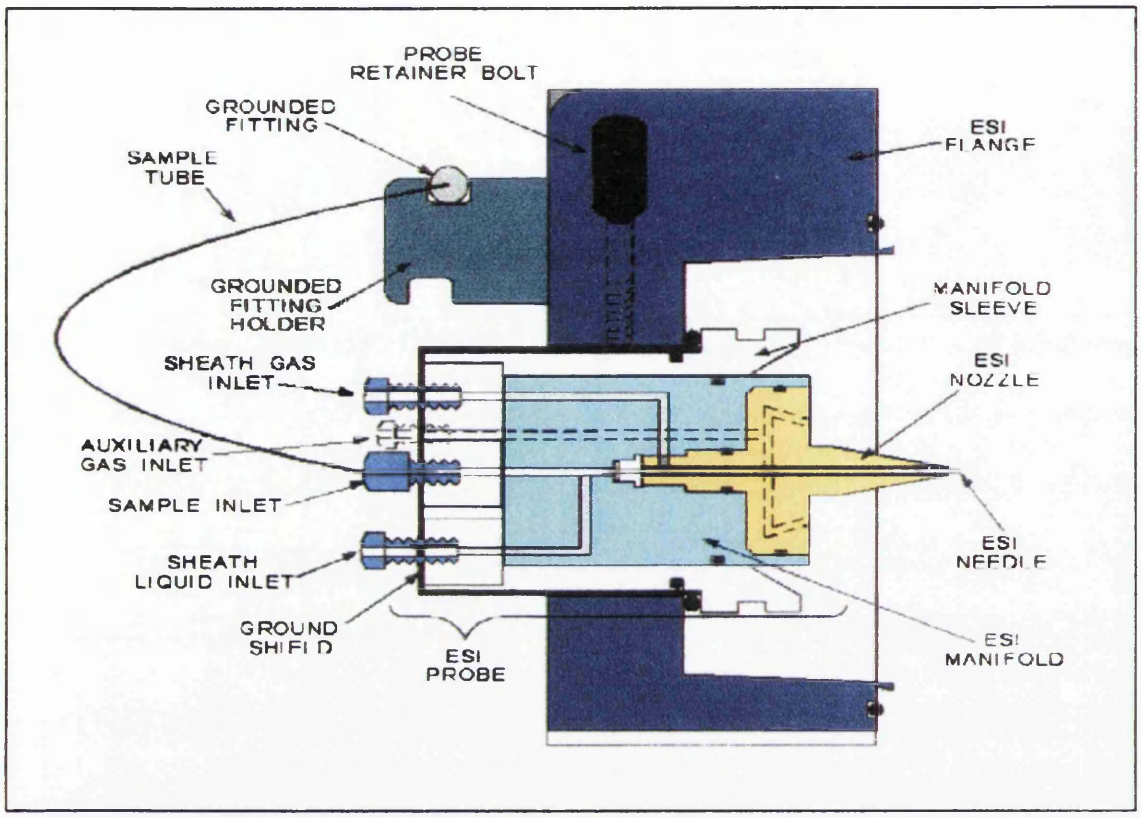



Figure 1.8 Cross sectional view of LCQ ESI source.

Ion then pass into the first lower pressure region of the mass spectrometer via the API stack. The stack consists of a spray shield that separates the API source and the first pressure region, a heated capillary, a tube lens and a skimmer. The heated capillary has a small hole through which ions travel, and is maintained at temperatures of up to 300°C to help desolvation of the ions. These ions are transported down the capillary by a decreasing pressure gradient. A potential is applied to the heated capillary that also assists in transporting the ions to the tube lens. The tube lens helps to focus the ions into the skimmer region but at the same time acts as a gate to deflect ions. This

occurs through the application of positive or negative voltages that deflect negative and positive ions, respectively. Ions pass to the skimmer, which is held at ground potential. This is offset from the exit of the heated capillary to reduce the number of large charged particles that pass through and create noise. Figure 1.9 shows a diagram of the API stack.

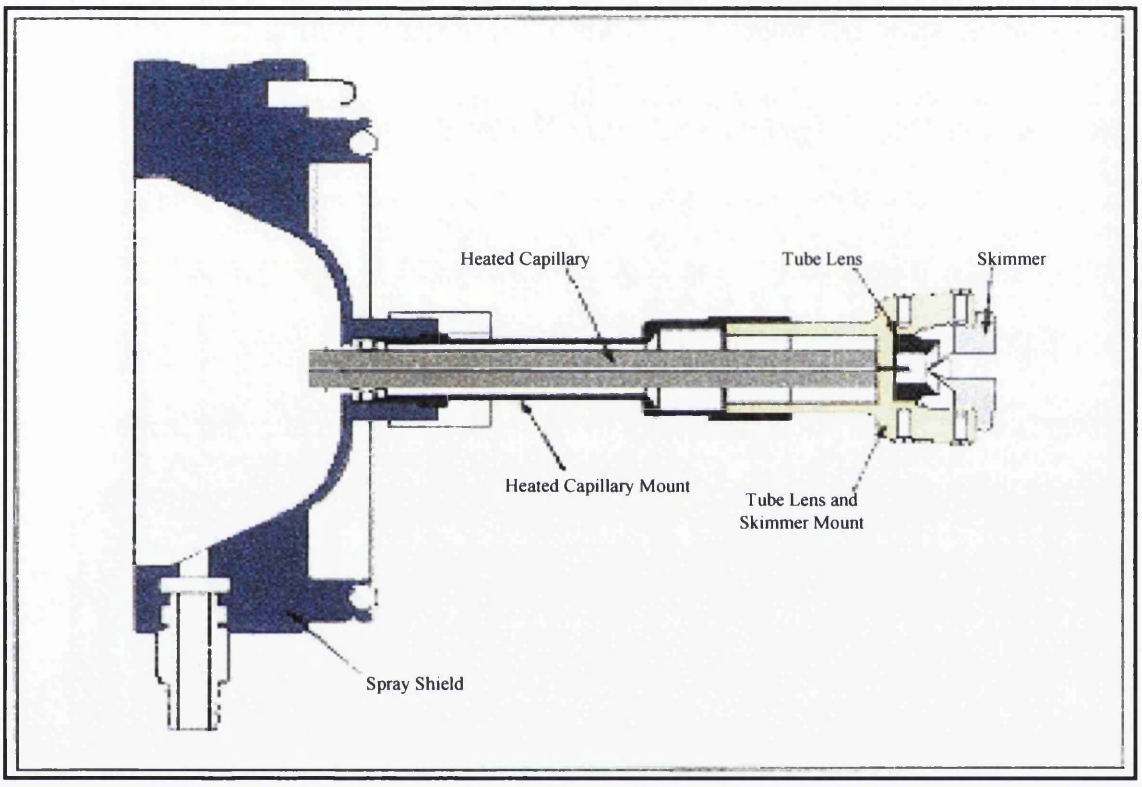



Figure 1.9 Schematic diagram of the API stack.

## Ion Optics

The ion optics consist of two octapoles separated by an interoctapole lens. Each Octapole is an octagonal array of cylindrical rods. Application of an Rf voltage to these rods gives rise to an electric field that transmits the ion through the centre of the octapoles. The Interoctapole lens serves to focus these ions but also controls the

amount of ions passing to the mass analyser. Figure 1.10 is a schematic of the ion optics.

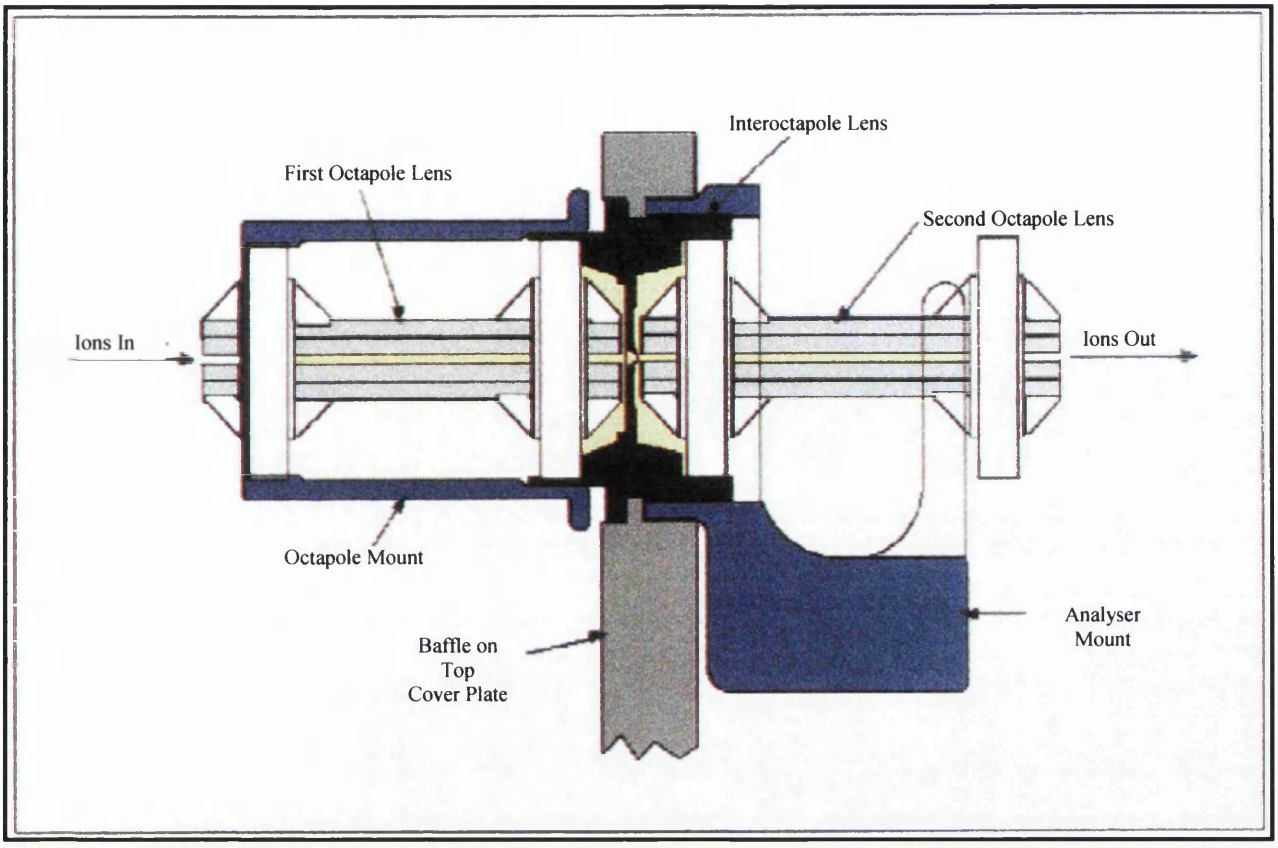



Figure 1.10 Schematic diagram of the ion optics.

## Ion Trap

Ions are drawn into the trap from the second octapole by the application of an ac voltage to the electrodes, usually +10V or –10V for negative or positive ions respectively. Once the ions are in the mass analyser cavity they are trapped by an Rf voltage applied to the ring electrode while the endcap electrodes are grounded. Trapping of ions is aided by the introduction of helium damping gas through a nipple at $10^{-3}$ Torr. Figure 1.11 below shows a schematic diagram of the ion trap.

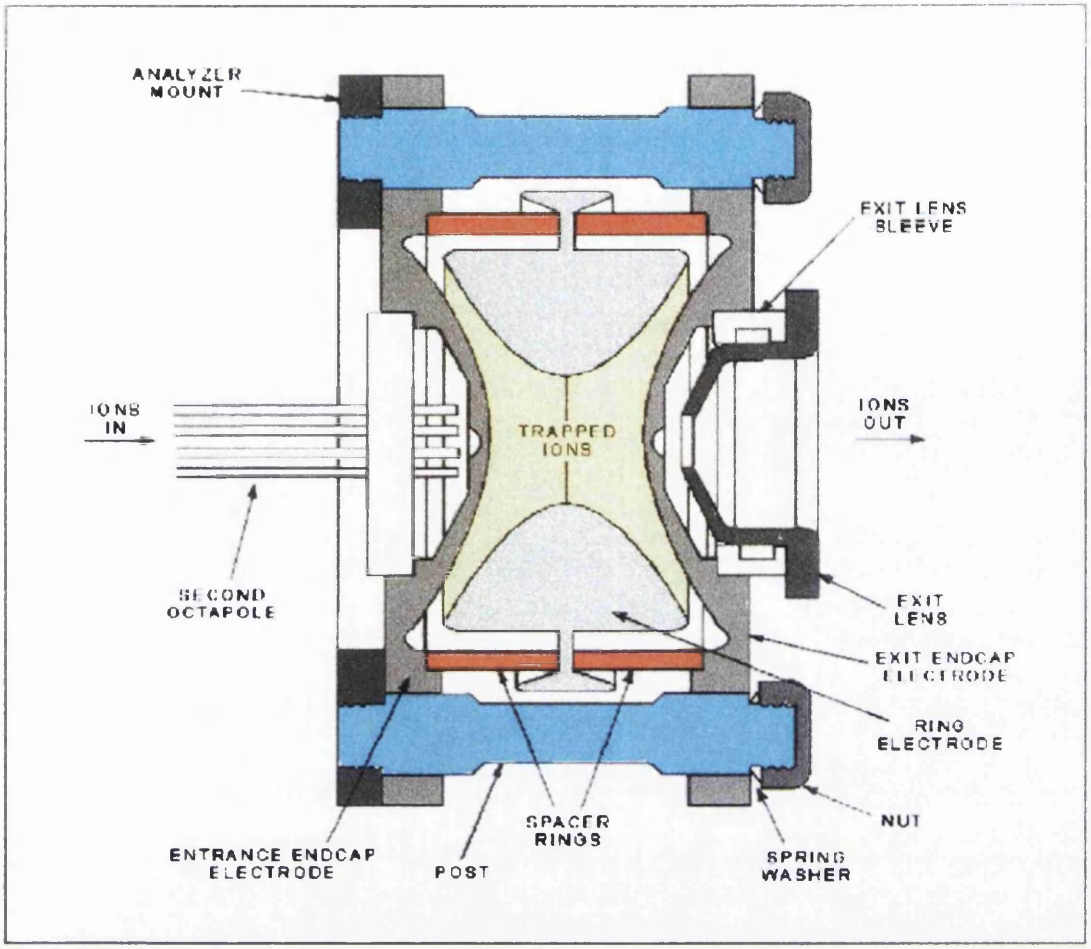



Figure 1.11 Schematic diagram of the ion trap.

Depending on the voltages a number of scanning modes can be achieved. Full scan mode gives a spectrum of all the ions contained in the mass analyser. Single ion monitoring (SIM) can be performed if the analyte of interest is known. This involves scanning for a particular mass while all the other ions are ejected from the trap. This is particularly useful in trace analysis when low level detection is required. As mentioned earlier various stages of MS-MS can be performed. A slight variation of MS-MS is selected reaction monitoring (SRS) which links precursor ions with the respective product ion pairs. Consecutive reaction monitoring (CRM) is the second

stage equivalent of SRM. Ions are then scanned out of the trap and only those that leave via the exit endcap electrode pass into the detector.

## Detector

The type of detector used by the LCQ is an electron multiplier. Ions emerge from the trap and strike the conversion dynode held at a right angle to the ion beam. This conversion dynode has a concave metal surface at positive or negative potentials for the detection of negative and positive ions respectively. When positive ions strike the negative surface of the conversion dynode secondary particles are produced consisting of negative ions and electrons (negative ions striking a positive surface produce positive ions). These secondary particles are focused by the curved surface of the conversion dynode and accelerated into the electron multiplier by a voltage gradient. The electron multiplier consists of a funnel shaped lead oxide restrictor that acts as a cathode and a 'cup' anode. Secondary particles strike the inner walls of the cathode with sufficient energy to eject electrons. These electrons are accelerated further into the cathode repeatedly striking the inner walls to produce a cascade of ejected electrons. These electrons are then collected by the anode to produce a measurable current and results in the construction of a mass spectrum. Figure 1.12 on the following page shows a schematic diagram of an electron multiplier.

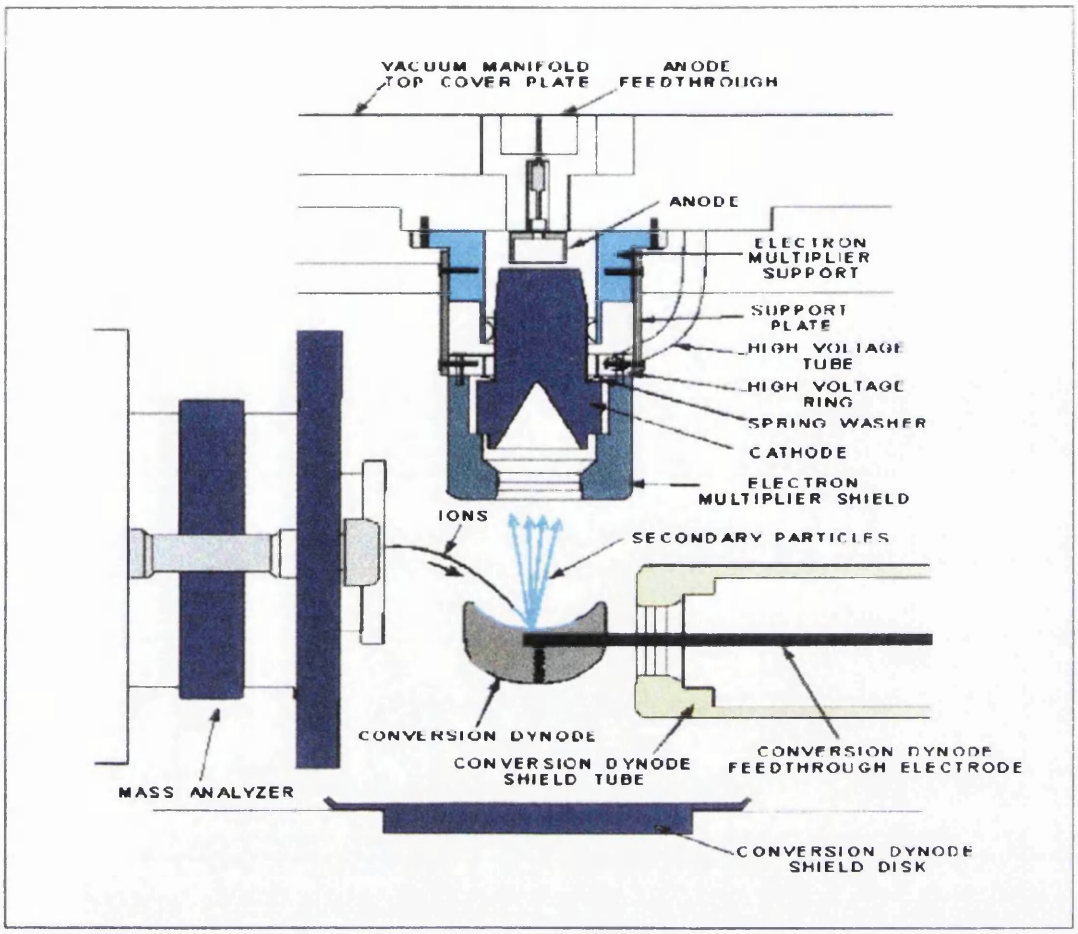



Figure 1.12 Schematic diagram of the electron multiplier.

## Vacuum System

There are three regions that need to be kept at variable pressures. Firstly, the capillary region is held at 1 Torr by a rotary vane pump or roughing pump. Secondly, a lower pressure of $10^{-3}$ Torr is required in the first octapole region and $10^{-5}$ Torr in the analyser region, both of which occur in two steps. Initially a reduced pressure is brought about by the roughing pump which then kick-starts the turbomolecular pump into action that in turn provides the lower pressure.

## Experimental

The molecular weights referred to in the mass spectrometric experiments conducted in the various studies contained in this thesis are the monoisotopic masses of the compounds of interest. This is due to analysis generally being centred on the most abundant ion produced during ionisation of the parent compound and this ion arising from the most prevalent isotope of the parent compound; hence the molecular weight of interest is that of the most abundant isotope of the parent and is therefore the monoisotopic mass related to this isotope.

The mass spectra presented in this thesis contain mass to charge (m/z) values that are given in Thompson units (Th), the instrument response is given in the top right hand corner of each mass spectrum or ion chromatogram to allow for the reader to make comparisons of the variation in response from one compound to another.

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# Chapter 2

# LC-MS Analysis of Polar Herbicides in River Water

# Chapter 2 LC-MS Analysis of Polar Herbicides

## Introduction to Polar Herbicides

The United States Environment Protection Agency describes a pesticide as "any substance or mixture of substances intended for preventing, destroying, repelling or mitigating any pest". The term pest refers to insects, unwanted plants, mice and other animals, fungi and microorganisms like bacteria and viruses. This definition causes some confusion since the term pesticide applies not only to insecticides, as most believe, but also to herbicides, fungicides and other substances used to control "pests".

Herbicides are chemicals that inhibit or interrupt normal plant growth and development. They are widely used in agriculture, industry and urban areas to control weeds. Herbicides can provide cost effective weed control with a minimum of labour (1). As well as their use in agriculture herbicides are also used to eliminate undesirable plants from roadsides, railways and power line rights of way, and so on, and sometimes to defoliate entire regions (2). Improper herbicide use can injure crops, damage the environment, and pose a threat to the applicator and others exposed to the chemical (1).

Herbicides kill plants in different ways; but a herbicide must meet certain requirements in order to be effective:

1. Contact the target weed.
2. Be absorbed by the weed.
3. Move to the site of action in the weed.
4. Accumulate sufficient levels at the site of action to kill the plant.

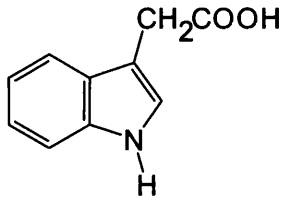
Weed control is unsatisfactory unless these requirements are met (1).

Herbicide usage has increased dramatically in the last three decades coinciding with changing farming practices and increasingly intensive agriculture. The annual application of herbicides for example in Italy increased from 9600 tonnes of active ingredients in 1971 to 33000 tonnes in 1987. The 36000 tonnes of herbicides used in France during 1988 represented 36% of the total pesticides used in the country endorsing the importance of this class of agrochemicals. Herbicides have been detected in many rivers around the world (3).

The compound groups investigated in our studies are all used as selective herbicides; they are extensively applied in agriculture to kill weeds without damage to vegetation. Away from agriculture they have been used to control broad leaf weeds on golf courses, railway tracks and house lawns.

## Acidic Herbicides

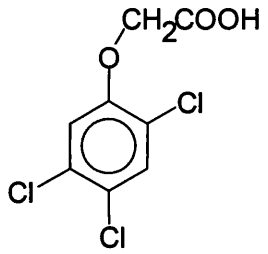
In 1928 the first plant hormone, indolylacetic acid (IAA) was discovered:




It might be expected that applying large quantities of a plant hormone such as IAA to a plant would cause it to grow excessively and die as a result; plants however can regulate the levels of IAA they contain through metabolism and as a result the chemical is ineffective as a weed killer. The hormone weed killers, which were discovered in 1940, are synthetic analogues of IAA. Plants are unable to metabolise these chemicals and as a result they do die of uncontrolled and grossly distorted growth (4).

The discovery of the phenoxyacetic acid herbicides during the Second World War heralded the modern era of agrochemicals. These herbicides are called growth regulators because they mimic natural growth hormones, and upset the natural hormone balance in plants. Growth hormones regulate cell elongation, protein synthesis and cell division. The killing action of growth regulating chemicals is not caused by any single factor, but rather by the disruption of several growth processes in the treated plant. Growth regulator herbicides may affect reproduction, resulting in sterile florets and non-viable seed production. These weed killers were introduced at

the end of the Second World War, 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) being one of the first and most important:




2,4,5-T is a phenoxy herbicide; environmentally the by products contained in phenoxy herbicides are often of greater concern than the herbicides themselves. Phenols are mildly acidic, in the presence of concentrated solutions of a strong base like sodium hydroxide the hydrogen of the OH group is lost as $H^+$ and the phenoxide anion, $C_6H_5O^-$, is produced in the form of a sodium salt:

$$C_6H_5OH + NaOH \longrightarrow C_6H_5O^-Na^+ + H_2O$$

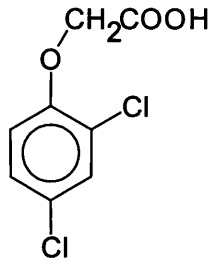
The phenoxide salt is very reactive and this property can be exploited in order to prepare molecules containing the C-O-C linkage. Thus if a R-Cl molecule is heated together with a salt containing the phenoxide ion, NaCl is eliminated and the phenoxy oxygen links the benzene ring to the R group:

$$C_6H_5O^-Na^+ + Cl{-}R \longrightarrow NaCl + C_6H_5{-}O{-}R$$

Such a reaction is the commercial route to the large-scale preparation of 2,4,5-T introduced in 1944. Here the R-group is acetic acid minus one of its methyl group hydrogen's, so that the Cl-R reactant is Cl-$CH_2COOH$. Thus according to the reaction

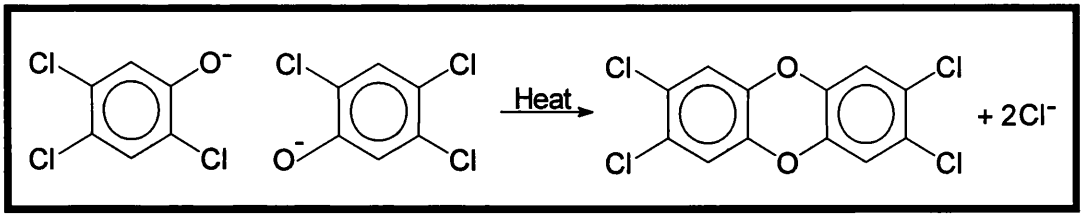
format we obtain phenoxyacetic acid as an intermediate in the production of the actual herbicides.

In the commercial herbicides chlorine atoms replace some of the five remaining hydrogen atoms of the benzene ring, for example 2,4,5-T shown previously and 2,4-dichlorophenoxyacetic acid (2,4-D) shown below:




2,4-D is used to kill broad leaf weeds in lawns, golf course fairways and greens, and agricultural fields. In contrast 2,4,5-T is effective in clearing brush, for instance, on roadsides and power line corridors.

Traditionally the industrial synthesis of 2,4,5-T started with 2,4,5-trichlorophenol, which was produced by reacting sodium hydroxide with the appropriate tetrachlorobenzene. Unfortunately, during the reaction in which the phenol is produced from tetrachlorobenzene, there occurs a side reaction that converts a very small portion of the trichlorophenol product into tetrachlorodibenzo-ρ-dioxin:



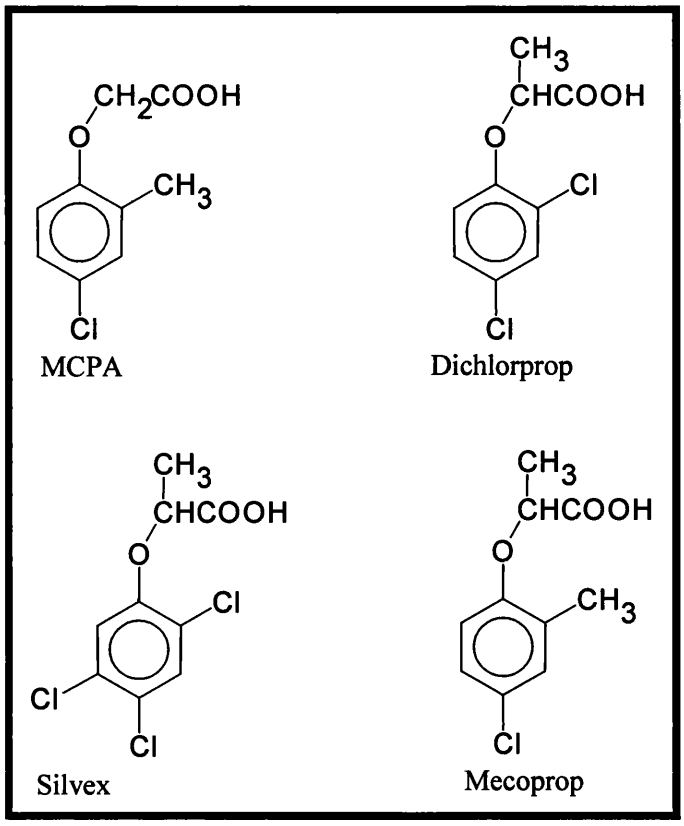

Tetrachlorodibenzo-ρ-dioxin is the most toxic of this class of compounds. The side reaction that produces the dioxin is rate dependent on the concentration of the chlorophenoxide ion; thus the rate of dioxin production increases dramatically as the initial chlorophenoxide ion concentration increases. The rate of this side reaction also increases rapidly with increasing reaction temperature. Therefore the extent to which the trichlorophenol and hence the herbicide become contaminated with the dioxin by-product can be minimised by controlling the concentration and temperature in the preparation of the original trichlorophenol. In modern synthesis the contamination of 2,4,5-T by the dioxin can be kept to about 0.1ppm by keeping both the phenoxide concentration and the temperature low. Nevertheless its manufacture and use in North America was phased out in the mid 1980's because of concerns about its dioxin content, however small.

A 1:1 mixture of 2,4-D and 2,4,5-T called Agent Orange was extensively used as a defoliant during the Vietnamese War. The mixture contained dioxin levels of around 10ppm so it is clear that the reaction used to produce the 2,4,5-T was not carefully controlled so as to minimise contamination. Environmental contamination also occurred as the result of an explosion in a chemical factory in Seveso, Italy in 1976. The factory produced 2,4,5-trichlorophenol from tetrachlorobenzene as described previously. The explosion was caused when the reaction was not brought to a

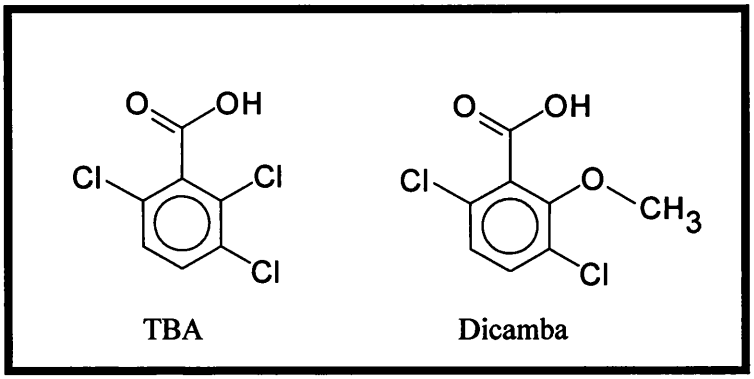
complete halt before the workers left for the weekend. The reaction continued unmonitored and the heat subsequently released resulted in the explosion. As the phenol had been heated to a high temperature a considerable amount of dioxin was produced and the explosion distributed the toxin extensively in the environment. Many wildlife deaths apparently resulted from the contamination and although a large number of humans were also exposed no serious health effects were initially found; however studies have established that the rates of a number of cancers has increased in people who lived in the regions most exposed to the dioxin.

Huge quantities of 2,4-D are used in developed countries for the control of weeds in both agricultural and domestic settings. In some communities its continued use on lawns has become a controversial practice because of its suspected effects on human health in particular, farmers in the mid western United States who mix and apply large quantities of 2,4-D to their crops are found to have an increased incidence of the cancer known as non-Hodgkin's lymphoma.

2-methyl 4-chlorophenoxyacetic acid (MCPA) is 2,4-D with the chlorine in the two position replaced by a methyl group; the herbicides called dichlorprop, silvex, and mecoprop are identical to 2,4-D, 2,4,5-T and MCPA respectively, except that their molecules have a methyl group replacing one of the hydrogen atoms in the $-CH_2-$ group of the acid chain; thus they are phenoxy herbicides based upon propionic acid rather than acetic acid (2):

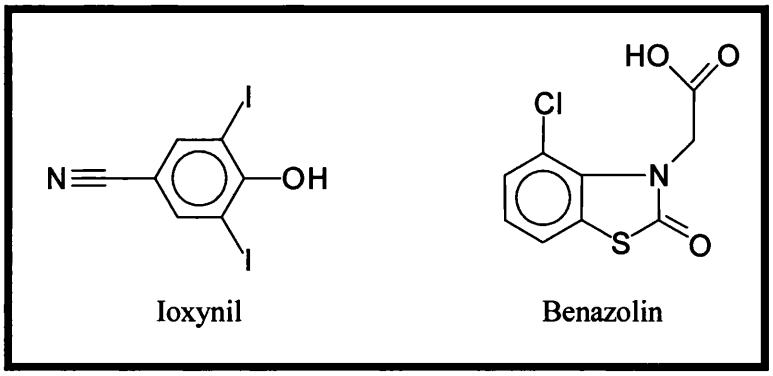



Other hormone type weed killers included under the acidic herbicide heading are the benzoic acid herbicides of which 2,3,6-trichlorobenzoic acid (TBA) and 3,6-dichloro 2-methoxybenzoic acid (dicamba) are representatives (4):




Classed within the same acidic herbicide category are other herbicides that are also aromatic in structure but with the use of halogens other than chlorine, for example

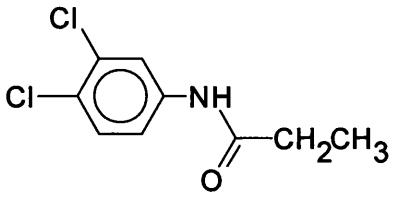
Ioxynil that is often used in the protection of cereal crops mixed with phenoxyacetic acid herbicides (4), and the presence of other active groups:



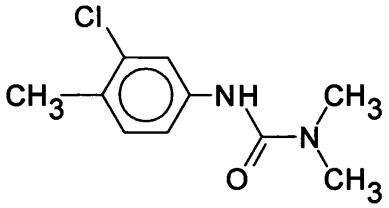

Ioxynil Benazolin

**Phenylurea and Triazine Herbicides**

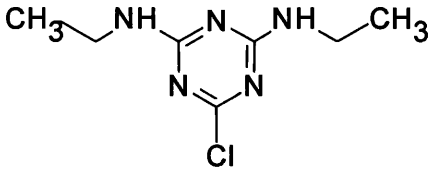
Photosynthesis is a vital process which takes place in plants enabling them to synthesise carbohydrates from carbon dioxide. The energy for this process is supplied by the photosynthetic electron flow system in the inner thylakoid membranes of the chloroplasts. In this system light causes electrons to be transferred from water, via the oxidation of water to oxygen, to physiological electron acceptors such as nicotinamide adenine dinucleotide phosphate (NADP) that are used for the reduction of carbon dioxide to carbohydrates. Many compounds are known that inhibit this process and they are not usually very toxic to mammals. Herbicides that appear to act primarily in this way can be divided into 3 major groups, amides, ureas and triazines, although other compounds are known (4, 5). Examples of the 3 major groups are shown on the following page:




Propanil an amide herbicide.

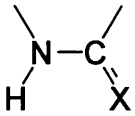



Chlortoluron a urea herbicide.




Simazine a triazine herbicide.

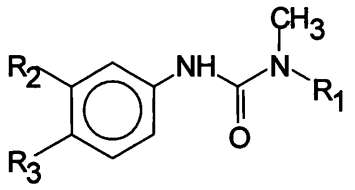
All of the herbicides inhibiting the photochemical oxidation of water, and hence inhibiting photosynthesis, have a common structural feature. This feature takes the form of the structure shown below where X is either oxygen or nitrogen and therefore possesses a lone pair of electrons:




It is believed that this part of the molecule is the toxophore responsible for binding to an enzyme involved in photochemical oxidation thereby inhibiting it (4).

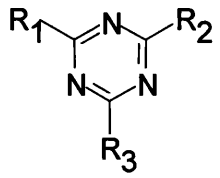
The phenylureas are a well-established class of herbicide originally developed by Du Pont. They can be used either pre or post emergent and are systematic being taken up into the plant via the roots and distributed around the plant to their point of action, photosystem II, in the chloroplasts. These herbicides are photosynthetic inhibitors, as

described earlier. Plants are not affected by the herbicide until after they emerge and begin photosynthesis. Even though photosynthesis is inhibited by these herbicides, susceptible plants do not die simply from starvation. Instead plants treated with a photosynthetic inhibitor die from a build up of highly reactive molecules that destroy cell membranes. The phenylureas are generally broad spectrum herbicides, though a few useful selectivities do exist in some crops due to species specific metabolism. Phenylureas are based on the structure shown below:

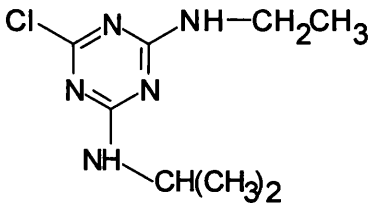



The triazines, first developed by Geigy, are similar in activity and scope to the phenylureas. They are broad spectrum herbicides taken up by the roots and inhibitors of photosystem II. The pathway by which they are metabolised and the rate of detoxification in a given plant primarily determine the selective action of triazine herbicides. Plant species such as corn and sorghum possess glutathione-s-transferase enzyme and can selectively metabolise triazine herbicides, via conjugation with glutathione, into non-toxic substances.

Triazines are based upon the symmetric, aromatic structure shown below, which has alternating carbon and nitrogen atoms in a six membered ring:




In triazines that are useful as herbicides, one carbon atom in the ring is bonded to either chlorine or a methylsulfinyl group and the other two to amino groups. The most well known member of this group is atrazine, which was introduced in 1958 and is used in huge quantities to destroy weeds in cornfields. Atrazine is shown below:




Usually it is applied to cultivated soils, at the rate of a few kilograms per hectare, in order to kill grassy weeds. In higher quantities it has been used to kill off all plant life, for example to create parking lots. Atrazine is not considered to be very toxic; however some preliminary surveys on the health of farmers and other individuals exposed to it in higher concentrations show disturbing links to higher cancer rates and higher incidents of birth defects. (1, 2, 6).

**Reason for analysis**

The agricultural usage of some of these pesticides is absolutely phenomenal; for example the consumption of Atrazine in the United States was once estimated at 34500 tonnes and 700 tonnes in the United Kingdom (7). The large-scale use of these toxic pesticides causes concern for environmental implications as well as their potential effect on human health. The water soluble and persistent nature of these compounds leads to their transportation and distribution into various aquatic environments through surface run off and leaching. Many studies have shown the presence of these herbicides in various types of ground and surface water (3, 8, 9, 10).

Limits on the concentrations of pesticides in water systems have been clearly set out by the European Union directive 80/788. Individual pesticides and by-products are limited to a concentration of 0.1μg/L and total pesticide concentrations of 0.5μg/L in drinking water, with surface water concentrations being 1μg/L and 5μg/L for individual and total concentrations respectively (11). Some of the herbicides used have been classified as possible carcinogens; it is therefore necessary to have a robust analytical procedure capable of meeting these low detection limit requirements.

We were requested to develop rapid LC-MS analysis methods to allow for the determination of low levels of polar herbicides in aqueous matrices by Hyder Environmental (Mid Glamorgan, UK). In the labs of Hyder Environmental analysis of the triazine and phenylurea herbicides was being carried out using the Ṣamos system which is performed by using on-line solid phase extraction and HPLC with UV diode

array detection; using this methodology analysis times in excess of 60 minutes are required for each sample. The analysis method used for the acidic herbicides involved derivitization of the compounds prior to GC-MS analysis (12). The use of LC-MS to analyse for the herbicides of interest negates the need for time consuming derivitization processes and should allow for a much shorter analysis time to be used.

# LC-UV For the Separation of 5 Triazine Herbicides

The structures of the triazine herbicides studied are shown in figure 2.1.

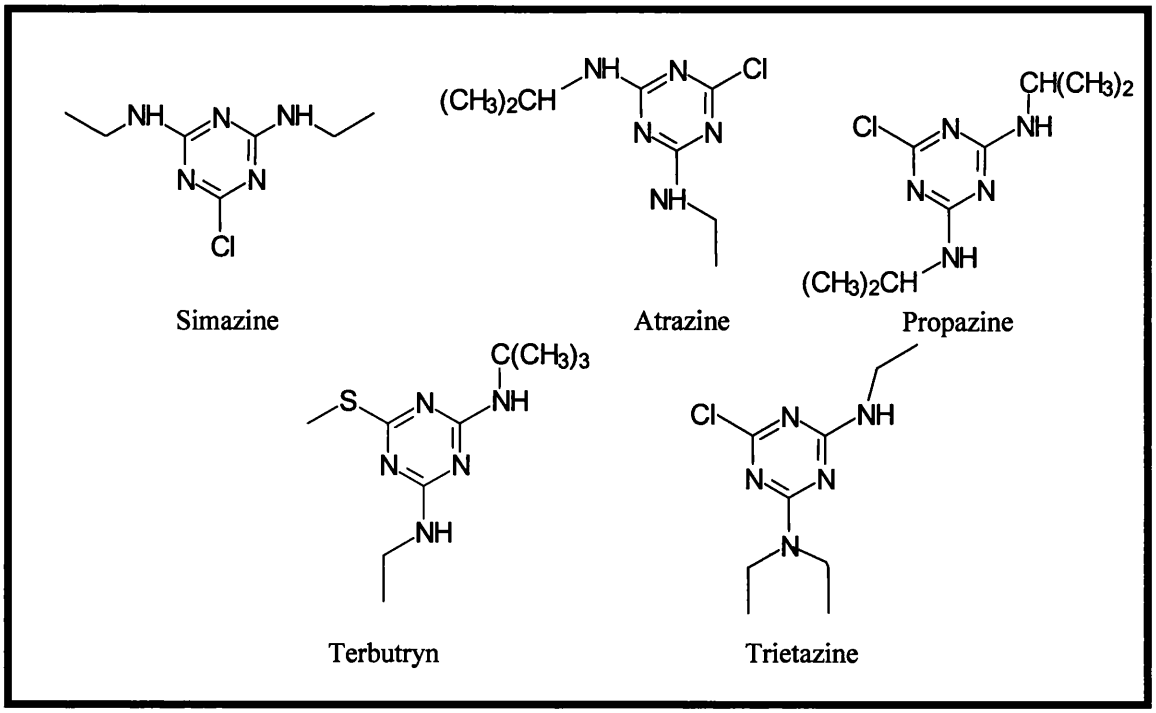



Figure 2.1 Structures of the Triazine herbicides investigated.

## Experimental

## Chemicals

HPLC grade solvents were purchased from Fisher Scientific (Loughborough, U.K.), gases from BOC Limited (Surrey, U.K.) and the following 5 triazine herbicides from Dr Ehrenstorfer GmbH (Augsburg, Germany).

| | |
|---|---|
| Simazine | Atrazine |
| Propazine | Terbutryn |
| Trietazine | |

**Sample Preparation**

1mg/ ml standard solutions were made up in 50/ 50 acetonitrile/water for each of the triazines and solutions for analysis were prepared and diluted from these as necessary.

**Instrumentation**

A Hewlett Packard 1100 HPLC fitted with a diode array detector (DAD) was used to perform the chromatography with a Zorbax 4.6x150mm 5μ C18 column. The mobile phase used for analysis was made up of acetonitrile and 1mM ammonium acetate run in an isocratic system.

**Results**

We developed our chromatography method for the analysis of the triazines from the details given to us of Hyder Environmental (Mid Glamorgan, UK) gradient methods (12). The mixture of the 5 triazines was separated on the Zorbax C18 column with the following chromatographic conditions:

| Time in minutes | $\%CH_3CN$ | $\%1mM\ NH_4Oac$ |
|---|---|---|
| 0.00 | 40 | 60 |
| 18.00 | 40 | 60 |

| | |
|---|---|
| Column Thermostat | 30°C |
| Flow Rate | 1.0mL/min |
| DAD | 220nm |

The following table gives the retention times for the compounds.

| Compound name | Peak label | RT minutes |
|---|---|---|
| Simazine | A | 4.427 |
| Atrazine | B | 6.289 |
| Propazine | C | 9.474 |
| Terbutryn | D | 15.585 |
| Trietazine | E | 17.171 |

Figure 2.2 shows the UV chromatogram of the separation of the 5 triazines.

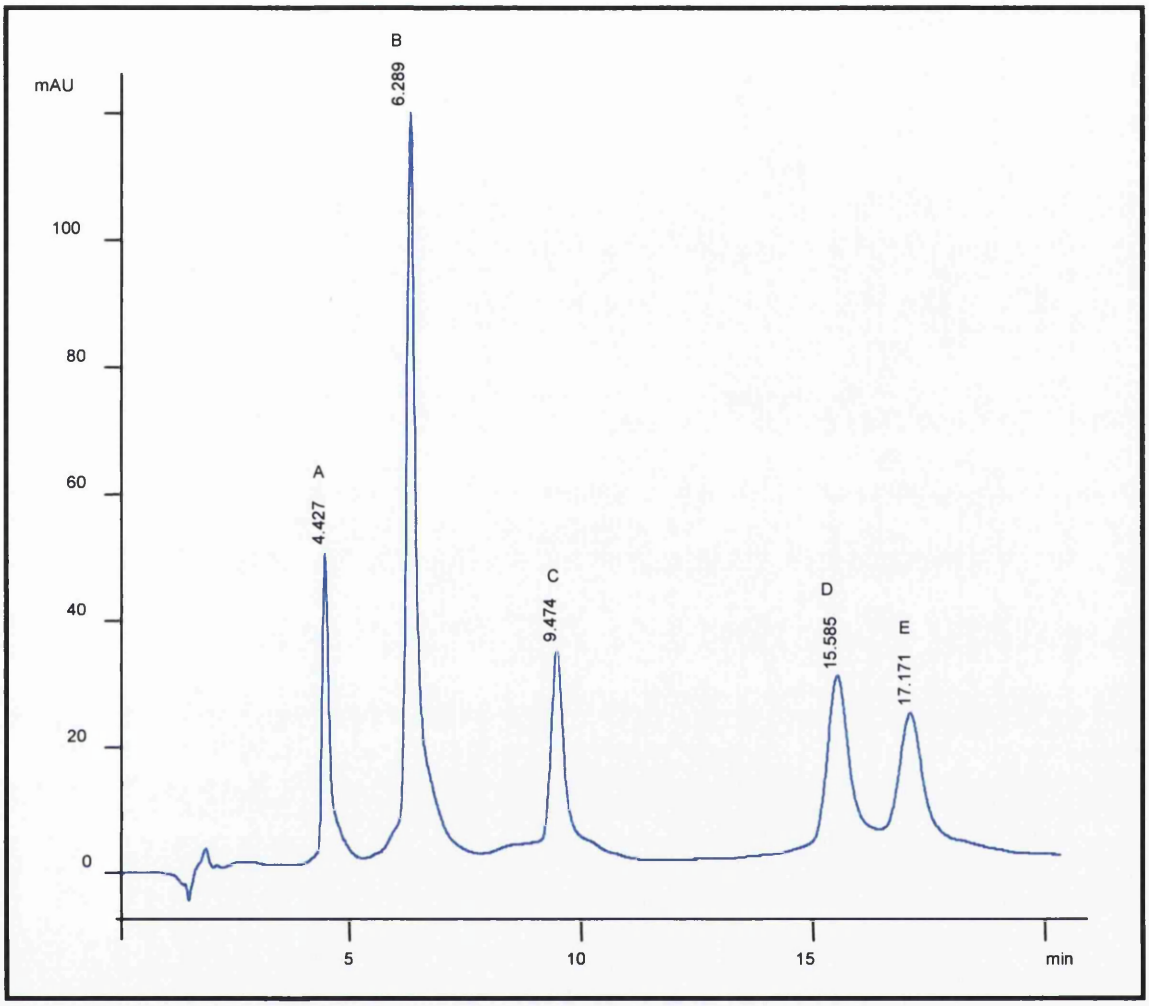



Figure 2.2 HPLC chromatogram of the separation of the 5 Triazines. 10 µL injection of a solution containing 10 µg/mL of each herbicide.

The following table gives the corresponding resolution values (Rs) for the separation shown above.

| Peaks | Rs Value |
|---|---|
| A+B | 3.50 |
| B+C | 5.33 |
| C+D | 7.50 |
| D+E | 1.50 |

## LC-UV For the Separation of 5 Phenylurea Herbicides

The structures of the phenylurea herbicides studied are shown in figure 2.3.

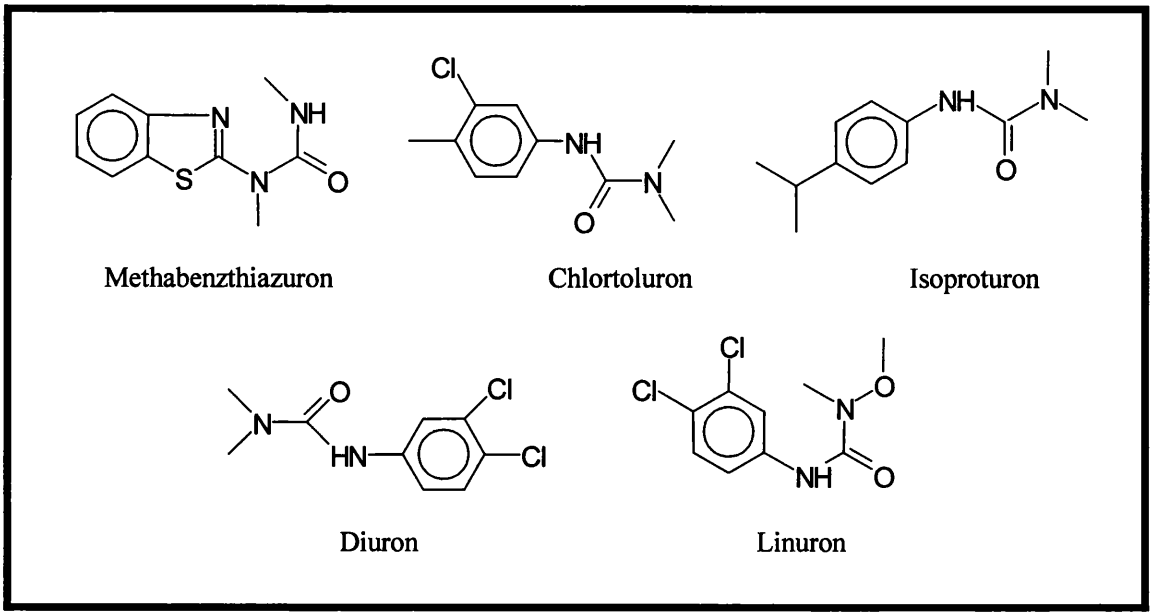



Figure 2.3 Structures of the Phenylurea herbicides investigated.

### Experimental

### Chemicals

HPLC grade solvents were purchased from Fisher Scientific (Loughborough, U.K.), gases from BOC Limited (Surrey, U.K.) and the following 5 phenylurea herbicides from Dr Ehrenstorfer GmbH (Augsburg, Germany).

| | |
|---|---|
| Methabenzthiazuron | Chlortoluron |
| Isoproturon | Diuron |
| Linuron | |

## Sample Preparation

1mg/ ml standard solutions were made up in 50/ 50 acetonitrile/water for each of the phenylureas and solutions for analysis were prepared and diluted from these as necessary.

## Instrumentation

A Hewlett Packard 1100 HPLC fitted with a diode array detector (DAD) was used to perform the chromatography with a Zorbax 4.6x150mm 5μ C18 column and a Jencons Scientific ltd RTE-110 cryostatic bath where reliable column temperatures more than 10°C below room temperature were required. The mobile phase used for analysis was made up of acetonitrile and 1mM ammonium acetate run in an isocratic system.

## Results

It was initially attempted to separate the 5 phenylureas on the Zorbax C18 column using the mobile phase conditions previously used with the LC-UV analysis of the triazines. The following chromatographic conditions were used:

| Time in minutes | %$CH_3CN$ | %1mM $NH_4Oac$ |
|---|---|---|
| 0.00 | 34 | 66 |
| 18.00 | 34 | 66 |

| | |
|---|---|
| Column Thermostat | Room Temperature |
| Flow Rate | 1.0mL/min |
| DAD | 220nm |

The following table gives the retention times for the compounds.

| Compound name | Peak label | RT minutes |
|---|---|---|
| Methabenzthiazuron | A | 5.858 |
| Chlortoluron | B | 6.204 |
| Isoproturon + Diuron | C+D | 7.739 |
| Linuron | E | 16.550 |

Figure 2.4 shows the UV chromatogram of the separation of the 5 phenylureas.

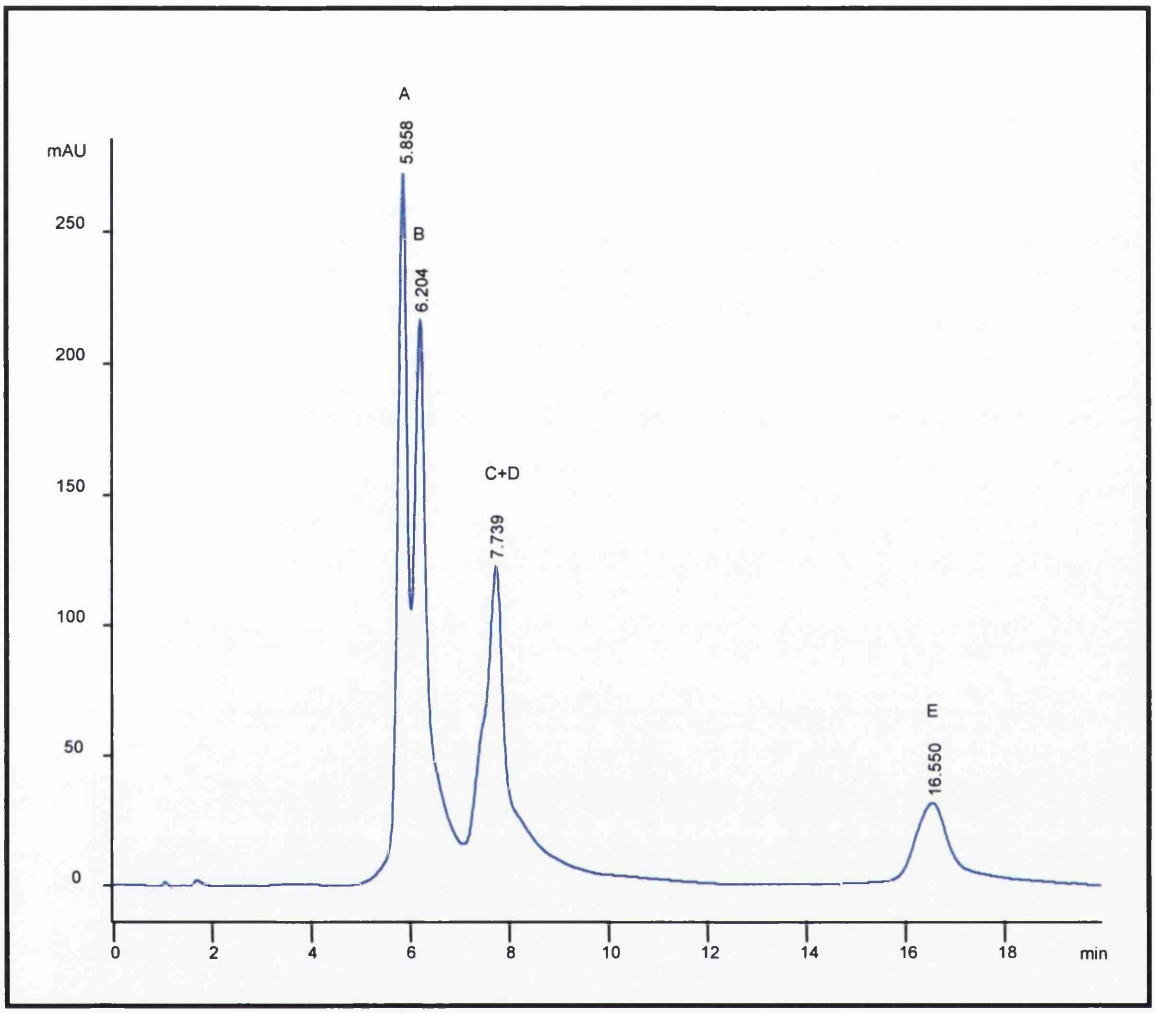



Figure 2.4 HPLC chromatogram of the separation of the 5 Phenylureas. 10 µL injection of a solution containing 10 µg/mL of each herbicide.

It can be seen from the chromatogram that isoproturon and diuron appear to co-elute; however when the same chromatographic run is viewed at a wavelength of 210nm it can be seen that there is a certain amount of "splitting" occurring in the peak responsible for these two compounds. (Figure 2.5)

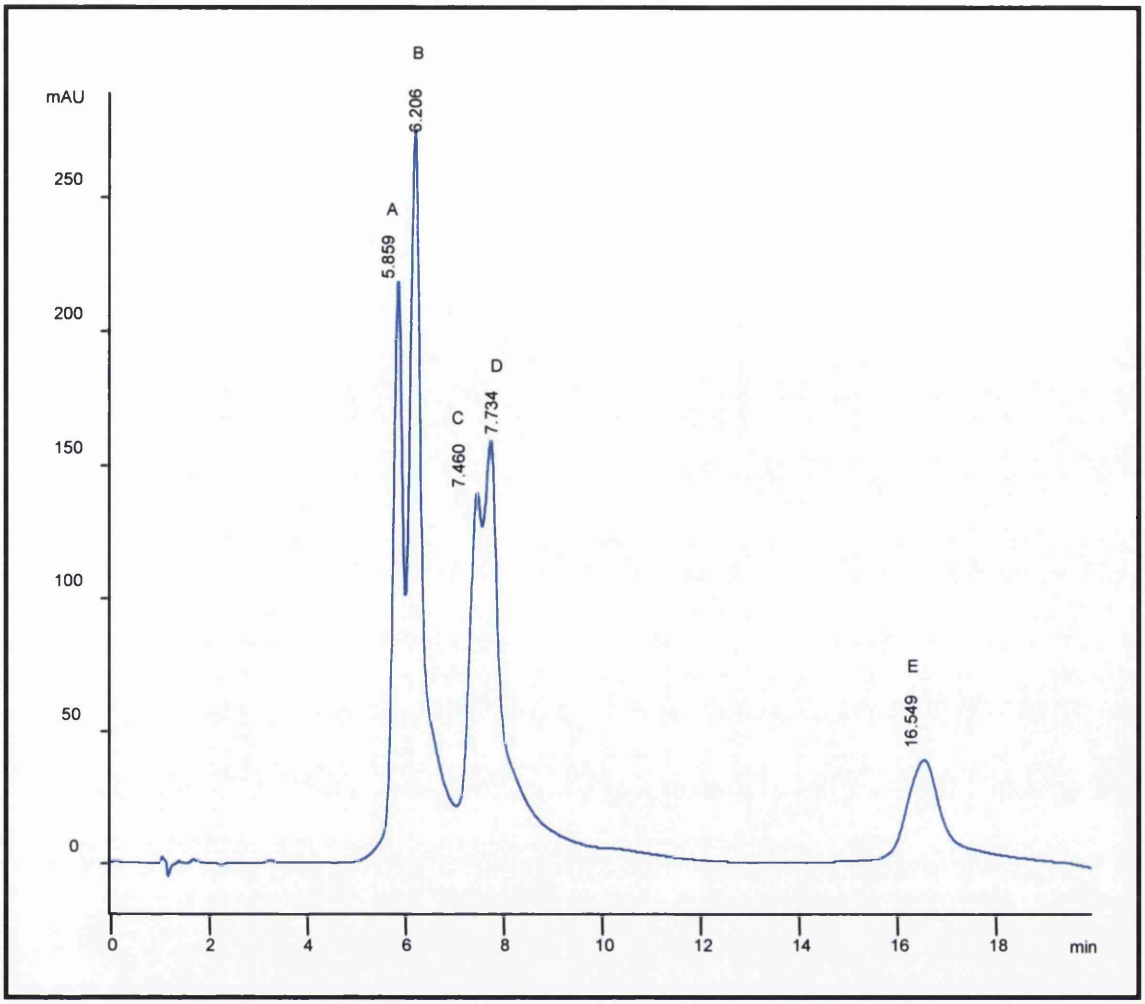



Figure 2.5 HPLC chromatogram of the separation of the 5 Phenylureas at a wavelength of 210nm. 10 µL injection of a solution containing 10 µg/mL of each herbicide.

As this did not occur when the temperature on the column thermostat was increased to temperatures higher than room temperature it was decided to cool the column using a cryostatic bath. The separation was progressively improved as the column was further cooled; this can be seen in the following two chromatograms. The first (figure 2.6) shows the separation under the same conditions as previously mentioned with the exception that the column was cooled to 14°C and the second (figure 2.7) with the column cooled to 0°C using the cryostatic bath.

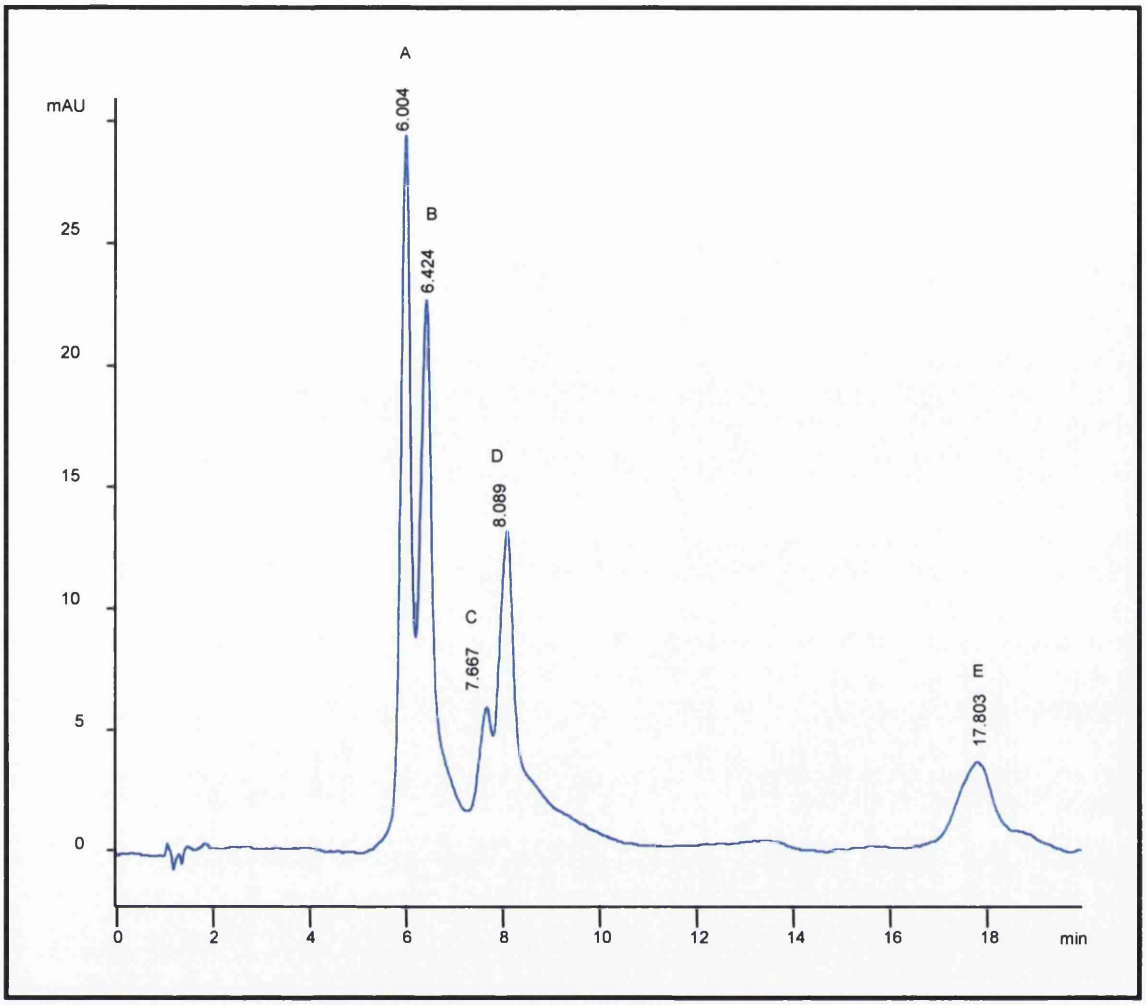



Figure 2.6 HPLC chromatogram of the separation of the 5 Phenylureas at 14°C. 10 µL injection of a solution containing 1 µg/mL of each herbicide.

The following table gives the retention times for the compounds.

| Compound name | Peak label | RT minutes |
|---|---|---|
| Methabenzthiazuron | A | 6.004 |
| Chlortoluron | B | 6.424 |
| Isoproturon | C | 7.667 |
| Diuron | D | 8.089 |
| Linuron | E | 17.803 |

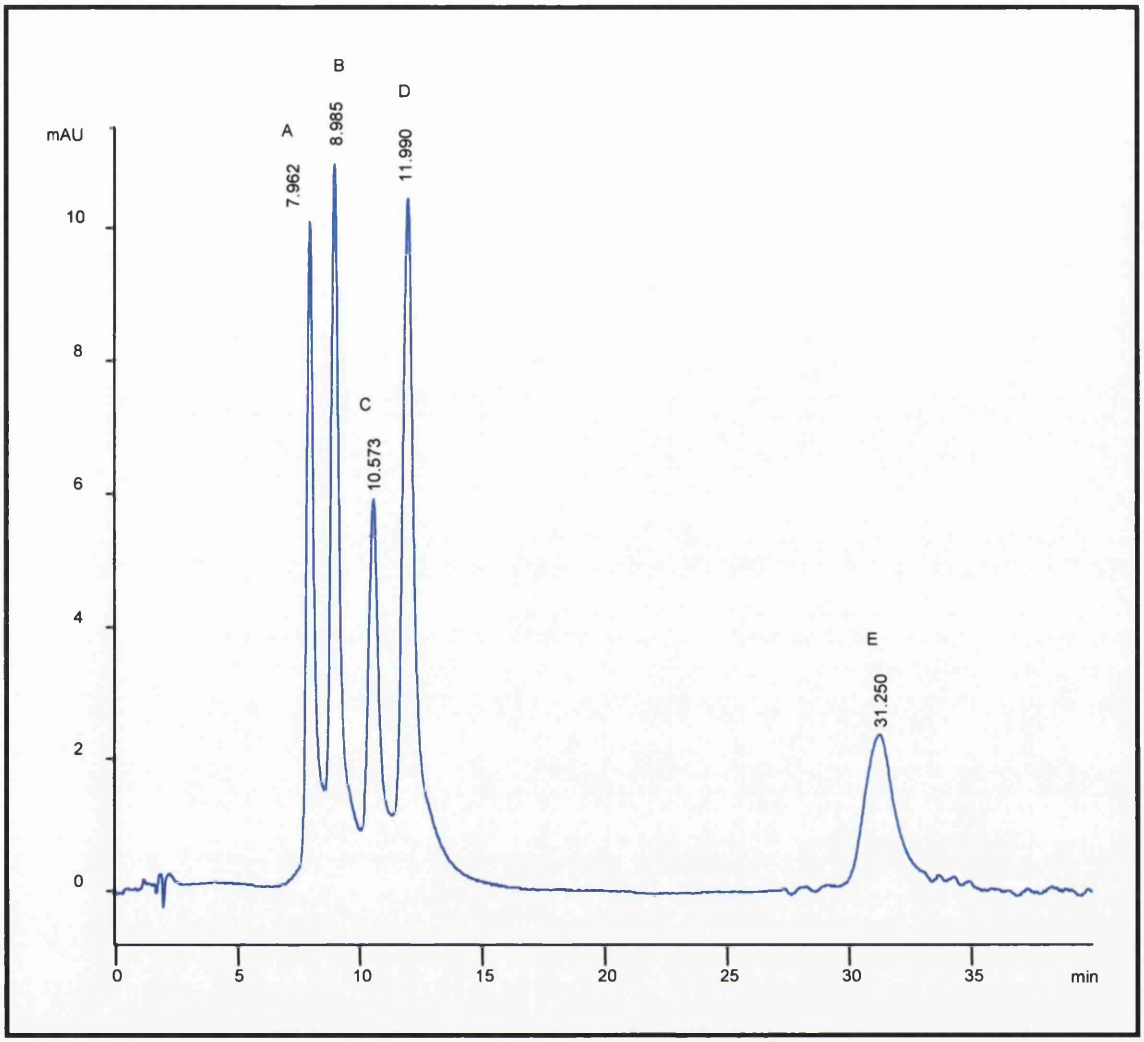



Figure 2.7 HPLC chromatogram of the separation of the 5 Phenylureas at 0°C. 10 µL injection of a solution containing 1 µg/mL of each herbicide.

The following table gives the retention times for the compounds.

| Compound name | Peak label | RT minutes |
|---|---|---|
| Methabenzthiazuron | A | 7.962 |
| Chlortoluron | B | 8.985 |
| Isoproturon | C | 10.573 |
| Diuron | D | 11.989 |
| Linuron | E | 31.250 |

The table below gives the corresponding resolution values (Rs) for the separation of the phenylureas at 0°C.

| Peaks | Rs Value |
|---|---|
| A+B | 1.07 |
| B+C | 1.33 |
| C+D | 1.20 |
| D+E | 10.27 |

The improvement in resolution achieved by decreasing the column temperature, although unexpected, has been investigated in the literature (13, 14, 15). Column selectivity has been found to vary continuously with temperature and the ability of the solid phase to separate closely related isomers observed to be greatest at sub ambient temperatures (13).

These changes in selectivity are believed to result from changes in the solid phase morphology occurring at low temperatures and have been explained in terms of the alkyl chain mobility and order. For pure long chain alkanes mobility decreases with temperature ultimately resulting in the formation of an ordered crystalline solid; in this crystalline state alkyl chains exist in the all trans conformation and are thus fully extended. While such a crystalline state is not possible for bonded phases, since the alkyl chain spacing is constrained by the covalent point of attachment to the surface, a reduction in bonded ligand mobility with temperature has been demonstrated and straightening of the alkyl chains with decreasing temperature has been shown to occur (13).

Alkyl bonded phases thus become more rigid and rod like at low temperatures and therefore take on some of the characteristics of liquid crystals which have been used

as a solid phase in gas chromatography (GC) and found to be highly shape selective allowing for the separation of isomers which could not be resolved with conventional GC phases (13).

The evidence found in the literature thus suggests that the increase in phase order that occurs at low temperatures increases the shape recognition ability of the C18 solid phase improving its ability to resolve structurally similar molecules.

## LC-UV For the Separation of a mixture of the 5 Triazines and 5 Phenylurea Herbicides

As the triazine and phenylurea herbicides have similar herbicidal properties, and are often used in conjunction with each other, it was decided to develop a chromatographic method to allow for the rapid analysis of the 10 previously mentioned compounds.

### Experimental

### Chemicals

HPLC grade solvents were purchased from Fisher Scientific (Loughborough, U.K.), gases from BOC Limited (Surrey, U.K.) and the following 10 herbicides from Dr Ehrenstorfer GmbH (Augsburg, Germany).

| | |
|---|---|
| Simazine | Methabenzthiazuron |
| Atrazine | Chlortoluron |
| Propazine | Isoproturon |
| Terbutryn | Diuron |
| Trietazine | Linuron |

### Sample Preparation

1mg/ ml standard solutions were made up in 50/ 50 acetonitrile/water for each of the herbicides. Solutions for analysis were prepared and diluted form these as necessary.

## Instrumentation

A Hewlett Packard 1100 HPLC fitted with a diode array detector (DAD) was used to perform the chromatography with a selection of columns (Zorbax 4.6x150mm 5μ C18, Phenomenex Luna 4.6x100mm 3μ C18 (2) and Phenomenex Luna 4.6x100mm 3μ Phenyl-Hexyl). A Jencons Scientific ltd RTE-110 cryostatic bath was used where reliable column temperatures more than 10°C below room temperature were required. The mobile phase used for analysis was made up of acetonitrile and either 1mM ammonium acetate or water run in a gradient system.

## Results

It was initially attempted to separate the 10 herbicides on the Zorbax C18 column using a gradient system developed from the isocratic mobile phase systems used for the analysis of the triazine and phenylurea herbicides when run separately. The following chromatographic conditions were used:

| Time in minutes | %$CH_3CN$ | %1mM $NH_4Oac$ |
|---|---|---|
| 0.00 | 34 | 66 |
| 13.5 | 34 | 66 |
| 14.0 | 66 | 34 |
| 20.0 | 66 | 34 |

| | |
|---|---|
| Column Thermostat | Room Temperature |
| Flow Rate | 1.0mL/min |
| DAD | 220nm |

The following table gives the retention times for the compounds.

| Compound name | Peak label | RT minutes |
|---|---|---|
| Simazine | A | 5.962 |
| Methabenzthiazuron | B | 8.829 |
| Chlortoluron | C | 9.487 |
| Atrazine | D | 10.489 |
| Isoproturon + Diuron | E+F | 12.200 |
| Propazine | G | 16.564 |
| Linuron | H | 17.162 |
| Terbutryn | I | 18.110 |
| Trietazine | J | 18.392 |

Figure 2.8 shows the UV chromatogram of the separation of the 10 herbicides.

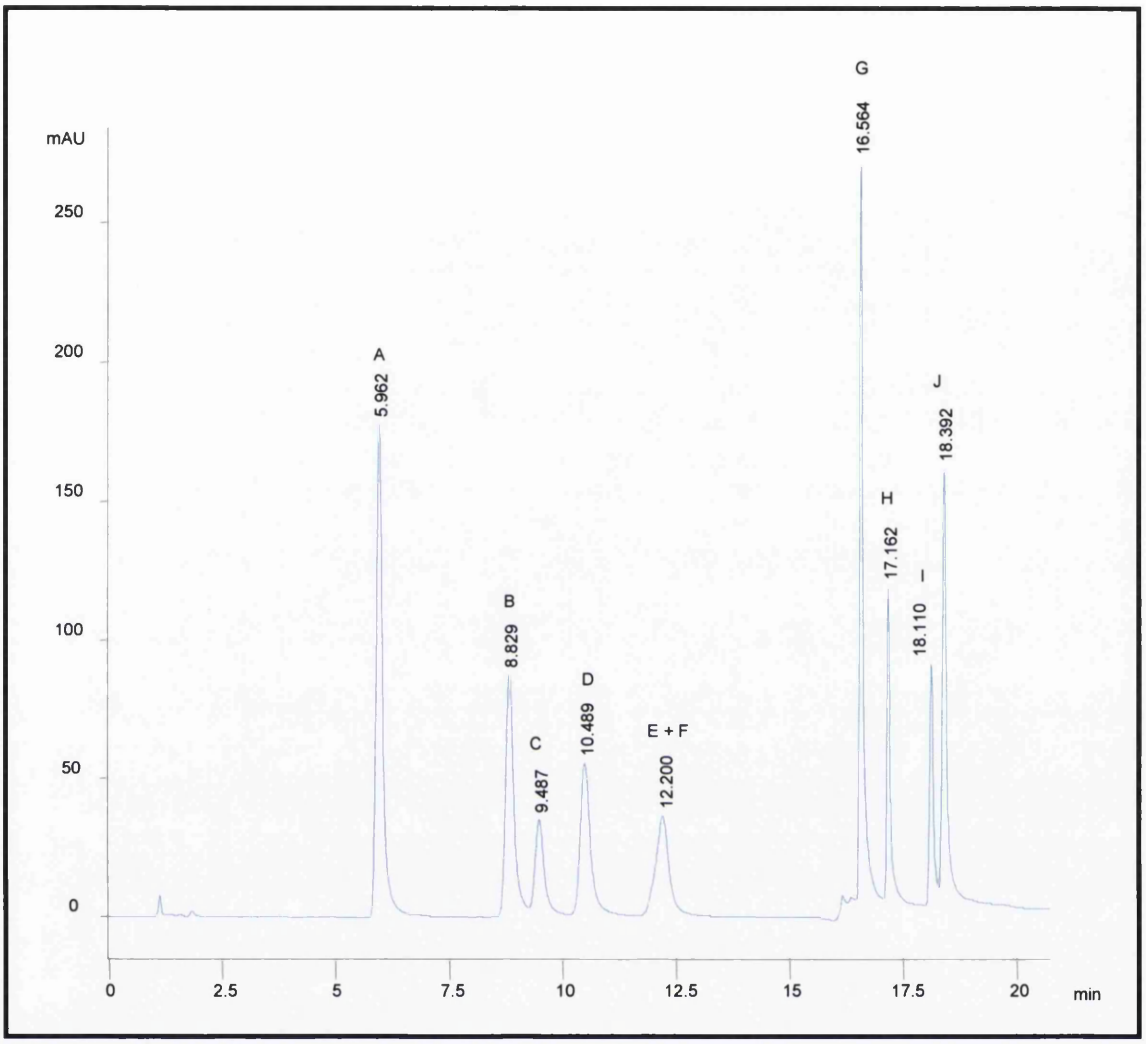



Figure 2.8 HPLC chromatogram of the separation of the 10 Herbicides at room temperature. 10 µL injection of a solution containing 10 µg/mL of each herbicide.

As can be seen from the chromatogram the problem of isoproturon and diuron co-eluting was again encountered. So it was decided to run the method at 0°C with the gradient altered to suit the longer retention times of the compounds. The following chromatographic conditions were used:

| Time in minutes | %$CH_3CN$ | %1mM $NH_4Oac$ |
|---|---|---|
| 0.00 | 34 | 66 |
| 16.5 | 34 | 66 |
| 17.0 | 66 | 34 |
| 23.0 | 66 | 34 |

Cryostatic bath 0°C

Flow Rate 1.0mL/min

DAD 220nm

The following table gives the retention times for the compounds.

| Compound name | Peak label | RT minutes |
|---|---|---|
| Simazine | A | 6.146 |
| Methabenzthiazuron | B | 9.618 |
| Chlortoluron + Atrazine | C+D | 10.954 |
| Isoproturon | E | 13.324 |
| Diuron | F | 14.994 |
| Propazine | G | 18.761 |
| Linuron | H | 20.215 |
| Terbutryn | I | 20.921 |
| Trietazine | J | 21.367 |

Figure 2.9 shows the UV chromatogram of the separation of the 10 herbicides.

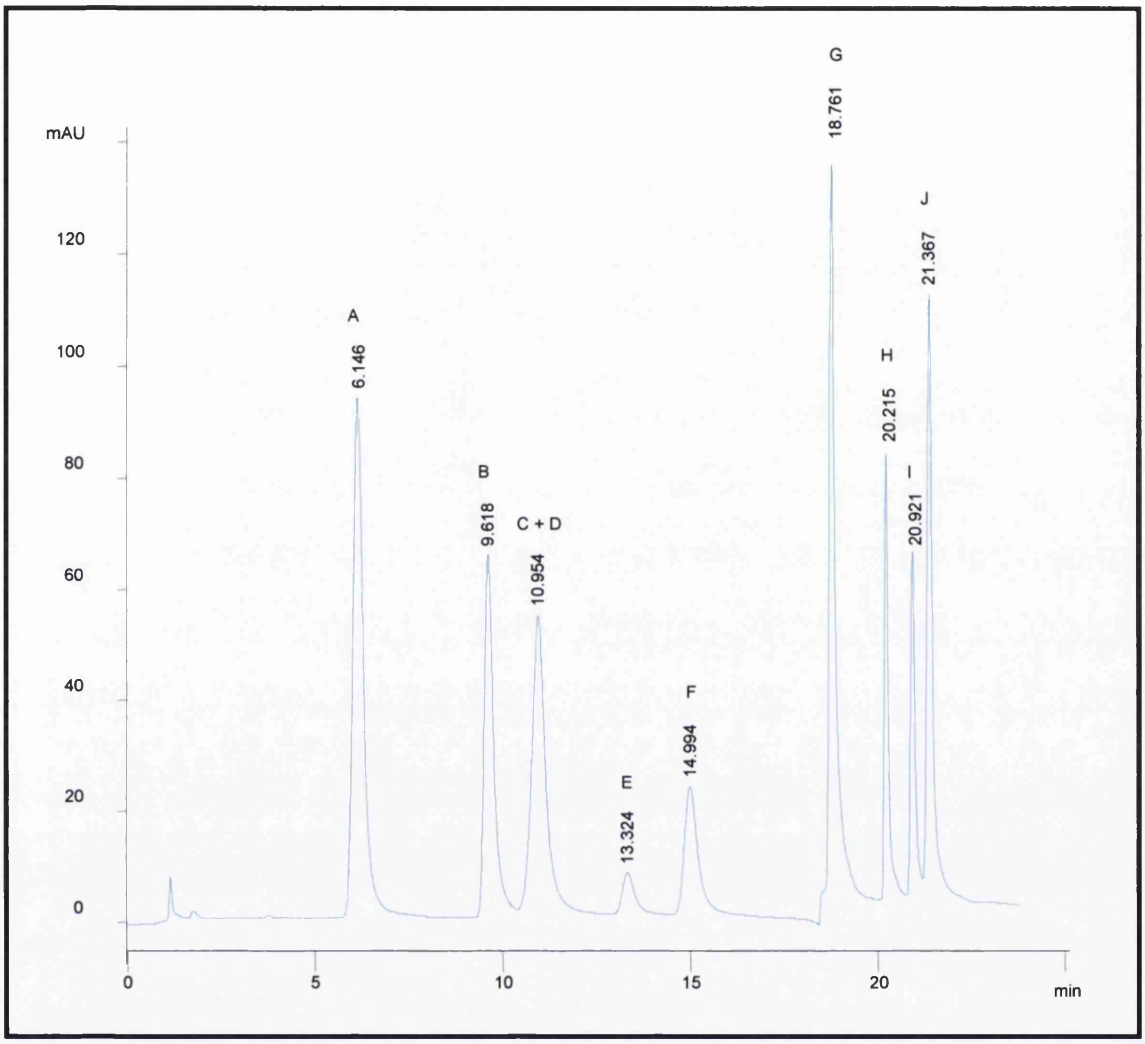



Figure 2.9 HPLC chromatogram of the separation of the 10 Herbicides at 0°C. 10 µL injection of a solution containing 10 µg/mL of each herbicide.

As can be seen from the chromatogram the problem of isoproturon and diuron co-eluting is solved by running the system at 0°C; however under these conditions chlortoluron and atrazine now co-elute. It was therefore decided to attempt to find a compromise between the two methods. This was achieved by running the chromatographic system at 18°C; the gradient set-up was altered to suit the new

conditions and also to reduce the run time of the separation. The following chromatographic conditions were used:

| Time in minutes | %$CH_3CN$ | %1mM $NH_4Oac$ |
|---|---|---|
| 0.00 | 34 | 66 |
| 11.5 | 34 | 66 |
| 12.0 | 66 | 34 |
| 18.0 | 66 | 34 |

Column Thermostat 18°C

Flow Rate 1.0mL/min

DAD 220nm

The following table gives the retention times for the compounds.

| Compound name | Peak label | RT minutes |
|---|---|---|
| Simazine | A | 6.042 |
| Methabenzthiazuron | B | 9.151 |
| Chlortoluron | C | 10.078 |
| Atrazine | D | 10.714 |
| Isoproturon | E | 12.598 |
| Diuron | F | 13.267 |
| Propazine | G | 14.848 |
| Linuron | H | 15.558 |
| Terbutryn | I | 16.418 |
| Trietazine | J | 16.795 |

Figure 2.10 shows the UV chromatogram of the separation of the 10 herbicides.

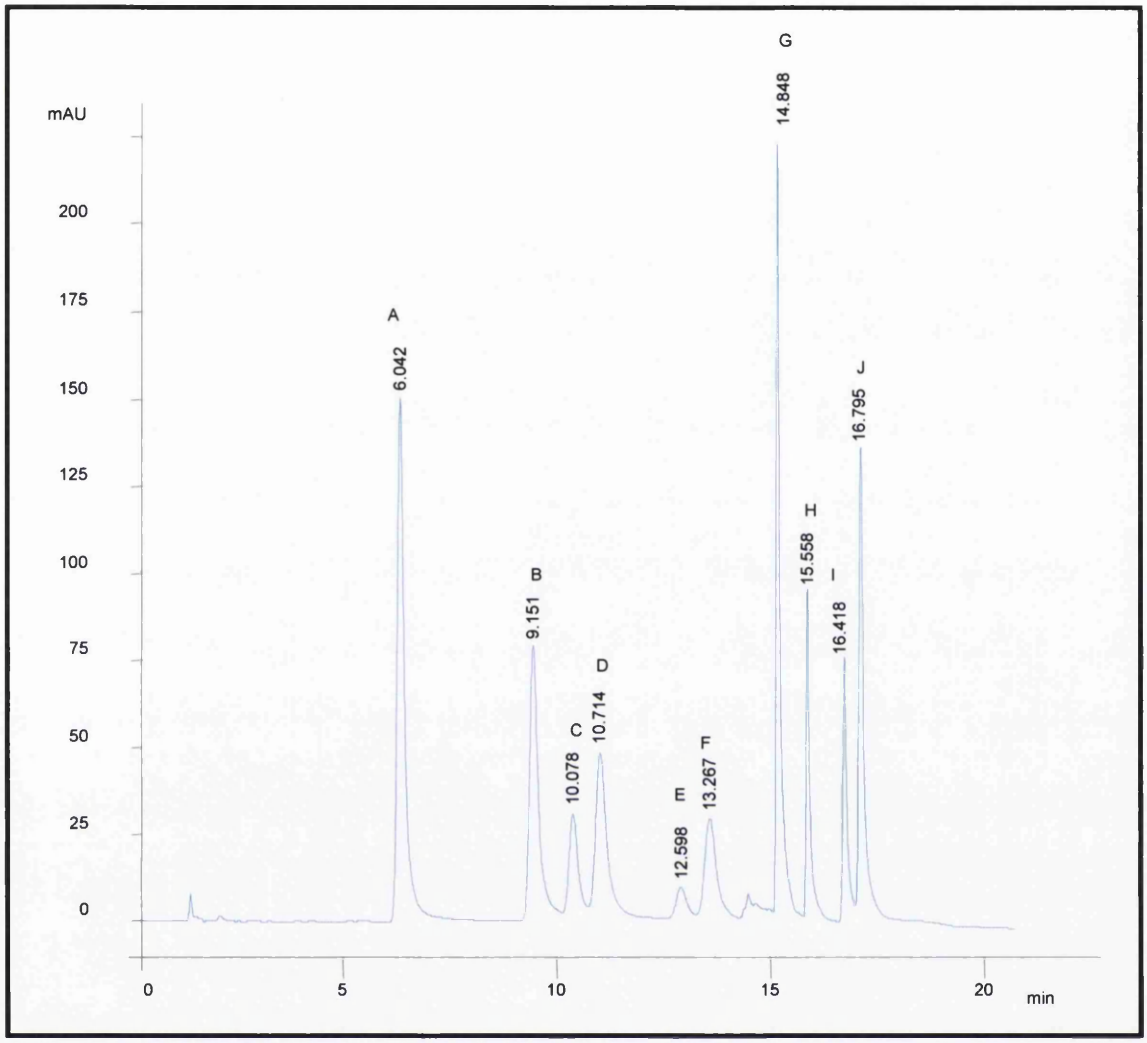



Figure 2.10 HPLC chromatogram of the separation of the 10 Herbicides at 18°C. 10 µL injection of a solution containing 10 µg/mL of each herbicide.

From the chromatogram it can be seen that we now have reasonably well resolved peaks within a run time of less than twenty minutes; however it was hoped that the analysis could be performed in less than 10 minutes. With this in mind it was decided to attempt the separation on a shorter column. The separation was transferred to a Phenomenex Luna 4.6x100mm 3µ C18 (2) column; it was found with the

Phenomenex C18 column that the presence of ammonium acetate was no longer required to aid the separation. The following chromatographic conditions were used:

| Time in minutes | %$CH_3CN$ | %$H_2O$ |
|---|---|---|
| 0.00 | 35 | 65 |
| 6.90 | 35 | 65 |
| 7.20 | 70 | 30 |
| 13.0 | 70 | 30 |

| | |
|---|---|
| Column Thermostat | 18°C |
| Flow Rate | 1.0mL/min |
| DAD | 220nm |

The following table gives the retention times for the compounds.

| Compound name | Peak label | RT minutes |
|---|---|---|
| Simazine | A | 4.787 |
| Methabenzthiazuron | B | 6.542 |
| Chlortoluron | C | 7.799 |
| Atrazine | D | 8.486 |
| Isoproturon | E | 9.524 |
| Diuron | F | 10.049 |
| Propazine | G | 10.734 |
| Linuron | H | 11.229 |
| Terbutryn | I | 11.822 |
| Trietazine | J | 12.053 |

Figure 2.11 shows the UV chromatogram of the separation of the 10 herbicides.

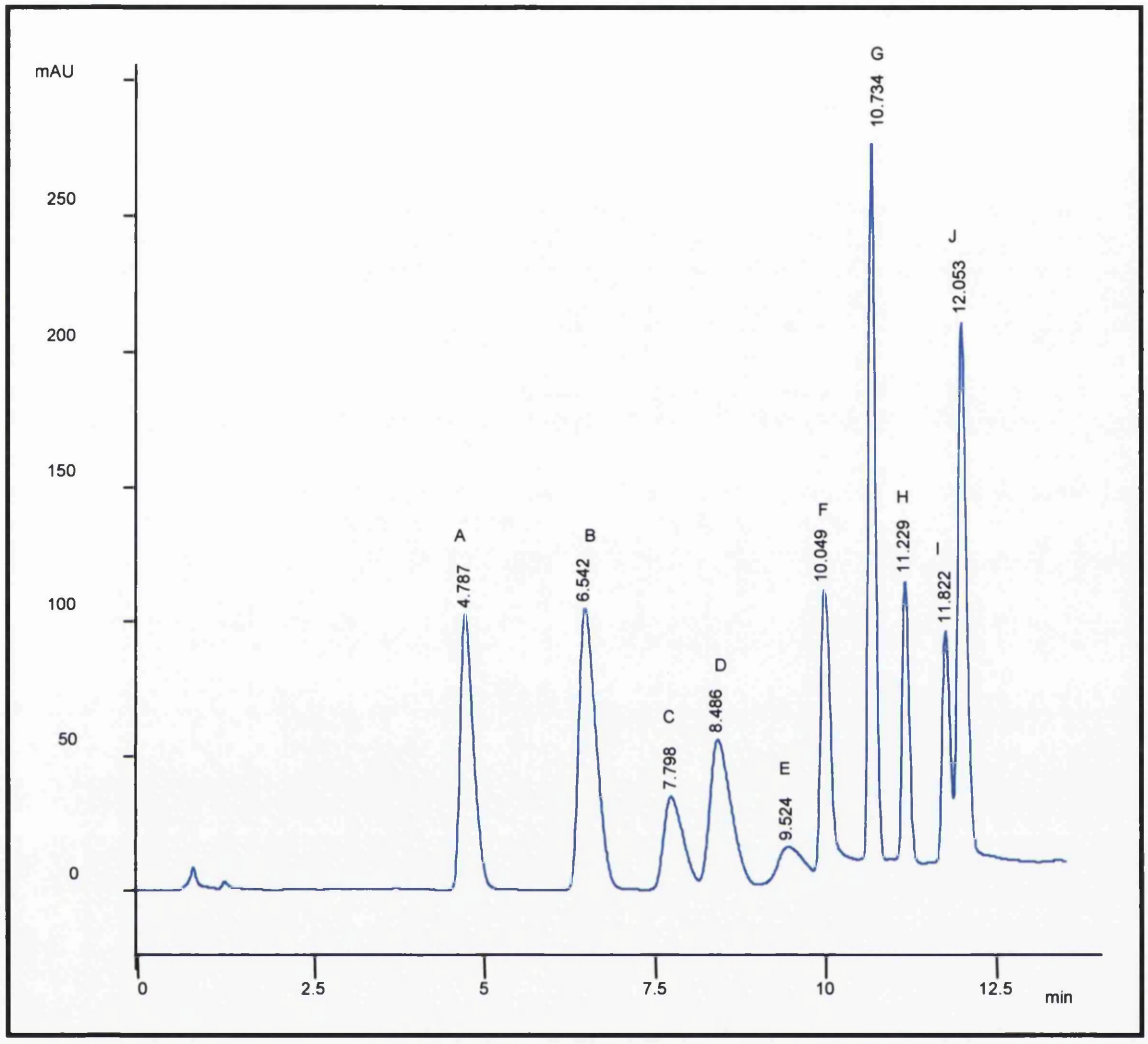



Figure 2.11 HPLC chromatogram of the separation of the 10 Herbicides on the Phenomenex C18 column. 10 µL injection of a solution containing 10 µg/mL of each herbicide.

Although the run time had now been reduced to less than thirteen minutes it was decided to transfer the separation to the Phenomenex Luna 4.6x100mm 3µ Phenyl-Hexyl column. It was expected that it would be possible to increase the strength of the mobile phase whilst still maintaining reasonable resolution of the peaks due to the ability of the phenyl-hexyl column to give greater resolving power when separating

aromatic compounds compared to a C18 column. The following chromatographic conditions were used:

| Time in minutes | %$CH_3CN$ | %$H_2O$ |
|---|---|---|
| 0.00 | 40 | 60 |
| 3.00 | 40 | 60 |
| 3.20 | 60 | 40 |
| 7.00 | 60 | 40 |

| | |
|---|---|
| Column Thermostat | Room Temperature |
| Flow Rate | 1.0mL/min |
| DAD | 220nm |

The following table gives the retention times for the compounds.

| Compound name | Peak label | RT minutes |
|---|---|---|
| Simazine | A | 3.327 |
| Methabenzthiazuron | B | 4.257 |
| Chlortoluron | C | 4.584 |
| Atrazine | D | 4.814 |
| Isoproturon | E | 5.071 |
| Diuron | F | 5.299 |
| Propazine | G | 5.740 |
| Linuron | H | 6.482 |
| Terbutryn | I | 6.774 |
| Trietazine | J | 6.934 |

Figure 2.12 shows the UV chromatogram of the separation of the 10 herbicides.

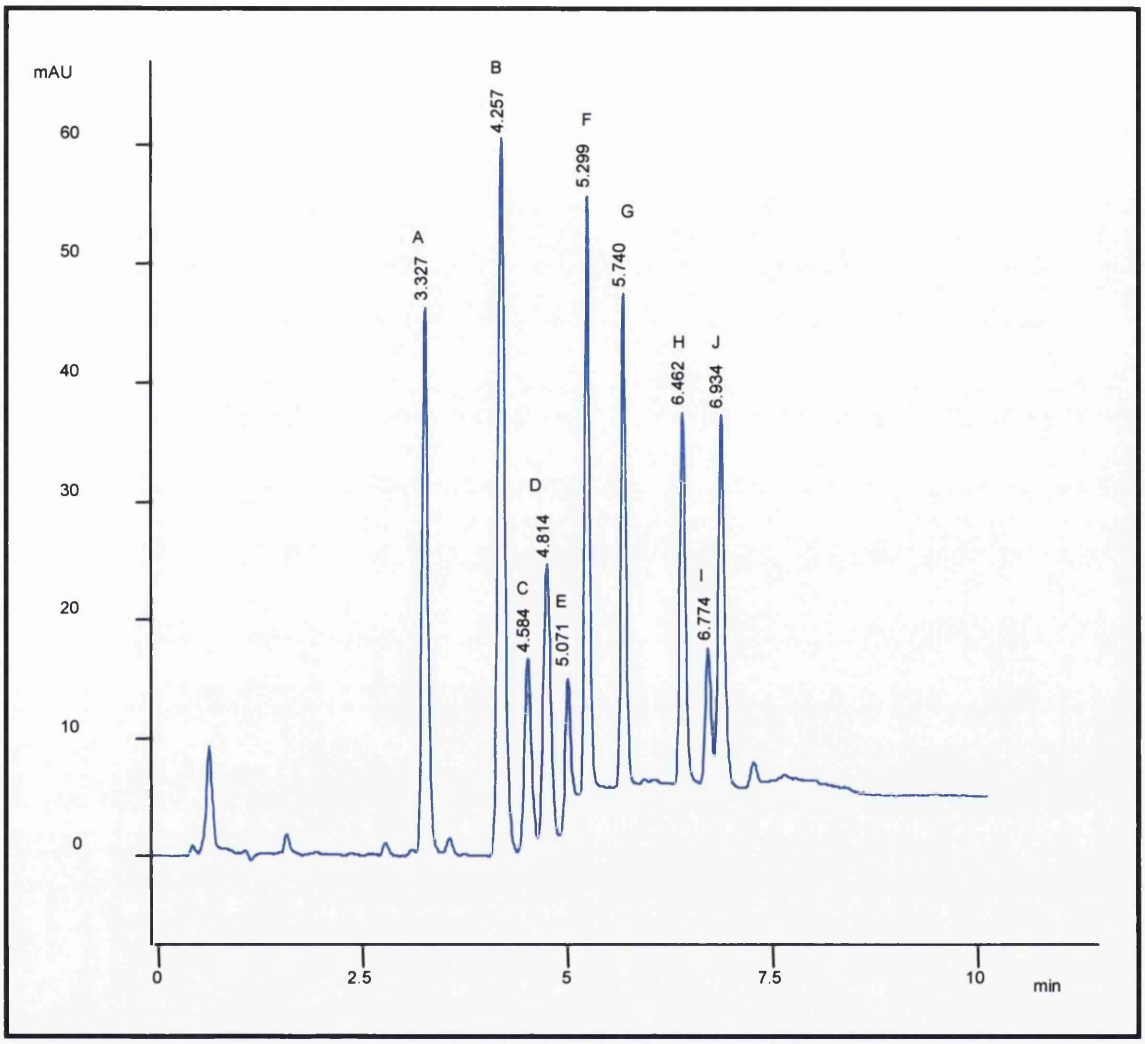



Figure 2.12 HPLC chromatogram of the separation of the 10 Herbicides on the Phenyl-Hexyl column. 10 µL injection of a solution containing 10 µg/mL of each herbicide.

Using the phenyl-hexyl column we were able to achieve a run time well within the ten minutes we were aiming for with adequate resolution of all the peaks of interest.

The following table gives the corresponding resolution values (Rs) for the separation shown above.

| Peaks | Rs Value |
|---|---|
| A+B | 4.67 |
| B+C | 1.33 |
| C+D | 1.25 |
| D+E | 1.25 |
| E+F | 1.71 |
| F+G | 2.86 |
| G+H | 3.75 |
| H+I | 1.75 |
| I+J | 1.00 |

## LC-MS For the Separation of the Mixture of the 5 Triazines and 5 Phenylurea Herbicides

The instrumentation and conditions used for LC-MS analysis of the ten herbicides was the same as that used in the LC-UV analysis performed using the phenyl-hexyl column. Mass spectrometric analysis was carried out using a Finnigan Mat LCQ ion trap; data was acquired in the positive ion-scanning mode.

Initial mass spectrometric analysis was carried out by using an infusion pump set to a flow rate of 5 to 10μL per minute to introduce standard solutions of each herbicide into the source; electrospray ionisation (ESI) was used to perform the analysis due to its suitability to low flow rates. From this analysis we were able to establish initial tune files for the compounds and identify m/z values associated with ionisation of the individual herbicides. LC-MS analysis was performed with both atmospheric pressure chemical ionisation (APCI) and ESI with the source conditions adjusted to suit the higher flow rates, below are the conditions used for both forms of ionisation.

**APCI**

| | |
|---|---|
| Polarity: Positive. | Sheath Gas Flow: 90psi. |
| Heated Capillary Temperature: 195°C. | Auxiliary Gas Flow: 50psi. |
| Electron Multiplier Voltage: 1150V. | Scan Range: 180-600amu. |
| Vaporiser Temperature: 450°C. | Corona Discharge Current: 5μ amps. |

**ESI**

Polarity: Positive.

Heated Capillary Temperature: 195°C.

Electron Multiplier Voltage: 1150V.

Needle Spray Voltage: 4.5kV.

Sheath Gas Flow: 60psi.

Auxiliary Gas Flow: 30psi.

Scan Range: 50-600amu.

For ESI analysis the flow from the HPLC system was split to allow approximately 200μL per minute into the ion source (a split of 20%).

It was found that both forms of ionisation yielded the same ions and hence the base ion remained the same with each technique. Figure 2.13 and 2.14 show comparisons of ESI and APCI mass spectra obtained for atrazine and isoproturon.

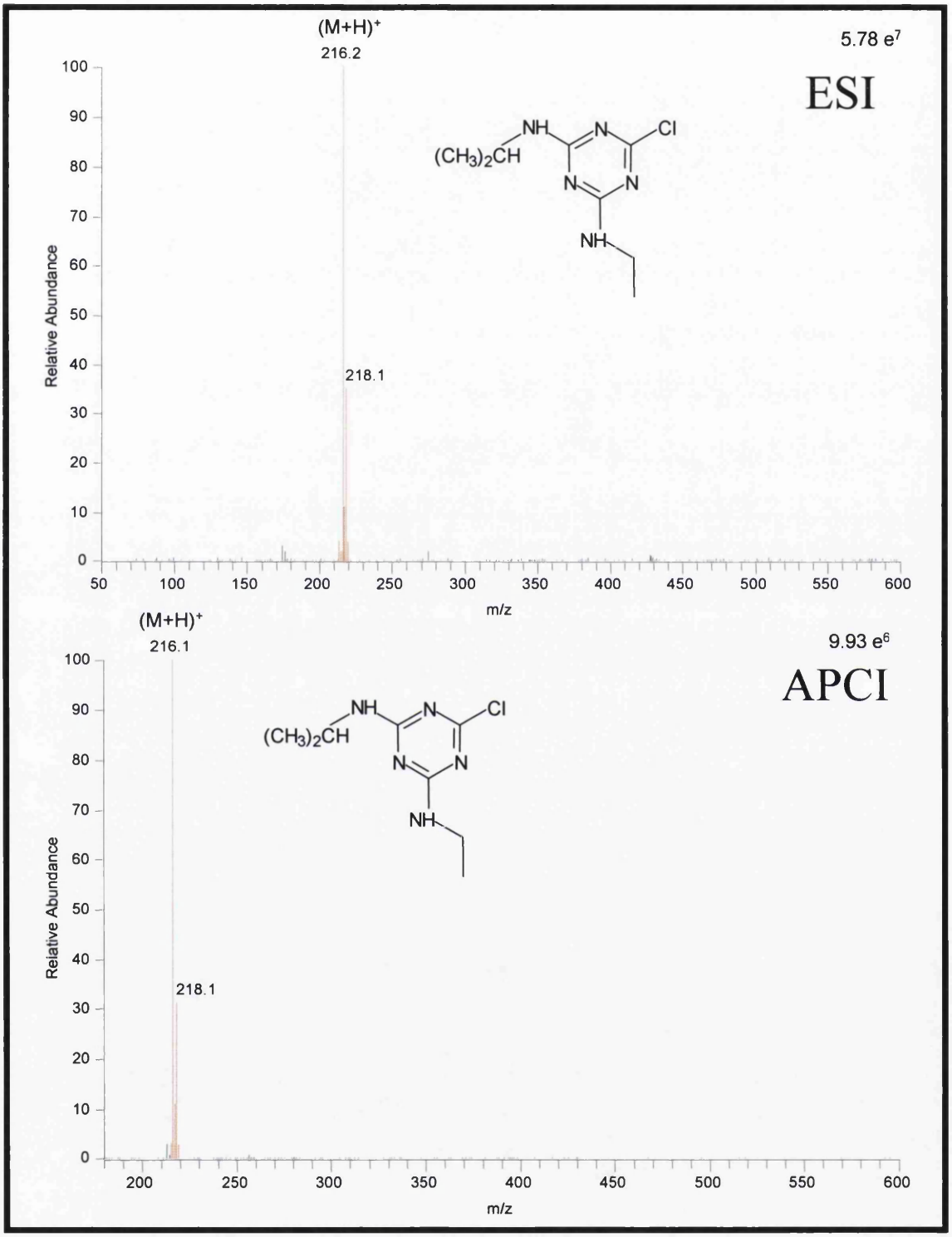



Figure 2.13 Positive ESI and APCI mass spectrum comparison of Atrazine. 10µL injection of a 10µg/mL solution; equal to 100ng on column.

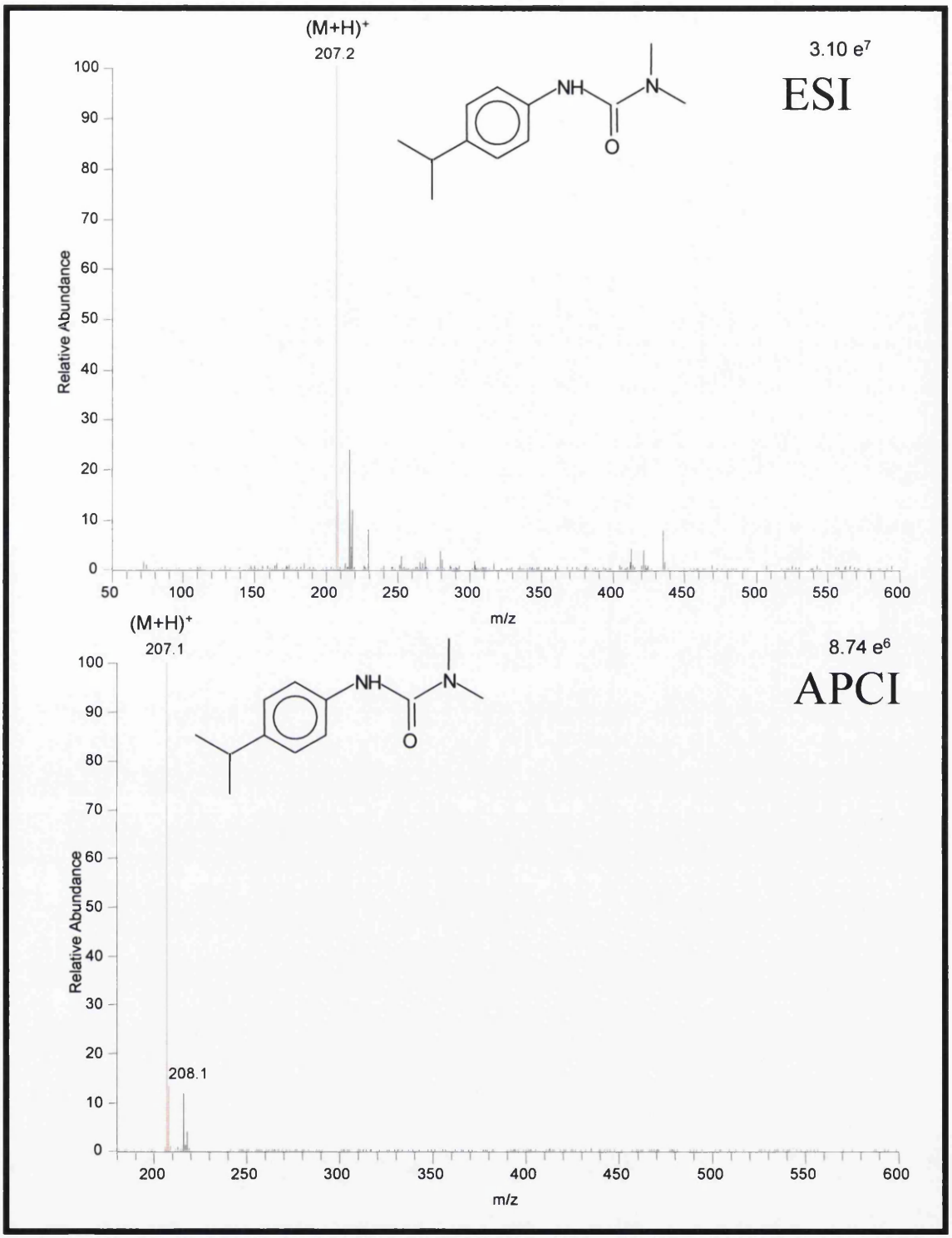



Figure 2.14 Positive ESI and APCI mass spectrum comparison of Isoproturon. 10µL injection of a 10µg/mL solution; equal to 100ng on column.

The difference between the scan ranges used in ESI and APCI is due to the presence of a high amount of background noise occurring below m/z 180 in the APCI spectra.

Figure 2.15 shows the total ion chromatogram (TIC) for the 10 separated herbicides using APCI and figure 2.16 the extracted ion chromatograms of the most abundant ion of each herbicide.

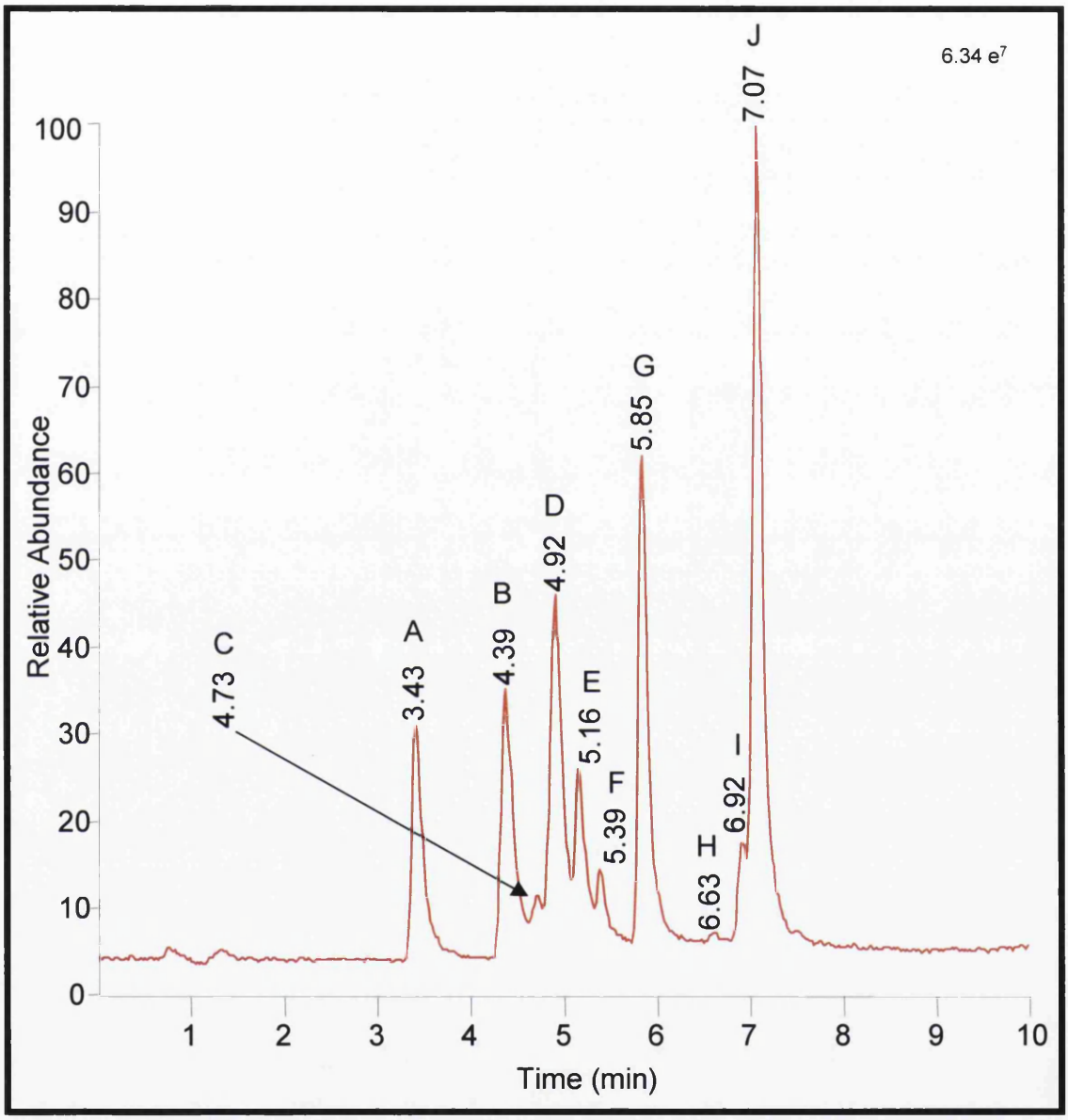



Figure 2.15 Positive LC-APCI-MS total ion chromatogram for the separation of the 10 herbicides. 10µL injection of a 10µg/mL solution of each herbicide; equal to 100ng on column of each.

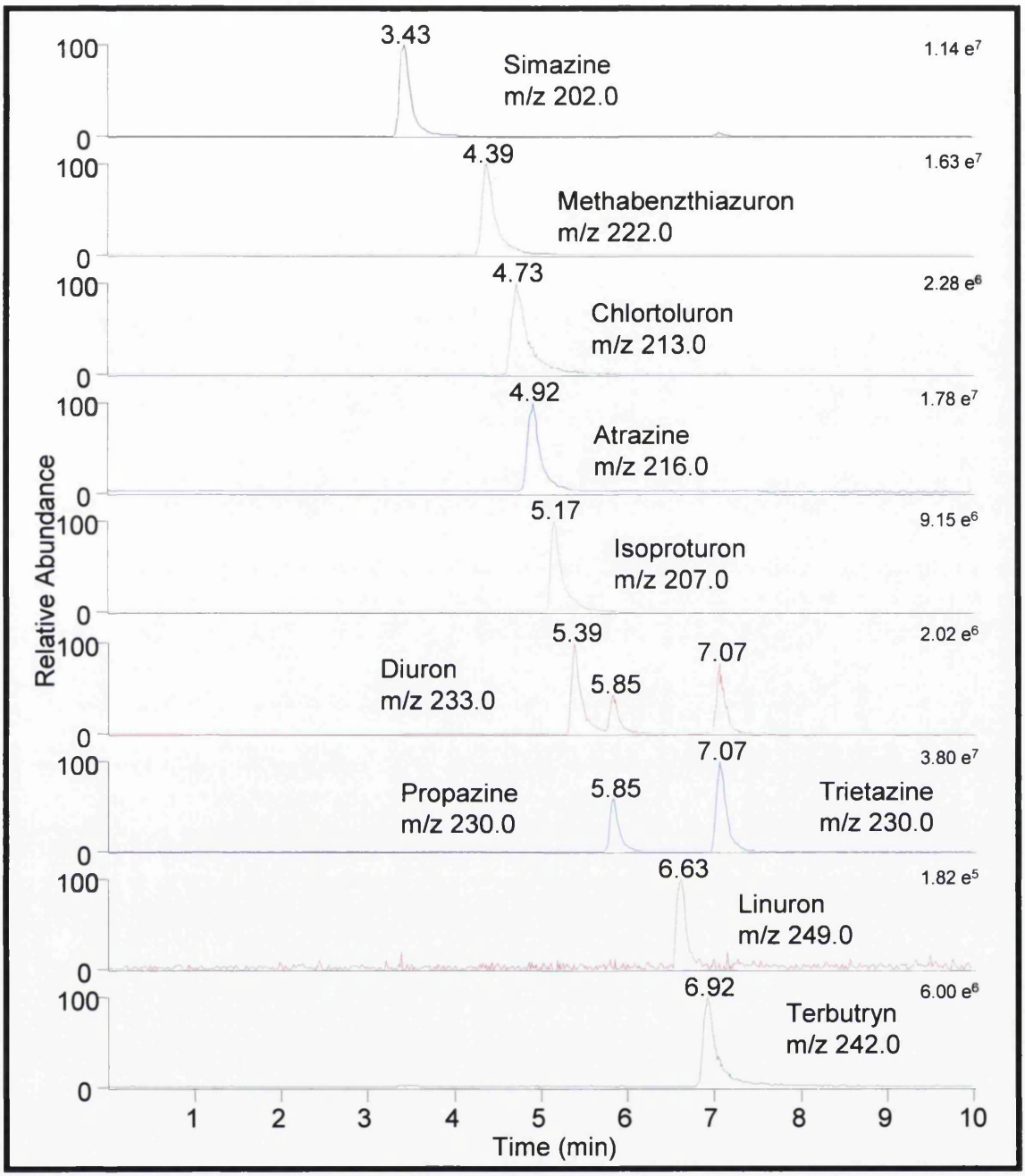



Figure 2.16 Extracted ion chromatograms of the base ion for each of the 10 herbicides. 10µL injection of a 10µg/mL solution of each herbicide; equal to 100ng on column of each.

The two peaks occurring in the extracted ion chromatogram of diuron, corresponding to the retention times of propazine and trietazine, are due to the presence of an ion of m/z 233 in the spectra of both these compounds. In the spectra of these compounds the ion occurring at m/z 230 is the most abundant, however the presence of isotopes at m/z 231, 232 and 233 can also be seen in their spectra. These are caused by the

presence of chlorine, giving rise to the 232 ion due to chlorine 37, and carbon 13 giving the isotope at 231 and also 233, which corresponds to the carbon 13 isotope of the 232 ion. Below is the mass spectrum of trietazine (figure 2.17), as can be seen the ratios of the various isotopes in relation to each other is as would be expected for the presence of chlorine within this compound. Figure 2.18 shows the predicted mass spectrum achieved using Micromass's Mass Lynx (version 4) for the empirical formula $C_9H_{17}N_5Cl$ which corresponds to both propazine and trietazine; as can be seen the ion abundances predicted using the software correspond well to the ratios seen in the actual mass spectrum.

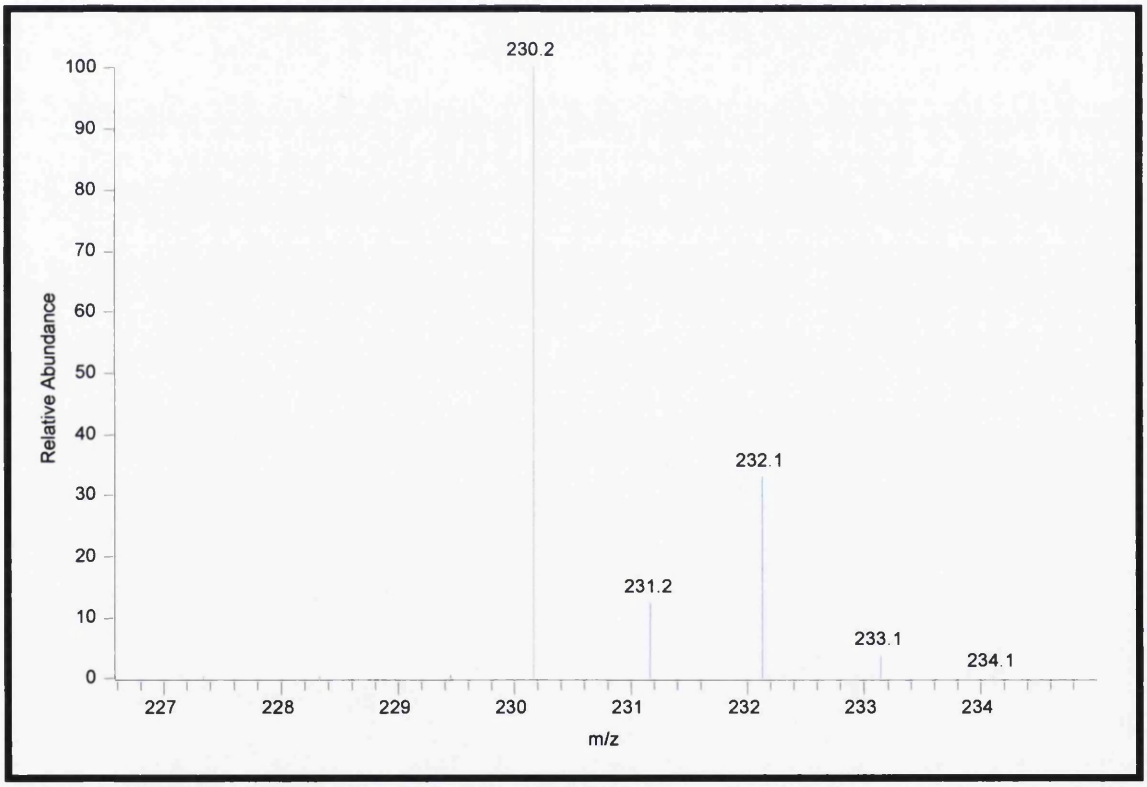



Figure 2.17 Positive APCI mass spectrum of Trietazine.

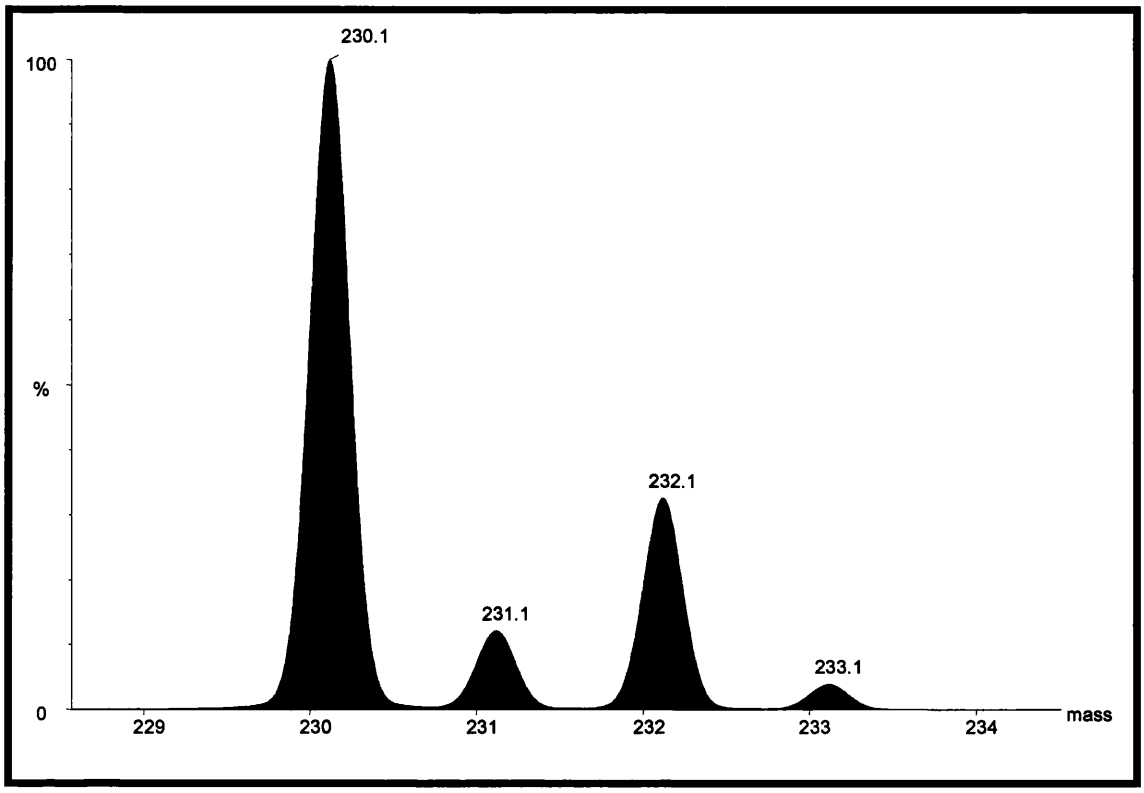



Figure 2.18 Mass Lynx predicted spectrum.

The table below lists the m/z used for the extracted ion chromatograms.

| Compound name | TIC Peak label | m/z |
|---|---|---|
| Simazine | A | 202.0 |
| Methabenzthiazuron | B | 222.0 |
| Chlortoluron | C | 213.0 |
| Atrazine | D | 216.0 |
| Isoproturon | E | 207.0 |
| Diuron | F | 233.0 |
| Propazine | G | 230.0 |
| Linuron | H | 249.0 |
| Terbutryn | I | 242.0 |
| Trietazine | J | 230.0 |

The following figures (2.19-2.28) show the mass spectrum, with ESI, for each of the herbicides and the proposed mass spectrum interpretation; the m/z values associated with ions other than the parent ion are assumed to be due to in source fragmentation

although this is not definitive and they may in fact be due to the presence of impurities in the standard materials.

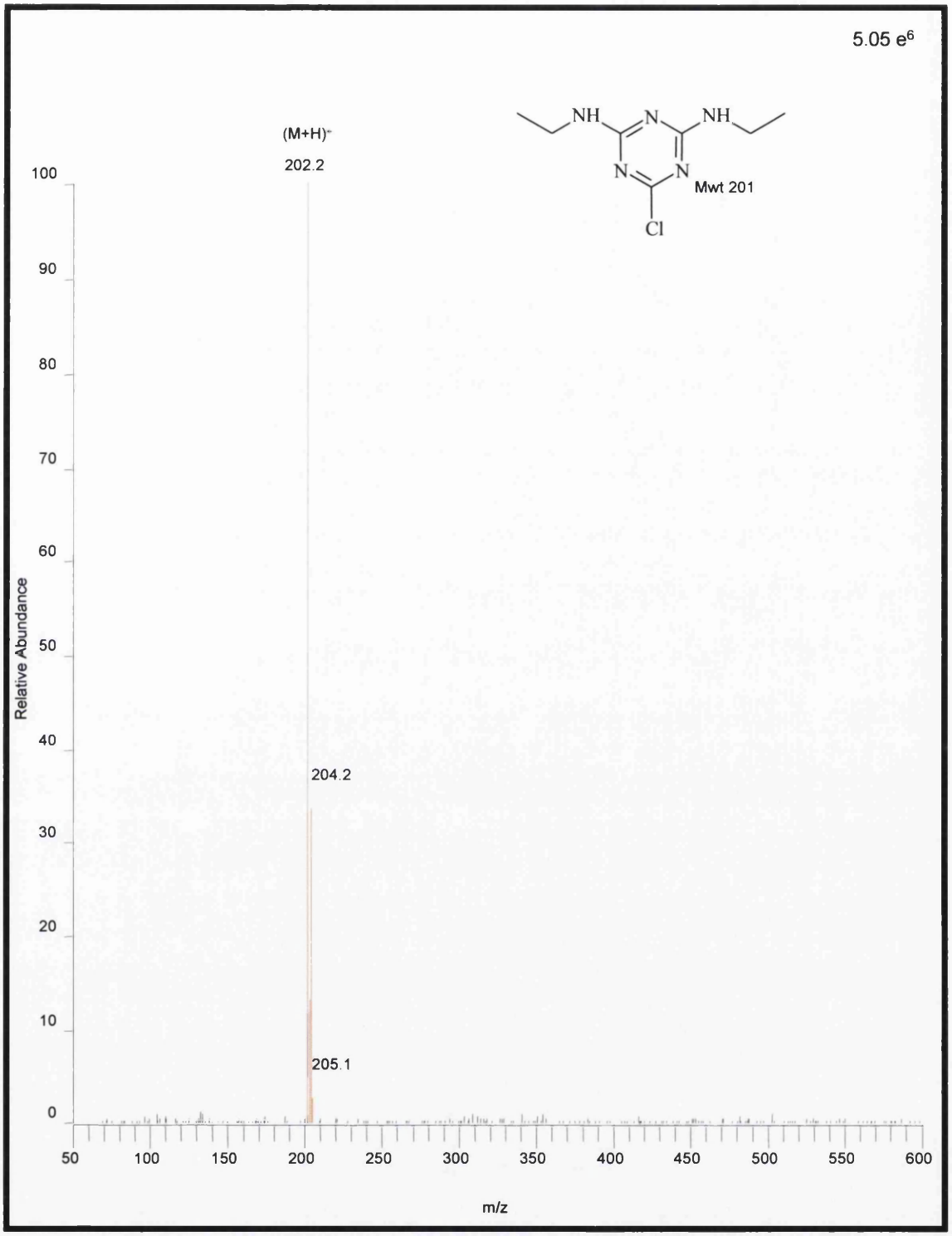



Figure 2.19 Positive LC-ESI-MS mass spectrum of Simazine. 10µL injection of a 1µg/mL solution; equal to 10ng on column.

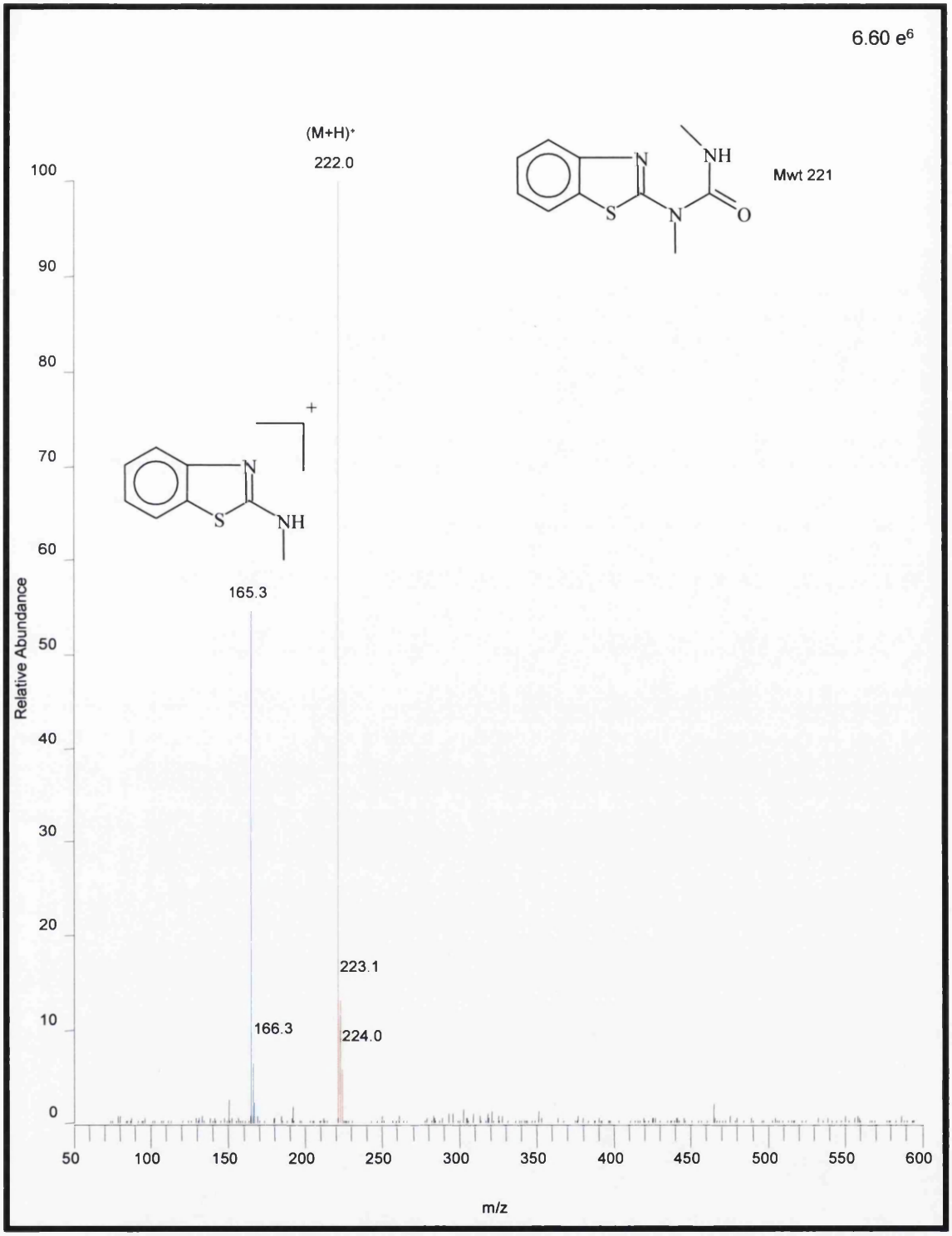



Figure 2.20 Positive LC-ESI-MS mass spectrum of Methabenzthiazuron. 10µL injection of a 1µg/mL solution; equal to 10ng on column.

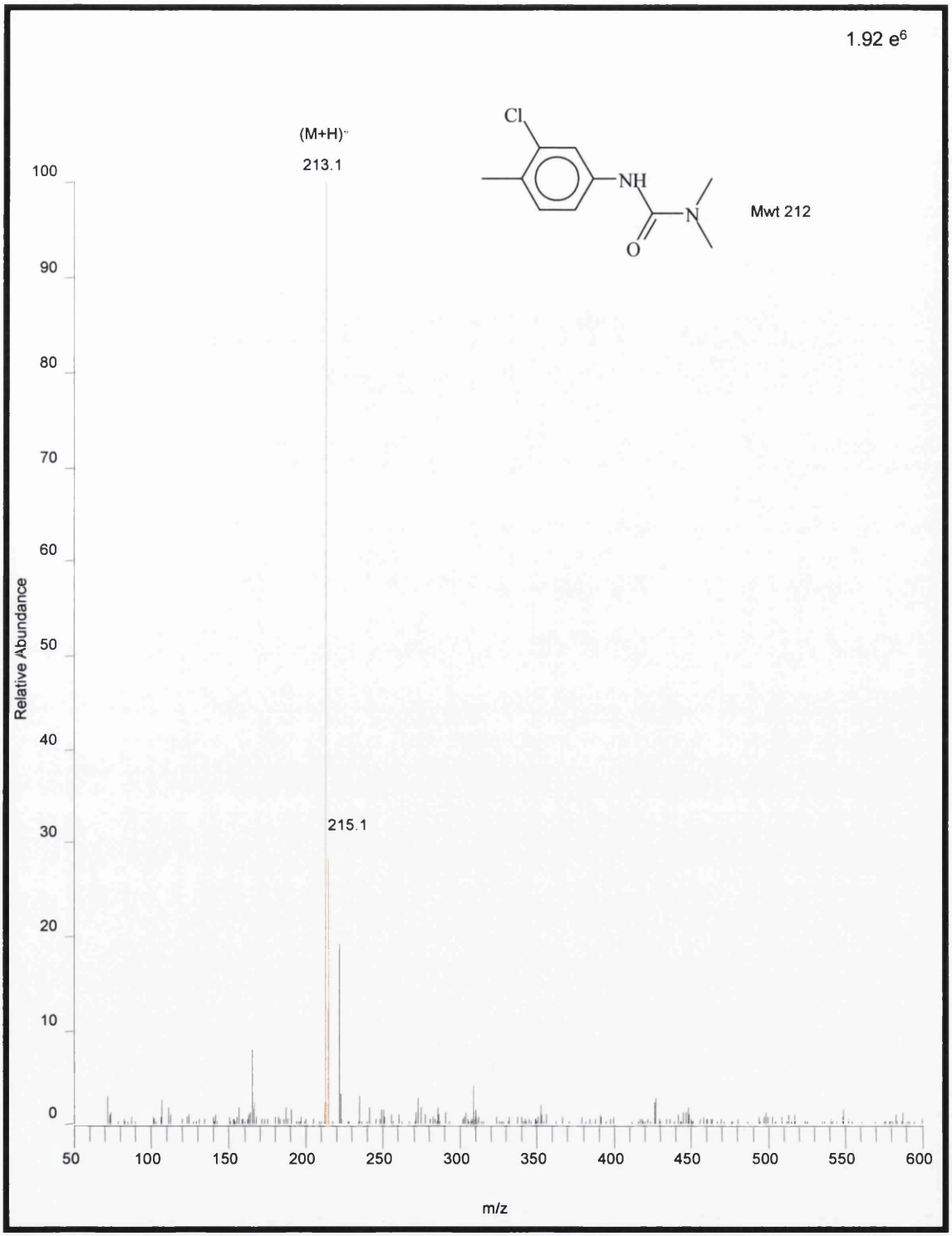



Figure 2.21 Positive LC-ESI-MS mass spectrum of Chlortoluron. 10µL injection of a 1µg/mL solution; equal to 10ng on column.

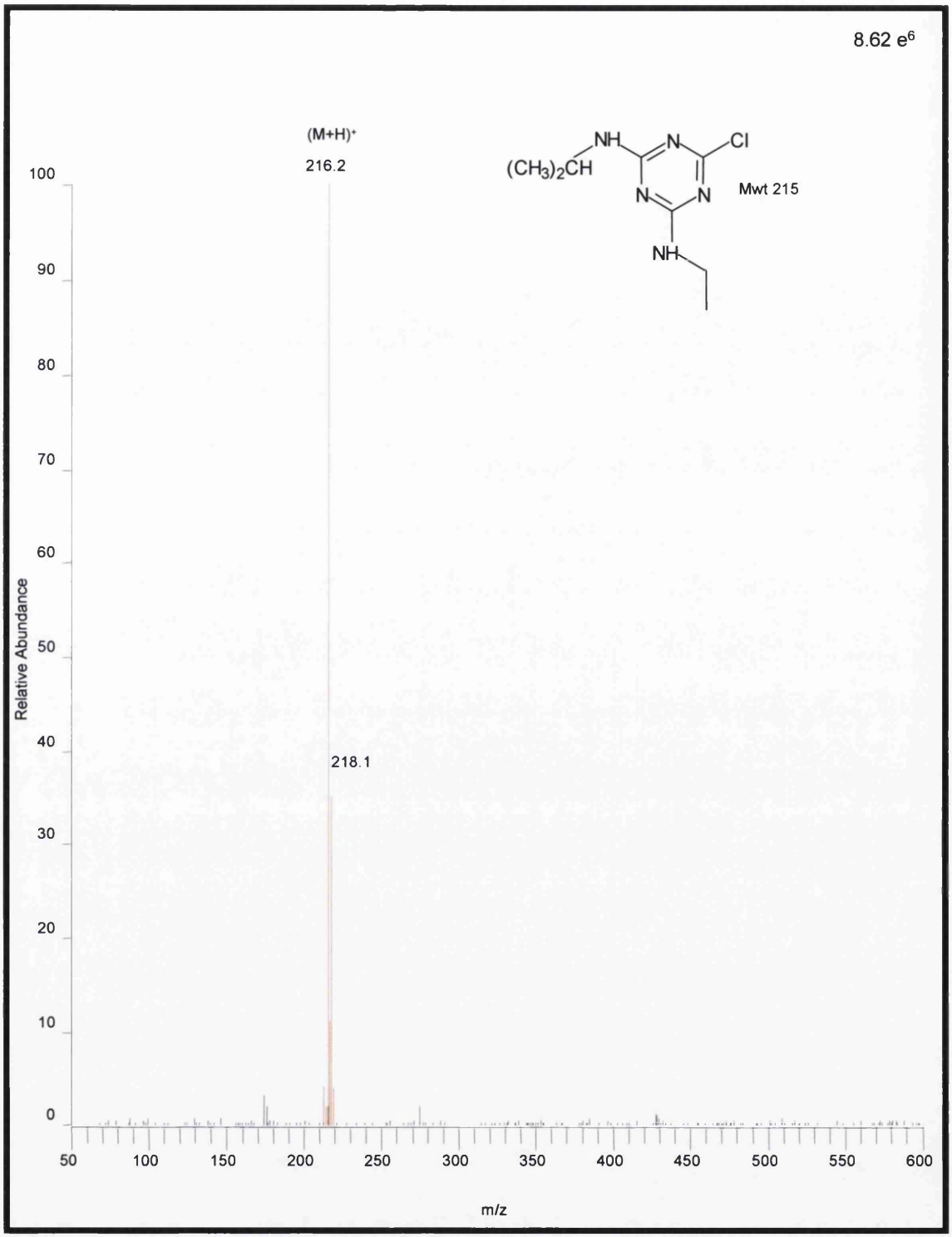



Figure 2.22 Positive LC-ESI-MS mass spectrum of Atrazine. 10µL injection of a 1µg/mL solution; equal to 10ng on column.

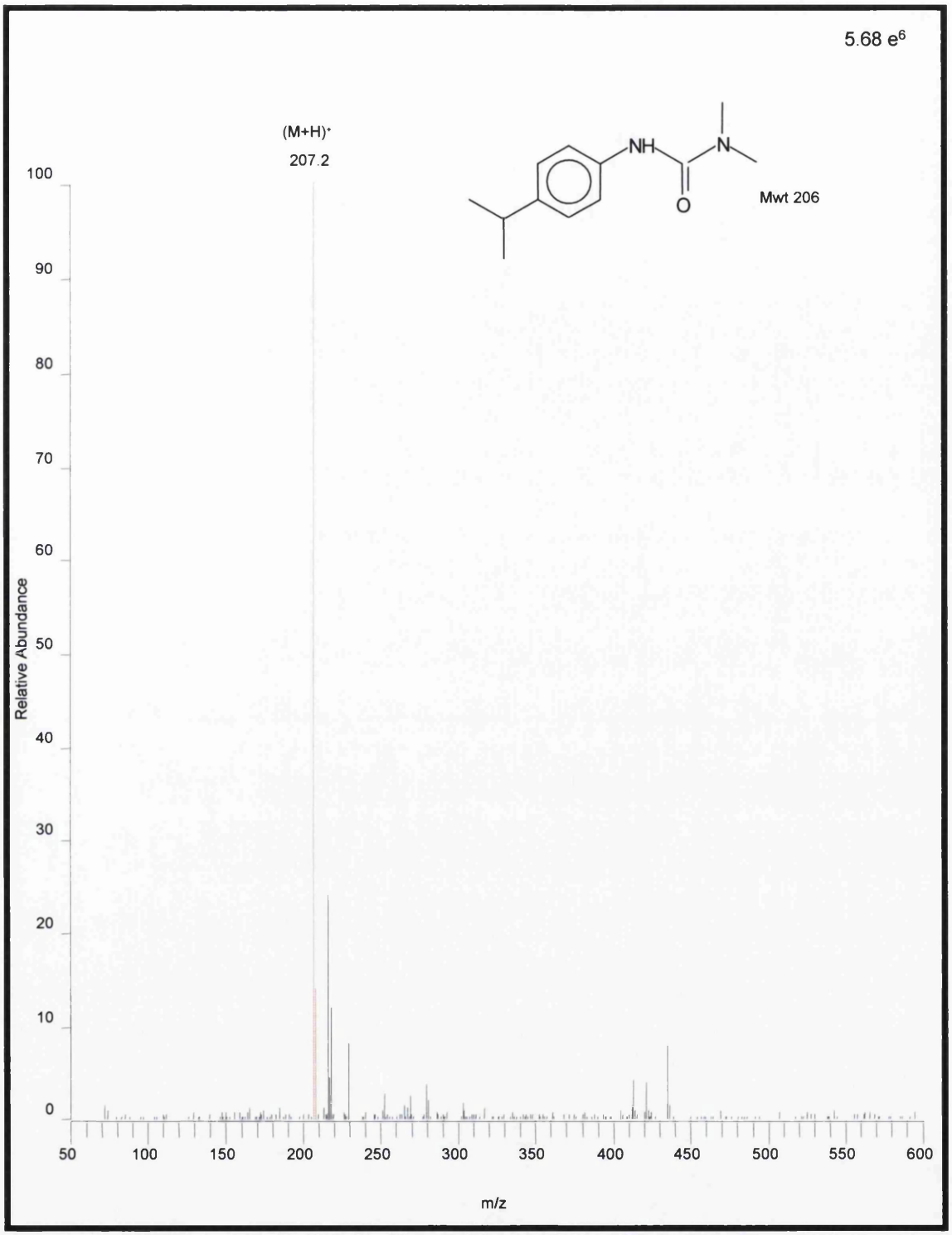



Figure 2.23 Positive LC-ESI-MS mass spectrum of Isoproturon. 10µL injection of a 1µg/mL solution; equal to 10ng on column.

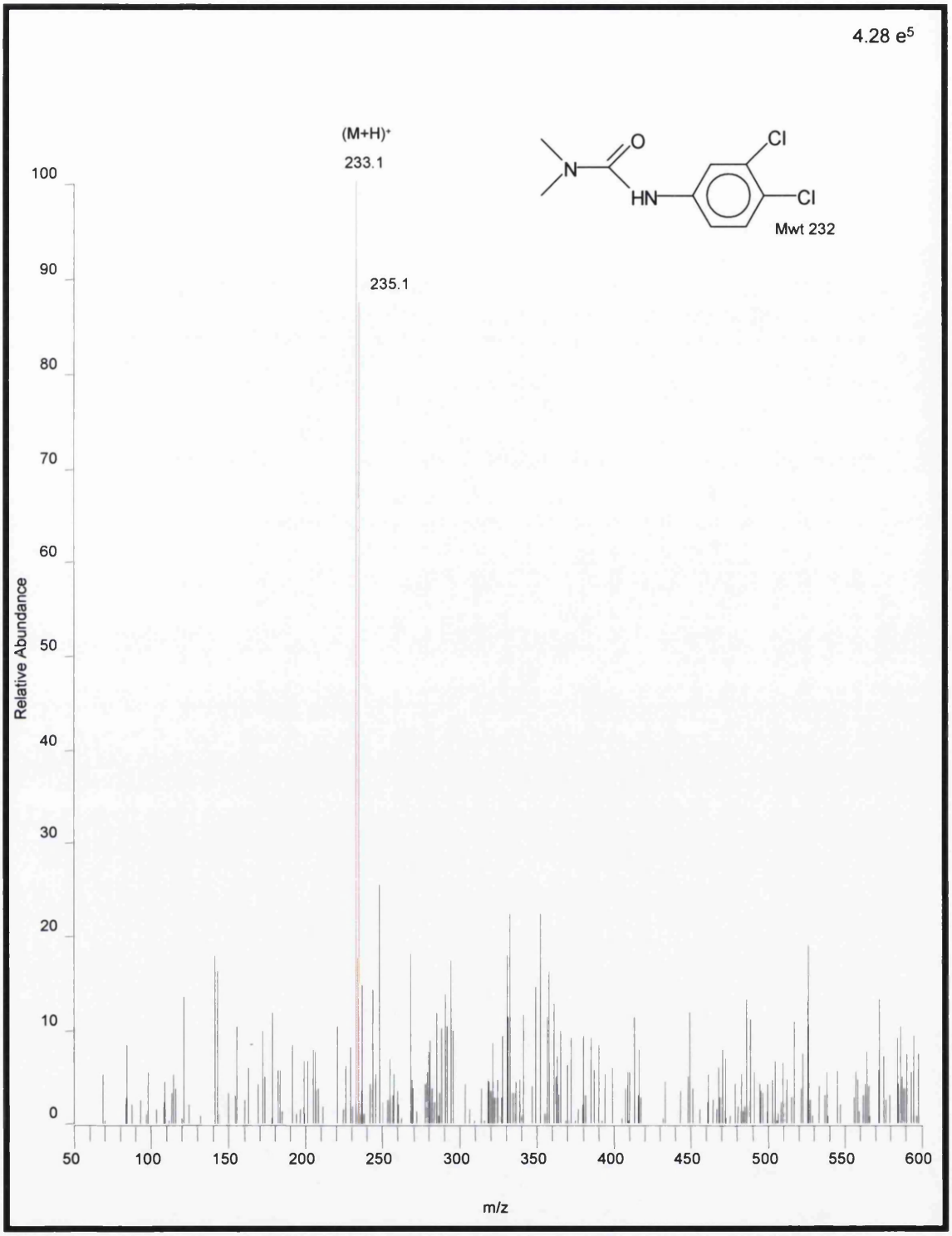



Figure 2.24 Positive LC-ESI-MS mass spectrum of Diuron. 10µL injection of a 1µg/mL solution; equal to 10ng on column.

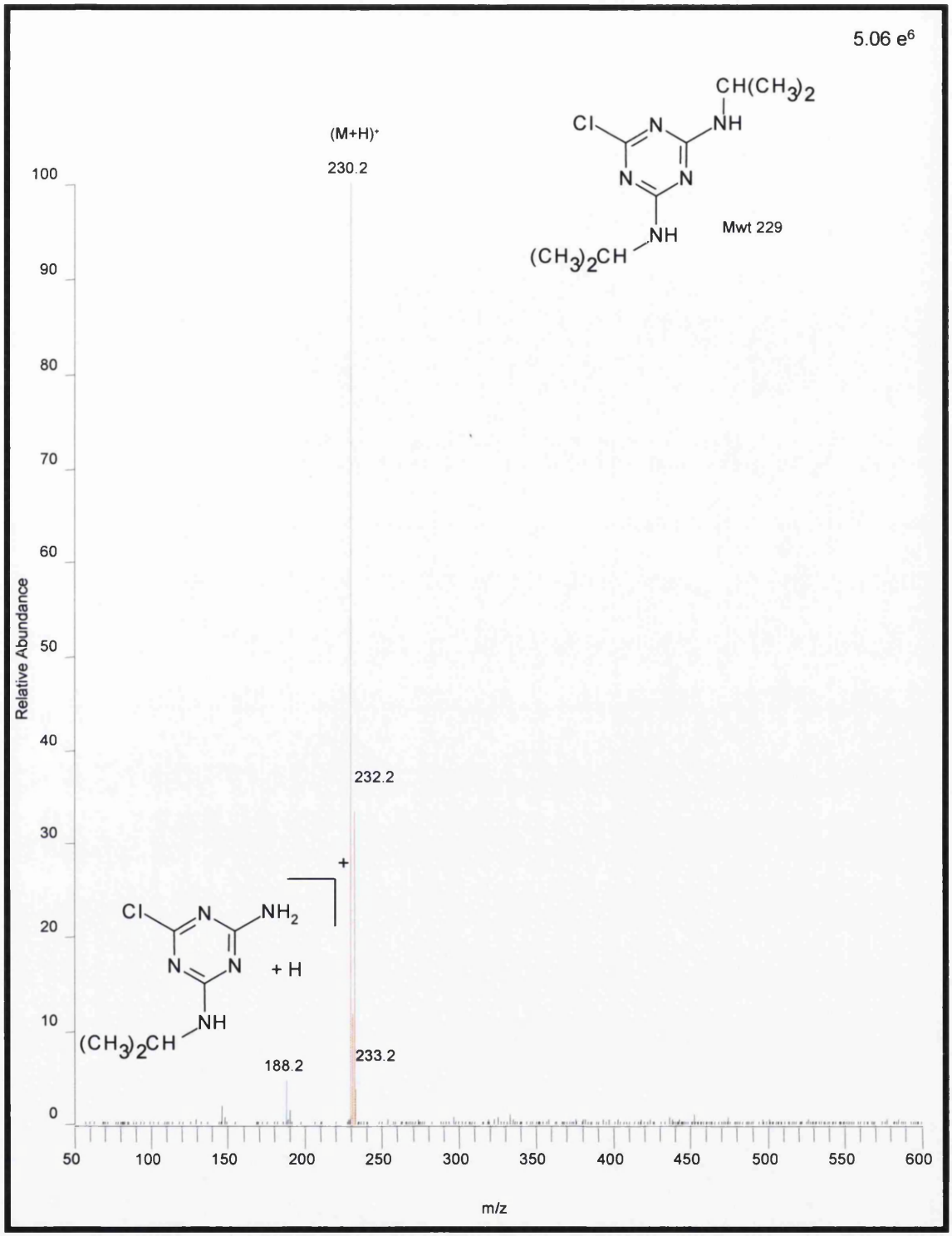



Figure 2.25 Positive LC-ESI-MS mass spectrum of Propazine. 10µL injection of a 1µg/mL solution; equal to 10ng on column.

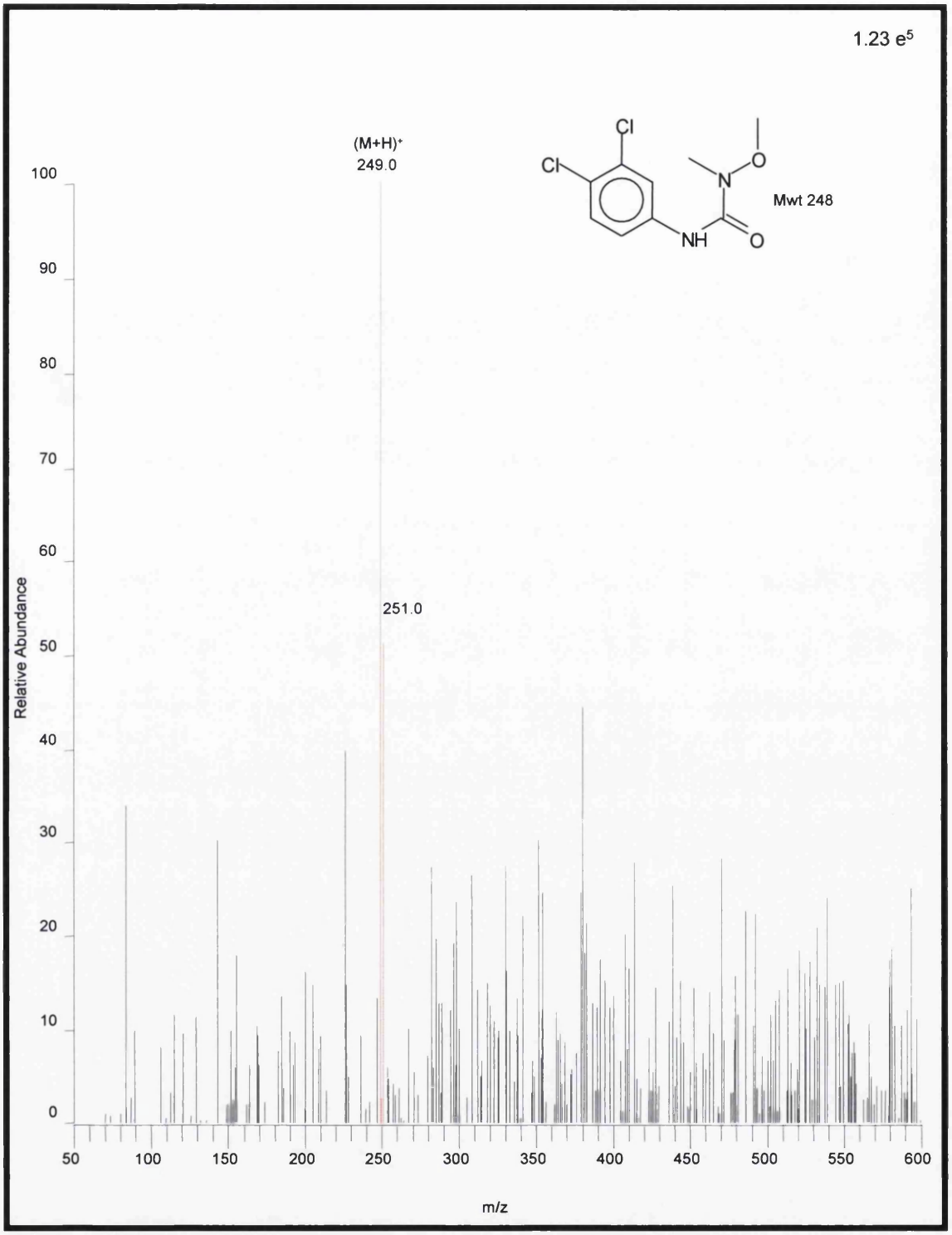



Figure 2.26 Positive LC-ESI-MS mass spectrum of Linuron. 10µL injection of a 1µg/mL solution; equal to 10ng on column.

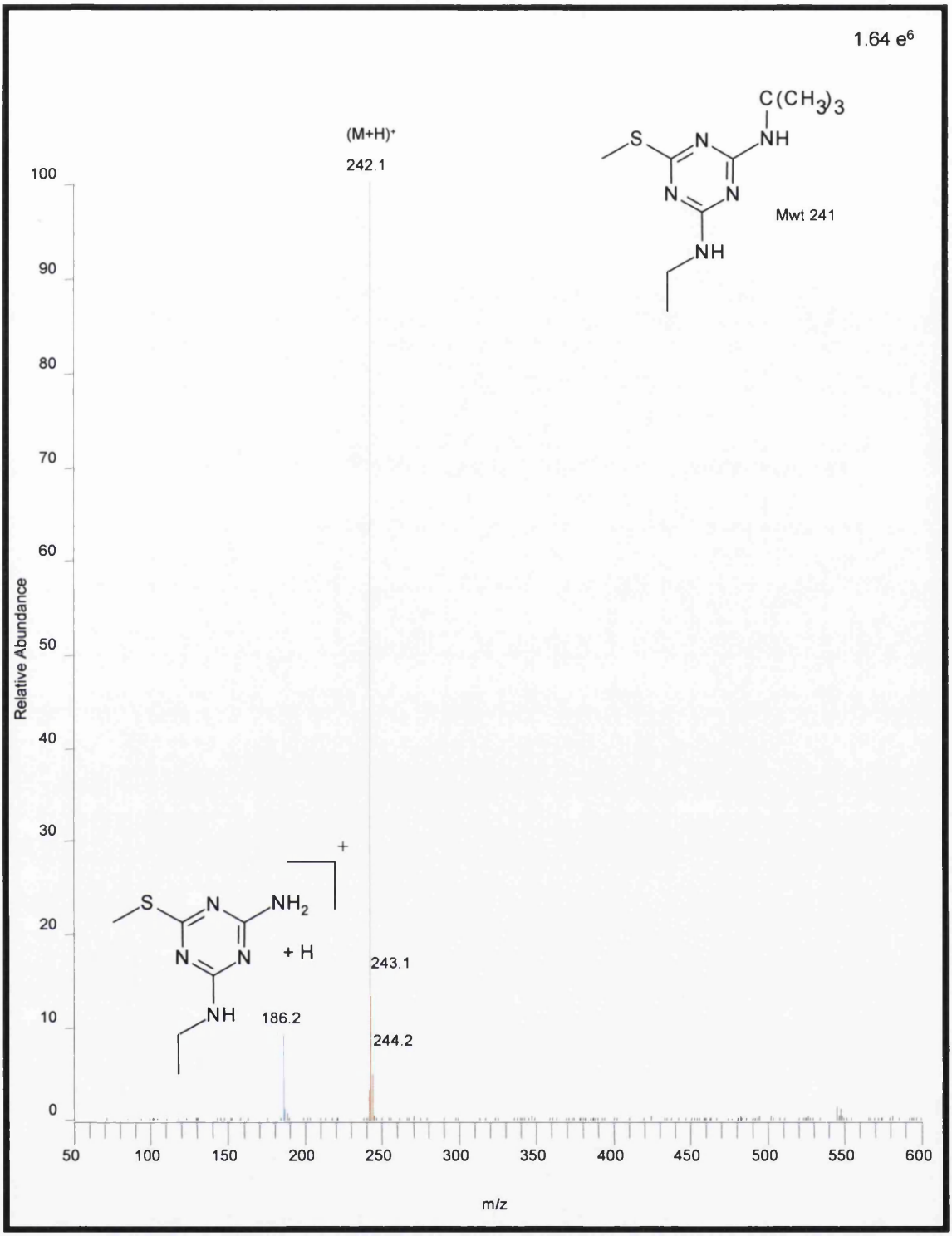



Figure 2.27 Positive LC-ESI-MS mass spectrum of Terbutryn. 10µL injection of a 1µg/mL solution; equal to 10ng on column.

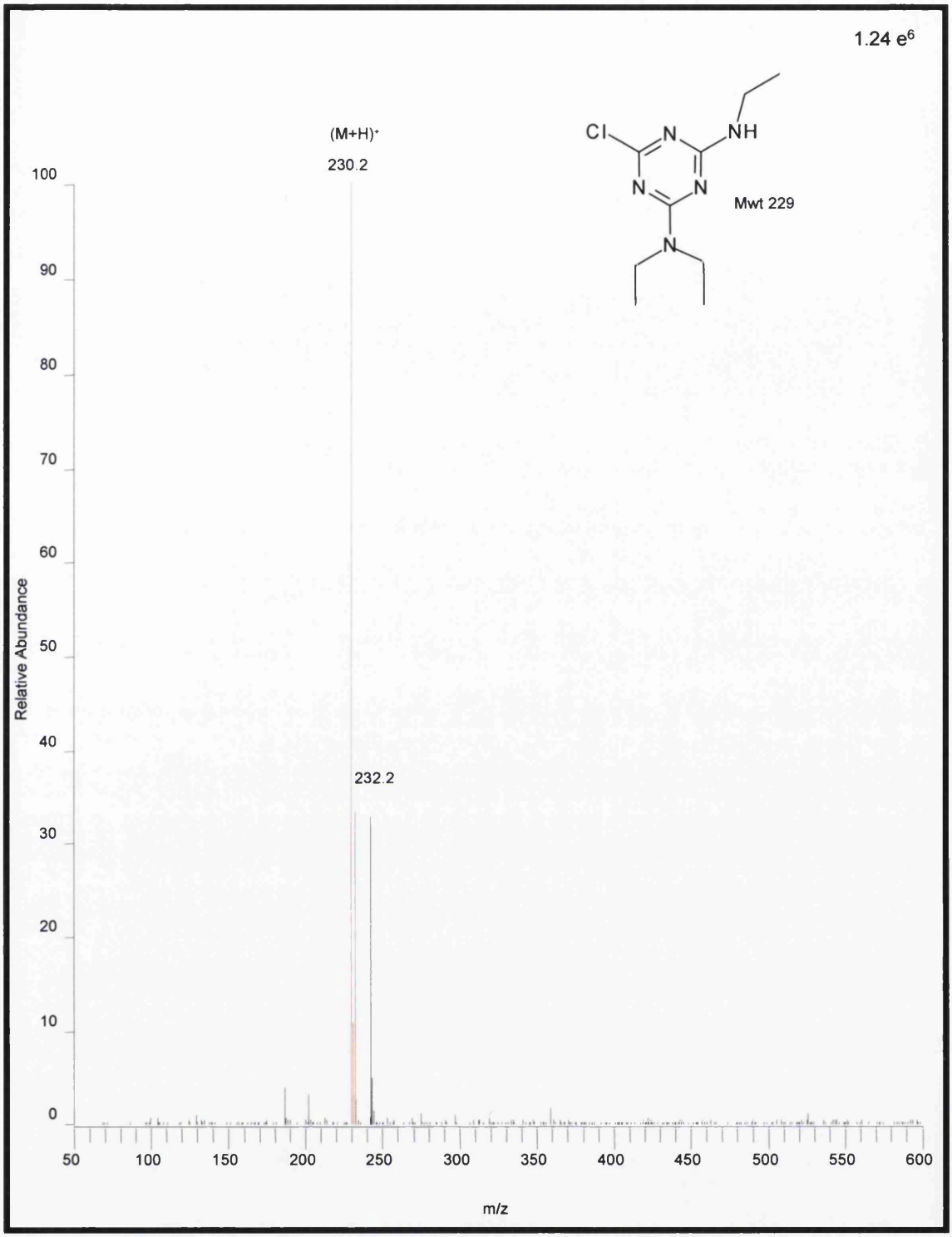



Figure 2.28 Positive LC-ESI-MS mass spectrum of Trietazine. 10µL injection of a 1µg/mL solution; equal to 10ng on column.

## Limits of Detection for the 10 Herbicides

Detection limits were obtained by taking extracted ion chromatograms of the m/z of the most abundant ion for each herbicide from the TIC. Limits of detection (LOD) were calculated by analysing a series of solutions at the following concentration levels; 0.1ng/mL, 1ng/mL, 10ng/mL, 100ng/mL and 1000ng/mL, the LOD was regarded as the concentration from this series at which a signal to noise ratio of at least 5 to 1 was achievable. The tables below show the LOD achieved with ESI and APCI; the amount of compound injected on column is also given.

**ESI**

| Compound name | m/z Monitored | LOD ESI | Amount on column |
|---|---|---|---|
| Simazine | 202.0 | 1ng/mL | 10pg |
| Methabenzthiazuron | 222.0 | 1ng/mL | 10pg |
| Chlortoluron | 213.0 | 10ng/mL | 100pg |
| Atrazine | 216.0 | 1ng/mL | 10pg |
| Isoproturon | 207.0 | 1ng/mL | 10pg |
| Diuron | 233.0 | 10ng/mL | 100pg |
| Propazine | 230.0 | 10ng/mL | 100pg |
| Linuron | 249.0 | 100ng/mL | 1ng |
| Terbutryn | 242.0 | 10ng/mL | 100pg |
| Trietazine | 230.0 | 10ng/mL | 100pg |

On column injection volume of 10μL. LOD is calculated as the concentration of the solution being injected.

**APCI**

| Compound name | m/z Monitored | LOD APCI | Amount on column |
|---|---|---|---|
| Simazine | 202.0 | 1ng/mL | 10pg |
| Methabenzthiazuron | 222.0 | 10ng/mL | 100pg |
| Chlortoluron | 213.0 | 1000ng/mL | 10ng |
| Atrazine | 216.0 | 10ng/mL | 100pg |
| Isoproturon | 207.0 | 10ng/mL | 100pg |
| Diuron | 233.0 | 1000ng/mL | 10ng |
| Propazine | 230.0 | 10ng/mL | 100pg |
| Linuron | 249.0 | 1000ng/mL | 10ng |
| Terbutryn | 242.0 | 10ng/mL | 100pg |
| Trietazine | 230.0 | 10ng/mL | 100pg |

On column injection volume of 10μL. LOD is calculated as the concentration of the solution being injected.

As can be seen from the table ESI was found to be more sensitive than APCI in the case of the majority of the 10 herbicides and as such was the chosen form of ionisation for $MS^n$ analysis. It was found with ESI that linuron was far less sensitive than the other herbicides, this may be due to the fact that linuron is an acid amide and is therefore acidic and so less easily protonated.

**Instrument Response**

The table below lists the instrument response in ESI-MS mode to a 10μL injection of a solution made up of a mixture of each of the herbicides at a concentration of 1μg/mL (the raw data has been presented earlier in this chapter in figures 2.19-2.28). From this data the response per nanogram on column of each herbicide has been calculated.

| Compound name | Instrument response | Response per ng on column |
|---|---|---|
| Simazine | 5.05 $e^6$ | 5.05 $e^5$ |
| Methabenzthiazuron | 6.60 $e^6$ | 6.60 $e^5$ |
| Chlortoluron | 1.92 $e^6$ | 1.92 $e^5$ |
| Atrazine | 8.62 $e^6$ | 8.62 $e^5$ |
| Isoproturon | 5.68 $e^6$ | 5.68 $e^5$ |
| Diuron | 4.28 $e^5$ | 4.28 $e^4$ |
| Propazine | 5.06 $e^6$ | 5.06 $e^5$ |
| Linuron | 1.23 $e^5$ | 1.23 $e^4$ |
| Terbutryn | 1.64 $e^6$ | 1.64 $e^5$ |
| Trietazine | 1.24 $e^6$ | 1.24 $e^5$ |

The response per nanogram on column could be used to give an indication of the amount of a herbicide present in an unknown sample; however without the use of an internal standard its value is limited.

## LC-ESI-MS$^n$ For the Separation of the Mixture of 5 Triazine and 5 Phenylurea Herbicides

Structural information was gained by the fragmentation of each of the most abundant ions obtained from LC-ESI-MS. This gives a specific method of identification for each of the herbicides for on-line analysis. $MS^2$ analysis was performed for all the herbicides and where possible $MS^3$ analysis also. The following table shows the precursor and product ions and the relative collision energies required to induce fragmentation.

| Compound name | Precursor ion | CE % | Product ions $MS^2$ | CE% | Product ions $MS^3$ |
|---|---|---|---|---|---|
| Simazine | 202.0 | 16.1 | 174.1, 166.2, 132.0, 124.0* | 11.1 | 96.0,68.2 |
| Methabenzthiazuron | 222.0 | 12.1 | 165.2 | | |
| Chlortoluron | 213.0 | 13.1 | 72.0 | | |
| Atrazine | 216.0 | 16.1 | 174.1 | 14.1 | 146.1, 138.1, 132.0, 96.0, 79.1 |
| Isoproturon | 207.0 | 14.1 | 165.1*, 72.1 | 12.1 | 120.1, 72.1 |
| Diuron | 233.0 | 14.1 | 72.1 | | |
| Propazine | 230.0 | 16.1 | 188.1*, 146.2 | 14.1 | 146.1 |
| Linuron | 249.0 | 13.1 | 187.9, 182.1*, 160.2 | 14.1 | 181.4, 165.4, 152.9 |
| Terbutryn | 242.0 | 16.1 | 186.2 | 15.1 | 158.2, 138.1, 91.1 |
| Trietazine | 230.0 | 17.1 | 202.2*, 132.1 | 16.1 | 174.2, 166.1, 132.0, 124.1 |

The * signifies the ion produced from $MS^2$ that was then used to conduct $MS^3$ analysis.

The following figures (2.29-2.55) show the LC-ESI-$MS^n$ mass spectra for each herbicide and the proposed fragmentation pattern interpretation.

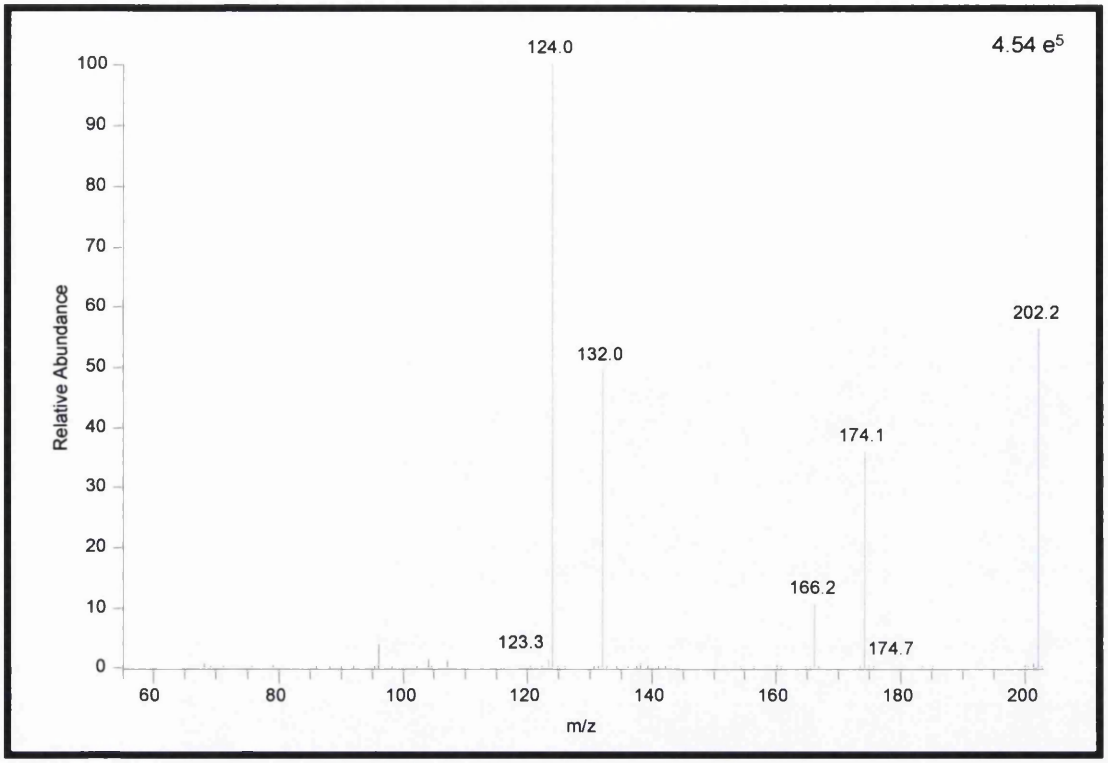



Figure 2.29 Positive LC-ESI-MS-MS spectrum of Simazine. 10µL injection of a 1µg/mL solution; equal to 10ng on column.

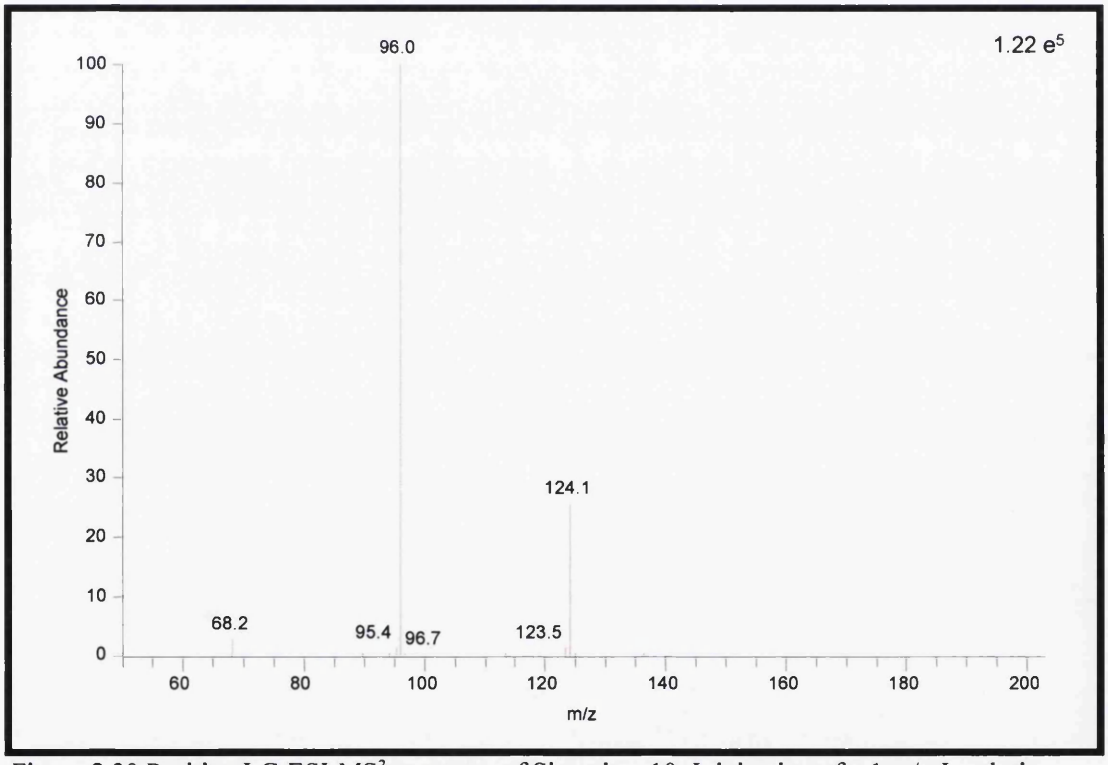



Figure 2.30 Positive LC-ESI-MS$^3$ spectrum of Simazine. 10µL injection of a 1µg/mL solution; equal to 10ng on column.

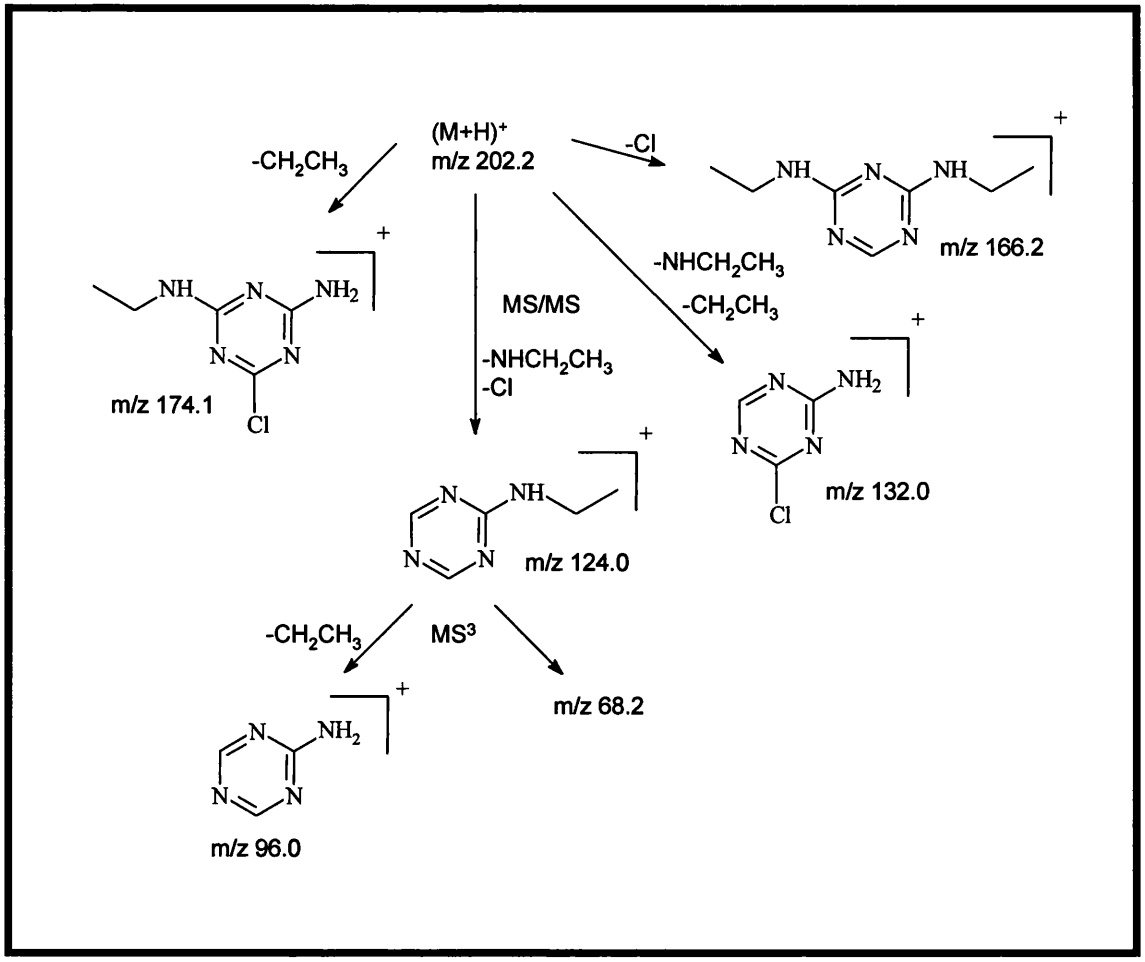



Figure 2.31 Proposed simazine fragmentation pattern interpretation.

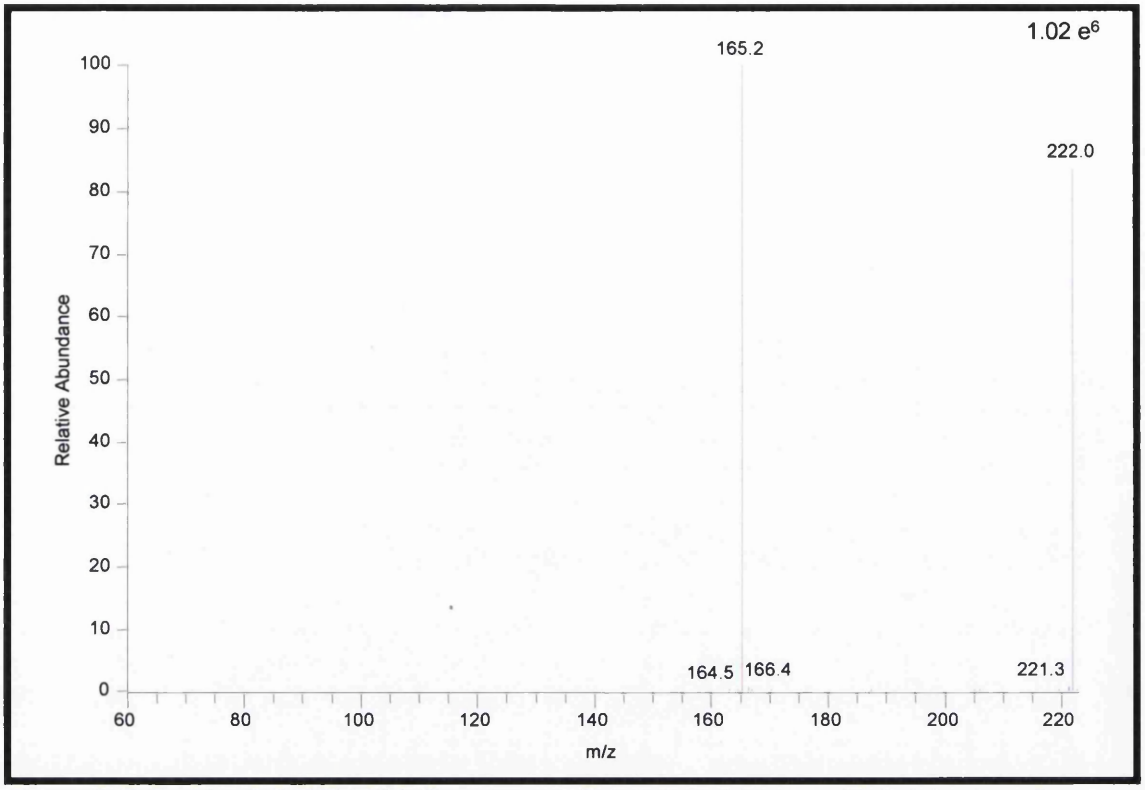



Figure 2.32 Positive LC-ESI-MS-MS spectrum of Methabenzthiazuron. 10µL injection of a 1µg/mL solution; equal to 10ng on column.

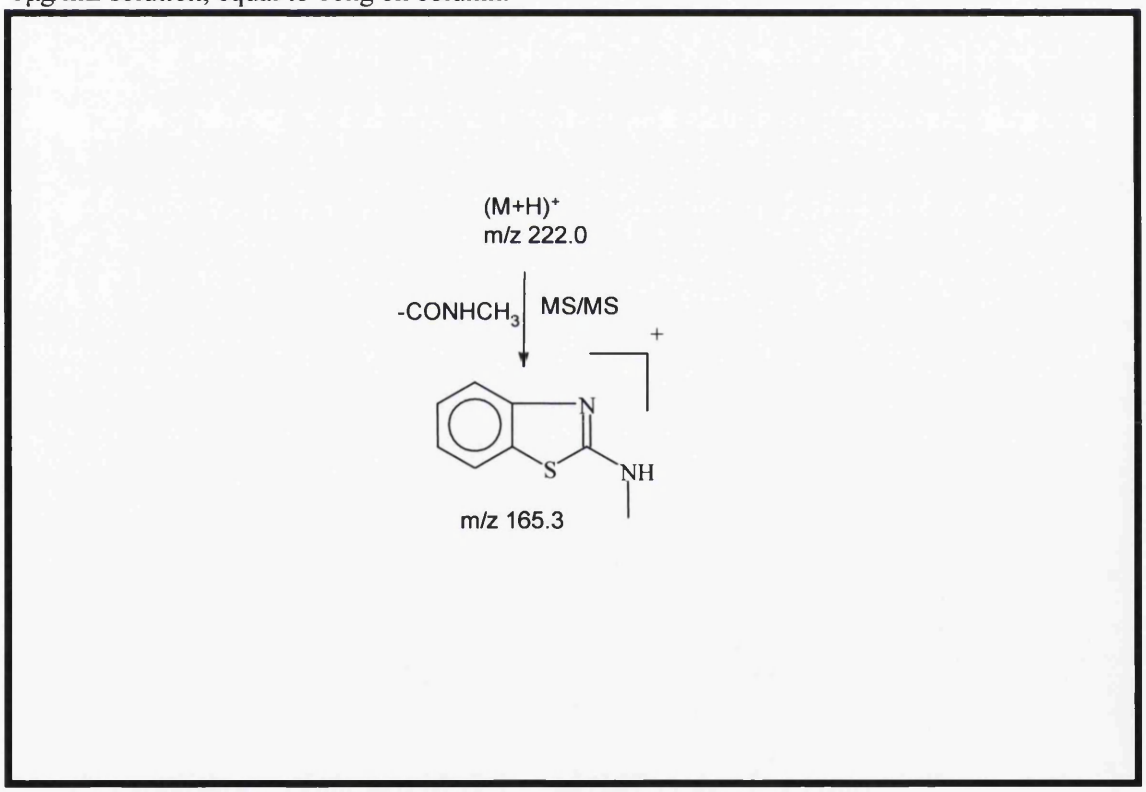



Figure 2.33 Proposed methabenzthiazuron fragmentation pattern interpretation.

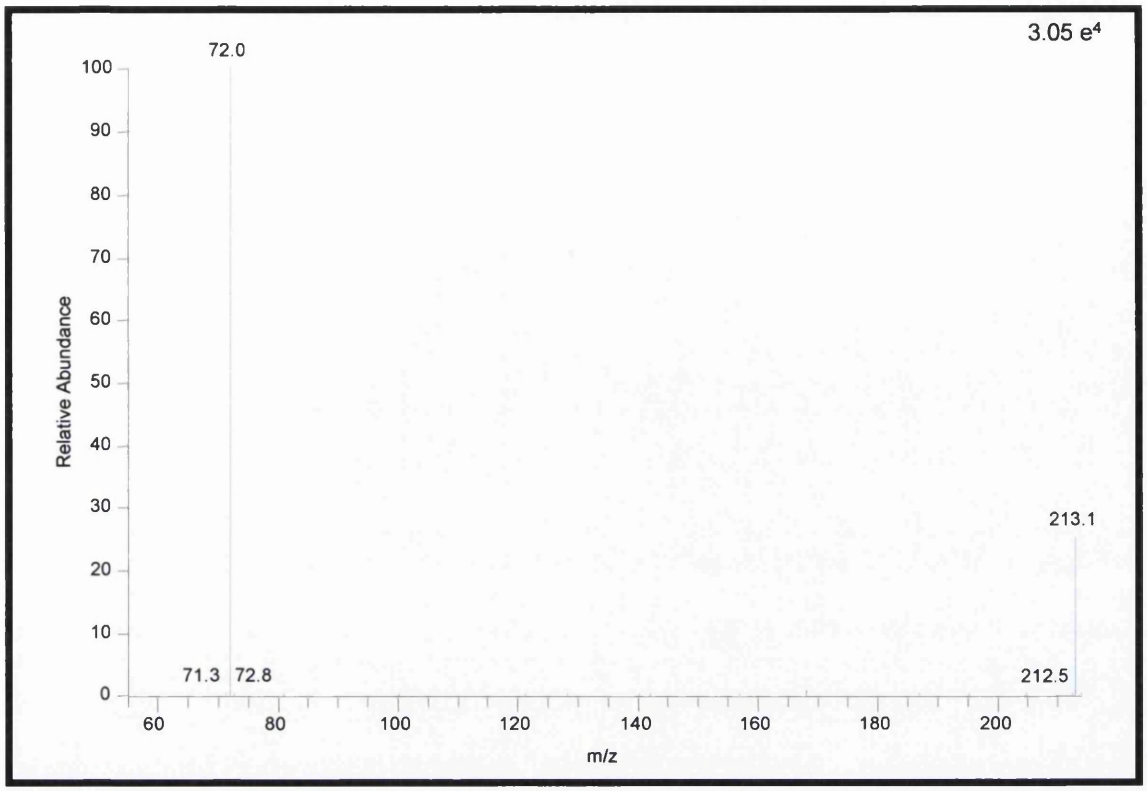



Figure 2.34 Positive LC-ESI-MS-MS spectrum of Chlortoluron. 10µL injection of a 1µg/mL solution; equal to 10ng on column.

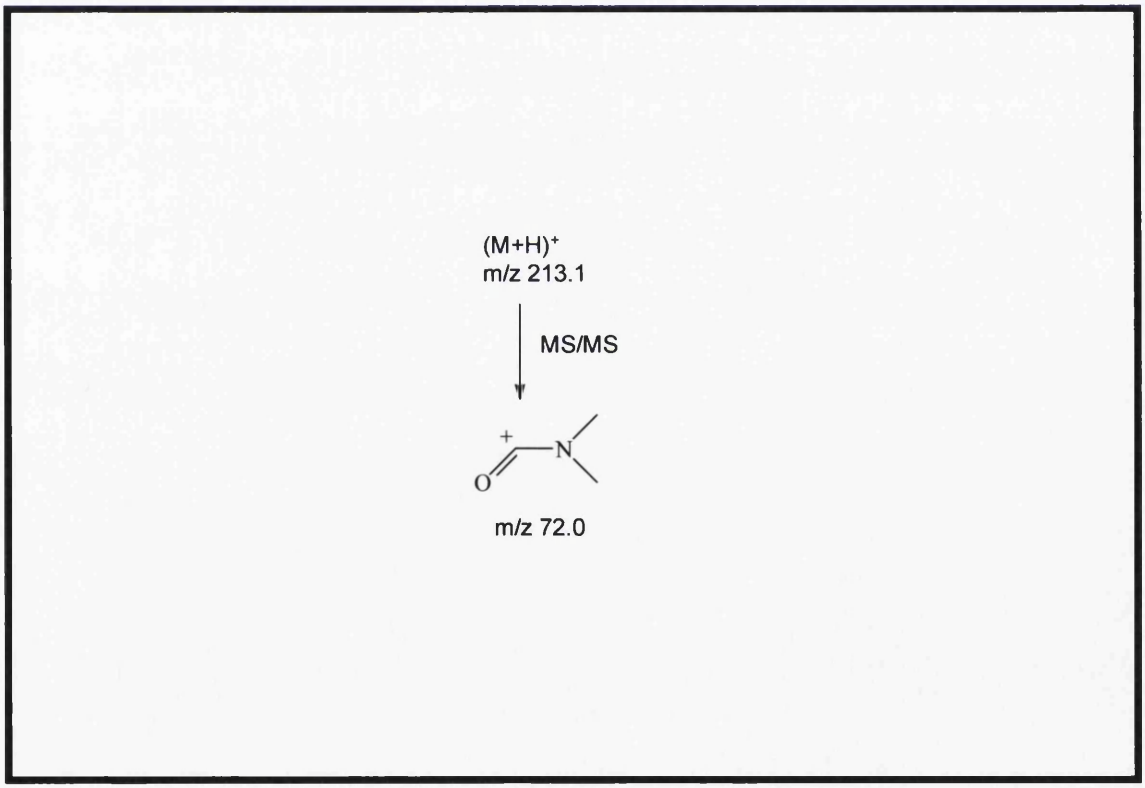



Figure 2.35 Proposed chlortoluron fragmentation pattern interpretation.

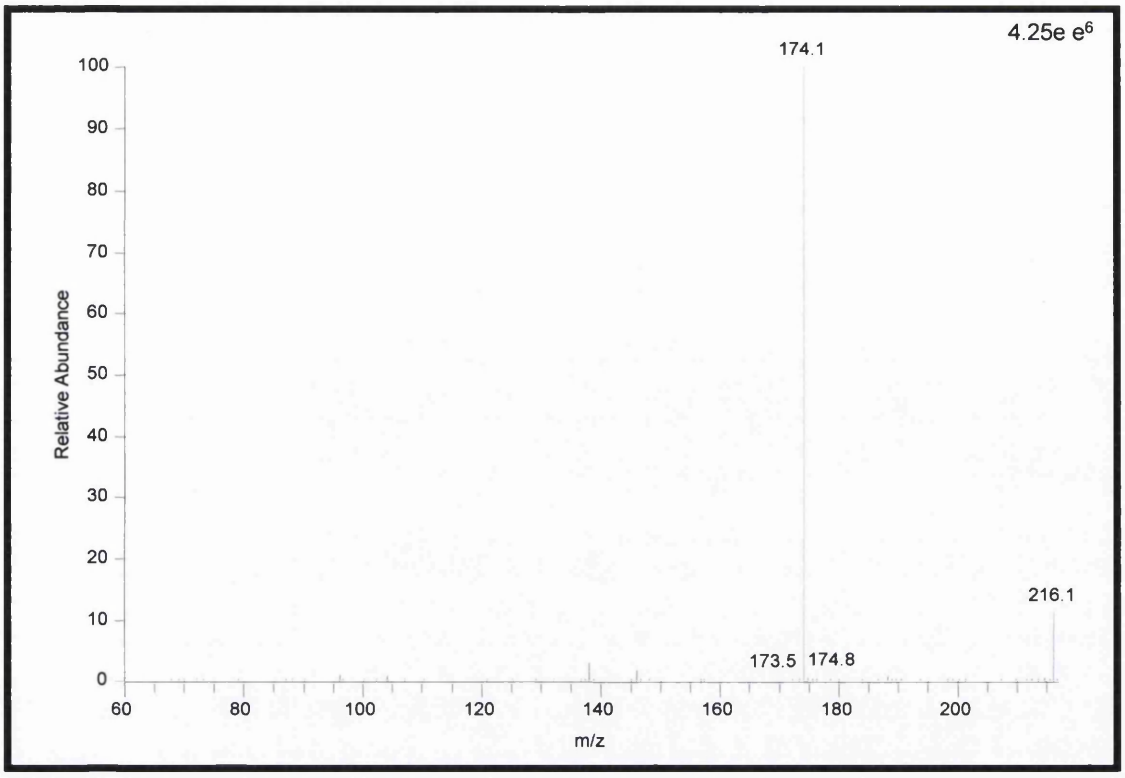



Figure 2.36 Positive LC-ESI-MS-MS spectrum of Atrazine. 10µL injection of a 1µg/mL solution; equal to 10ng on column.

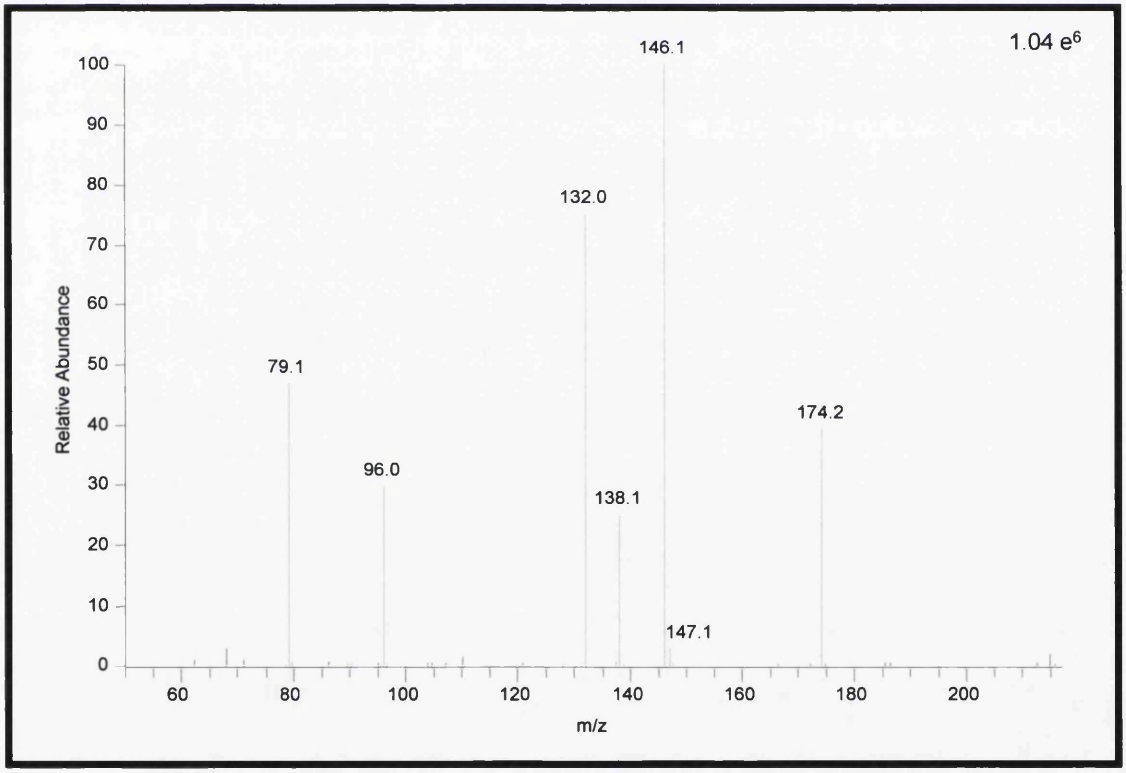



Figure 2.37 Positive LC-ESI-MS$^3$ spectrum of Atrazine. 10µL injection of a 1µg/mL solution; equal to 10ng on column.

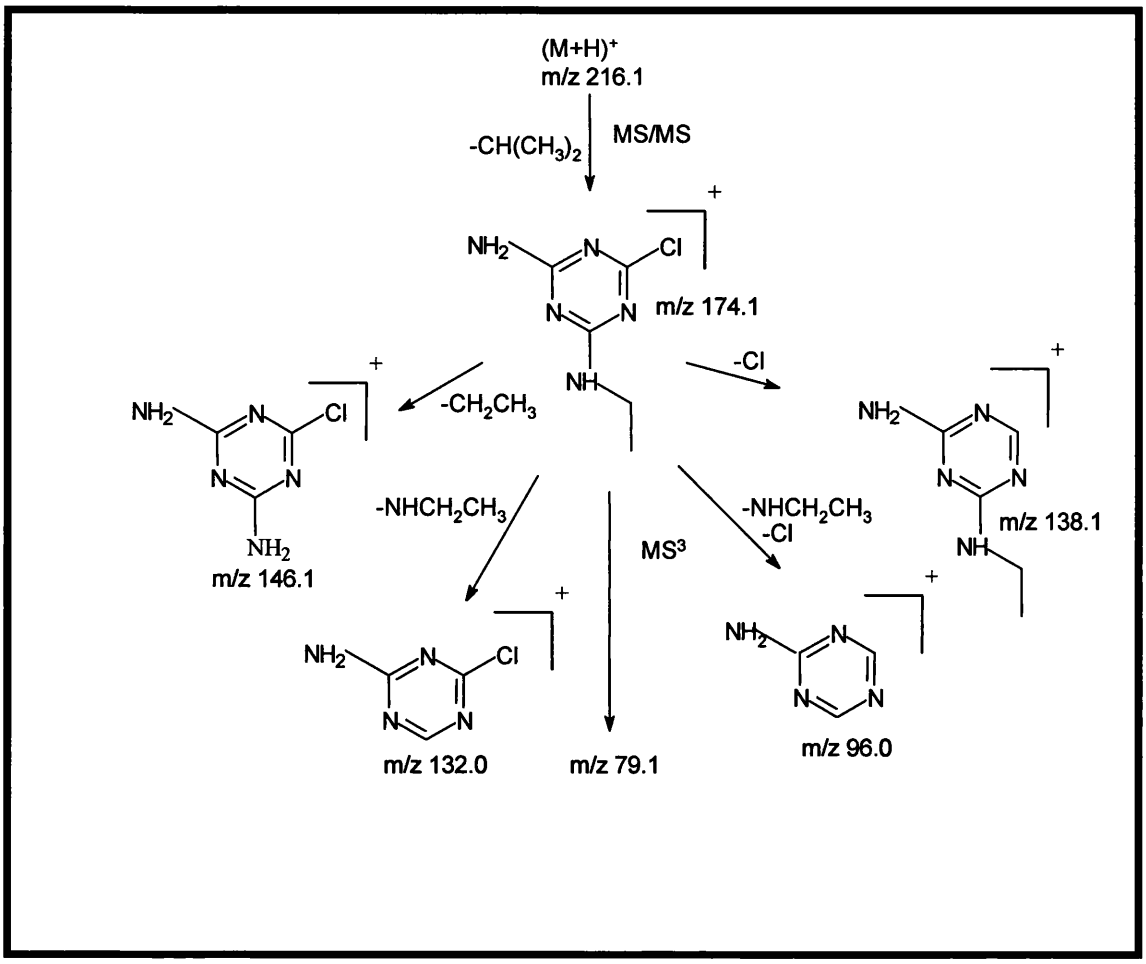



Figure 2.38 Proposed atrazine fragmentation pattern interpretation.

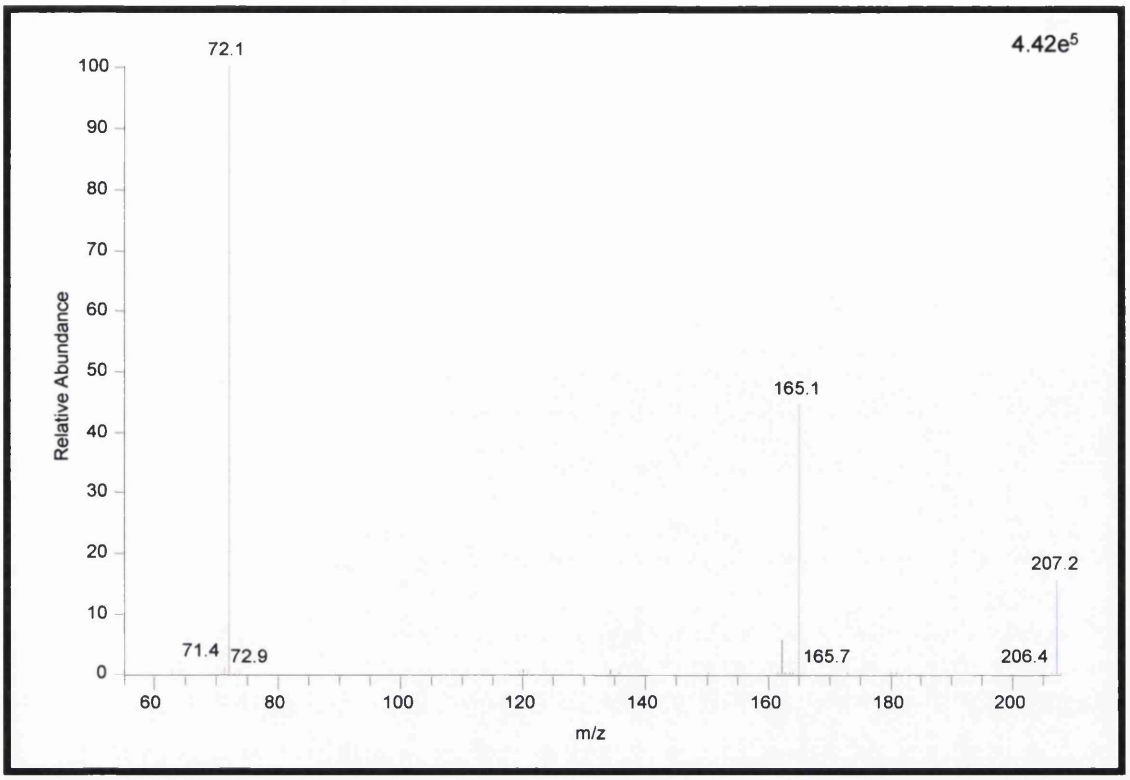



Figure 2.39 Positive LC-ESI-MS-MS spectrum of Isoproturon. 10µL injection of a 1µg/mL solution; equal to 10ng on column.

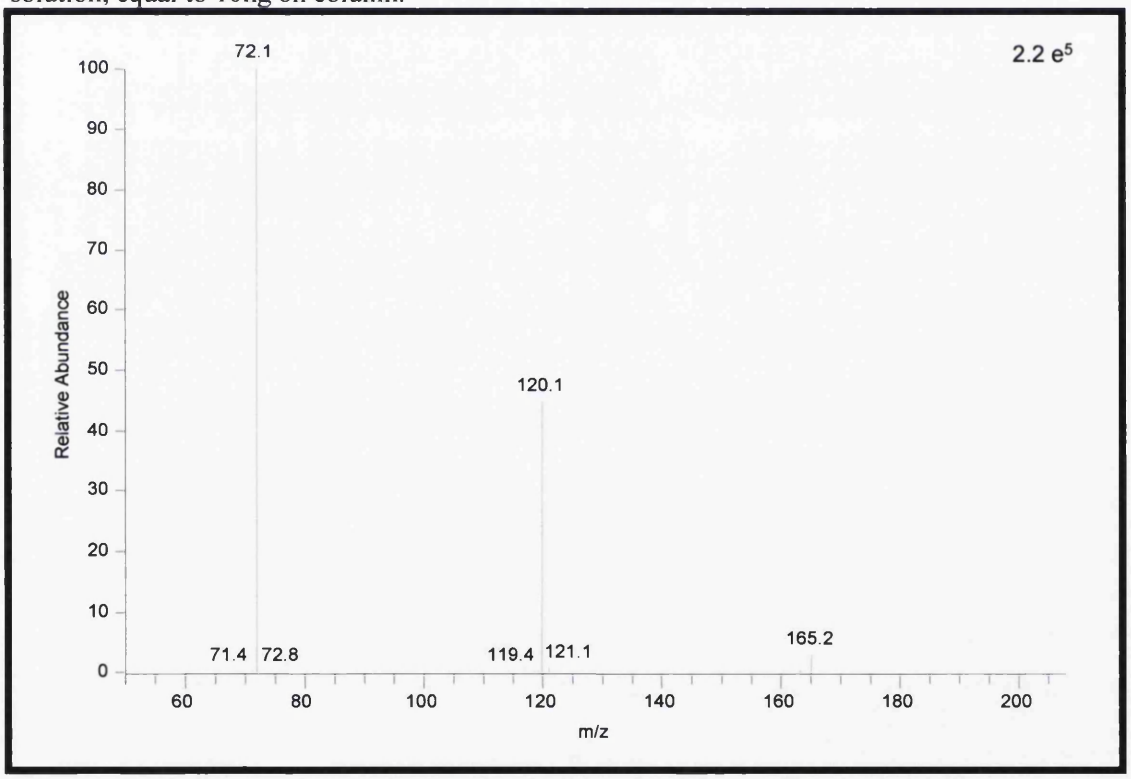



Figure 2.40 Positive LC-ESI-MS$^3$ spectrum of Isoproturon. 10µL injection of a 1µg/mL solution; equal to 10ng on column.

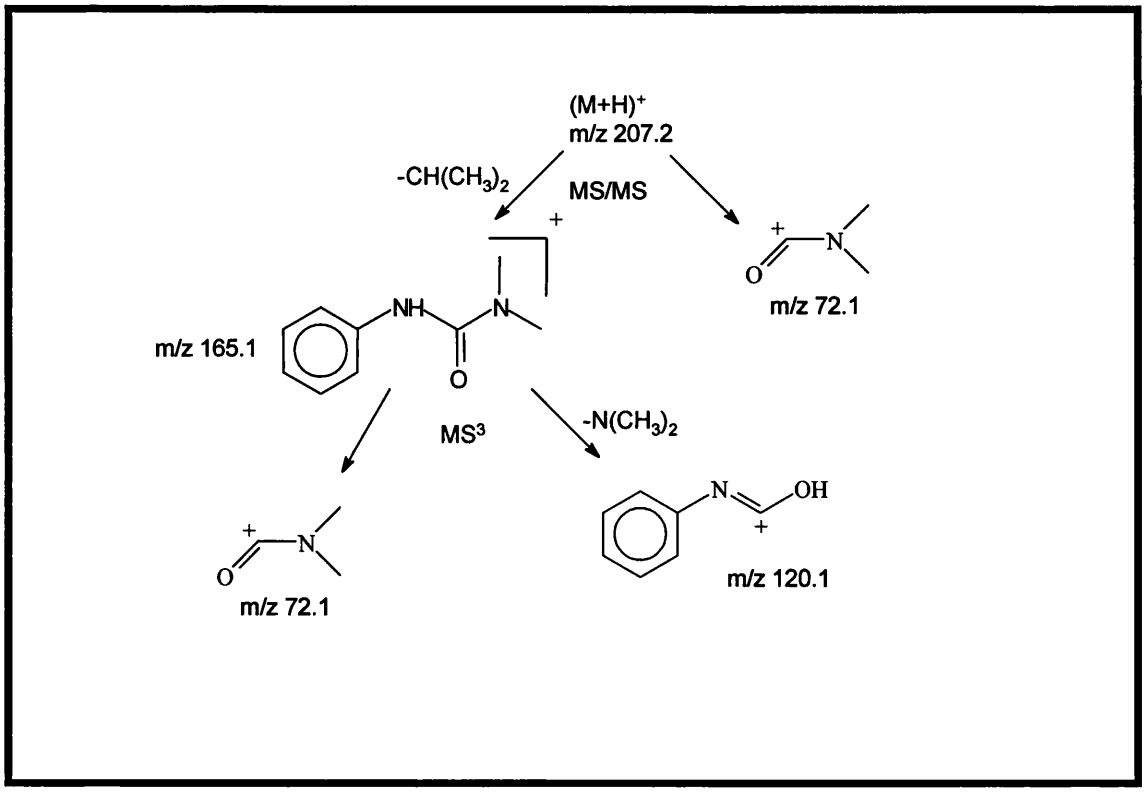



Figure 2.41 Proposed isoproturon fragmentation pattern interpretation.

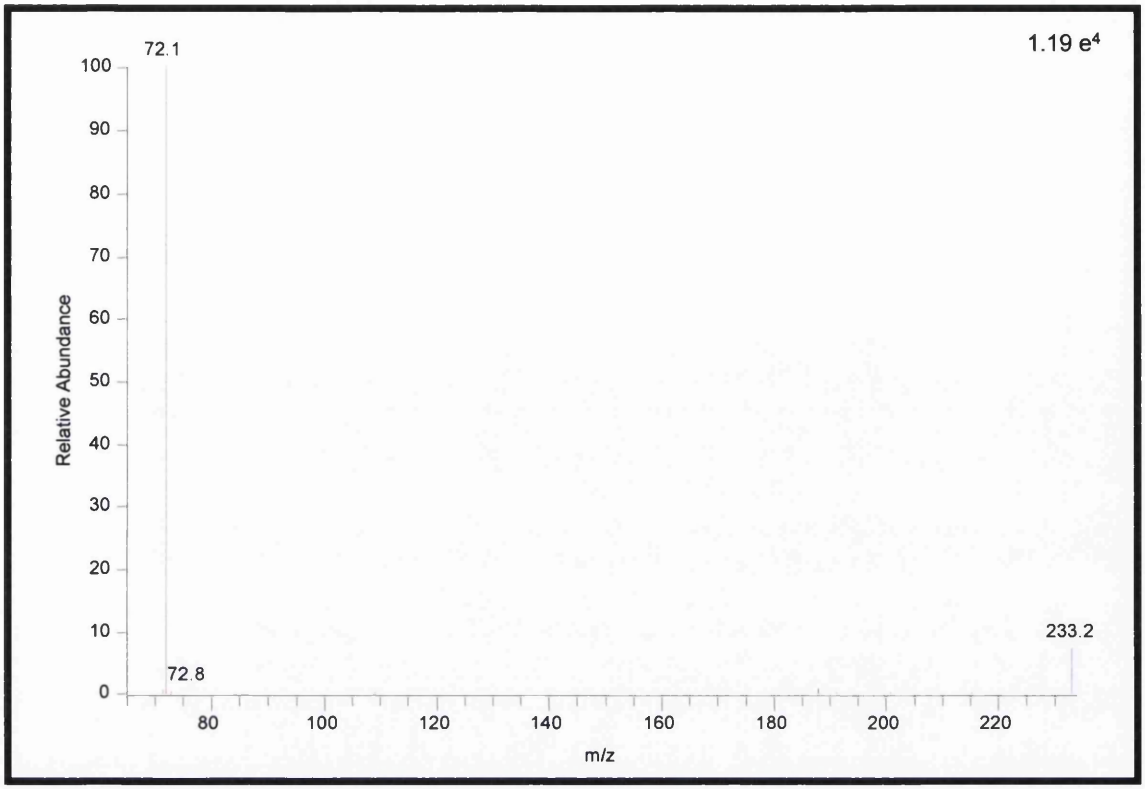



Figure 2.42 Positive LC-ESI-MS-MS spectrum of Diuron. 10µL injection of a 1µg/mL solution; equal to 10ng on column.

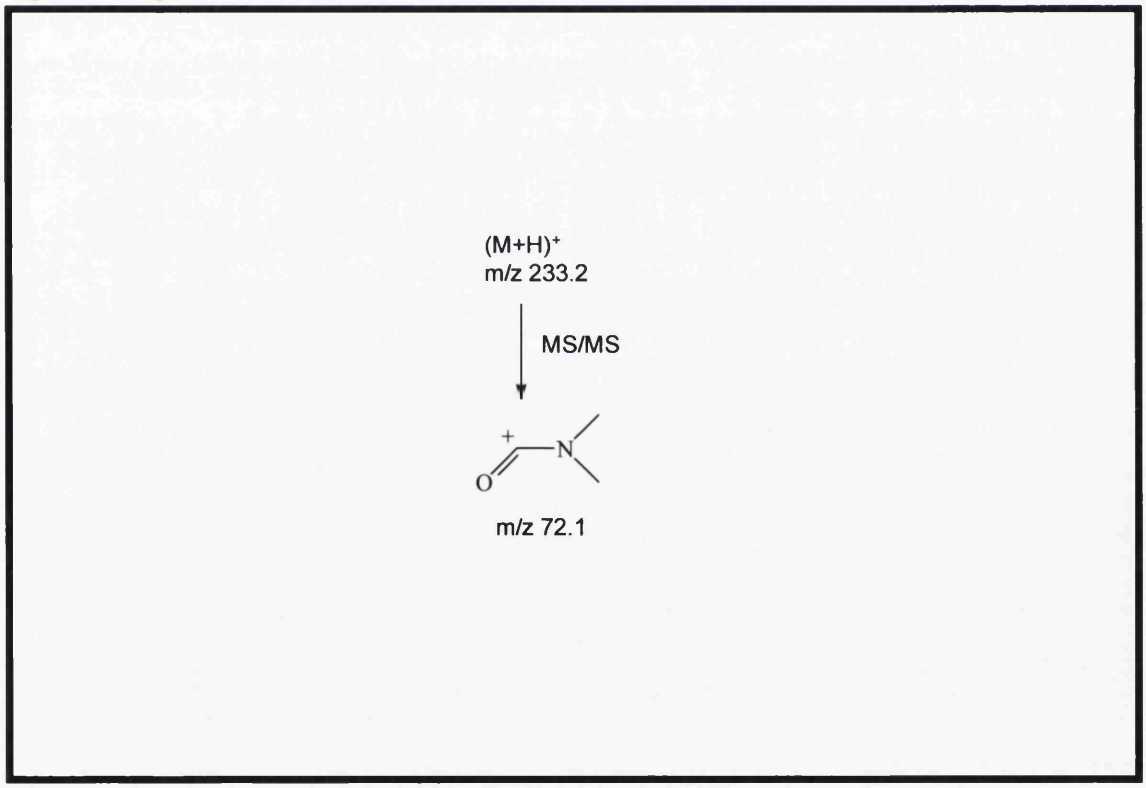



Figure 2.43 Proposed diuron fragmentation pattern interpretation.

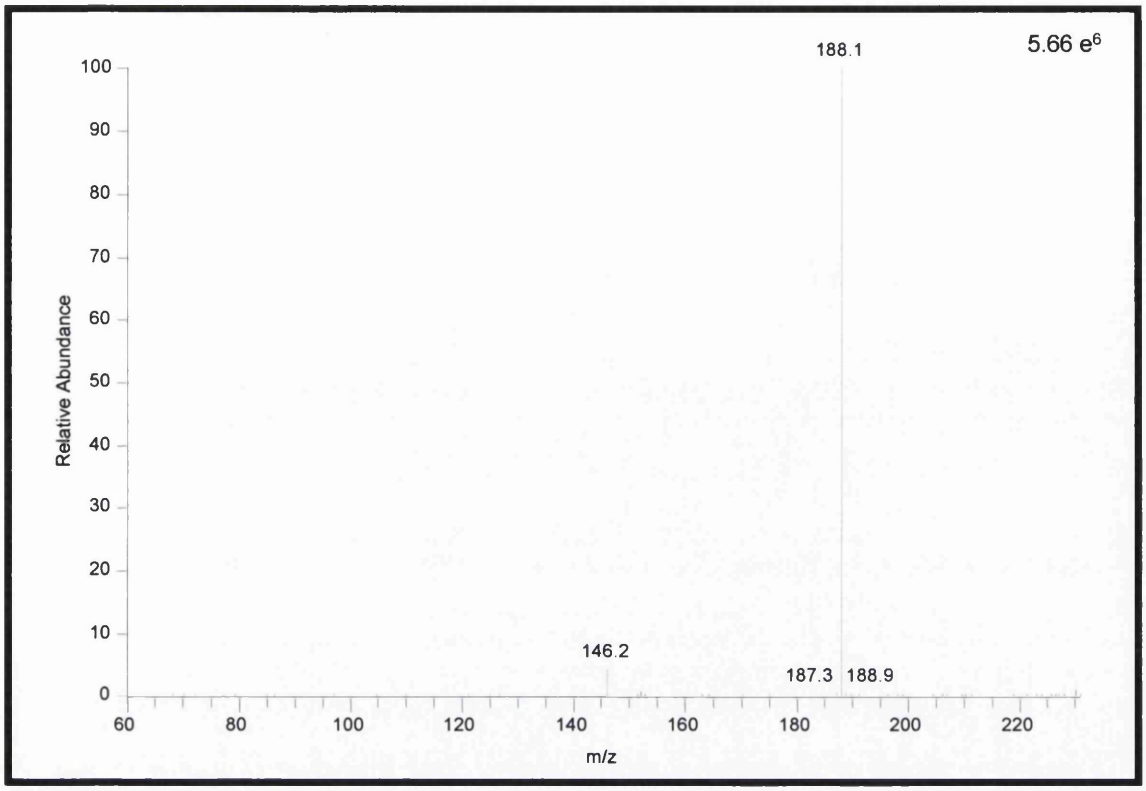



Figure 2.44 Positive LC-ESI-MS-MS spectrum of Propazine. 10µL injection of a 1µg/mL solution; equal to 10ng on column.

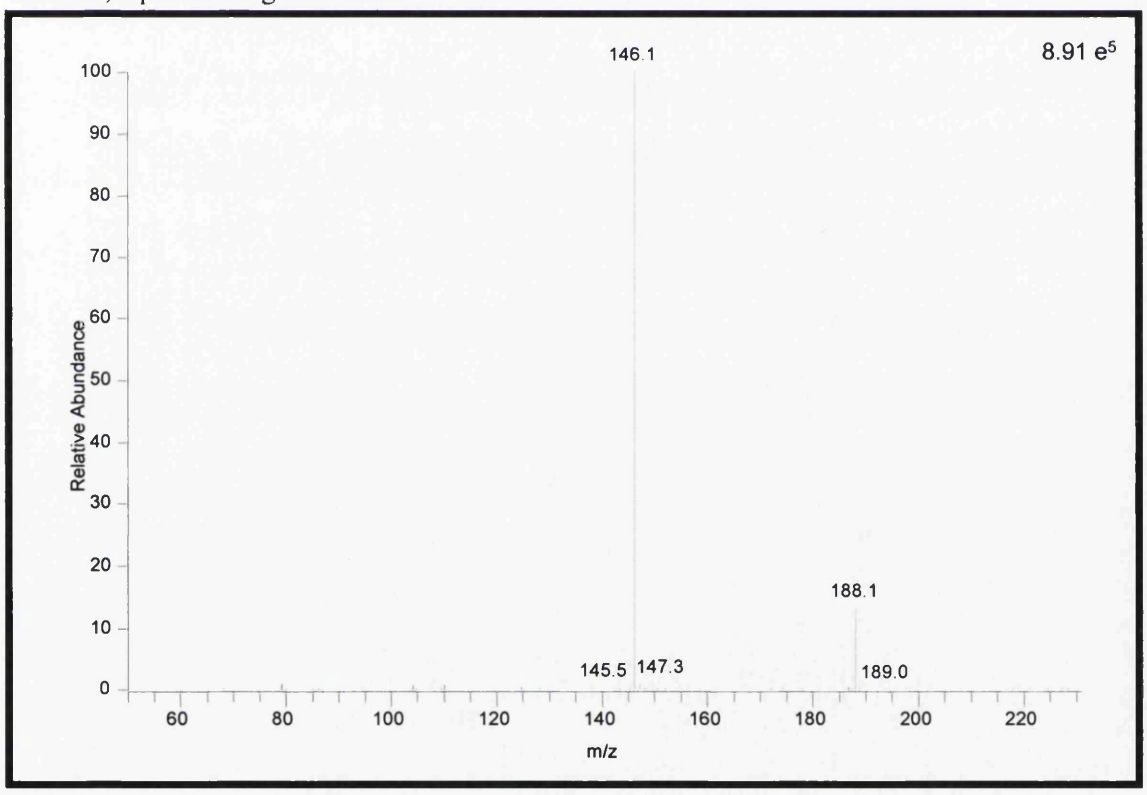



Figure 2.45 Positive LC-ESI-MS$^3$ spectrum of Propazine. 10µL injection of a 1µg/mL solution; equal to 10ng on column.

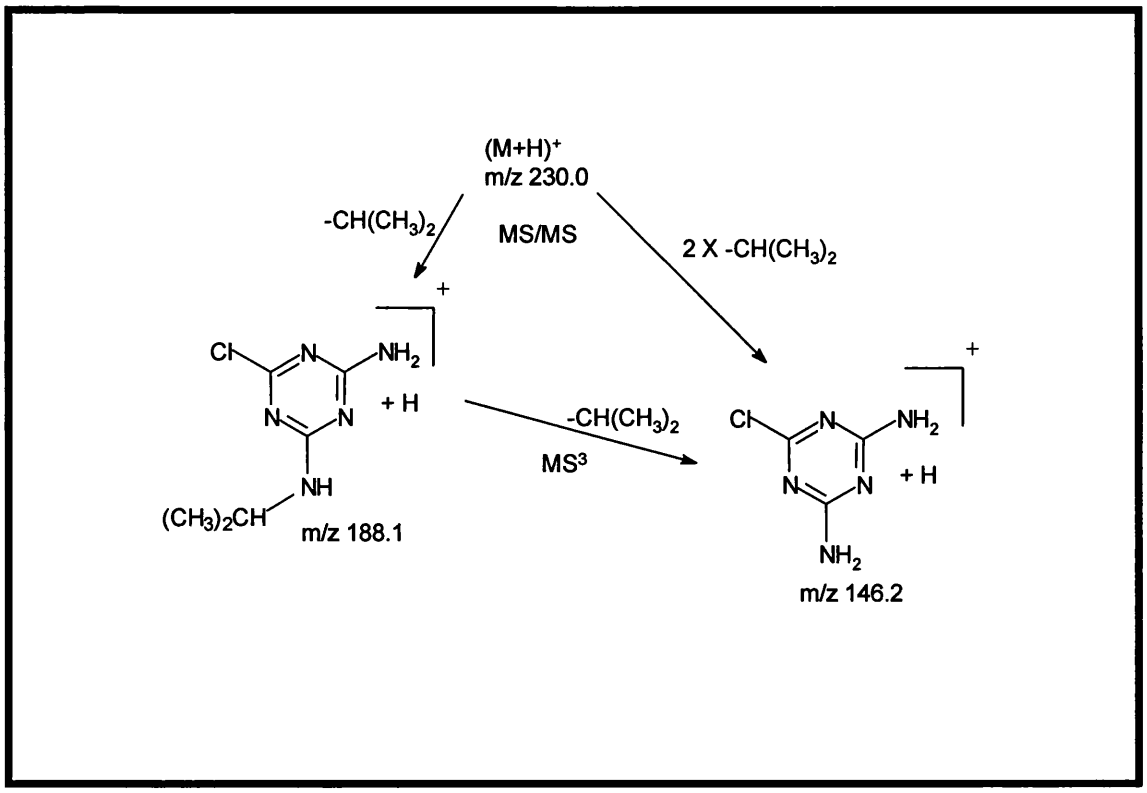



Figure 2.46 Proposed propazine fragmentation pattern interpretation.

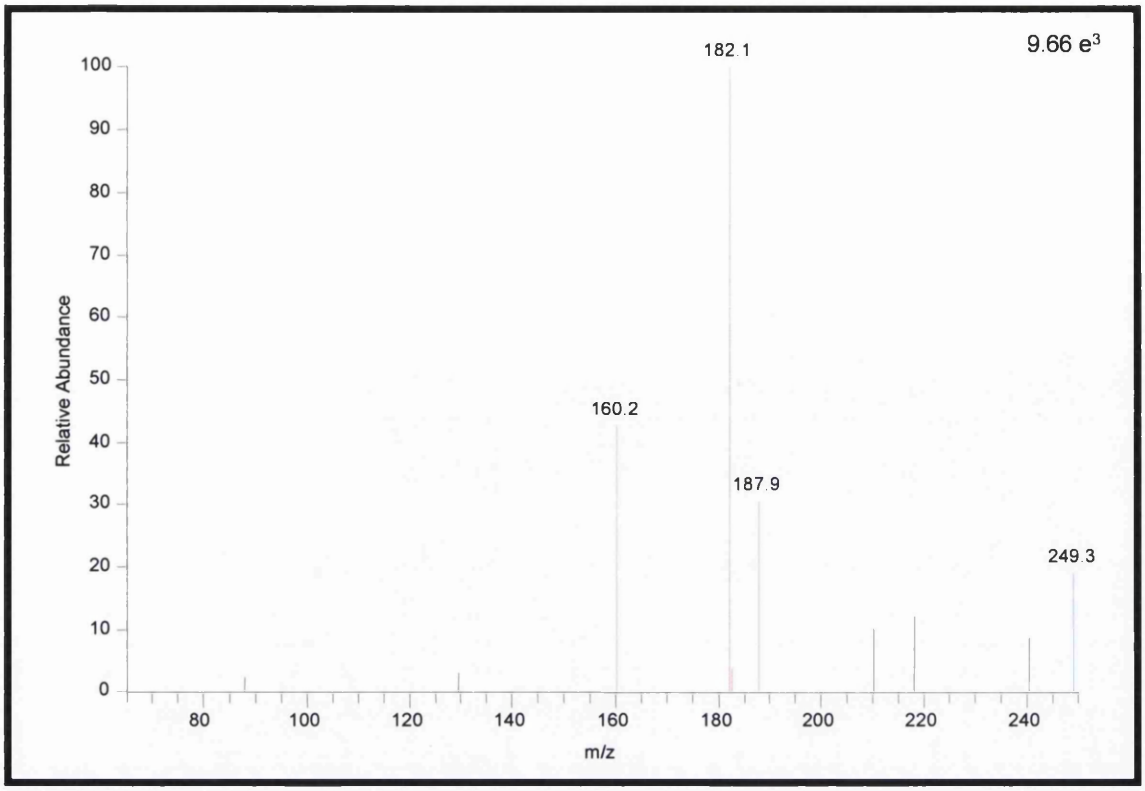



Figure 2.47 Positive LC-ESI-MS-MS spectrum of Linuron. 10µL injection of a 1µg/mL solution; equal to 10ng on column.

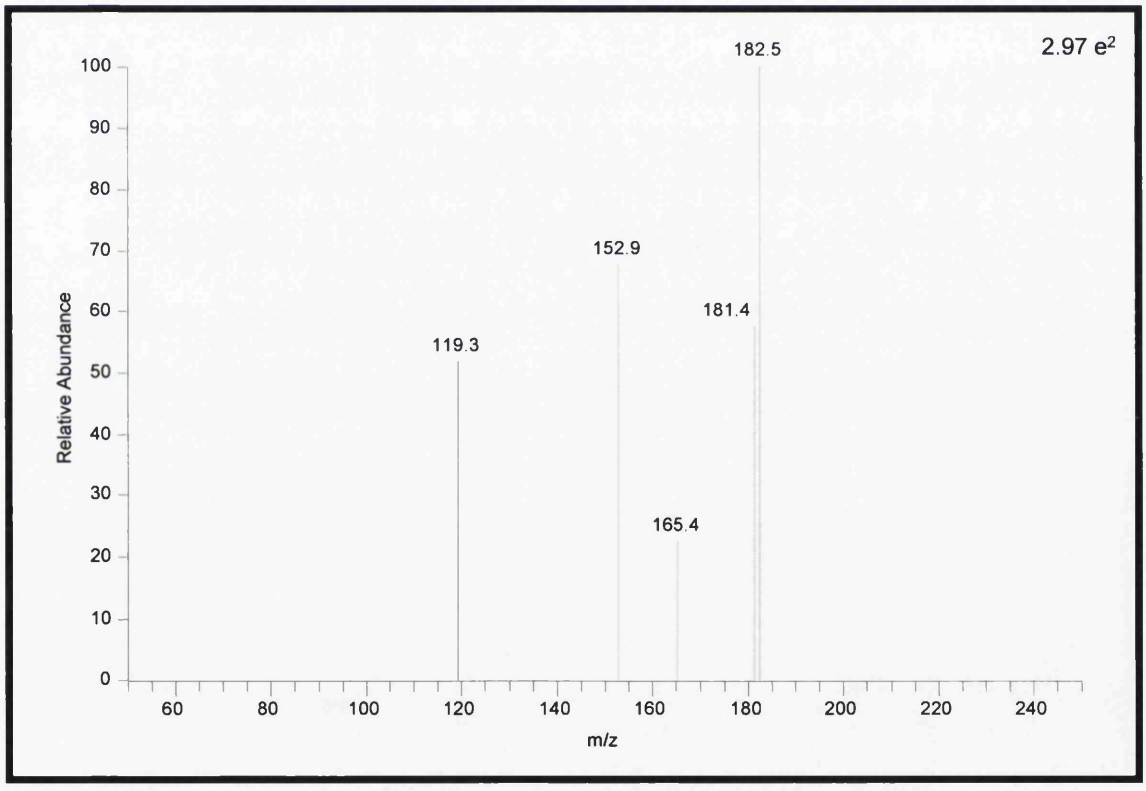



Figure 2.48 Positive LC-ESI-MS$^3$ spectrum of Linuron. 10µL injection of a 1µg/mL solution; equal to 10ng on column.

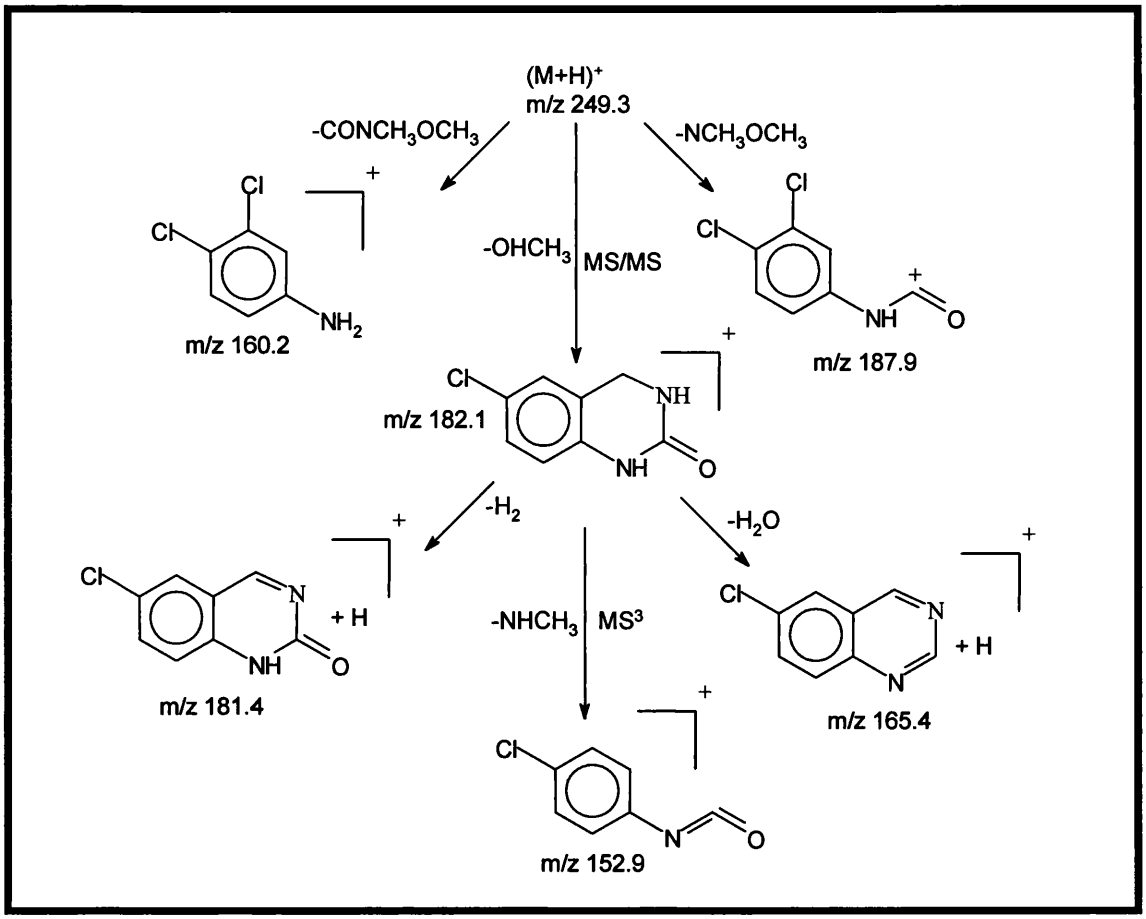



Figure 2.49 Proposed linuron fragmentation pattern interpretation.

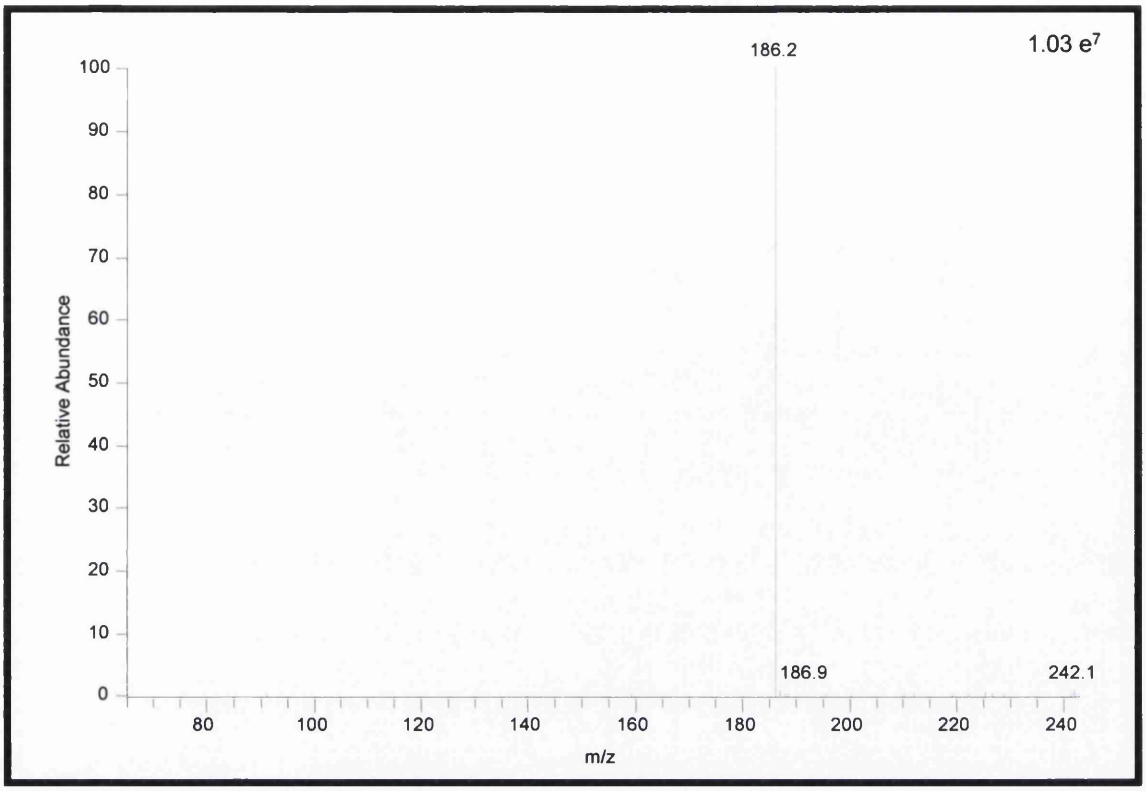



Figure 2.50 Positive LC-ESI-MS-MS spectrum of Terbutryn. 10µL injection of a 1µg/mL solution; equal to 10ng on column.

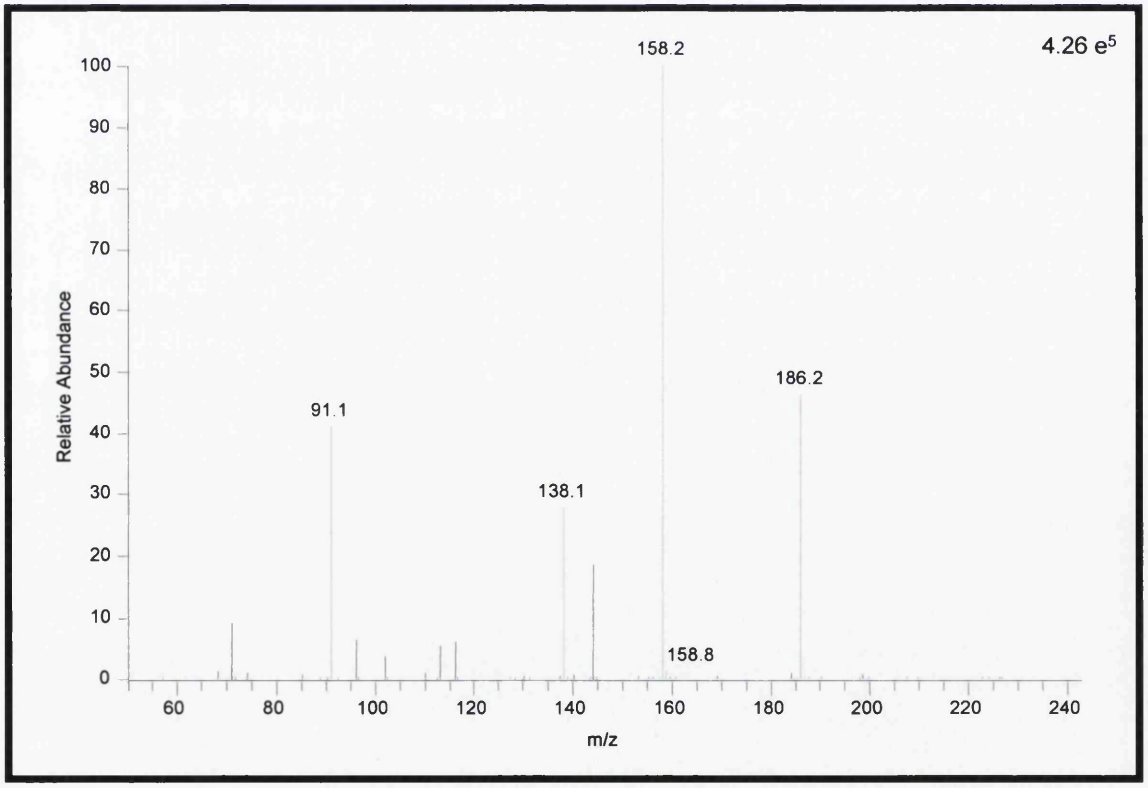



Figure 2.51 Positive LC-ESI-MS$^3$ spectrum of Terbutryn. 10µL injection of a 1µg/mL solution; equal to 10ng on column.

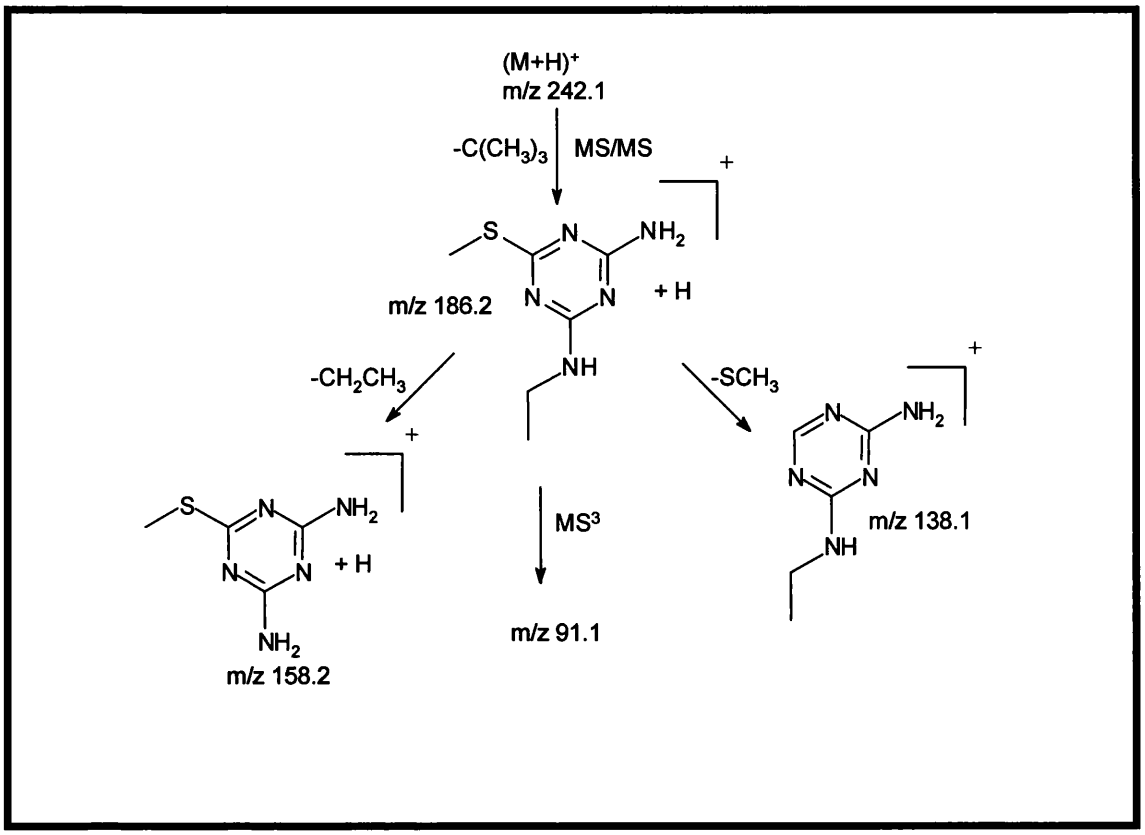



Figure 2.52 Proposed terbutryn fragmentation pattern interpretation.

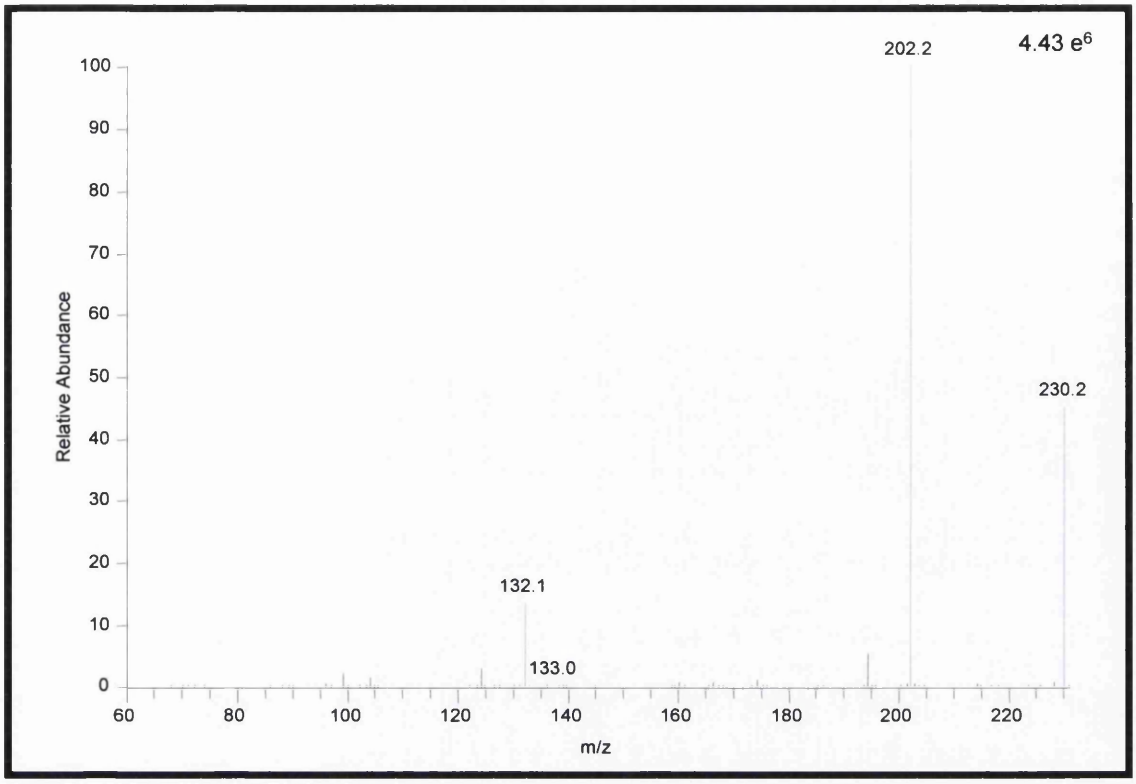



Figure 2.53 Positive LC-ESI-MS-MS spectrum of Trietazine. 10µL injection of a 1µg/mL solution; equal to 10ng on column.

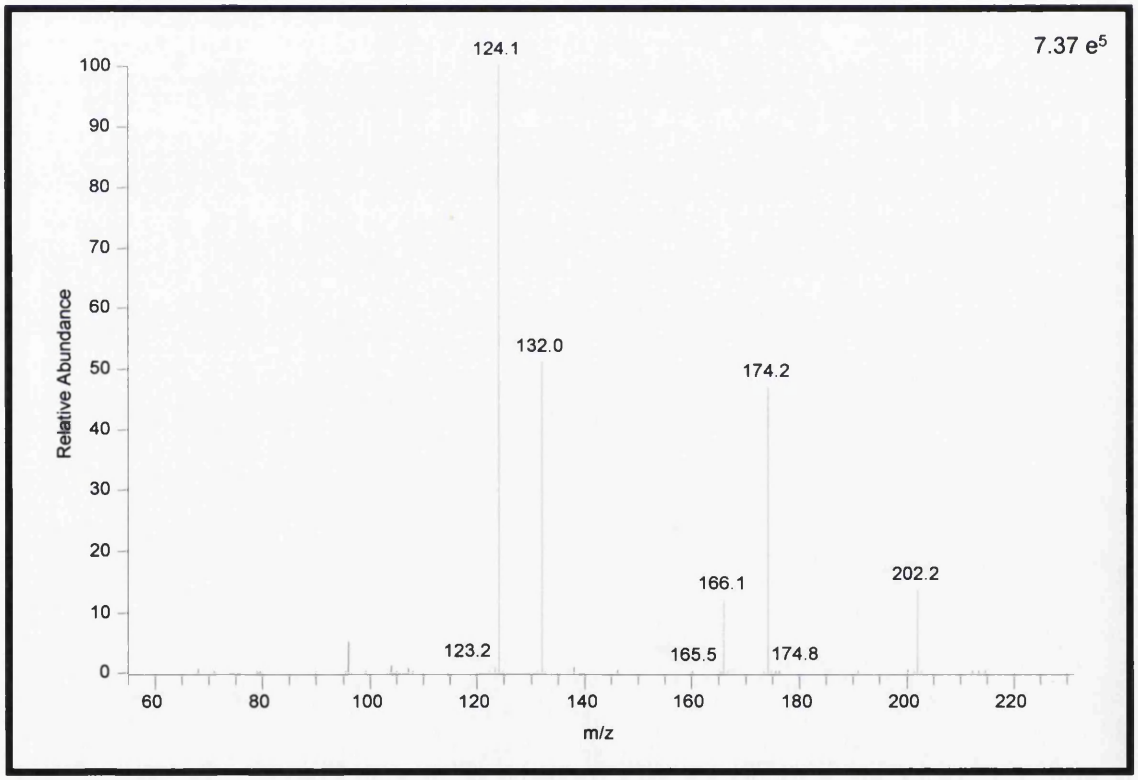



Figure 2.54 Positive LC-ESI-MS$^3$ spectrum of Trietazine. 10µL injection of a 1µg/mL solution; equal to 10ng on column.

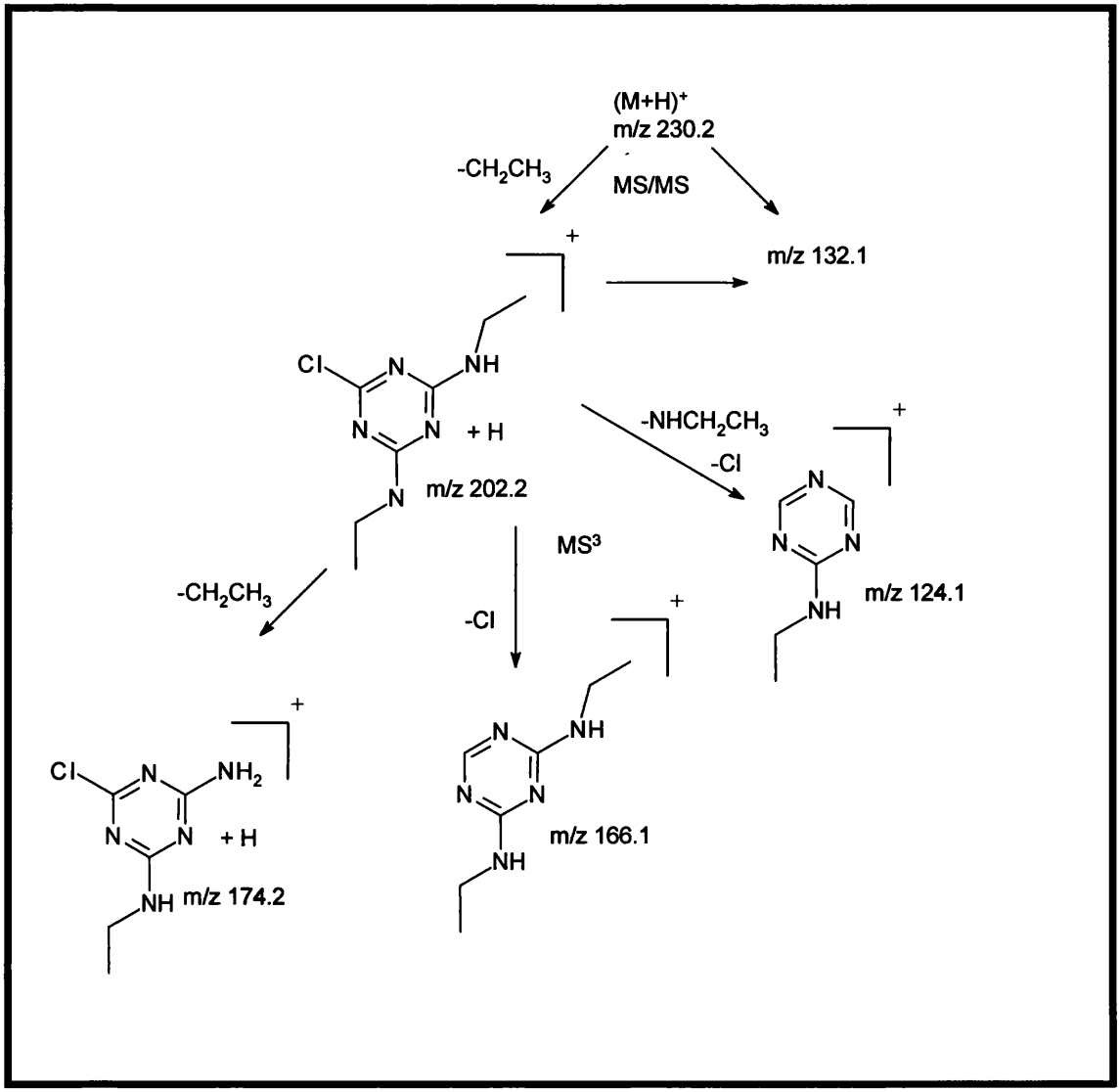



Figure 2.55 Proposed trietazine fragmentation pattern interpretation.

# Solid Phase Extraction of the Triazine and Phenylurea Herbicides

## Experimental

### Chemicals

HPLC grade solvents were purchased from Fisher Scientific (Loughborough, U.K.), gases from BOC Limited (Surrey, U.K.) and solid phase extraction (SPE) columns from Jones Chromatography Limited (Mid Glamorgan, U.K.).

### Instrumentation

SPE was performed on an International Sorbent Technology VacMaster extraction system using Isolute C18 500mg/3mL SPE columns.

### Results

Initial extraction experiments were conducted by adapting extraction methods found in the literature (16, 17, 18). An extraction method was developed which allowed for the detection of the 10 triazines and phenylureas, via LC-ESI-MS analysis, in river water samples. The table below sets out the method used for the detection of the 10 herbicides:

**Extraction Procedure:**

| Step | Procedure |
|---|---|
| Sample Pre-treatment | Sample untreated prior to extraction. |
| Column Solvation | Column conditioned with 6mL acetonitrile followed by 6mL water at a flow rate of 5mL/min. |
| Sample Application | Sample applied to column at a flow rate of 5mL/min. |
| Interference Elution | Interferences eluted with 6mL of water at a flow rate of 5mL/min. |
| Column Drying | Dry column under full vacuum for 60 seconds. |
| Analyte Elution | Herbicides eluted with 2mL of acetonitrile, allowed to soak for 10 minutes and then "dripped" through. |
| Reconstitution | Dry down under nitrogen and reconstitute in 50:50 (v/v) acetonitrile/water to a volume of 0.5mL. |

The following table shows the LOD achieved for each of the herbicides in spiked river water.

| Compound name | m/z Monitored | LOD |
|---|---|---|
| Simazine | 202.0 | 0.1ng/L |
| Methabenzthiazuron | 222.0 | 1ng/L |
| Chlortoluron | 213.0 | 1ng/L |
| Atrazine | 216.0 | 0.1ng/L |
| Isoproturon | 207.0 | 0.1ng/L |
| Diuron | 233.0 | 1ng/L |
| Propazine | 230.0 | 0.1ng/L |
| Linuron | 249.0 | 100ng/L |
| Terbutryn | 242.0 | 0.1ng/L |
| Trietazine | 230.0 | 0.1ng/L |

On column injection volume of 50μL. LOD is calculated as the concentration of the herbicide spiked into the blank river water.

The volume of river water extracted for analysis was 2 litres; to ensure that there would be no interference from herbicides already present in the water used for determining the LOD it was collected from the source of the river Lougher in South Wales. Figure 2.56 is an extraction of river water spiked at 100ng/L for each herbicide.

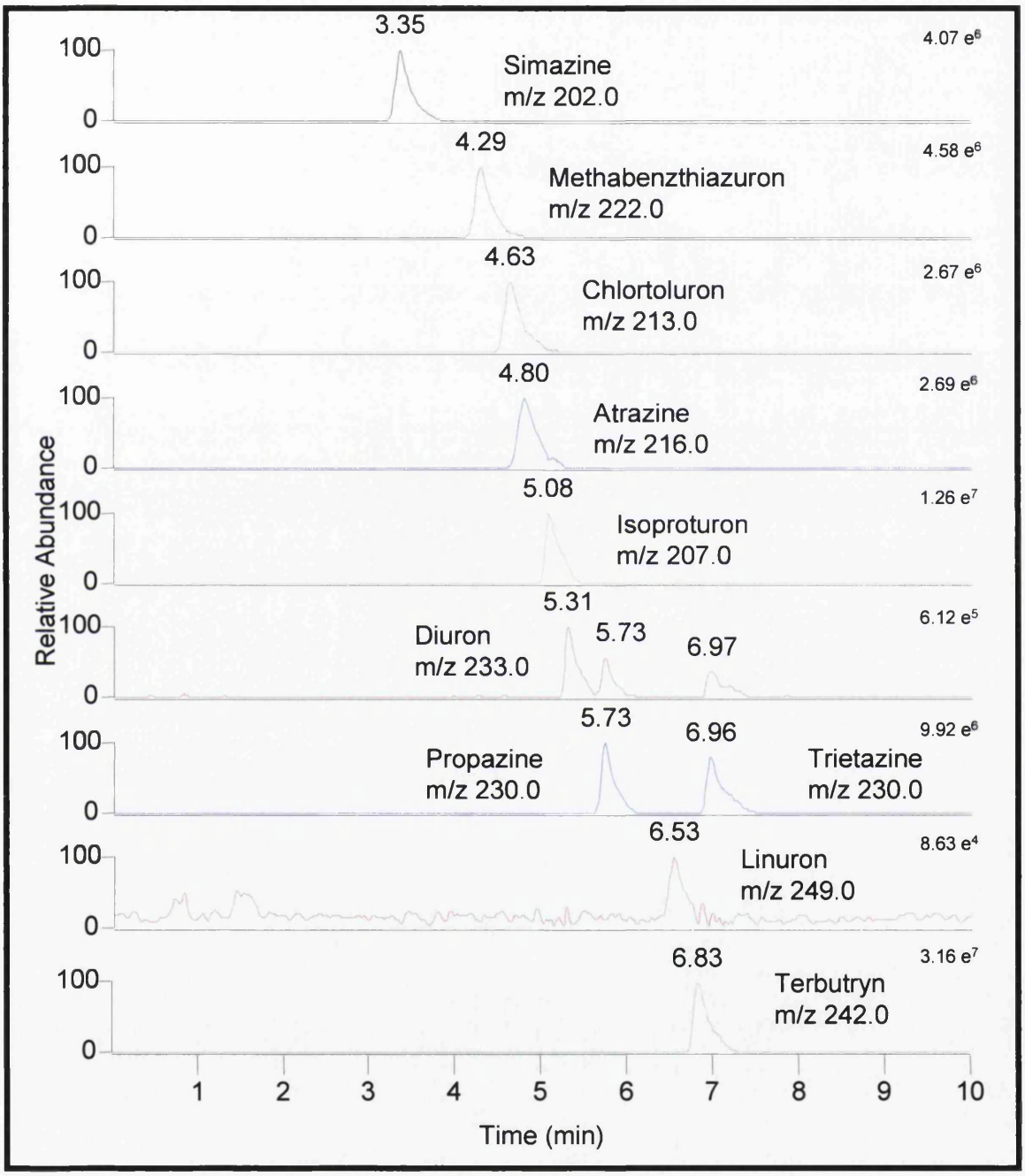



Figure 2.56 Positive LC-ESI-MS extracted ion chromatograms of the 10 herbicides. SPE of river water spiked with 100ng/L of each herbicide.

## Analysis of River Water Samples

River Water samples were collected from points along the rivers Lougher and Bryn where they ran along either side of Glynhir golf course, Llandybie in South Wales; at each site 4 litres of river water was collected. 2 litres of river water was extracted using the technique described previously from each site and in the case of positive results the second 2 litres were then extracted and also analysed as confirmation of the positive result. Herbicides were shown to be present in both rivers. The following two figures (2.57 and 2.58) show the positive results achieved.

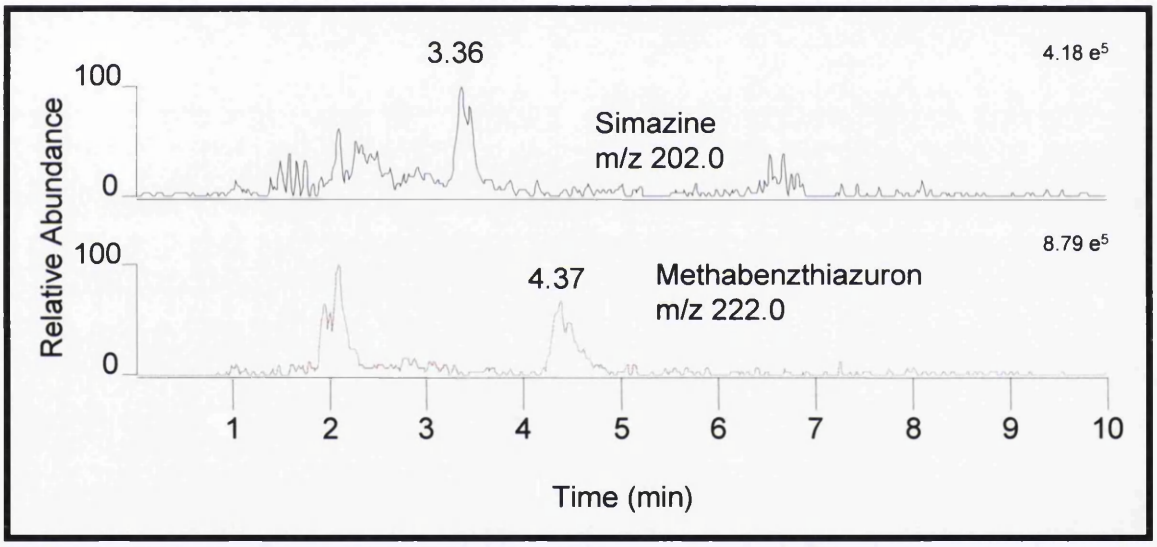



Figure 2.57 Extracted ion chromatogram of the herbicide positive results from the river Lougher site.

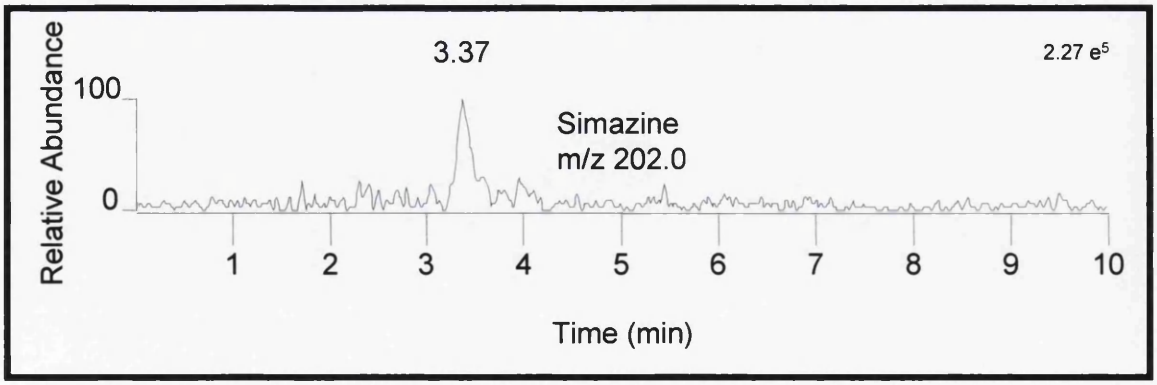



Figure 2.58 Extracted ion chromatogram of the herbicide positive result from the river Bryn site.

From the chromatograms of the extracted river water samples it can be seen that the river Lougher gave positive results for both simazine and methabenzthiazuron, whereas the river Bryn only gave a positive result for simazine. This is unexpected as all fairways on the course would be treated with the same herbicides; however by performing MS-MS analysis it was found that the methabenzthiazuron result found on the river Lougher was not in fact due to the herbicide but to an interference. Figure 2.59 shows the MS-MS chromatograms and figure 2.60 the MS-MS spectrum obtained for the river Lougher simazine result.

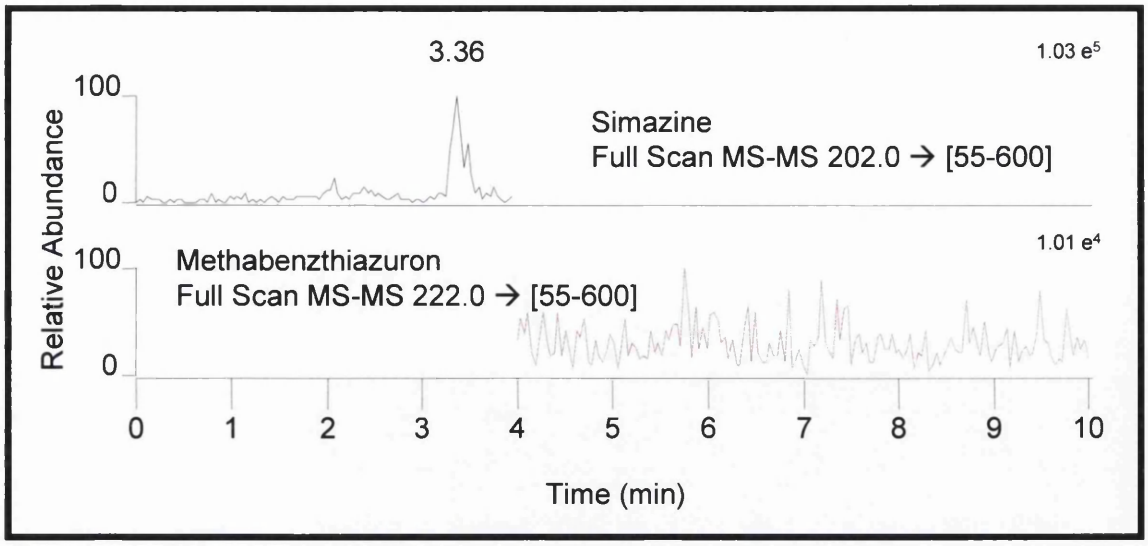



Figure 2.59 Positive LC-ESI-MS-MS chromatogram of the herbicide positive results from the river Lougher site.

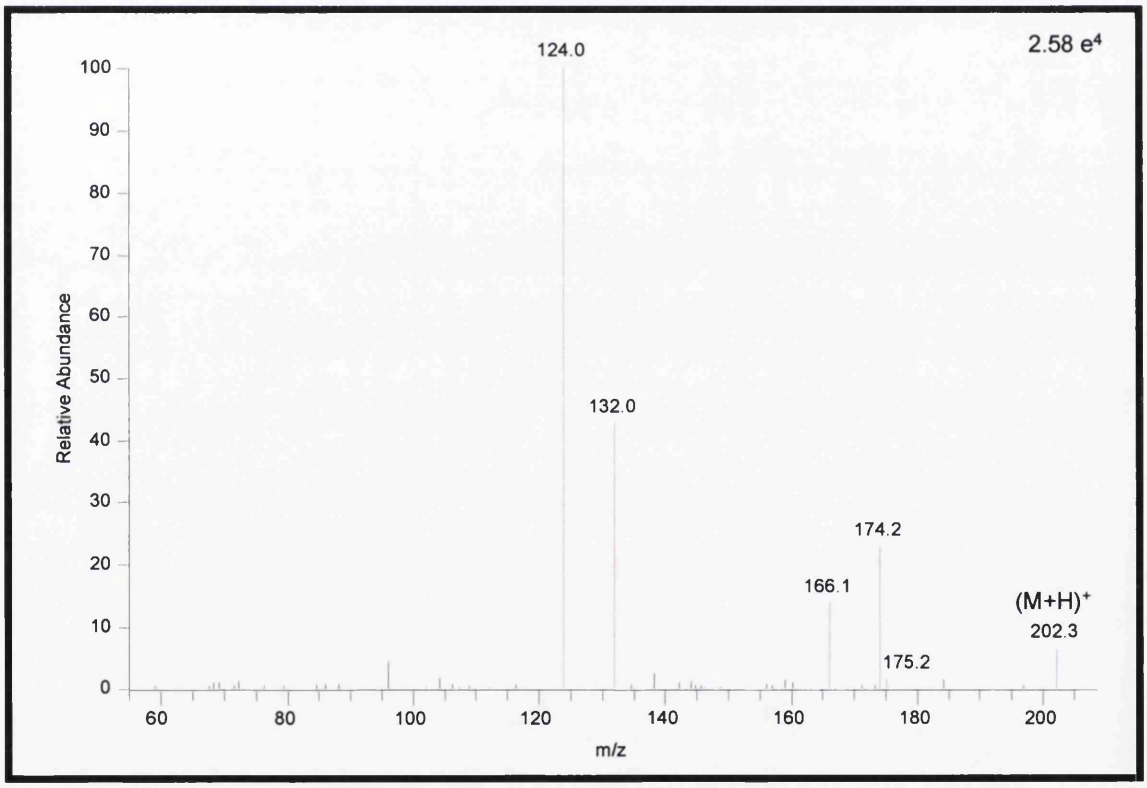



Figure 2.60 Positive LC-ESI-MS-MS spectrum of Simazine from river Lougher sample.

## LC-UV For the Separation of 9 Acidic Herbicides

The structures of the acidic herbicides studied are shown in figure 2.61.

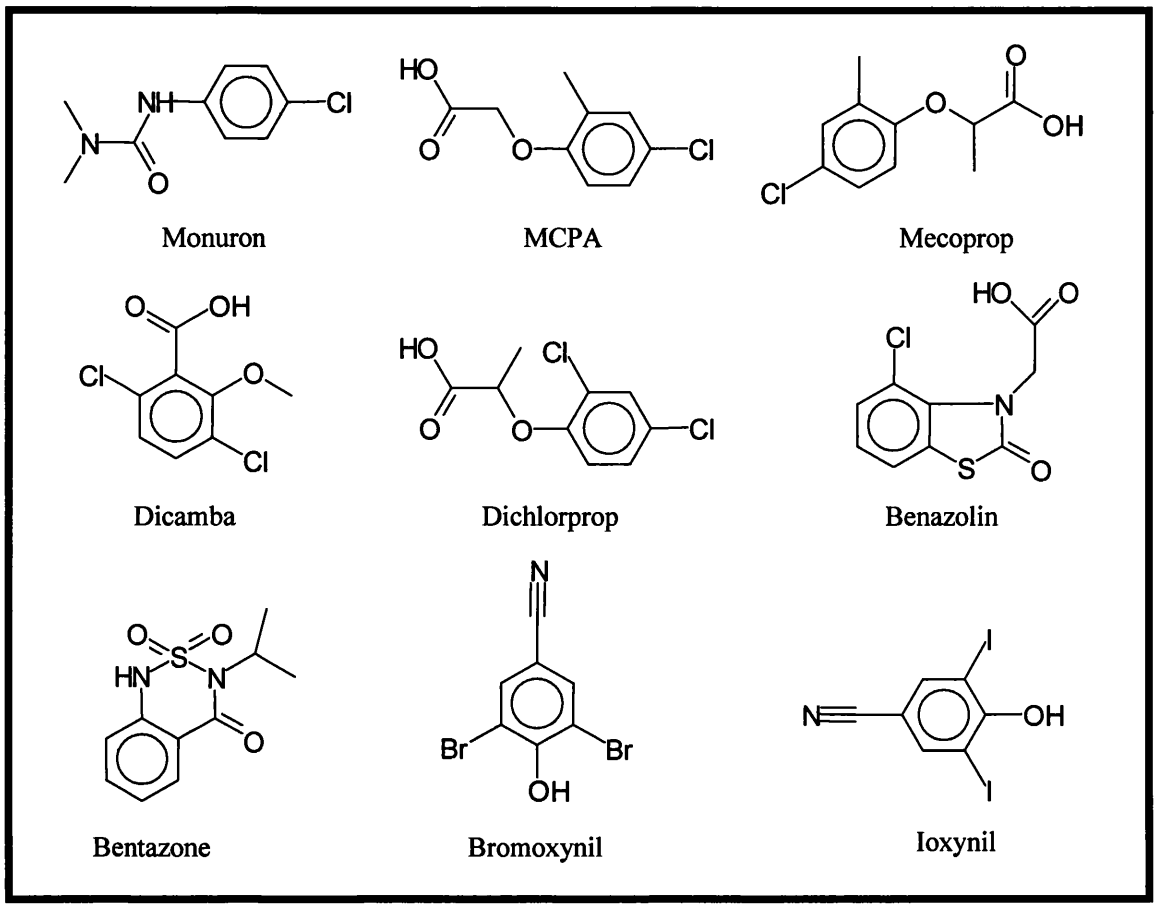



Figure 2.61 Structures of the Acidic herbicides investigated.

### Experimental

### Chemicals

HPLC grade solvents were purchased from Fisher Scientific (Loughborough, U.K.), gases from BOC Limited (Surrey, U.K.) and the following 9 acidic herbicides from Dr Ehrenstorfer GmbH (Augsburg, Germany).

| | |
|---|---|
| Monuron | Benazolin |
| MCPA | Bentazone |
| Mecoprop | Bromoxynil |
| Dicamba | Ioxynil |
| Dichlorprop | |

**Sample Preparation**

1mg/ ml standard solutions were made up in 50/ 50 acetonitrile/ water for each of the acidic herbicides and solutions for analysis were prepared and diluted from these as necessary.

**Instrumentation**

A Hewlett Packard 1100 HPLC fitted with a diode array detector (DAD) was used to perform the chromatography with a Phenomenex Luna 4.6x100mm 3μ Phenyl-Hexyl column. The mobile phase used for analysis was made up of acetonitrile and water acidified to pH 3.15 with acetic acid run in a gradient system.

**Results**

It was found from the literature that the use of a C18 column with an acidified aqueous buffer had been used for the successful LC-MS analysis of acidic herbicides (19); however this work had been performed on a 250mm HPLC column and the run times required for analysis were in excess of 50 minutes. With this in mind we attempted to develop a method on the Phenomenex Luna 4.6x100mm 3μ C18 (2) column which we had previously used in the chromatography of the triazine and

phenylurea herbicides; our attempts to develop a suitable separation on this column were unsuccessful so we switched to the phenyl-hexyl column. The mixture of the 9 acidic herbicides was separated on the phenyl-hexyl column with the following chromatographic conditions:

| Time in minutes | %$CH_3CN$ | %$H_2O$ pH 3.15 |
|---|---|---|
| 0.00 | 30 | 70 |
| 12.00 | 30 | 70 |
| 22.00 | 70 | 30 |

| | |
|---|---|
| Column Thermostat | Room Temperature |
| Flow Rate | 0.1mL/min to 1.0mL/min from 0 to 12 minutes then held for the remainder of the run. |
| DAD | 230nm |

The following table gives the retention times for the compounds.

| Compound name | Peak label | RT minutes |
|---|---|---|
| Dicamba | A | 8.294 |
| Benazolin | B | 9.556 |
| Monuron | C | 11.781 |
| Bentazone | D | 12.134 |
| MCPA | E | 15.455 |
| Dichlorprop | F | 16.802 |
| Mecoprop + Bromoxynil | G+H | 17.284 |
| Ioxynil | I | 18.954 |

Figure 2.62 shows the UV chromatogram of the separation of the 9 herbicides.

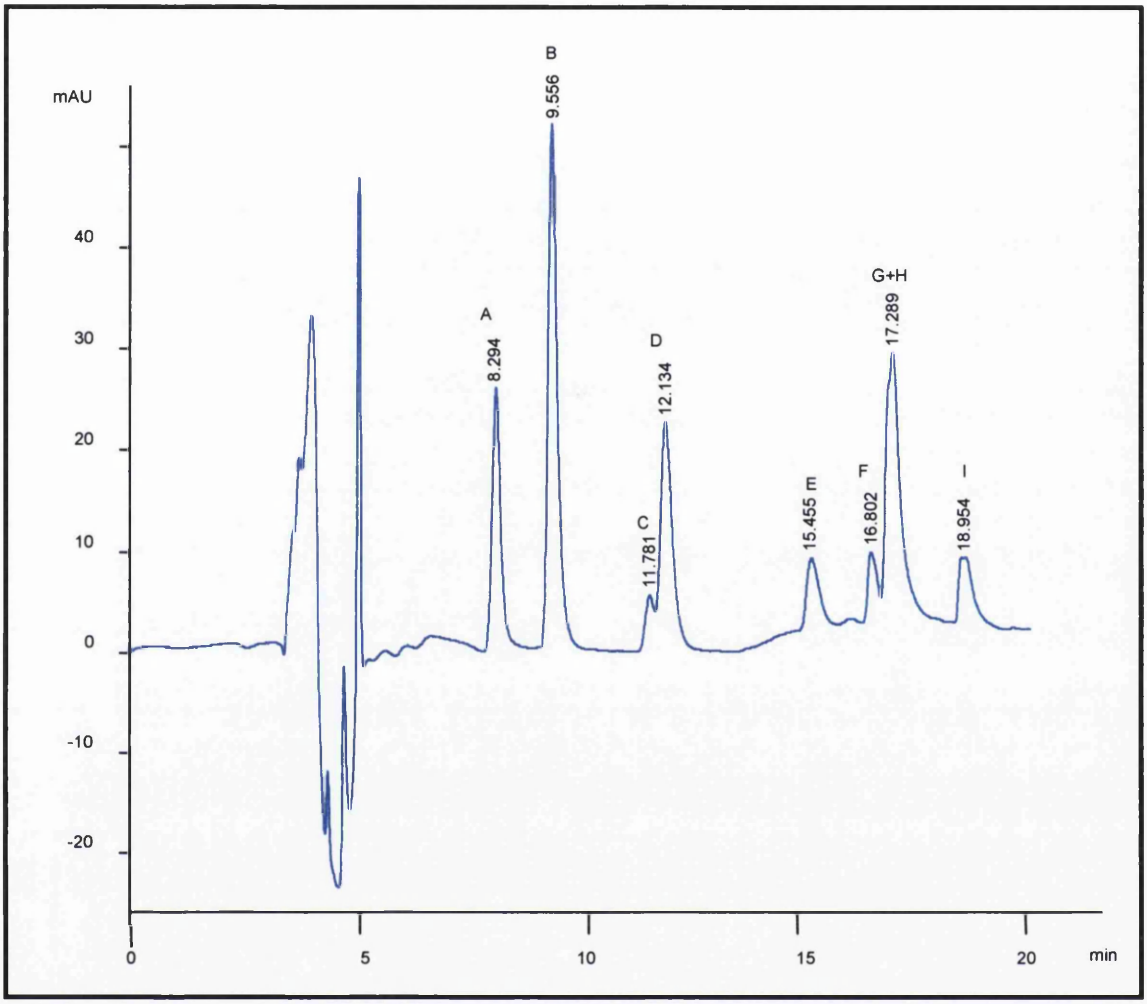



Figure 2.62 HPLC chromatogram of the separation of the 9 Acidic herbicides. 10 µL injection of a solution containing 10 µg/mL of each herbicide.

As can be seen from the chromatogram we were unable to develop the method to allow for the separation of mecoprop and bromoxynil; it was also necessary to incorporate a flow gradient to facilitate the separation of the acidic herbicides.

The following table gives the corresponding resolution values (Rs) for the separation shown above.

| Peaks | Rs Value |
|---|---|
| A+B | 2.57 |
| B+C | 4.29 |
| C+D | 0.75 |
| D+E | 6.00 |
| E+F | 2.67 |
| F+(G+H) | 0.71 |
| (G+H)+I | 2.44 |

## LC-MS For the Separation of the 9 Acidic Herbicides

The instrumentation and conditions used for LC-MS analysis of the nine herbicides was the same as that used in the LC-UV analysis performed using the phenyl-hexyl column. Mass spectrometric analysis was carried out using a Finnigan Mat LCQ ion trap; data was acquired in the negative ion-scanning mode.

Initial mass spectrometric analysis was carried out by using an infusion pump set to a flow rate of 5 to 10μL per minute to introduce standard solutions of each herbicide into the source; electrospray ionisation (ESI) was used to perform the analysis due to its suitability to low flow rates. From this analysis we were able to establish initial tune files for the compounds and identify m/z values associated with ionisation of the individual herbicides. LC-MS analysis was performed with both atmospheric pressure chemical ionisation (APCI) and ESI with the source conditions adjusted to suit the higher flow rates, below are the conditions used for both forms of ionisation.

**APCI**

| | |
|---|---|
| Polarity: Negative. | Sheath Gas Flow: 90psi. |
| Heated Capillary Temperature: 200°C. | Auxiliary Gas Flow: 50psi. |
| Electron Multiplier Voltage: 1150V. | Scan Range: 180-600amu. |
| Vaporiser Temperature: 450°C. | Corona Discharge Current: 5μ amps. |

**ESI**

Polarity: Negative.

Heated Capillary Temperature: 200°C.

Electron Multiplier Voltage: 1150V.

Needle Spray Voltage: 4.5kV.

Sheath Gas Flow: 90psi.

Auxiliary Gas Flow: 10psi.

Scan Range: 180-600amu.

For ESI analysis the flow from the HPLC system was split to allow approximately 200μL per minute into the ion source (a split of 20%).

It was found that both forms of ionisation yielded the same base ions, although in the case of APCI some of the acidic herbicides had a m/z charge occurring at an increase of 82 m/z units; we believe that this may be due to some form of adduct occurring due to the addition of 2 acetonitrile molecules, you would however expect to also see an ion at an increase of 41 m/z units associated with the addition of 1 acetonitrile molecule to the parent ion. Figure 2.63 shows a comparison of the ESI and APCI spectra of MCPA as an example.

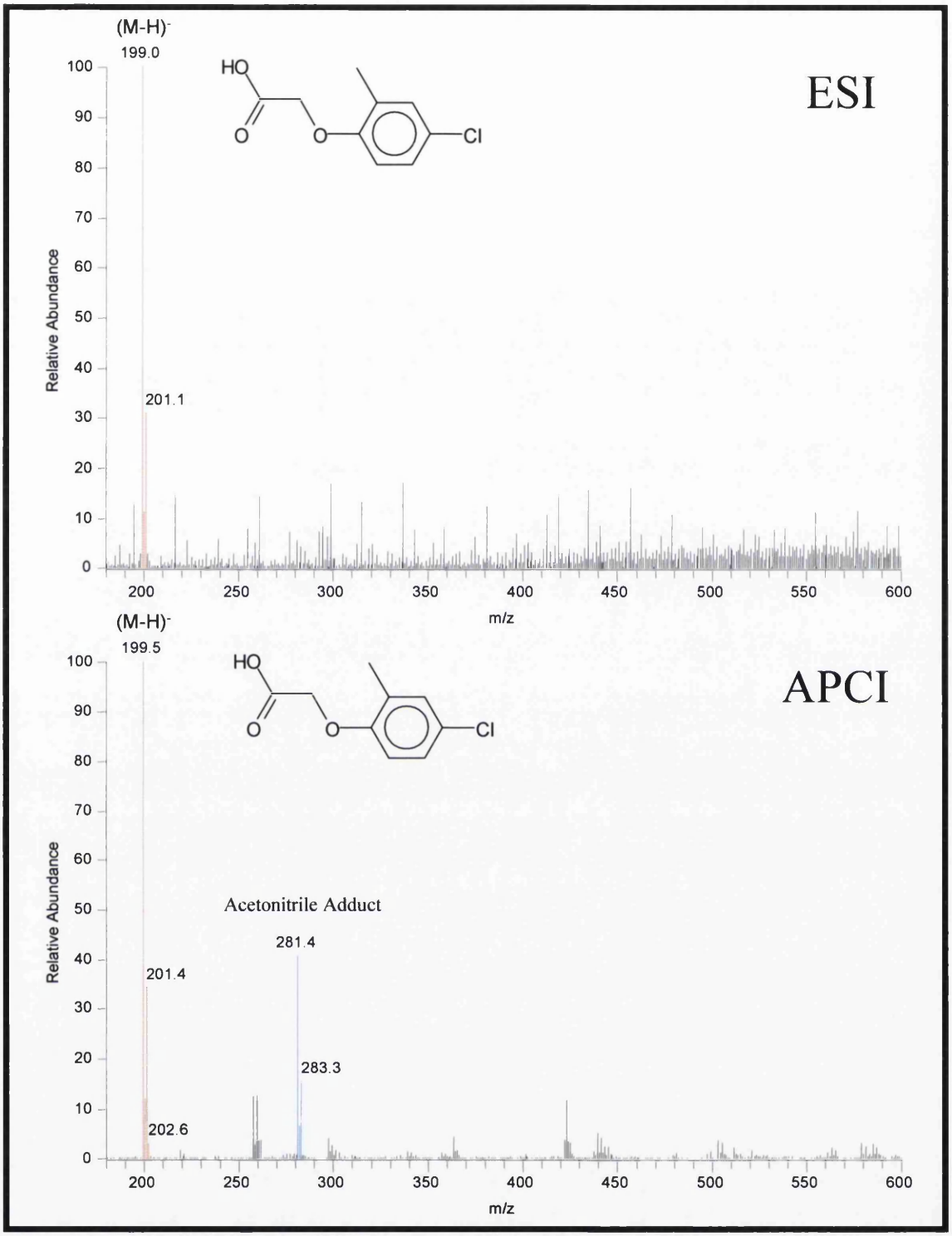



Figure 2.63 Negative ESI and APCI mass spectrum comparison of MCPA. 10µL injection of a 10µg/mL solution; equal to 100ng on column.

The m/z occurring at an increase of 82 m/z units may be due to the addition of 2 acetonitrile molecules to the $[M-H]^-$ base ion.

Figure 2.64 shows the total ion chromatogram (TIC) for the separation of the 9 herbicides and figure 2.65 the extracted ion chromatograms of the most abundant ion of each herbicide (except in the case of benazolin where the M-H ion was monitored for).

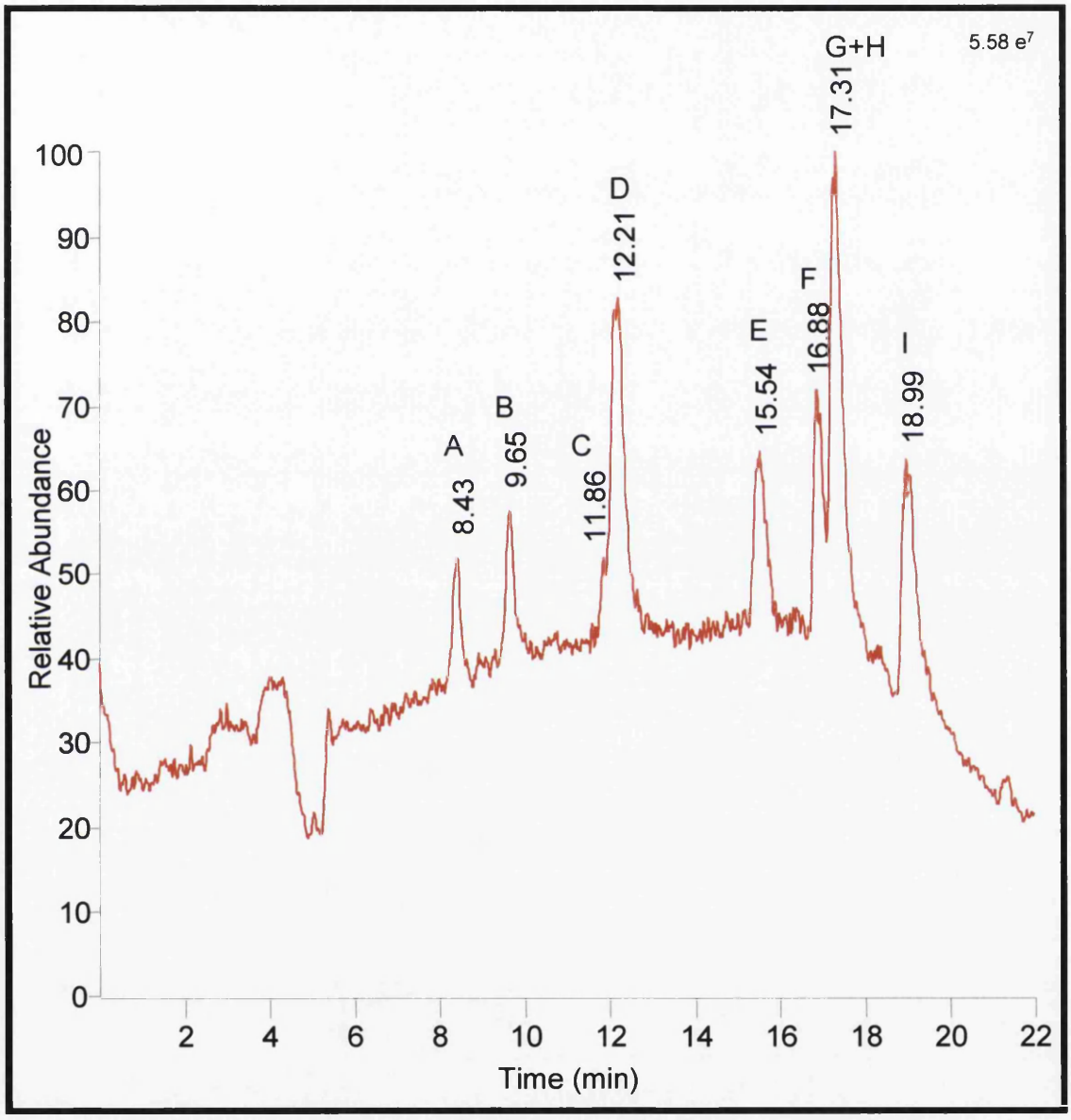



Figure 2.64 Negative LC-ESI-MS total ion chromatogram for the separation of the 9 herbicides. 10µL injection of a 10µg/mL solution of each herbicide; equal to 100ng on column of each.

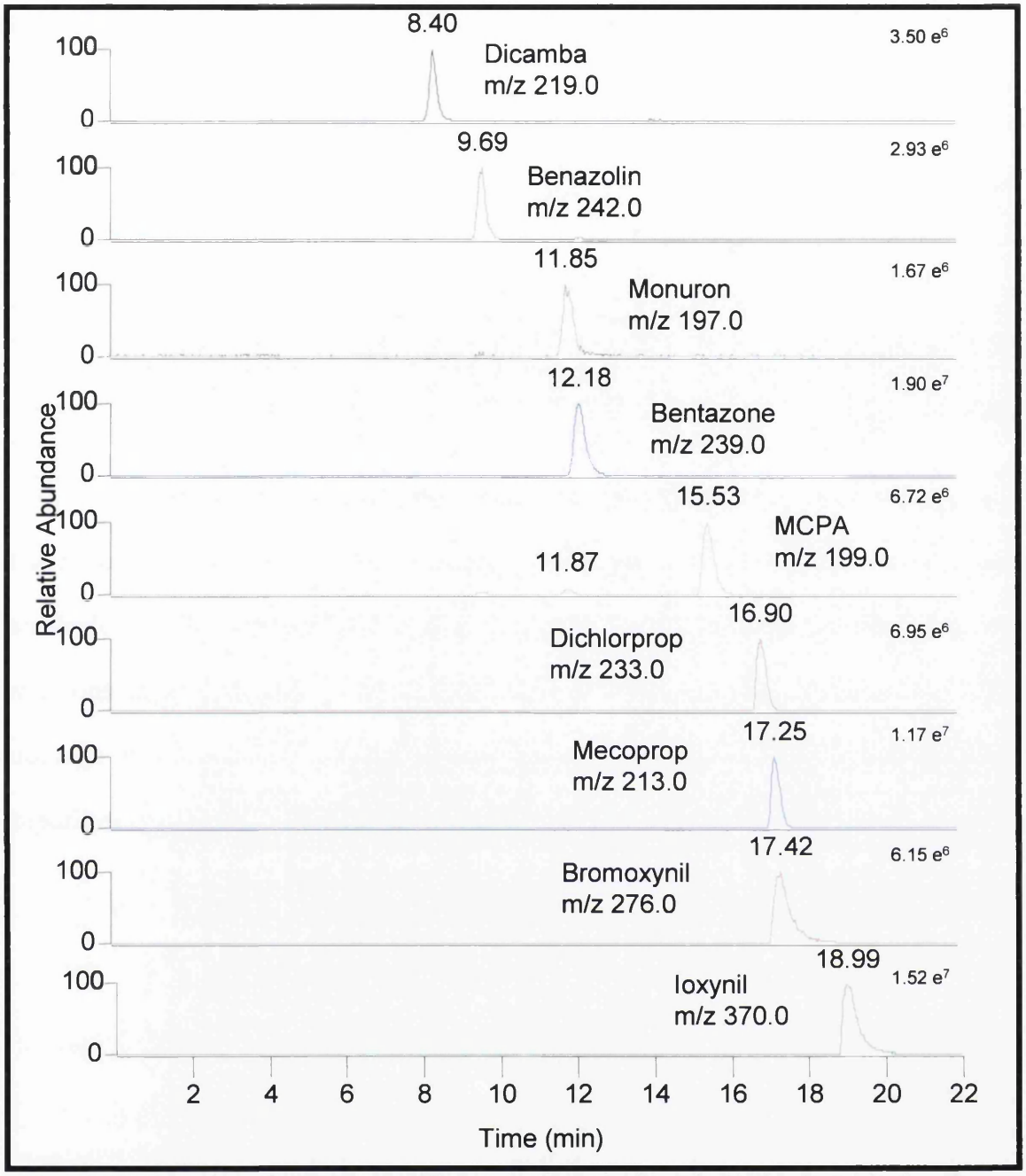



Figure 2.65 Extracted ion chromatograms of the base ion for each of the 9 herbicides. 10µL injection of a 10µg/mL solution of each herbicide; equal to 100ng on column of each.

The peak occurring at 11.87 minutes in the background subtracted spectrum of MCPA is due to the presence of an ion of m/z 199 in the spectrum of monuron; the presence of chlorine in monuron causes this due to the chlorine 37 isotope.

The table below lists the m/z values used for the extracted ion chromatograms.

| Compound name | TIC Peak label | m/z |
|---|---|---|
| Dicamba | A | 219.0 |
| Benazolin | B | 242.0 |
| Monuron | C | 197.0 |
| Bentazone | D | 239.0 |
| MCPA | E | 199.0 |
| Dichlorprop | F | 233.0 |
| Mecoprop | H | 213.0 |
| Bromoxynil | I | 276.0 |
| Ioxynil | J | 370.0 |

The following figures (2.66-2.74) show the mass spectrum, with ESI, for each of the herbicides and the proposed mass spectrum interpretation; the m/z values associated with ions other than the parent ion are assumed to be due to in source fragmentation although this is not definitive and they may in fact be due to the presence of impurities in the standard materials.

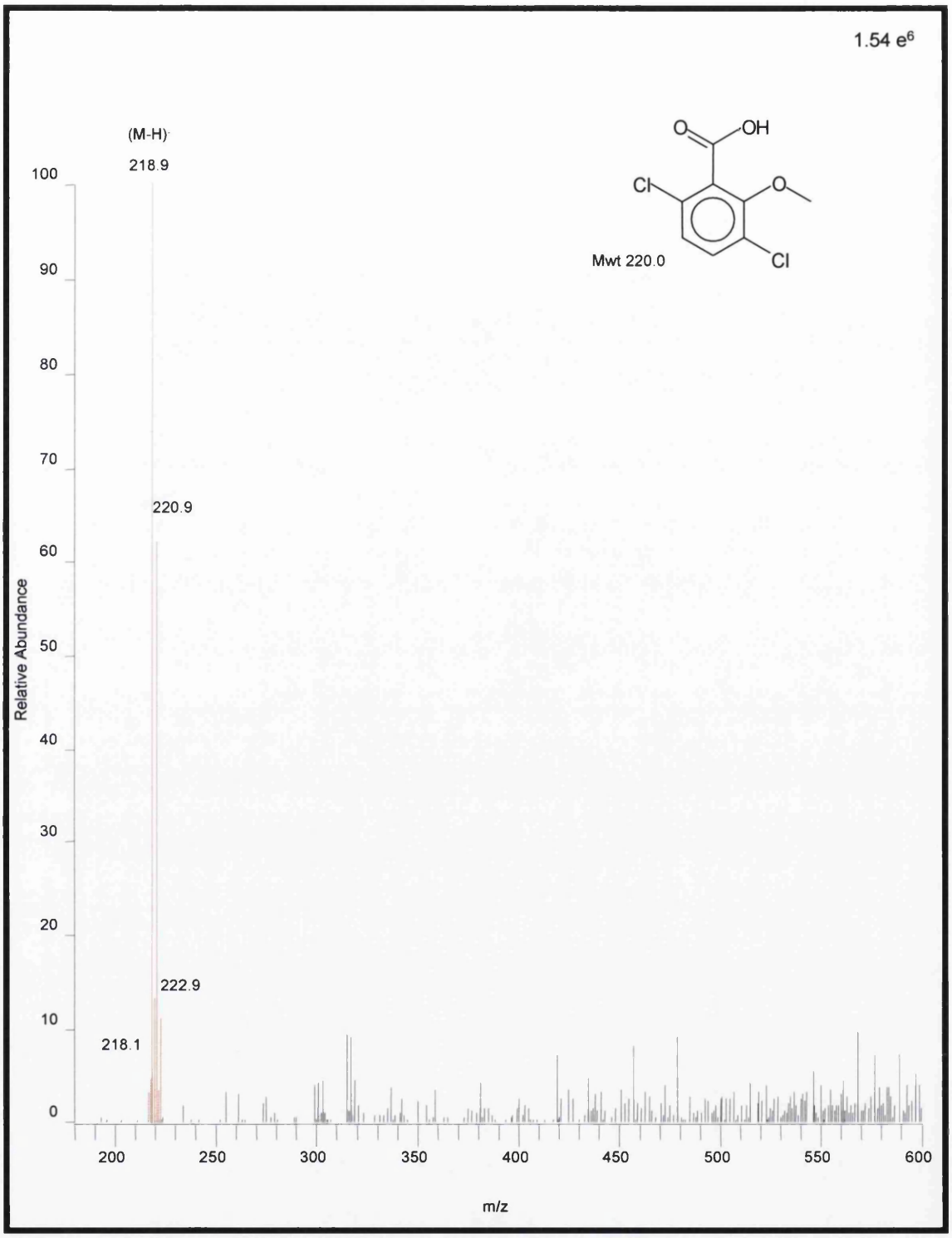



Figure 2.66 Negative LC-ESI-MS mass spectrum of Dicamba. 10µL injection of a 10µg/mL solution; equal to 100ng on column.

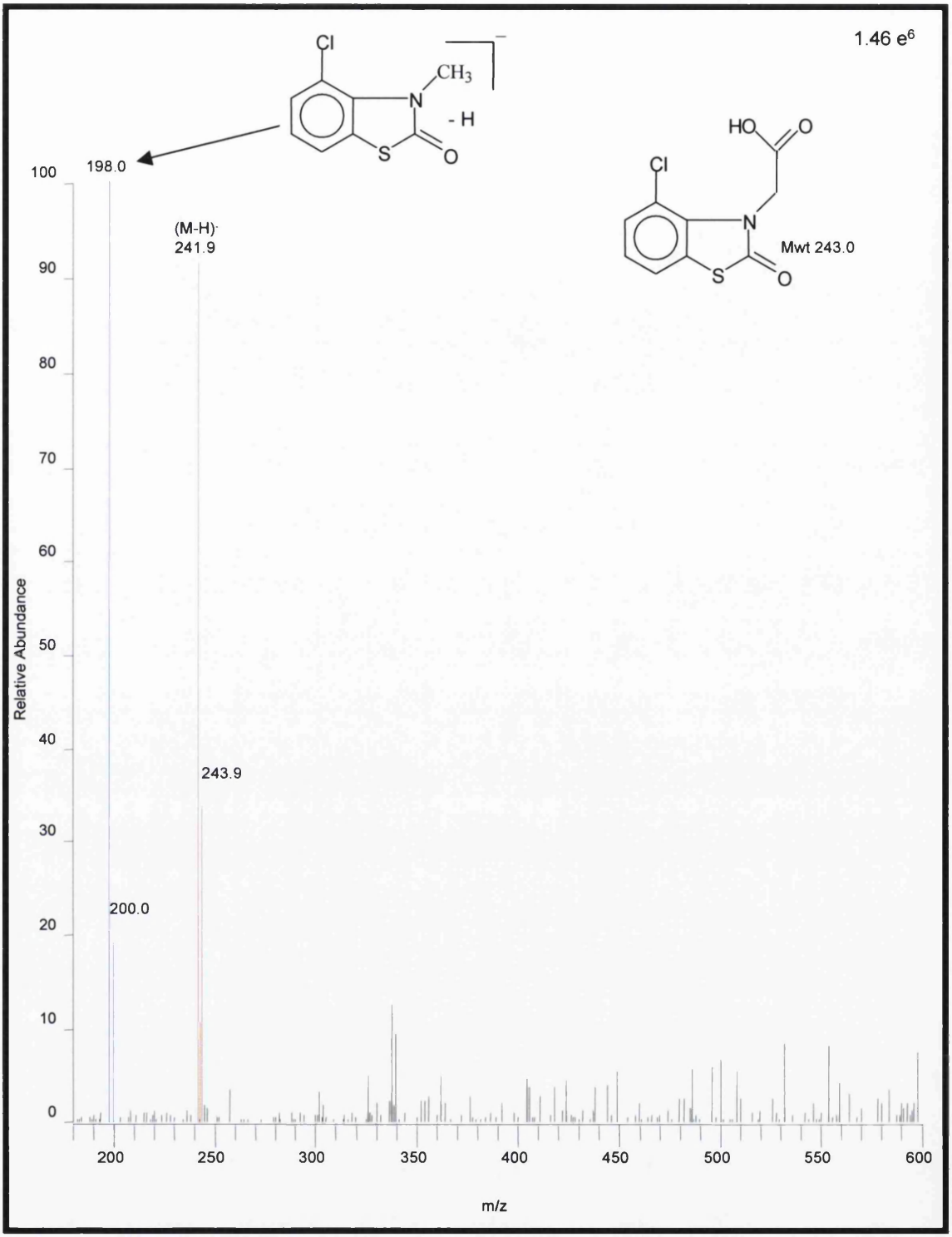



Figure 2.67 Negative LC-ESI-MS mass spectrum of Benazolin. 10µL injection of a 10µg/mL solution; equal to 100ng on column.

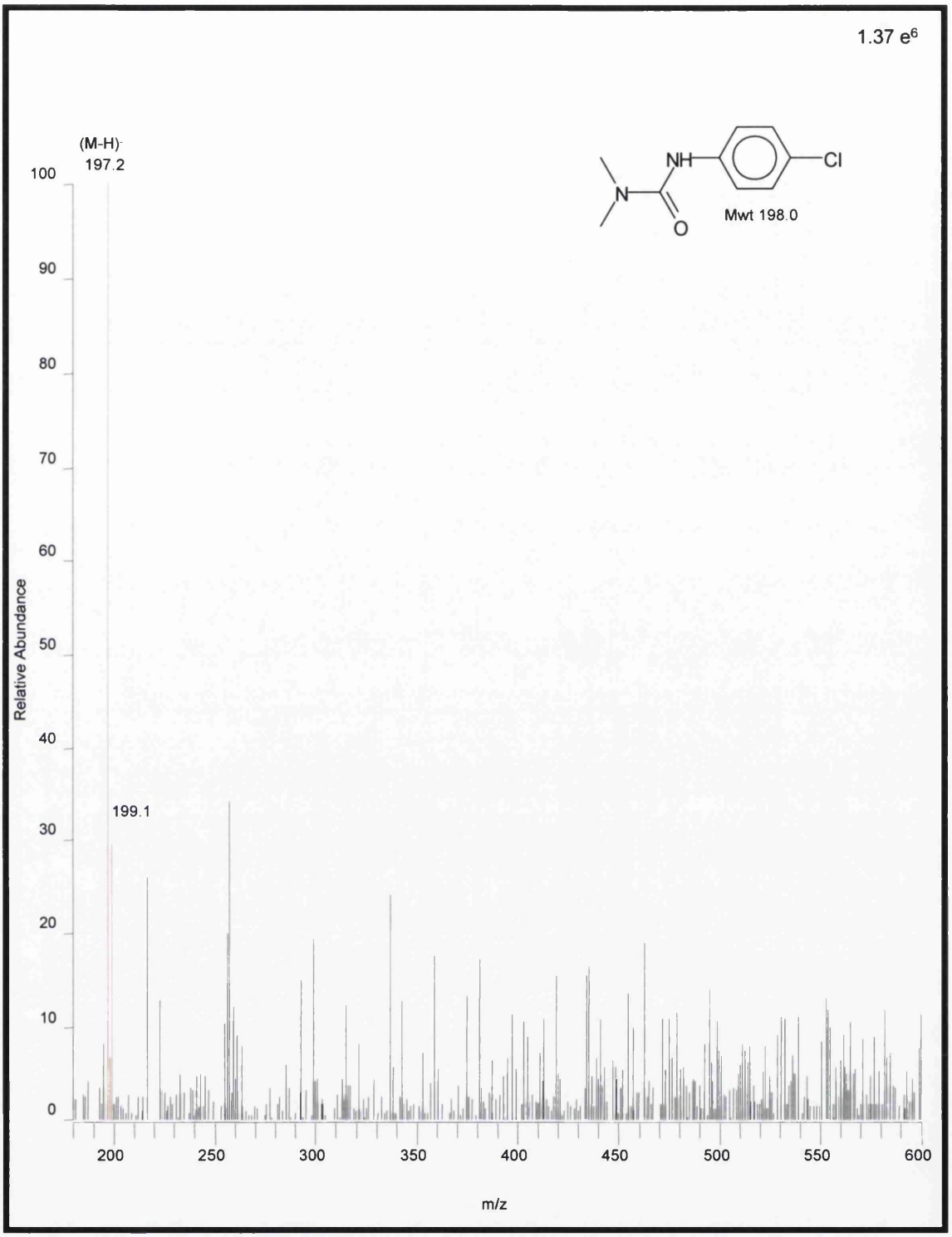



Figure 2.68 Negative LC-ESI-MS mass spectrum of Monuron. 10µL injection of a 10µg/mL solution; equal to 100ng on column.

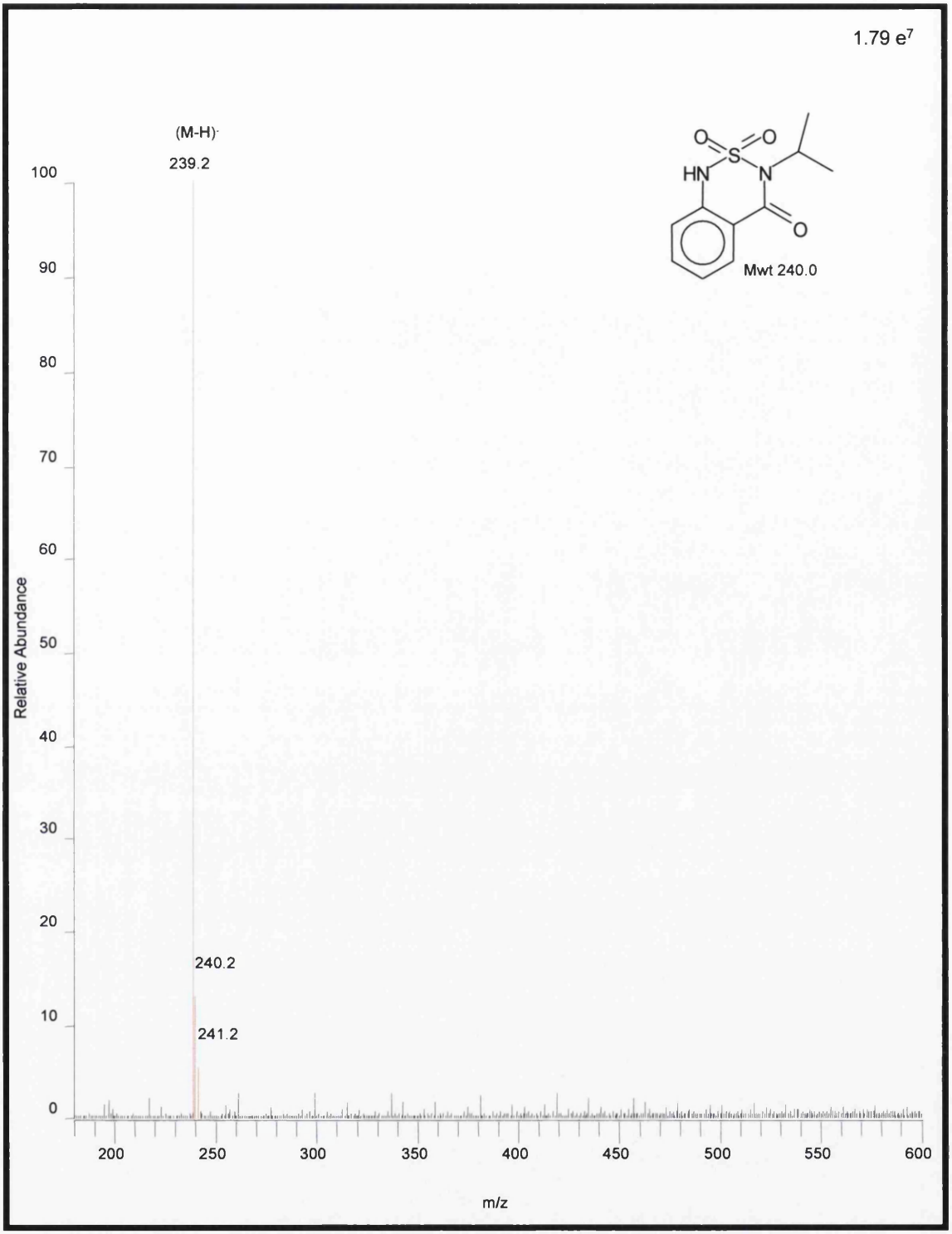



Figure 2.69 Negative LC-ESI-MS mass spectrum of Bentazone. 10µL injection of a 10µg/mL solution; equal to 100ng on column.

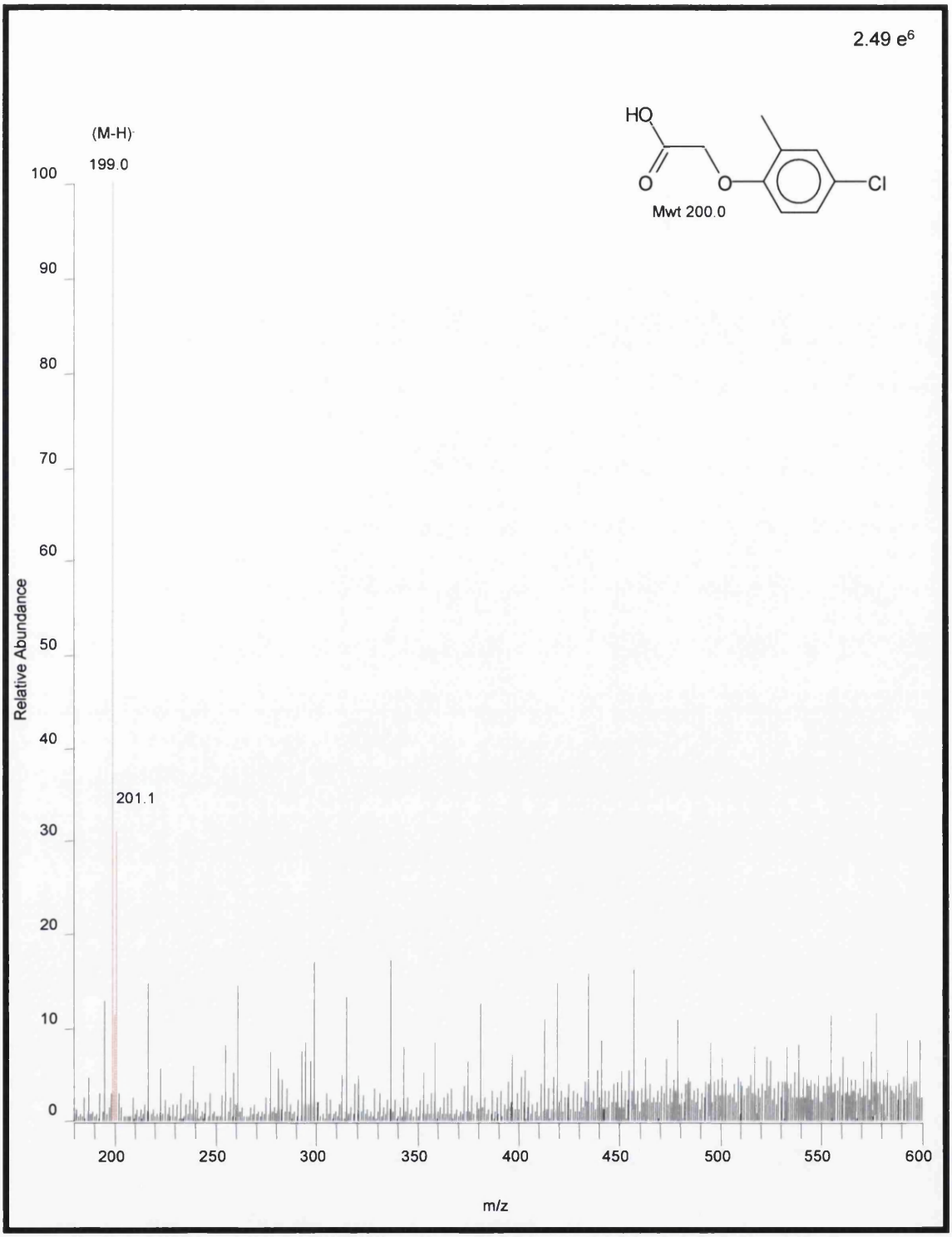



Figure 2.70 Negative LC-ESI-MS mass spectrum of MCPA. 10µL injection of a 10µg/mL solution; equal to 100ng on column.

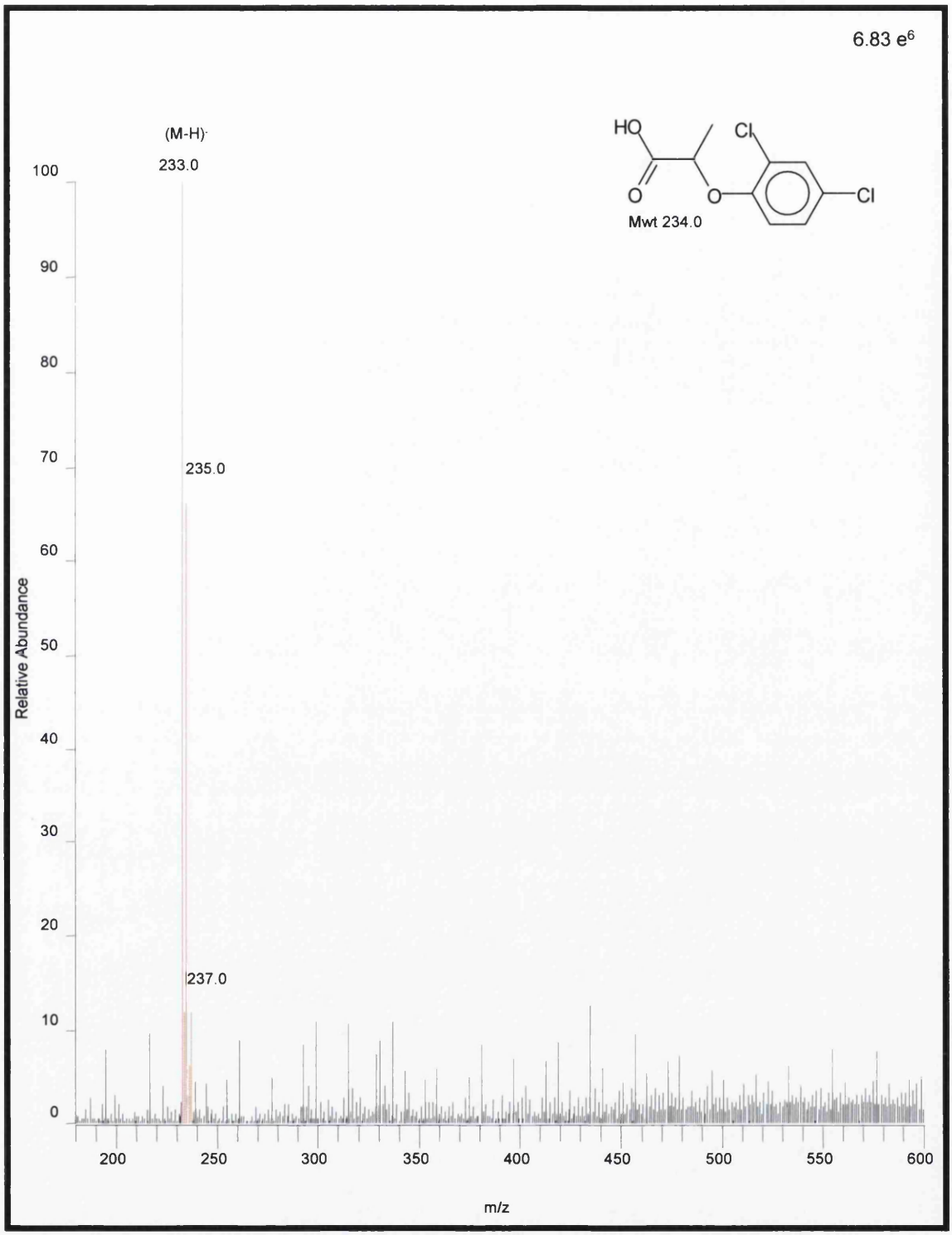



Figure 2.71 Negative LC-ESI-MS mass spectrum of Dichlorprop. 10µL injection of a 10µg/mL solution; equal to 100ng on column.

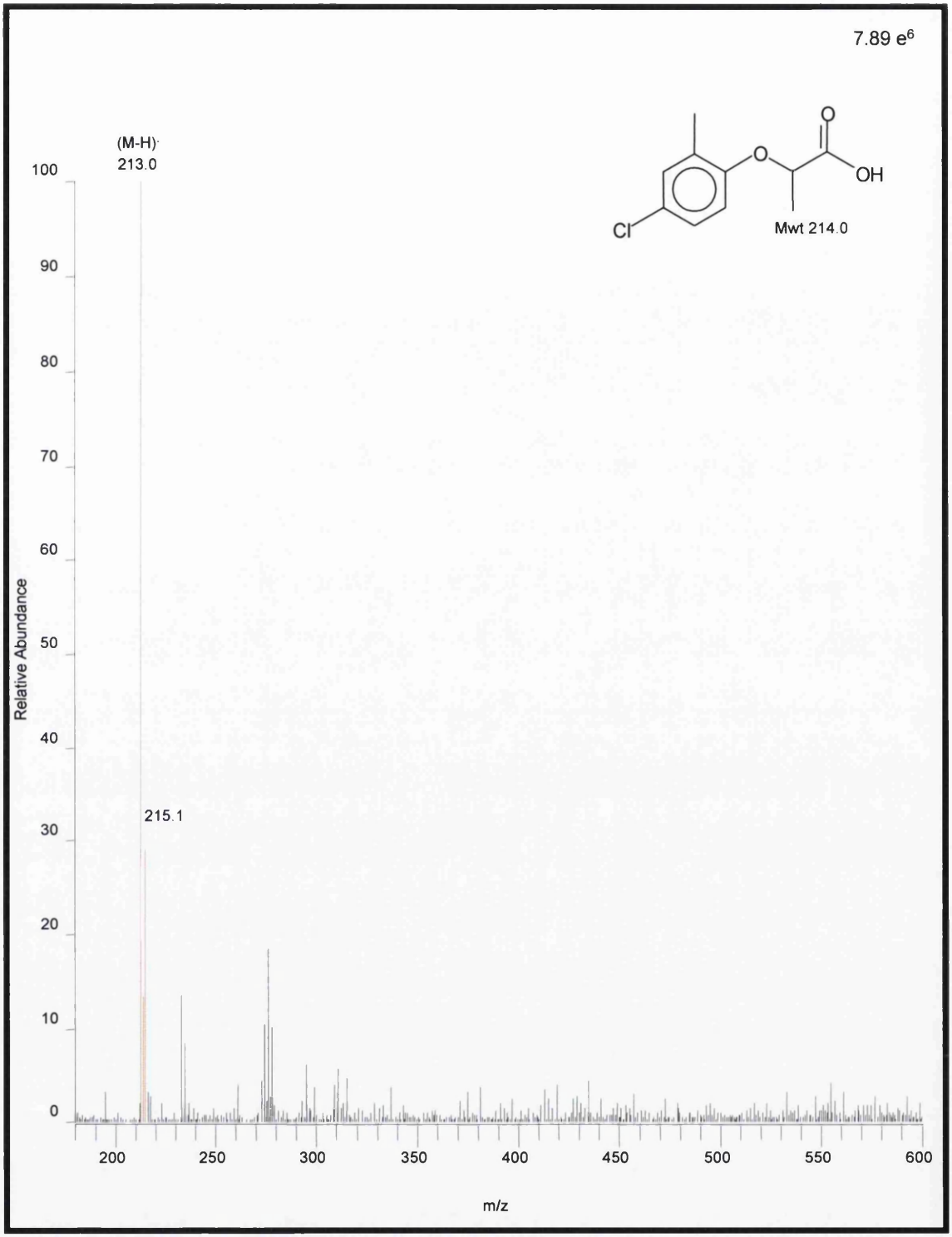



Figure 2.72 Negative LC-ESI-MS mass spectrum of Mecoprop. 10µL injection of a 10µg/mL solution; equal to 100ng on column.

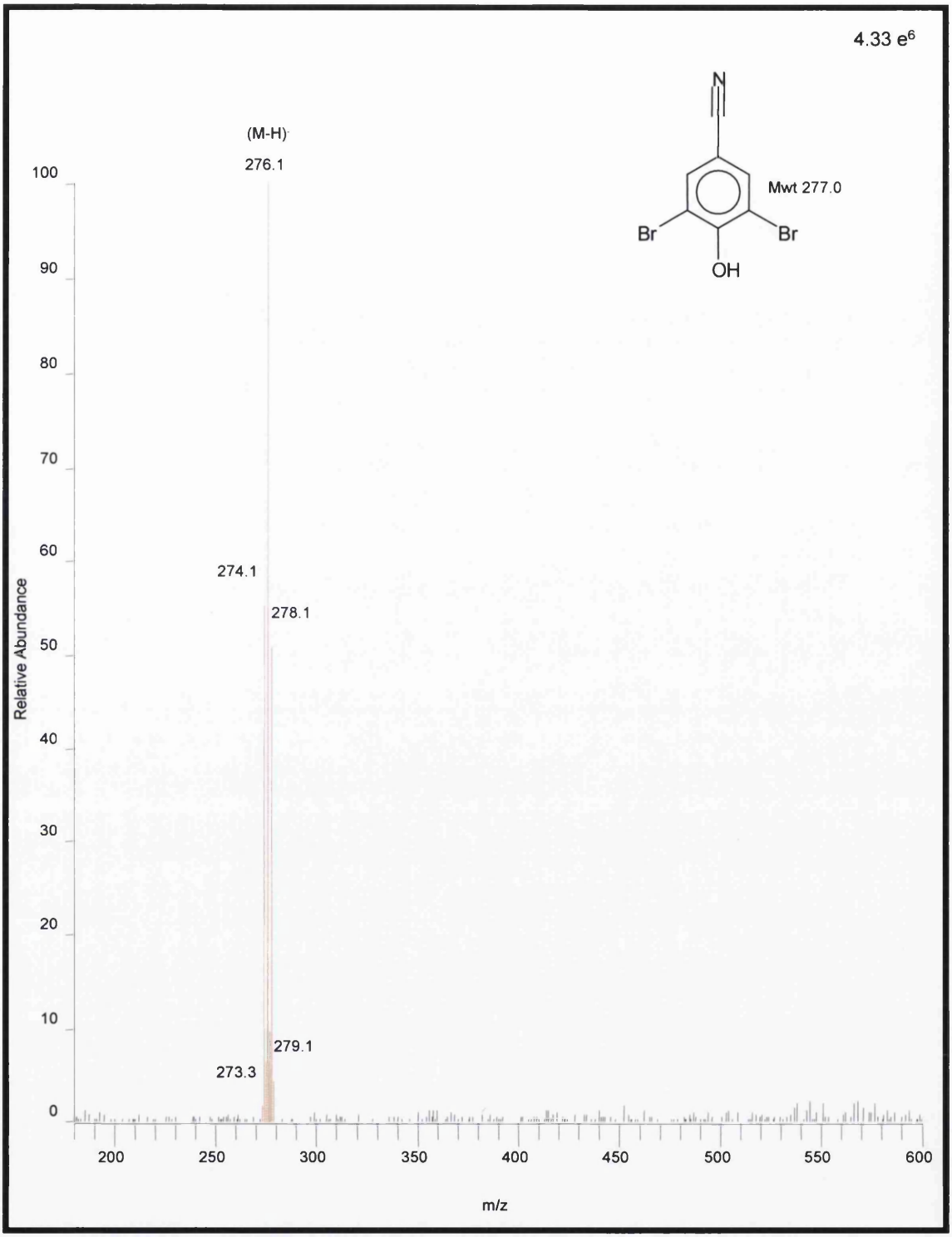



Figure 2.73 Negative LC-ESI-MS mass spectrum of Bromoxynil. 10µL injection of a 10µg/mL solution; equal to 100ng on column.

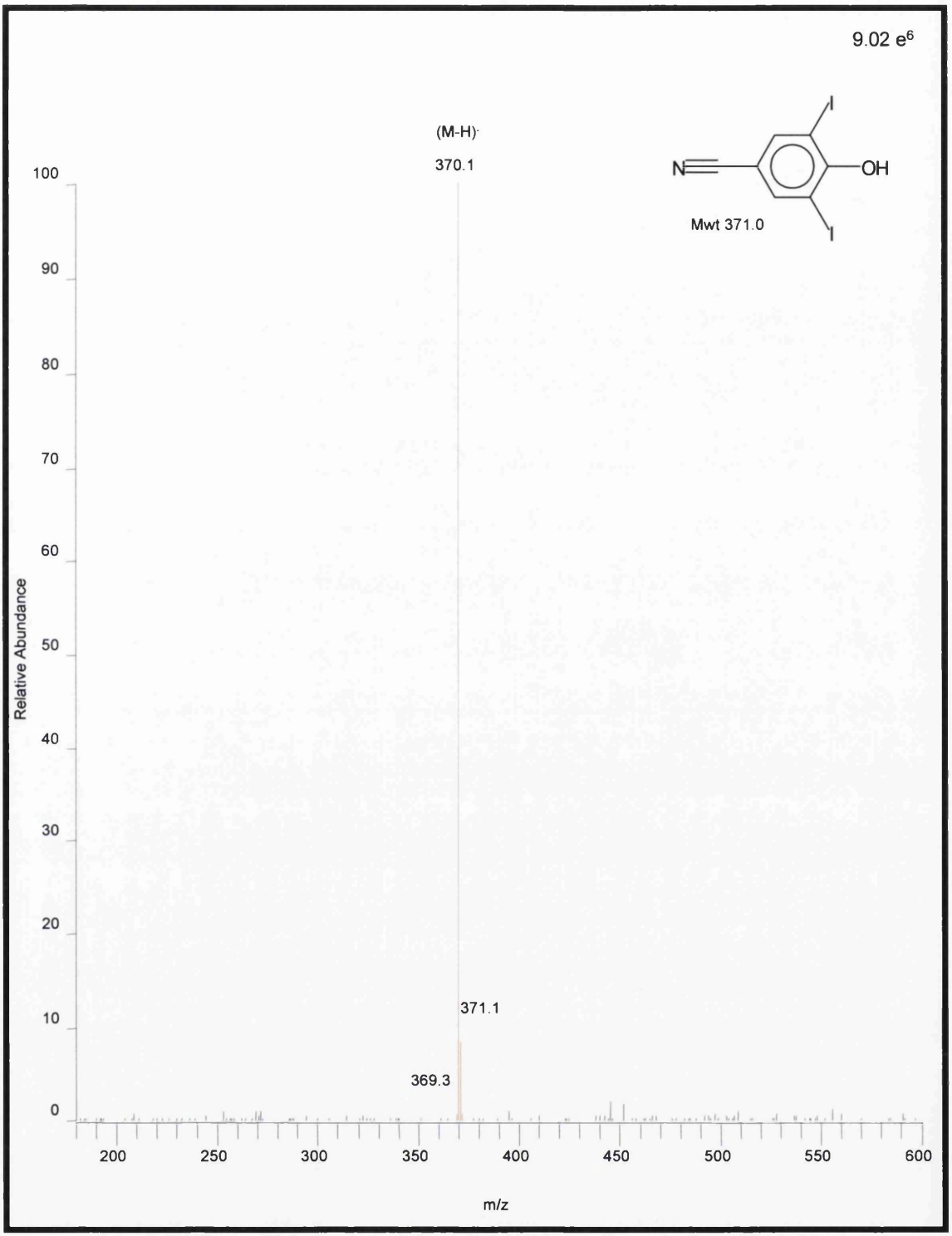



Figure 2.74 Negative LC-ESI-MS mass spectrum of Ioxynil. 10µL injection of a 10µg/mL solution; equal to 100ng on column.

## Limits of Detection for the 9 Herbicides

Detection limits were obtained by taking extracted ion chromatograms of the m/z of the most abundant ion for each herbicide (except in the case of benazolin where the M-H ion was monitored for) from the TIC. Limits of detection (LOD) were calculated by analysing a series of solutions at the following concentration levels; 1ng/mL, 10ng/mL, 100ng/mL, 1μg/mL, and 10μg/mL, the LOD was regarded as the concentration from this series at which a signal to noise ratio of at least 5 to 1 was achievable. The tables below show the LOD achieved with ESI and APCI; the amount of compound injected on column is also given.

**ESI**

| Compound name | m/z Monitored | LOD ESI | Amount on column |
|---|---|---|---|
| Dicamba | 219.0 | 100ng/mL | 1ng |
| Benazolin | 242.0 | 1μg/mL | 10ng |
| Monuron | 197.0 | 10μg/mL | 100ng |
| Bentazone | 239.0 | 100ng/mL | 1ng |
| MCPA | 199.0 | 100ng/mL | 1ng |
| Dichlorprop | 233.0 | 10ng/mL | 100pg |
| Mecoprop | 213.0 | 10ng/mL | 100pg |
| Bromoxynil | 276.0 | 100ng/mL | 1ng |
| Ioxynil | 370.0 | 10ng/mL | 100pg |

On column injection volume of 10μL. LOD is calculated as the concentration of the solution being injected.

**APCI**

| Compound name | m/z Monitored | LOD APCI | Amount on column |
|---|---|---|---|
| Dicamba | 219.0 | 1μg/mL | 10ng |
| Benazolin | 242.0 | 1μg/mL | 10ng |
| Monuron | 197.0 | 1μg/mL | 10ng |
| Bentazone | 239.0 | 100ng/mL | 1ng |
| MCPA | 199.0 | 100ng/mL | 1ng |
| Dichlorprop | 233.0 | 100ng/mL | 1ng |
| Mecoprop | 213.0 | 100ng/mL | 1ng |
| Bromoxynil | 276.0 | 100ng/mL | 1ng |
| Ioxynil | 370.0 | 100ng/mL | 1ng |

On column injection volume of 10μL. LOD is calculated as the concentration of the solution being injected.

As can be seen from the tables ESI was found to be more sensitive than APCI in the case of the majority of the 9 herbicides and as such was the chosen form of ionisation for $MS^n$ analysis.

**Instrument Response**

The table below lists the instrument response in ESI-MS mode to a 10μL injection of a solution made up of a mixture of each of the herbicides at a concentration of 10μg/mL (the raw data has been presented earlier in this chapter in figures 2.66-2.74). From this data the response per nanogram on column of each herbicide has been calculated.

| Compound name | Instrument response | Response per ng on column |
|---|---|---|
| Dicamba | 1.54 $e^6$ | 1.54 $e^4$ |
| Benazolin | 1.46 $e^6$ | 1.46 $e^4$ |
| Monuron | 1.37 $e^6$ | 1.37 $e^4$ |
| Bentazone | 1.79 $e^7$ | 1.79 $e^5$ |
| MCPA | 2.49 $e^6$ | 2.49 $e^4$ |
| Dichlorprop | 6.83 $e^6$ | 6.83 $e^4$ |
| Mecoprop | 7.84 $e^6$ | 7.84 $e^4$ |
| Bromoxynil | 4.33 $e^6$ | 4.33 $e^4$ |
| Ioxynil | 9.02 $e^6$ | 9.02 $e^4$ |

The response per nanogram on column could be used to give an indication of the amount of a herbicide present in an unknown sample; however without the use of an internal standard its value is limited.

## LC-ESI-MS$^n$ For the Separation of the 9 Acidic Herbicides

Structural information was gained by the fragmentation of each of the most abundant ions obtained from LC-ESI-MS (except in the case of benazolin). This gives a specific method of identification for each of the herbicides for on-line analysis. The following table shows the precursor and product ions and the relative collision energies required to induce fragmentation.

| Compound name | Precursor ion | CE % | Product ions |
|---|---|---|---|
| Dicamba | 219.0 | 12.1 | 175 |
| Benazolin | 242.0 | 16.1 | 198 |
| Monuron | 197.0 | 17.1 | 152 |
| Bentazone | 239.0 | 18.1 | 197, 175, 132 |
| MCPA | 199.0 | 14.1 | 155, 141 |
| Dichlorprop | 233.0 | 12.1 | 161 |
| Mecoprop | 213.0 | 13.1 | 169, 141, 71 |
| Bromoxynil | 276.0 | 21.1 | 81, 79 |
| Ioxynil | 370.0 | 24.1 | 243, 127 |

The following figures (2.75-2.92) show the LC-ESI-MS-MS mass spectra for each herbicide and the proposed fragmentation pattern interpretation.

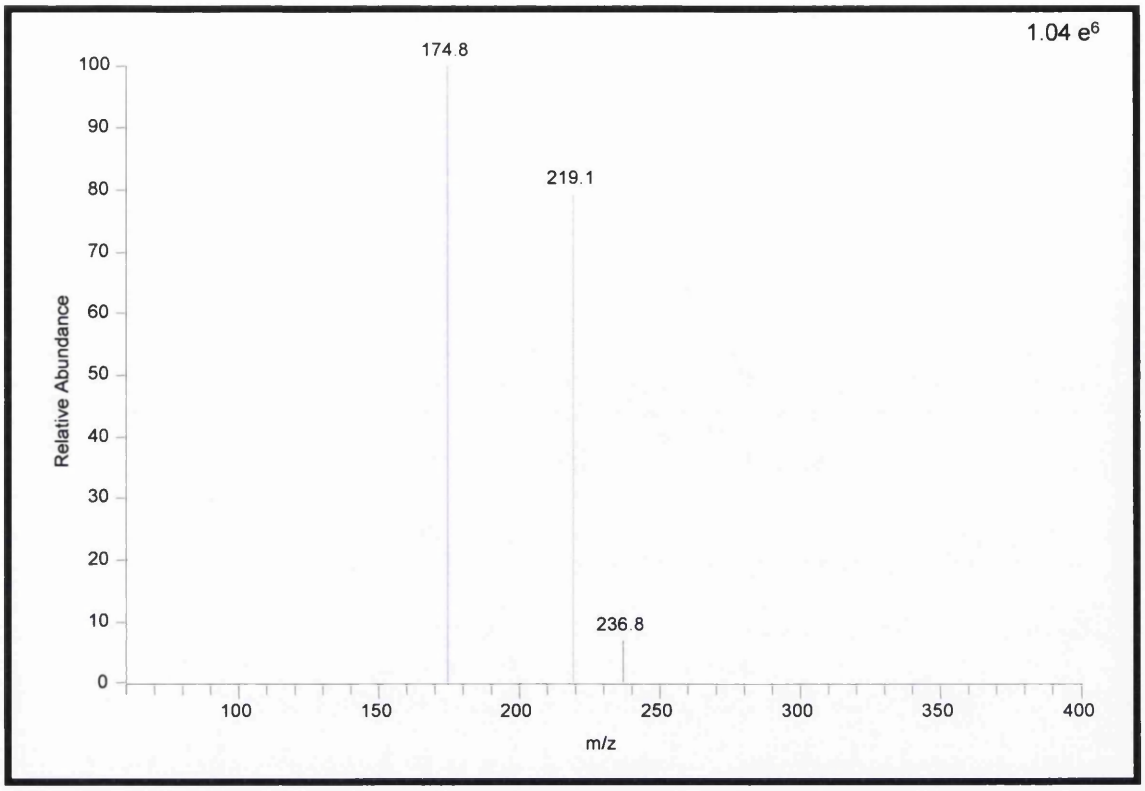



Figure 2.75 Negative LC-ESI-MS-MS spectrum of Dicamba. 10µL injection of a 10µg/mL solution; equal to 100ng on column.

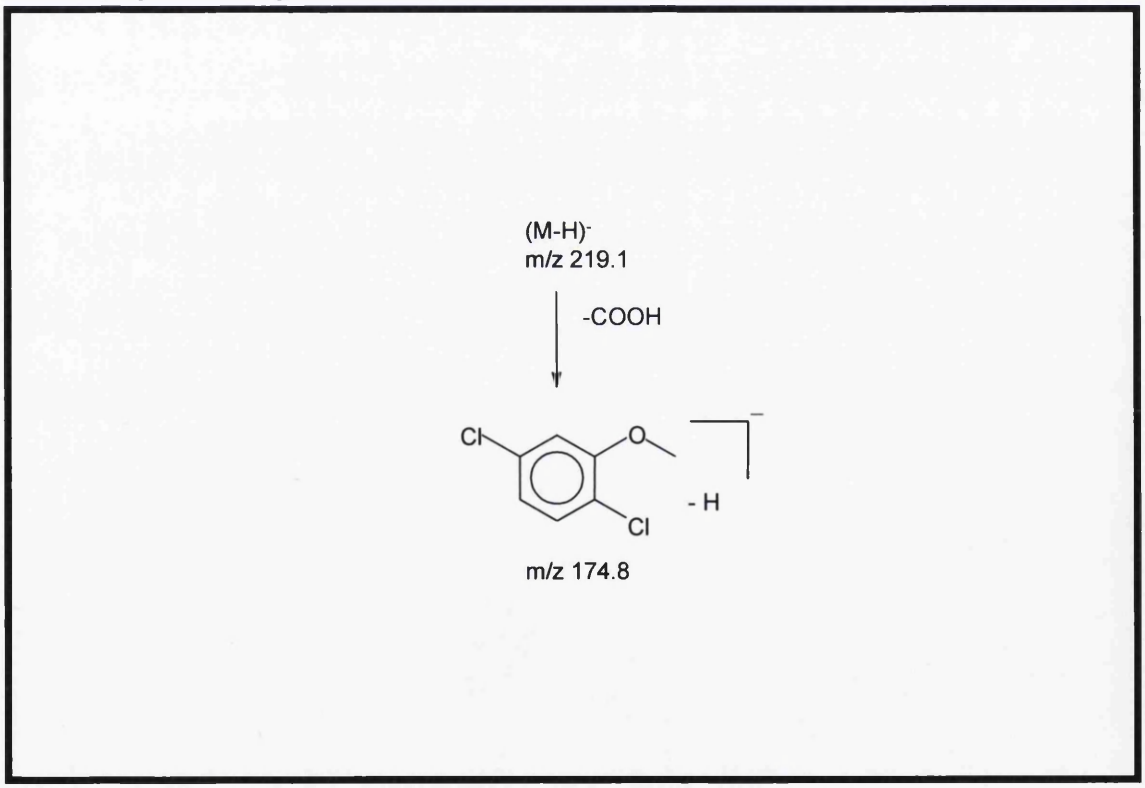



Figure 2.76 Proposed dicamba fragmentation pattern interpretation.

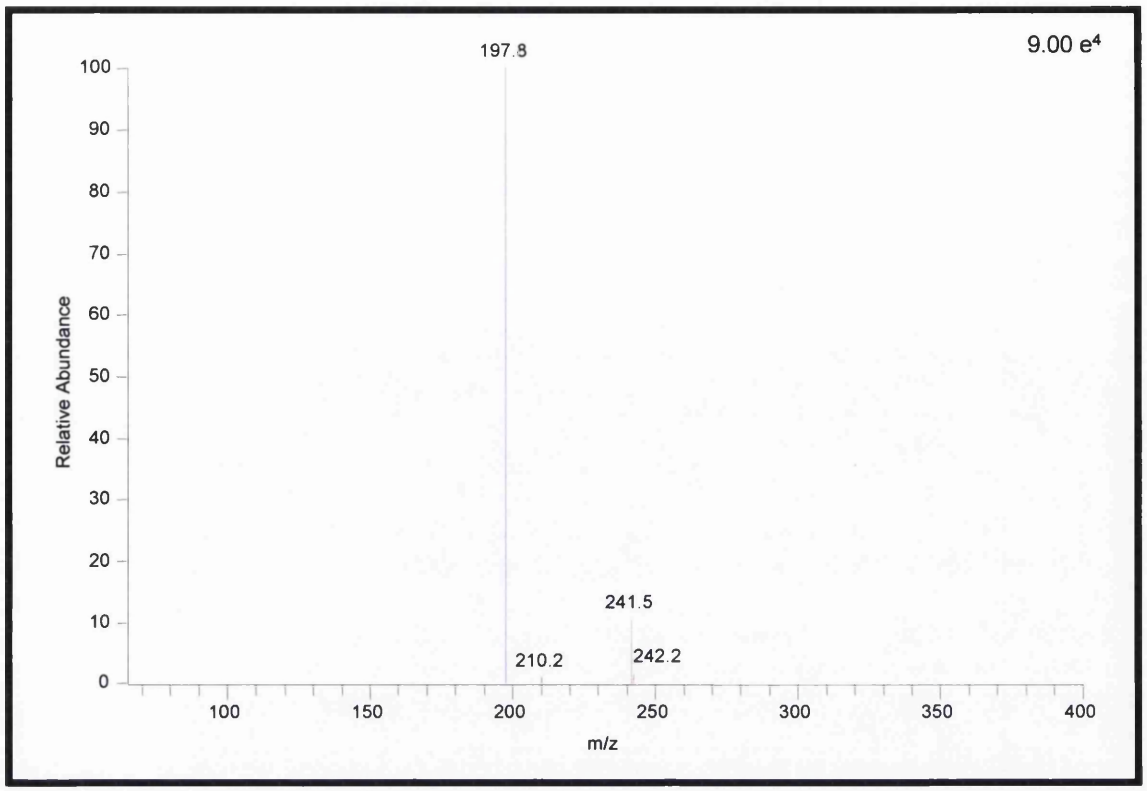



Figure 2.77 Negative LC-ESI-MS-MS spectrum of Benazolin. 10µL injection of a 10µg/mL solution; equal to 100ng on column.

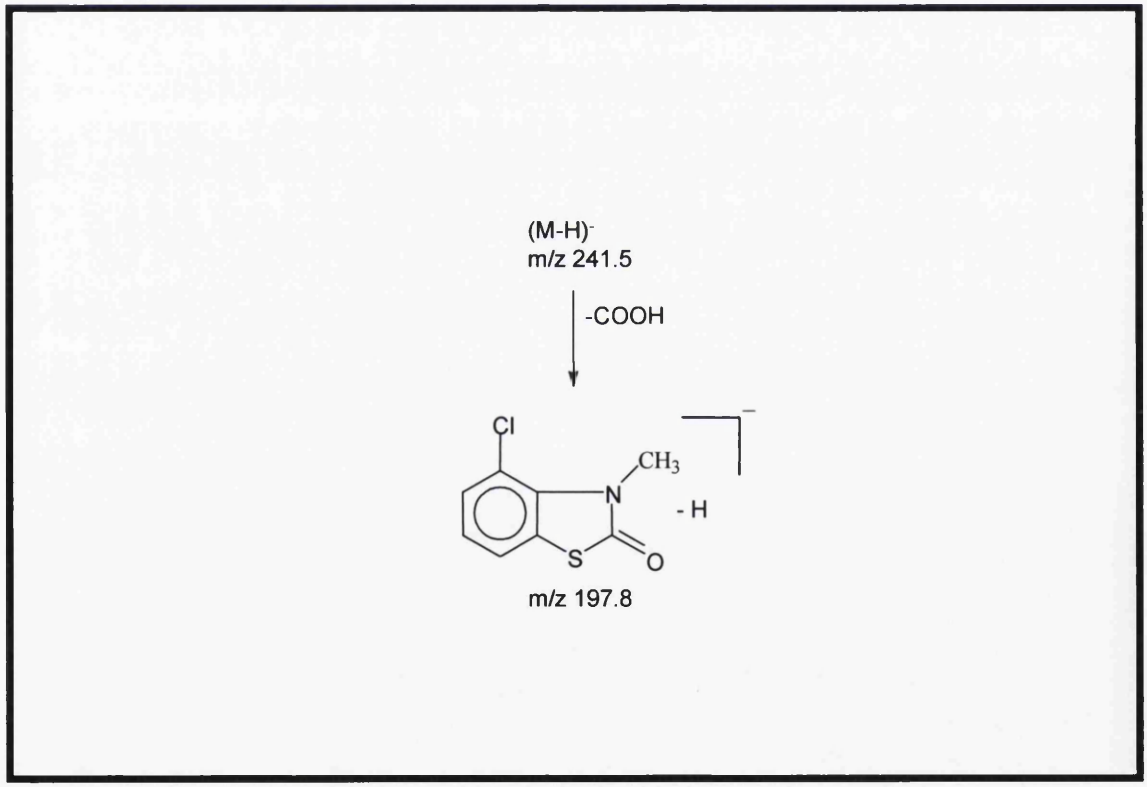



Figure 2.78 Proposed benazolin fragmentation pattern interpretation.

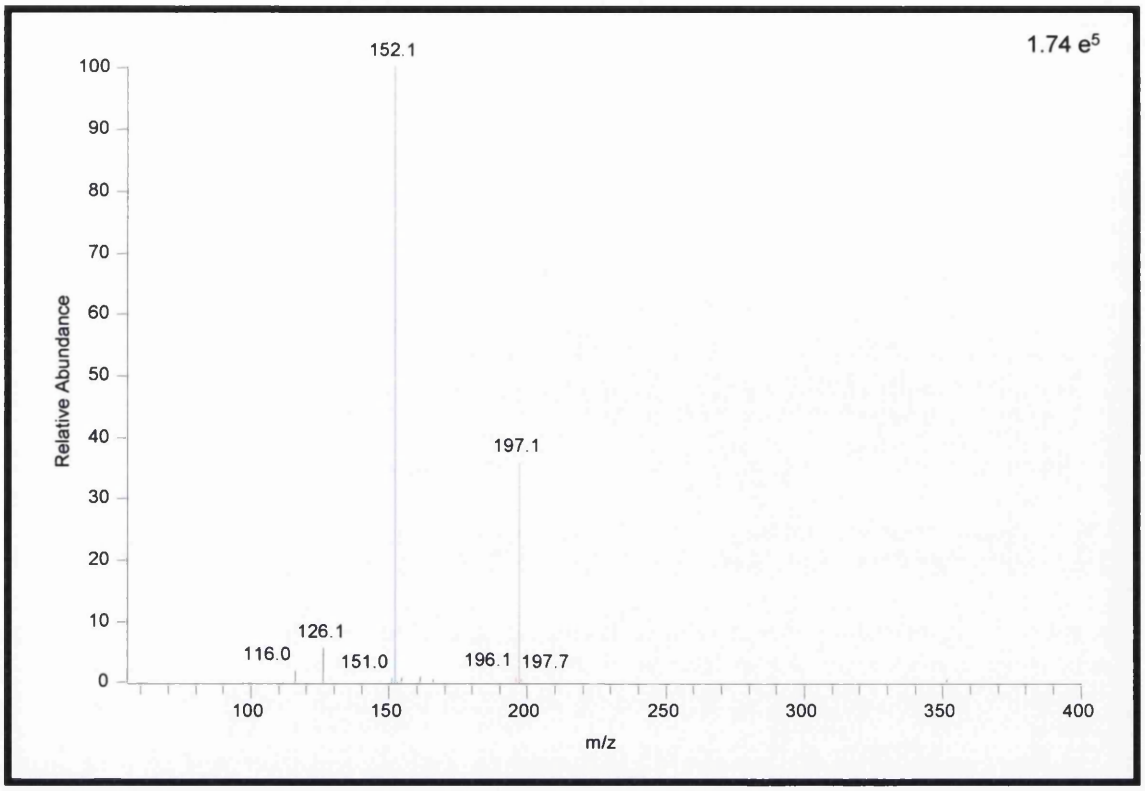



Figure 2.79 Negative LC-ESI-MS-MS spectrum of Monuron. 10µL injection of a 10µg/mL solution; equal to 100ng on column.

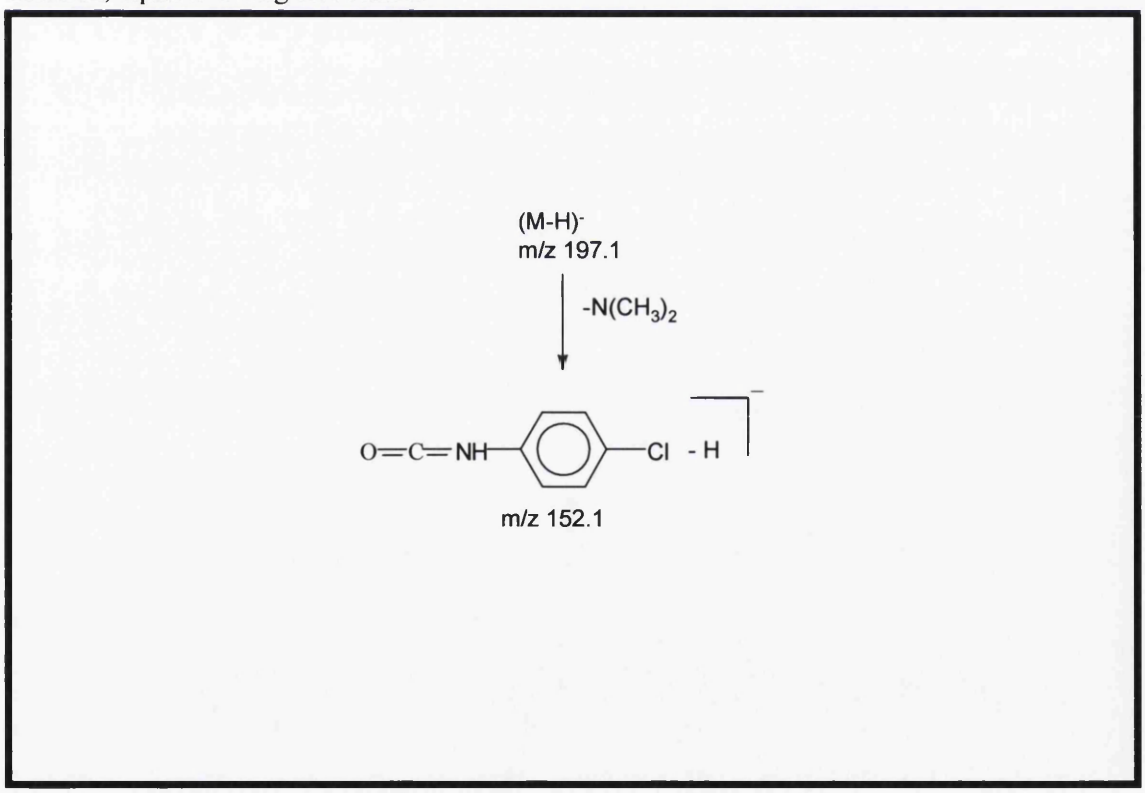



Figure 2.80 Proposed monuron fragmentation pattern interpretation.

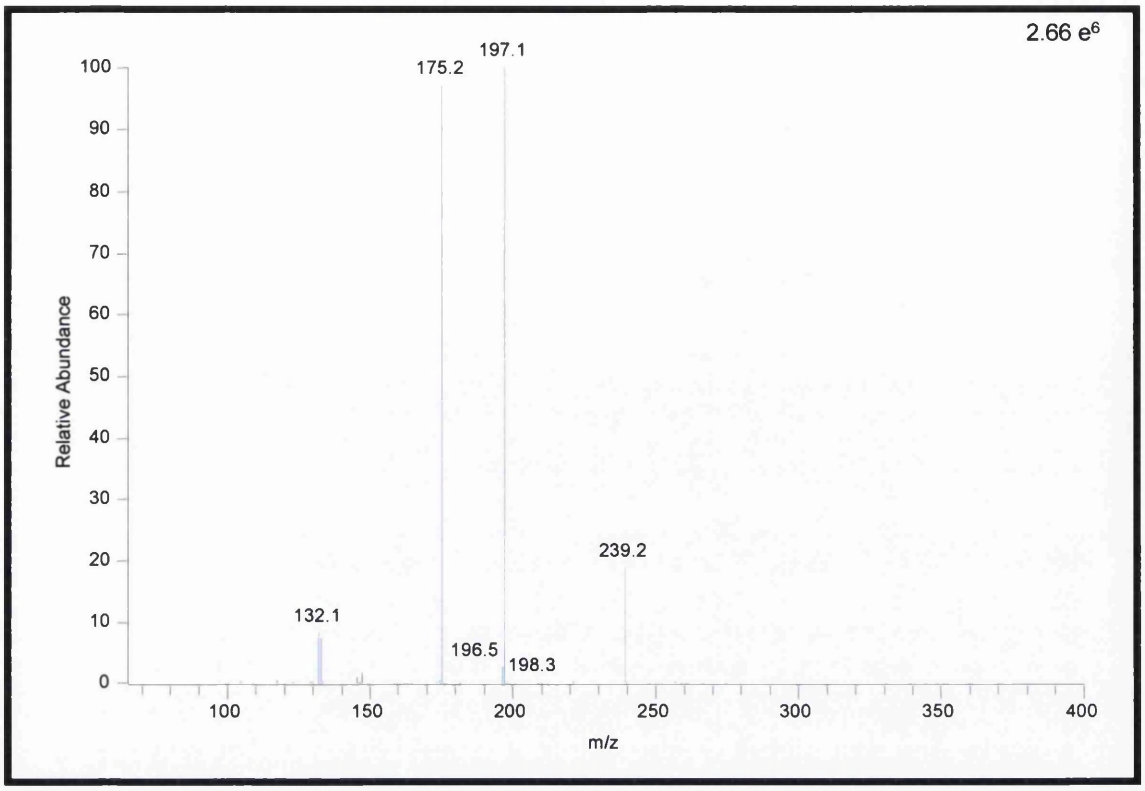



Figure 2.81 Negative LC-ESI-MS-MS spectrum of Bentazone. 10µL injection of a 10µg/mL solution; equal to 100ng on column.

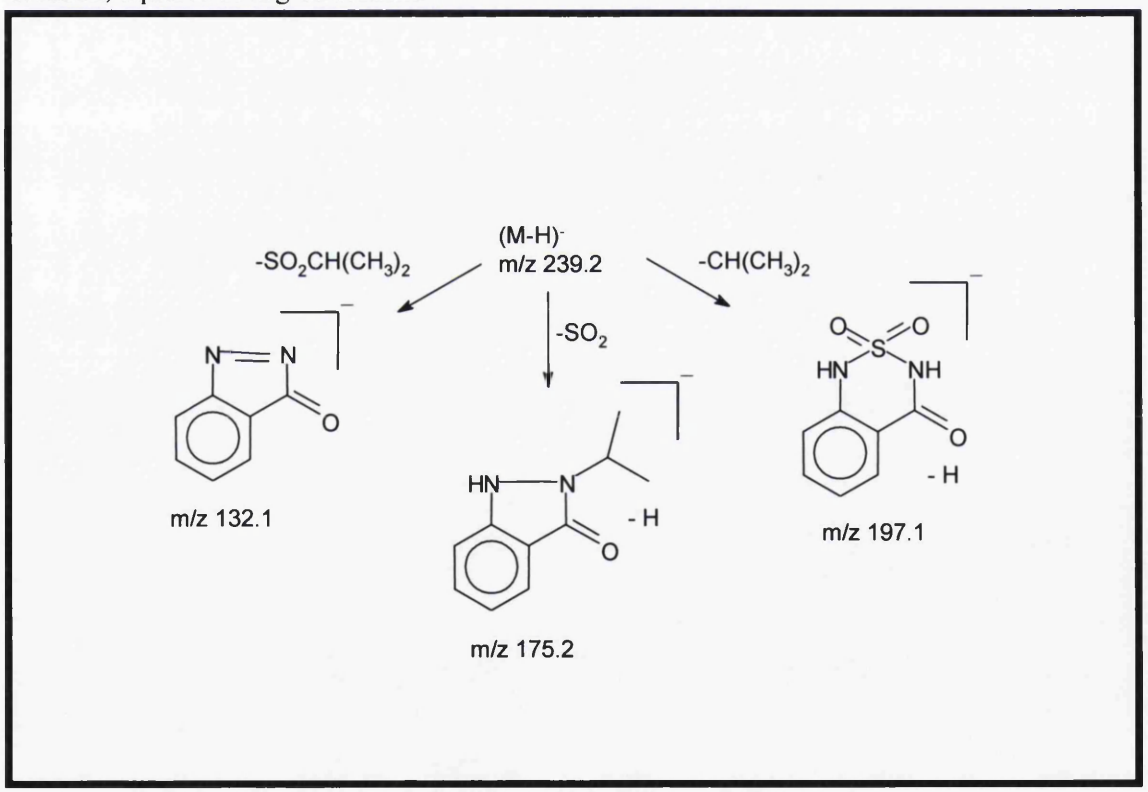



Figure 2.82 Proposed bentazone fragmentation pattern interpretation.

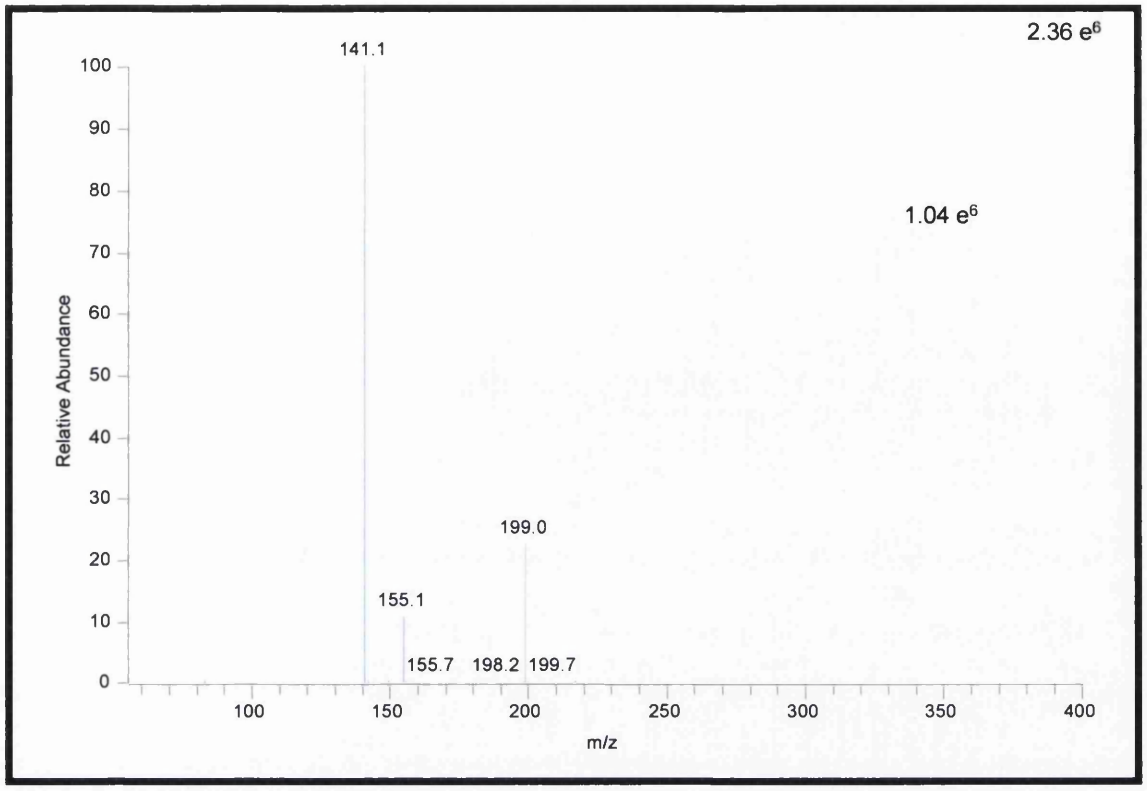



Figure 2.83 Negative LC-ESI-MS-MS spectrum of MCPA. 10µL injection of a 10µg/mL solution; equal to 100ng on column.

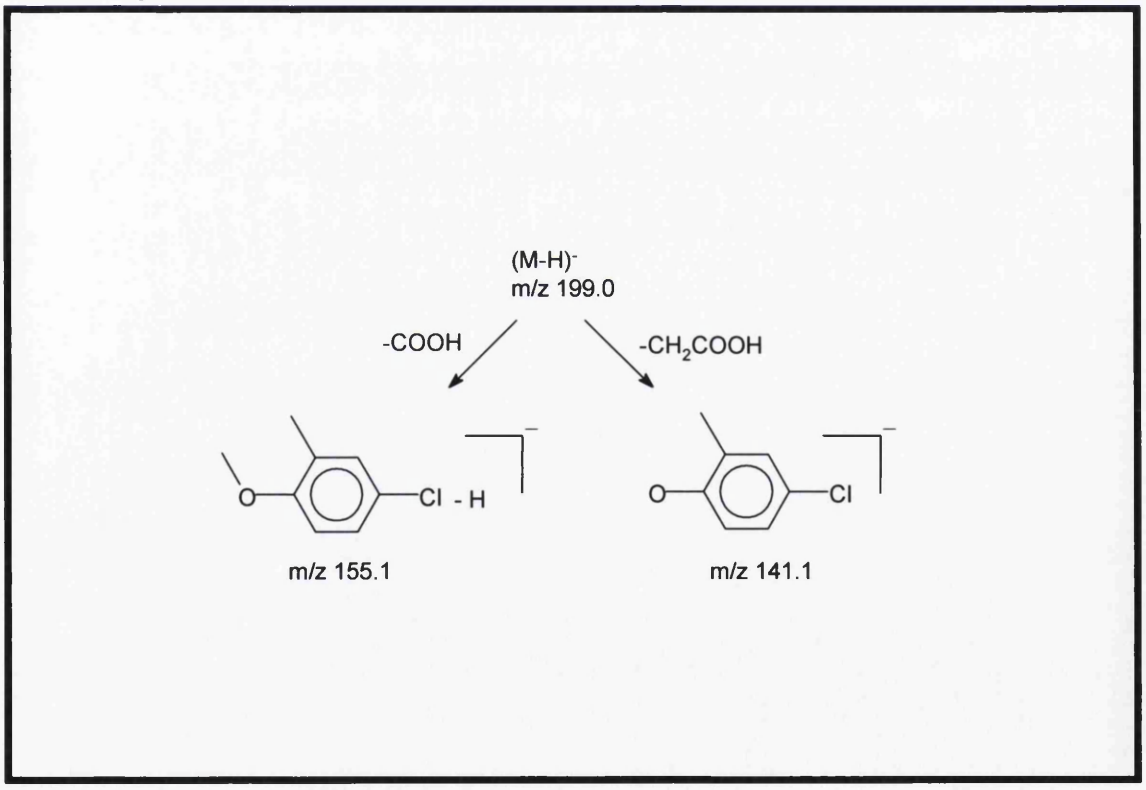



Figure 2.84 Proposed MCPA fragmentation pattern interpretation.

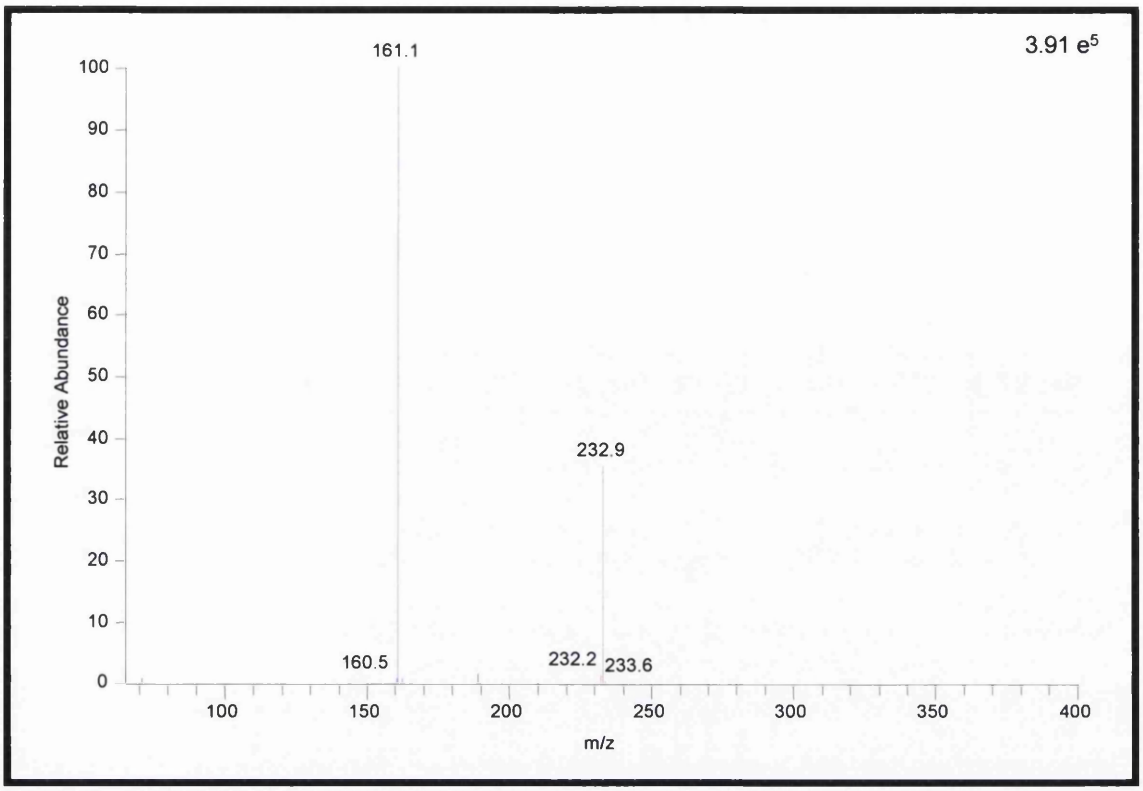



Figure 2.85 Negative LC-ESI-MS-MS spectrum of Dichlorprop. 10µL injection of a 10µg/mL solution; equal to 100ng on column.

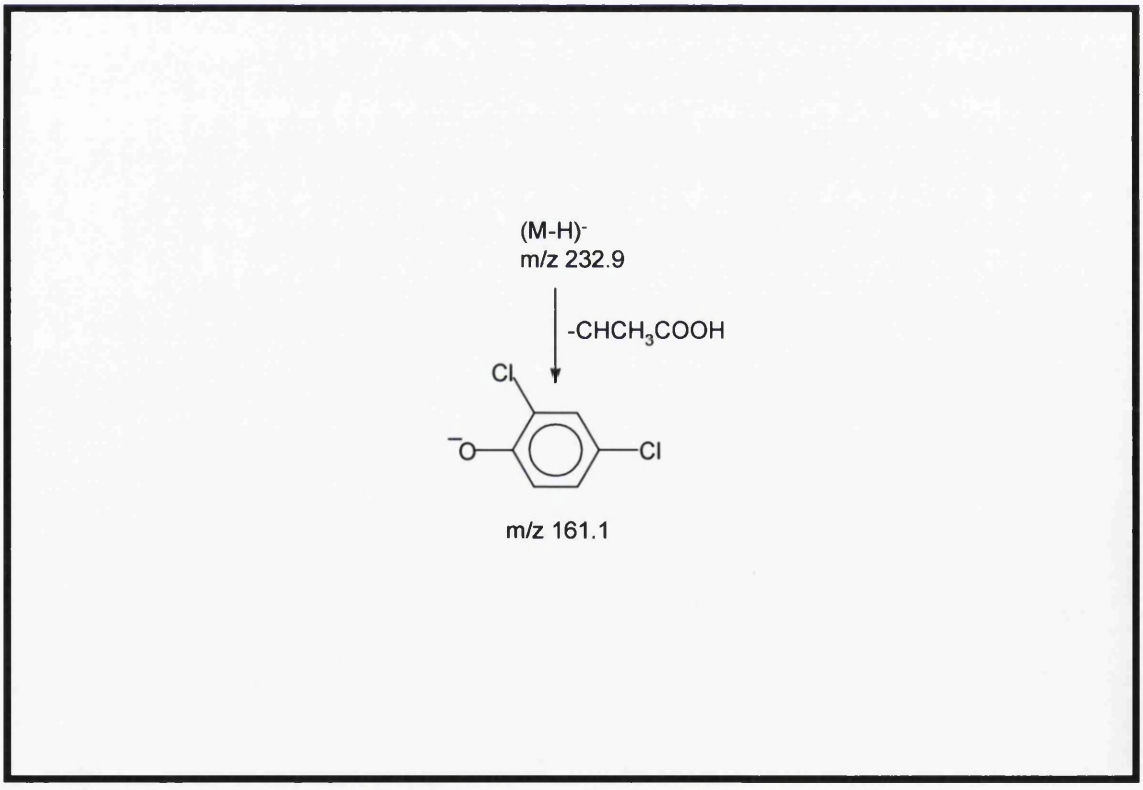



Figure 2.86 Proposed dichlorprop fragmentation pattern interpretation.

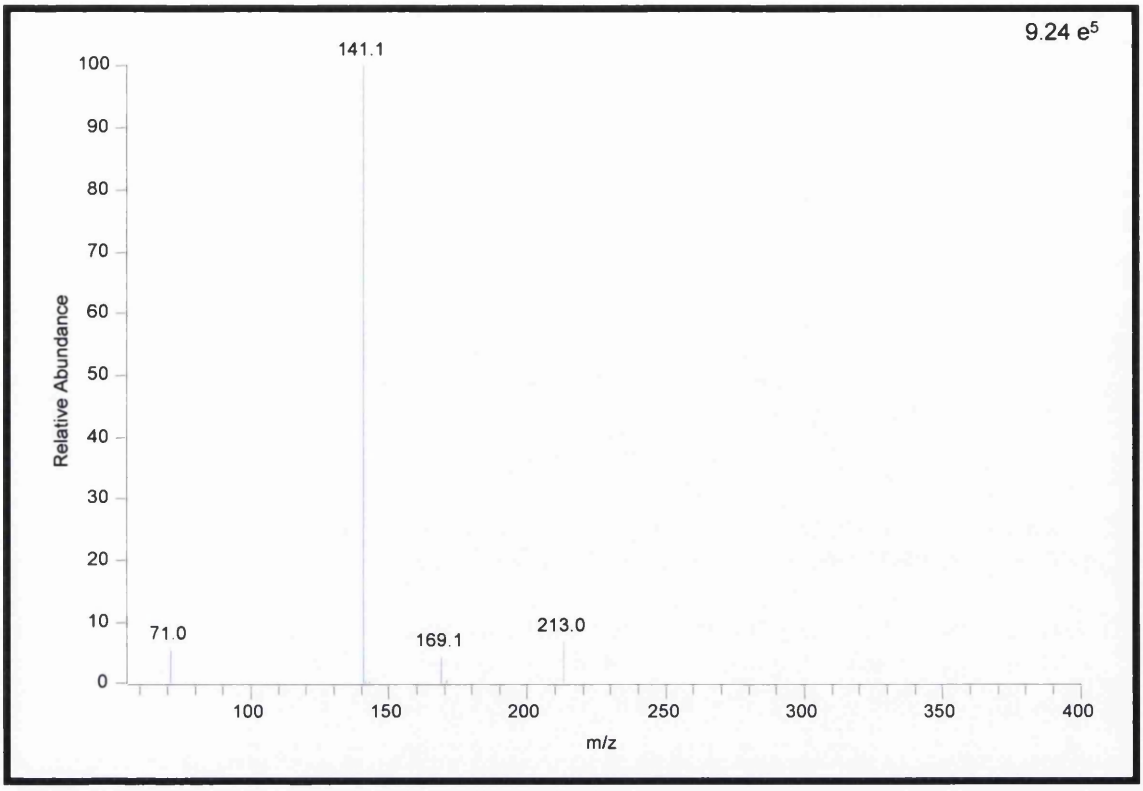



Figure 2.87 Negative LC-ESI-MS-MS spectrum of Mecoprop. 10µL injection of a 10µg/mL solution; equal to 100ng on column.

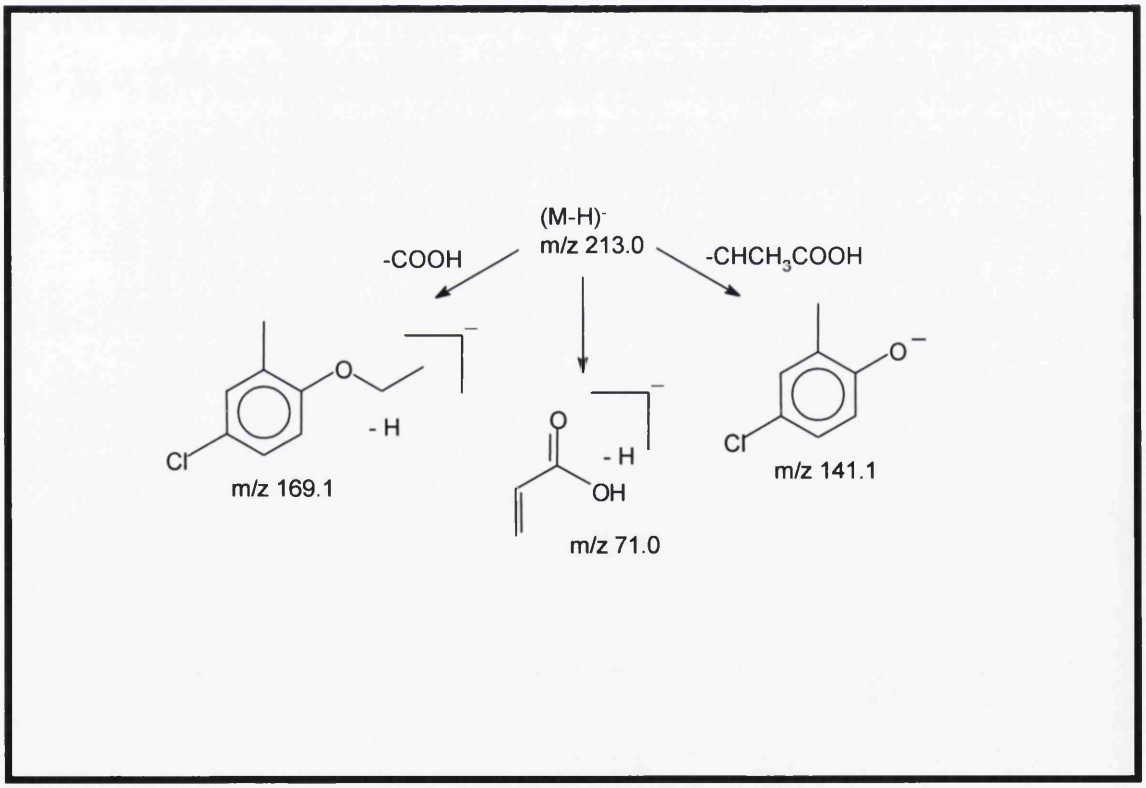



Figure 2.88 Proposed mecoprop fragmentation pattern interpretation.

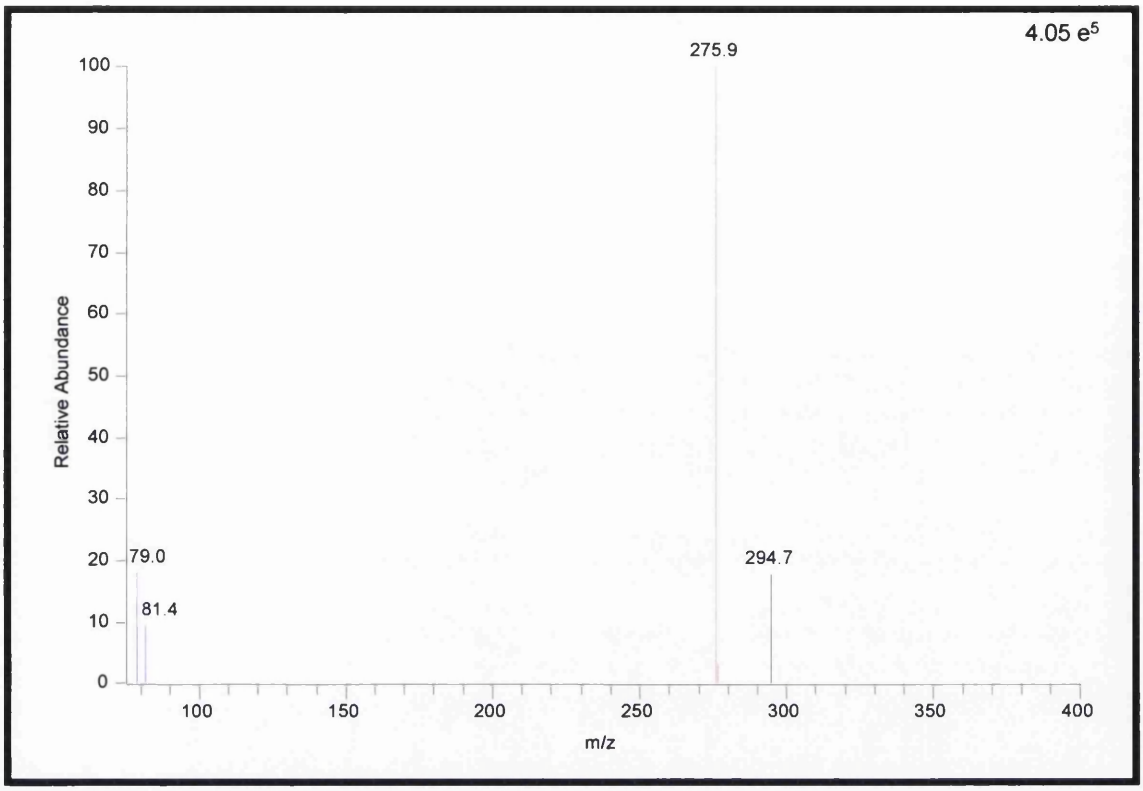



Figure 2.89 Negative LC-ESI-MS-MS spectrum of Bromoxynil. 10µL injection of a 10µg/mL solution; equal to 100ng on column.

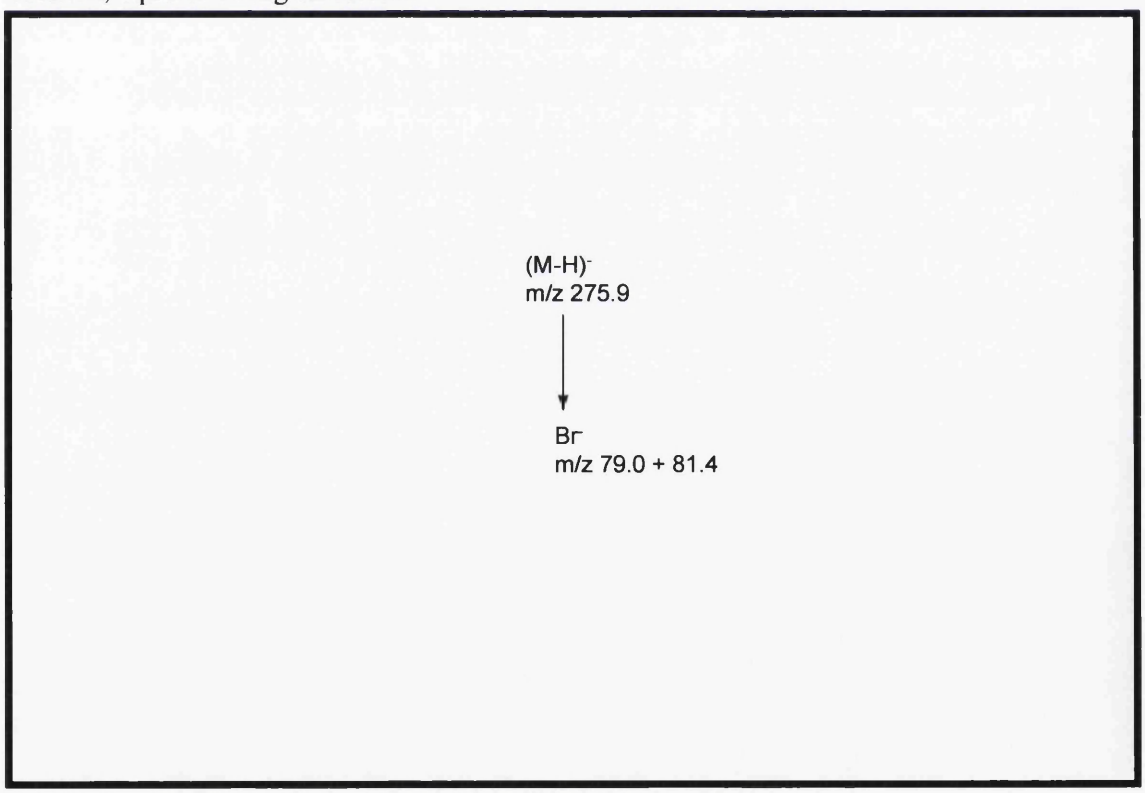



Figure 2.90 Proposed bromoxynil fragmentation pattern interpretation.

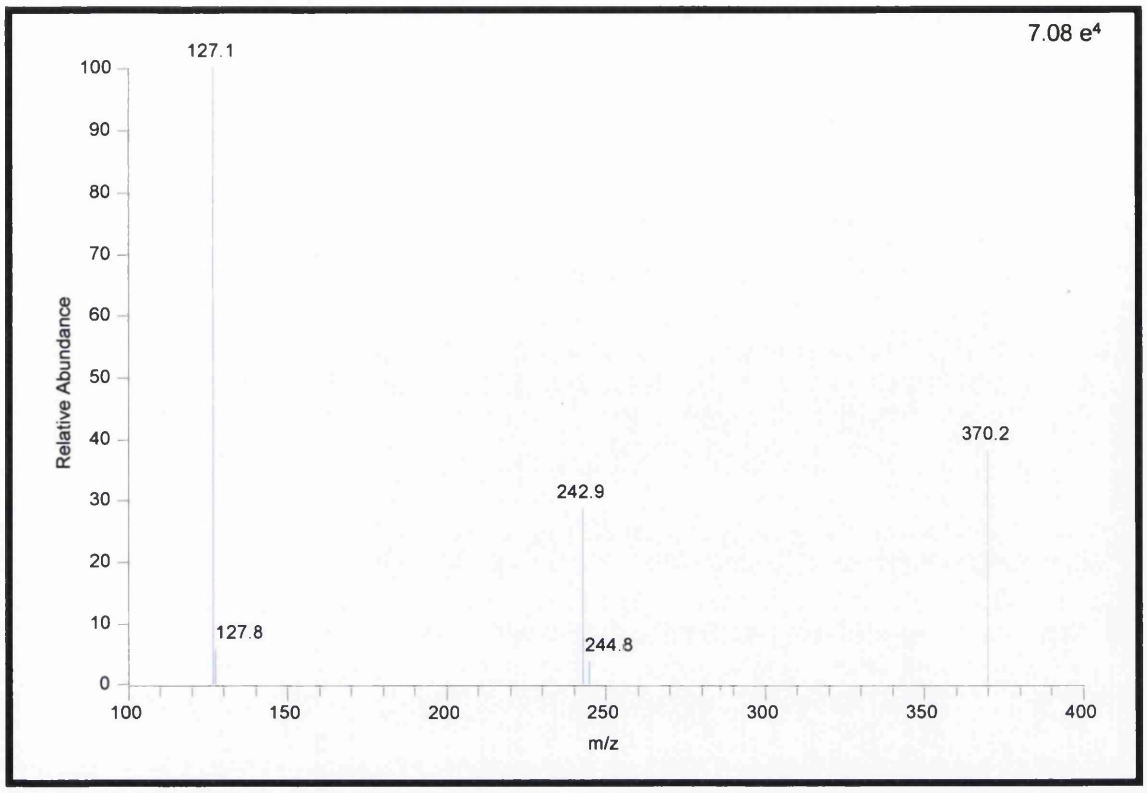



Figure 2.91 Negative LC-ESI-MS-MS spectrum of Ioxynil. 10µL injection of a 10µg/mL solution; equal to 100ng on column.

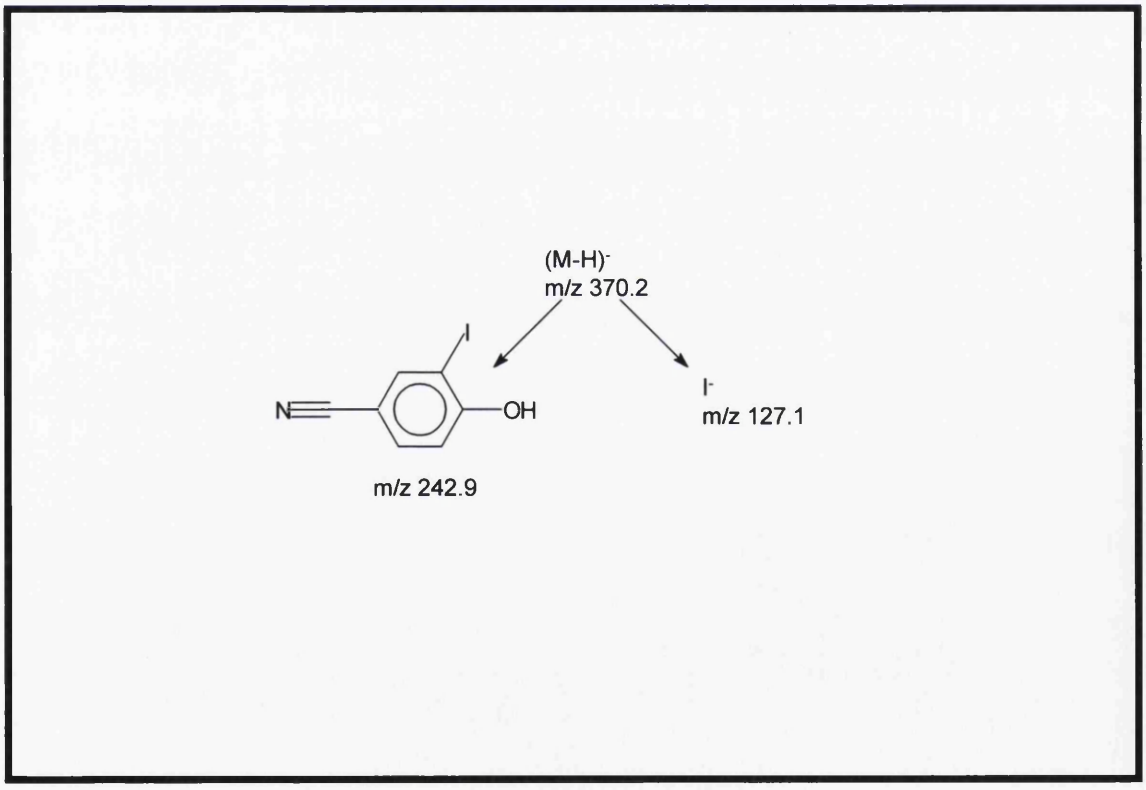



Figure 2.92 Proposed ioxynil fragmentation pattern interpretation.

$MS^3$ analysis was also attempted for the acidic herbicides but we were only able to achieve this for three of the nine herbicides; monuron, dichlorprop and ioxynil, and the data obtained was not sufficiently sensitive so it was decided not to pursue this further. Figure 2.93 below shows the fragmentation pattern interpretations for the $MS^3$ data obtained.

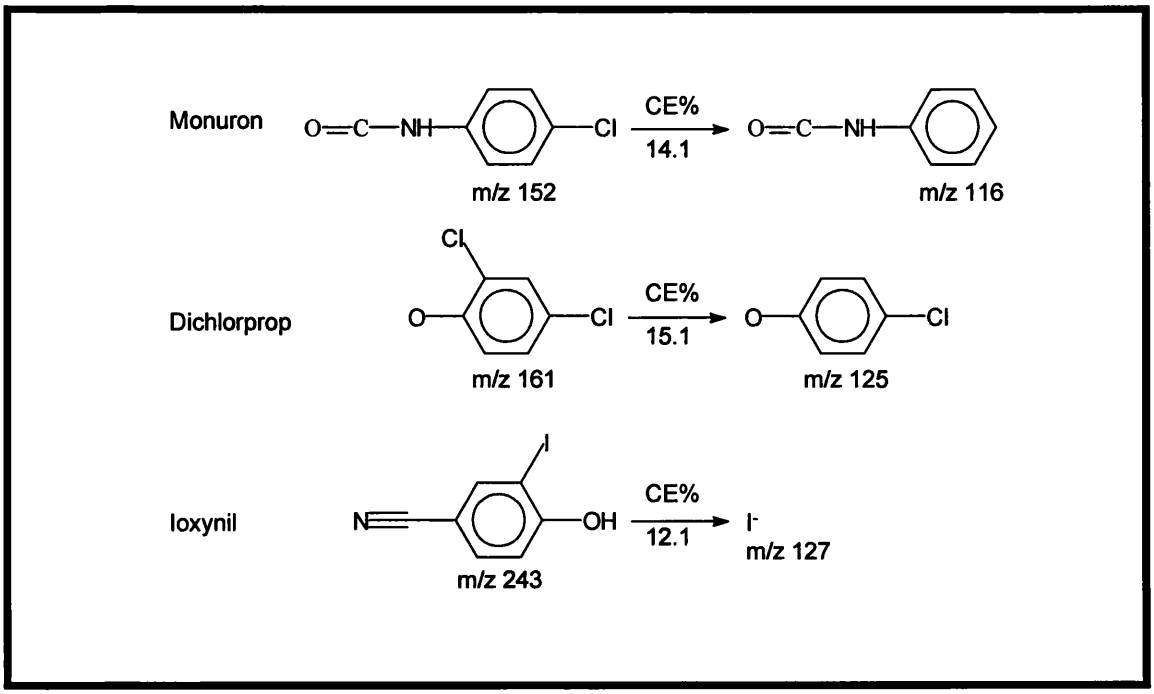



Figure 2.93 Mass spectrum interpretation of acidic herbicide $MS^3$ results.

# Solid Phase Extraction of the Acidic Herbicides

## Experimental

## Chemicals

HPLC grade solvents were purchased from Fisher Scientific (Loughborough, U.K.), gases from BOC Limited (Surrey, U.K.) and solid phase extraction (SPE) columns from Jones Chromatography Limited (Mid Glamorgan, U.K.).

## Instrumentation

SPE was performed on an International Sorbent Technology VacMaster extraction system using Isolute C18 500mg/3mL SPE columns.

## Results

Initial experiments were conducted by adapting extraction methods found in the literature (18, 19, 20). An extraction method was developed which allowed for the detection of the 9 acidic herbicides, via LC-ESI-MS analysis, in river water samples. The table below sets out the method used for the detection of the 9 herbicides.

**Extraction Procedure:**

| Step | Procedure |
|---|---|
| Sample Pre-treatment | Sample acidified to pH2 using acetic acid. |
| Column Solvation | Column conditioned with 10mL acetone followed by 10mL methanol and then 10mL of water acidified to pH2 at a flow rate of 5mL/min. |
| Sample Application | Sample applied to column at a flow rate of 5mL/min. |
| Interference Elution | Interferences eluted with 5mL of water acidified to pH2 at a flow rate of 5mL/min. |
| Column Drying | Dry column under full vacuum for 60 seconds. |
| Analyte Elution | Herbicides eluted with 2.5mL of methanol, allowed to soak for 10 minutes and then "dripped" through. |
| Reconstitution | Dry down under nitrogen and reconstitute in 50:50 (v/v) acetonitrile/water to a volume of 0.5mL. |

The following table shows the LOD achieved for each of the herbicides in spiked river water.

| Compound name | m/z Monitored | LOD |
|---|---|---|
| Dicamba | 219.0 | 1ng/L |
| Benazolin | 242.0 | 10ng/L |
| Monuron | 197.0 | 10ng/L |
| Bentazone | 239.0 | 1ng/L |
| MCPA | 199.0 | 1ng/L |
| Dichlorprop | 233.0 | 1ng/L |
| Mecoprop | 213.0 | 1ng/L |
| Bromoxynil | 276.0 | 10ng/L |
| Ioxynil | 370.0 | 1ng/L |

On column injection volume of 50µL. LOD is calculated as the concentration of the herbicide spiked into the blank river water.

The volume of river water extracted for analysis was 2 litres; to ensure that there would be no interference from herbicides already present in the water used for determining the LOD it was collected from the source of the river Lougher in South Wales. Figure 2.94 is an extraction of river water spiked at 10ng/L for each herbicide.

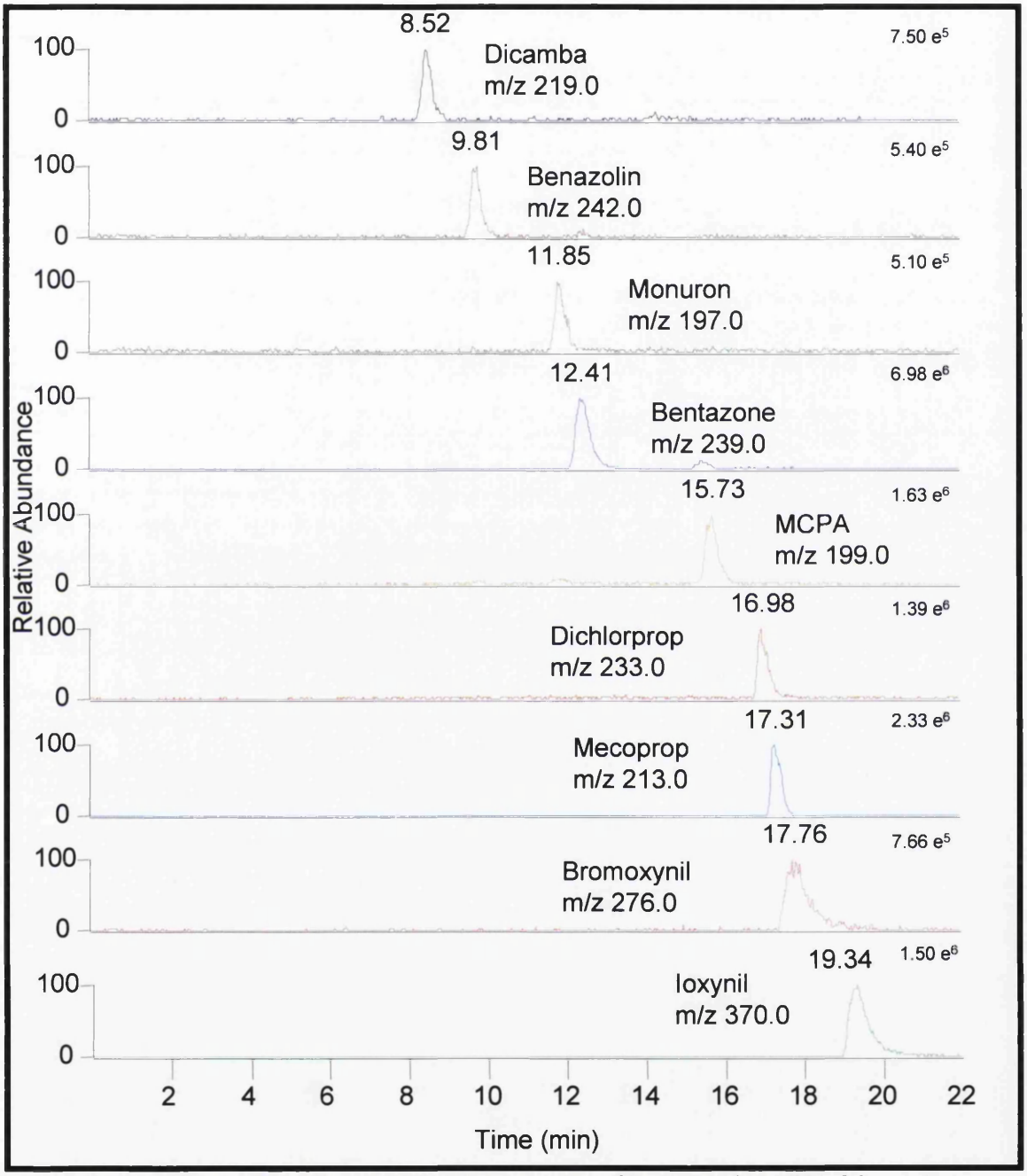



Figure 2.94 Negative LC-ESI-MS extracted ion chromatograms of the 9 herbicides. SPE of river water spiked with 10ng/L of each herbicide.

## Analysis of River Water Samples

River Water samples were collected from points along the rivers Lougher and Bryn where they ran along either side of Glynhir golf course, Llandybie in South Wales; at each site 4 litres of river water was collected. 2 litres of river water was extracted using the technique described previously from each site. We were however unable to detect the presence of any of the 9 acidic herbicides.

## Discussion

The main goal of this study was to develop a relatively fast, reliable and sensitive determination method for polar herbicides in river water using LC-MS analysis.

LC-UV analysis has been used in the labs of Hyder Environmental as the technique of choice for the analysis of triazines and phenylureas for some time. Whilst they have found it to be a reliable and adequately sensitive analysis tool the instrument time required to perform the analysis, in excess of 60 minutes, has made it costly and time consuming (12). The first stage of our studies therefore involved the development of a faster chromatographic separation method; this was achieved with the use of a phenyl-hexyl column, which allowed us to perform separation times of less than 10 minutes.

The LC-MS analysis performed on the mixture of the 10 triazines and phenylureas proved to be more sensitive in the ESI mode than in the APCI mode. This may in part be due to ESI being a softer ionisation technique than APCI and thus less degradation occurring. The spectra obtained for APCI differed very little from the ESI spectra and did not show signs of degradation taking place however this may be because we were only scanning over a mass range of 180 to 600 for the APCI analysis due to the amount of background noise occurring below this range, from the data obtained for the ESI-MS-MS analysis you would expect the majority of degradation products to fall below 180 m/z units so this could explain the lack of evidence to support the idea of APCI causing greater amounts of degradation to take place. The LC-ESI-MS data obtained for the analysis of the phenylureas and triazines is comparable to results found in the literature (21).

The acidic herbicides have been analysed in the labs of Hyder Environmental by the use of GC-MS. To perform GC-MS analysis of these compounds they first have to undergo methylation with the use of diazomethane. LC-MS analysis allows for the acidic herbicides to be analysed directly without the need for any time-consuming derivitization steps.

The LC-UV method we developed for the acidic herbicides relied on the use of a flow gradient; while this is not a widely used technique it can be found in the literature (22). A flow gradient is a technique that is used for increasing the front-end resolution of a sample run; unfortunately this tends to be at the expense of the back end resolution in an analytical run. In the case of the acidic herbicides as we were unable to prevent the co-elution of mecoprop and bromoxynil using other techniques this did not pose a significant problem and the use of a flow gradient provided the best resolution we were able to obtain.

The LC-MS analysis performed on the mixture of 9 acidic herbicides proved again to be more sensitive in the ESI mode than the APCI mode. This could again be due to less degradation taking place in ESI, but the formation of an adduct ion occurring with an increase of 82 m/z units shows that the lower sensitivities achieved with ESI when compared to APCI could in part be due to the fact that we only took selected ion chromatograms for one ion and if we had taken them to include the adduct ion also then we may have seen results with APCI which were more akin to those achieved with ESI.

The SPE methods we developed for both sets of compounds were similar to extraction procedures found in the literature (16, 17, 18, 19, 20) and allowed for determination of all the compounds of interest down to levels that were within the European Unions legal requirements (11).

## Conclusion

Currently the technique of choice for the analysis of polar herbicides in environmental matrices tends to be GC-MS. Analysis of these compounds with GC-MS offers a high degree of sensitivity with limits of detection in the high picogram per litre region being reported in the literature for the analysis of acidic herbicides (23). However due to their highly polar nature, low volatility and, particularly in the case of the phenylureas, the fact that they undergo thermal degradation under the conditions required to perform gas chromatography polar herbicides must be derivatised prior to GC-MS analysis (24, 25). Derivitization processes are time consuming and tend to have low reproducibilities (26).

LC-MS allows for the direct analysis of these compounds without the need for derivitization and while the methods of analysis we have developed do not offer the same sensitivities that can be gained with GC-MS these sensitivities could be improved upon by the use of a smaller reconstitution volume to give a more concentrated final extract for analysis. Another approach which could be taken would be the use of solid phase micro extraction which has been shown to offer sensitivities akin to those achieved with GC-MS when used in conjunction with LC-MS analysis of herbicides (27, 28). Another disadvantage of GC-MS when compared to LC-MS is

the instrument run times required for analysis; GC-MS run times for the analysis of polar herbicides tend to be 40 minutes or longer (29, 30) whereas the LC-MS methods we have established are substantially less than this.

Immunoassay is another technique that has been used in more recent years for the analysis of polar herbicides in the environment. Immunoassay is an immunochemical detection method based on a selective reaction between a target analyte and its antibody. An immunoassay does not require a sample pre-treatment step, is very simple to perform and has a high degree of sensitivity (31, 32, 33). Immunoassay has been used to detect a wide variety of compounds including environmental pollutants.

Immunoassay has major advantages over LC-MS; primarily it is rapid allowing large volumes of samples to be analysed in a relatively short period of time (400 samples analysed in 6 hours for triazine herbicides (32)), inexpensive and sensitive with limits of detection in the low picogram per litre region being reported in the literature (32). Another advantage of immunoassay is that it is portable and can therefore easily be used in the field allowing for the possibility of an at site early warning system to monitor for sudden changes in the levels of environmental pollutants (33).

The main disadvantage of immunoassay is the cross reactivity of similar compounds; in cases where cross reactivity cannot be avoided immunoassays only give a sum parameter and cannot be used for the quantification of a single compound. This can give rise to false positive results due to the presence of pesticides other than those of interest and also due to matrix effects, which in the case of environmental samples can vary dramatically. This feature of immunoassay does however allow for it to be a

useful tool for the rapid screening of samples to detect for the presence of any compound from within a family of compounds; and although there is the possibility of getting false positive results false negatives do not occur (34).

LC-MS allows for far more specificity than immunoassay giving rise to the use of the two techniques in compliment to each other. Immunoassay is used as a screening tool; being inexpensive, rapid and sensitive, and LC-MS is used to establish the identity of the compounds giving rise to the positive results obtained with immunoassay. This allows for a faster turn around of samples and greatly reduces the cost of sample analysis. So while immunoassay is unlikely to replace the use of either LC-MS or GC-MS in the definitive identification of polar herbicides present in environmental matrices it is likely to greatly reduce the number of analyses that will require analysis with these techniques.

For the three compound groups studied in this chapter LC-ESI-MS was on the whole found to be more sensitive than LC-APCI-MS. MS-MS data, and in some cases $MS^3$ data, was acquired which allowed for more definitive identification of the polar herbicides.

The solid phase extraction methods we developed allowed for the sensitive analysis of river water samples containing nanogram per litre, and in the case of the triazines and isoproturon sub nanogram per litre, levels of the herbicides proving the value of the methods used. The sensitivities achieved with linuron were only down to the 100 nanogram per litre level and this may in part be due to linuron being an acid amide making the compound acidic and therefore less easily protonated.

The results of this chapter show that LC-MS can be used as a reliable and sensitive technique for the analysis of triazine, phenylurea and acidic herbicides at levels that are below the limits set for drinking and surface water.

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# Chapter 3

# LC-MS Analysis of Estrogen Steroids in River Water

# Chapter 3 LC-MS Analysis of Estrogen Steroids

## Introduction to Estrogen Steroids

The estrogen steroids are responsible for the development of the female sex organs and the secondary sex characteristics; the main action of these steroids is on the growth and function of the reproductive tract in order to prepare it to receive the fertilized ovum. In humans the naturally occurring estrogenic hormone is estradiol, estrone is also important but has less activity than estradiol and its activity probably depends upon its metabolic conversion to estradiol. Estriol is a weak estrogen but assumes importance during pregnancy (1, 2).

In most species the naturally occurring estrogens contain a phenolic A ring; while estrogen has been identified in some plants, more often estrogenic activity in plants is due to non-steroidal compounds. Thus estrogenicity is not restricted to the steroid structure, many synthetic compounds, in particular polyvinyl chloride and the phthalate group of compounds, also have estrogenic activity (figure 3.1).

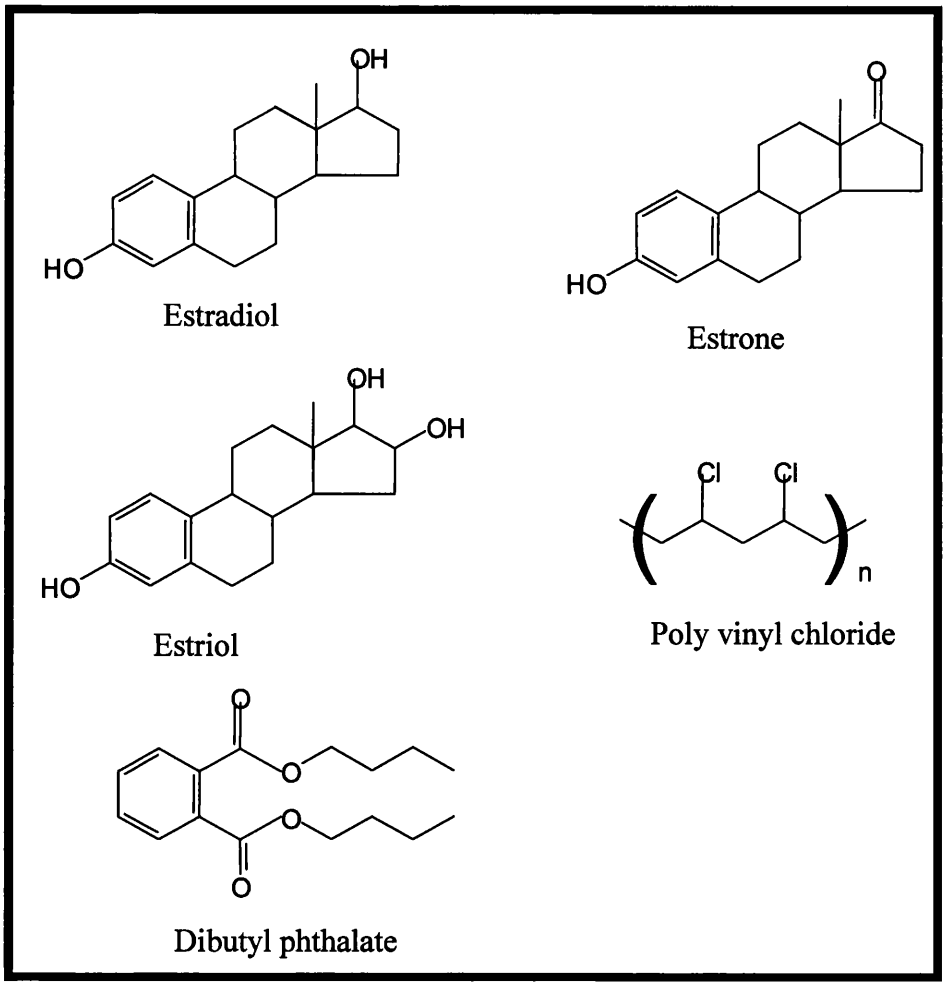



Figure 3.1 Examples of Estrogenically active compounds.

**Estrogen biosynthesis**

In humans estrogens may arise from five sources:

1. The adrenal cortex that produces estrone in small amounts and hence does not significantly contribute to the estrone pool in the body.
2. The testes, it has been shown that direct secretion of estrogen from the testes occurs and may account for up to 30% of total estrogen produced in some males.

3. The ovaries, in pre-menopausal women the ovaries are the main source of estrogens.
4. Other tissues, peripheral tissues are capable of producing estrogens from $C_{19}$ precursors. In post-menopausal women most of the estrogen produced is derived from plasma androstenedione.
5. The fetoplacental unit is responsible for large amounts of estrogens produced during pregnancy.

The ovaries are the main source of estrogen steroids in pre-menopausal women; both the follicular and the corpora leuteal tissues are responsible for the biosynthesis of estradiol-17β, estrone and estriol. The parent compound, cholesterol, is converted to pregnenolone and this subsequently gives rise to testosterone and 4-androstenedione by way of the 5-ene-3β-hydroxy (figure 3.2) and 4-en-3-oxo pathways (figure 3.3) (2).

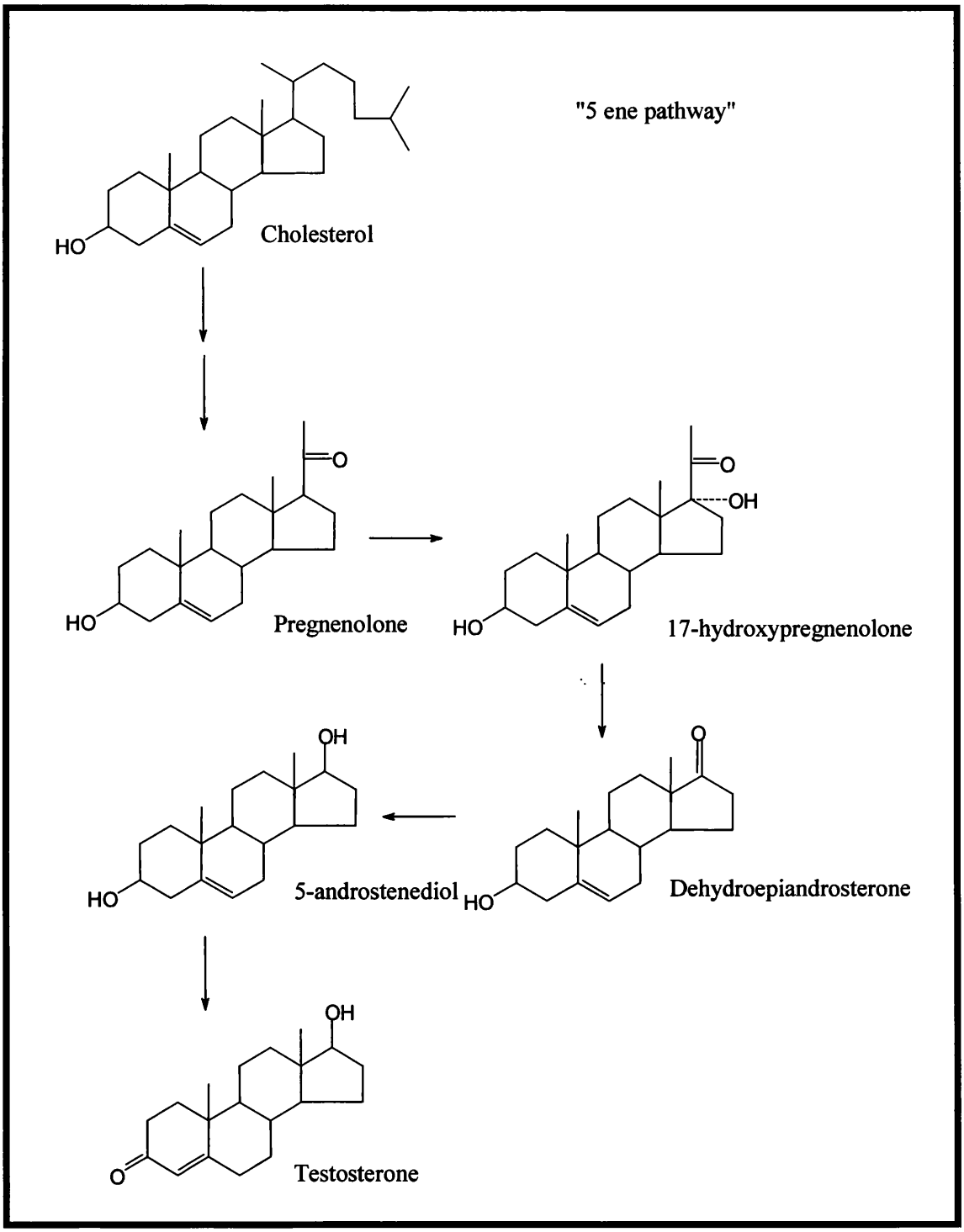



Figure 3.2 The 5-ene-3β-hydroxy pathway for steroid synthesis.

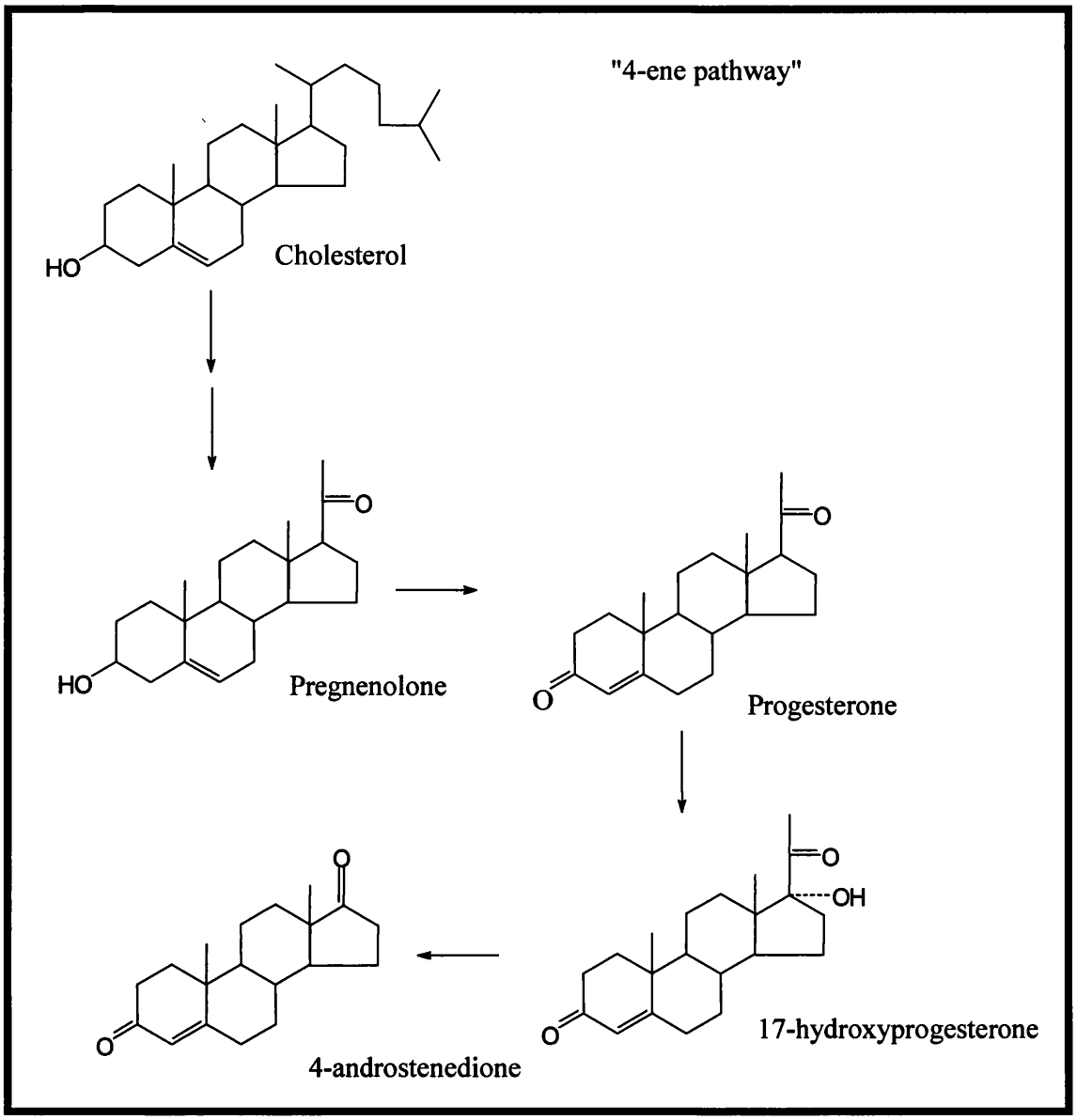



Figure 3.3 The 4-ene-3-oxo pathway for steroid synthesis.

Ovarian tissue contains a series of enzymes, which are responsible for the conversion of these androgens into estrogens. 4-androstenedione and testosterone are 19-hydroxylated first (microsomal 19-hydroxylase); the 19-hydroxy groups are then oxidised to aldehyde groups (microsomal 19-hydroxysteroid dehydrogenase) and finally these are removed as methanal (microsomal C-10, 19 lyase). Spontaneous rearrangement results in an aromatic ring A, the final products being estrone from 4-androstenedione and estradiol-17β from testosterone; microsomal 16α-hydroxylase

catalyses the conversion of estradiol-17β to estriol (see figure 3.4). All four enzymes involved in estrogen biosynthesis require NADPH and oxygen for activity and are cytochrome P-450 dependent (1).

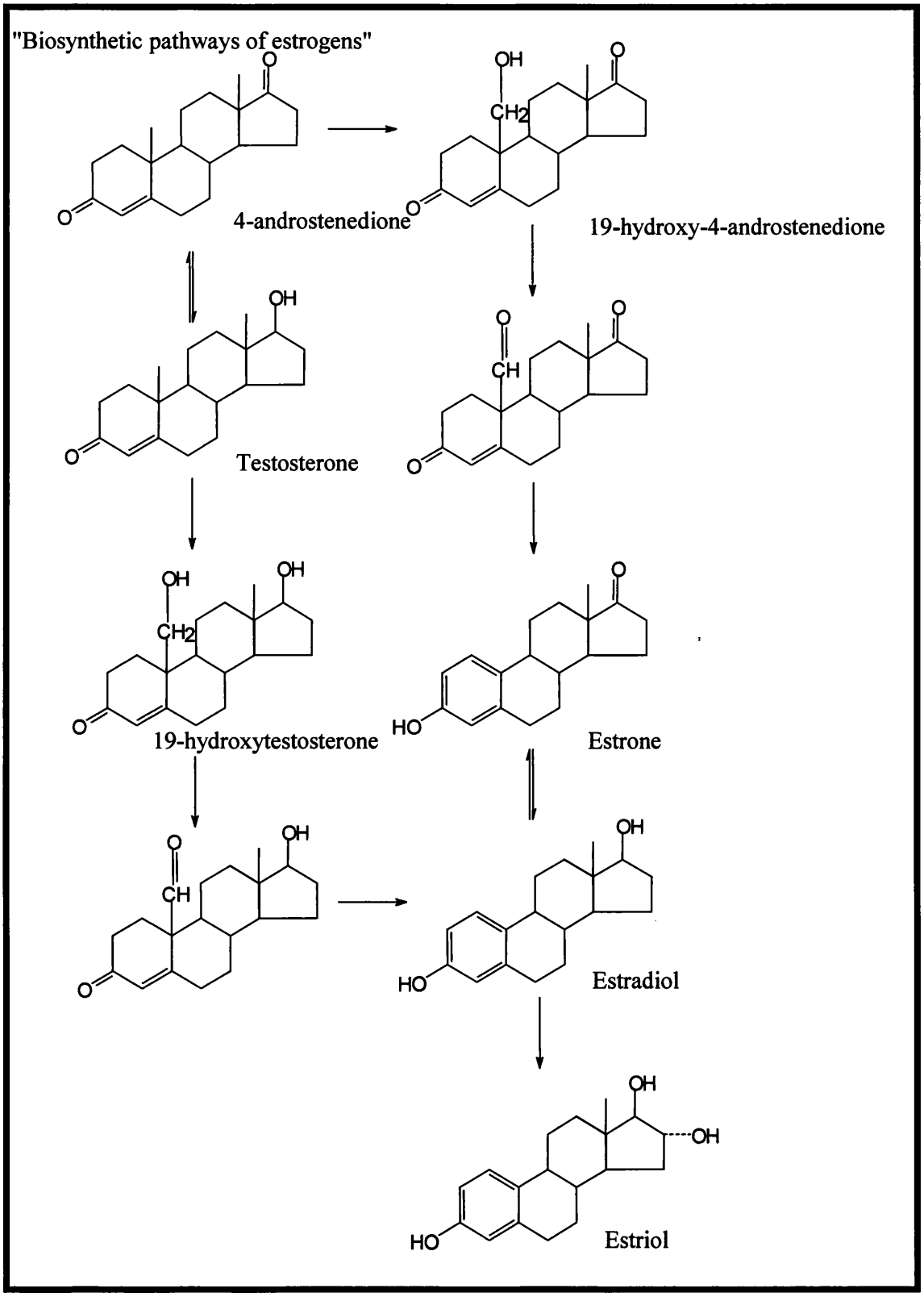



Figure 3.4 The biosynthetic pathway leading to the formation of the estrogen steroids.

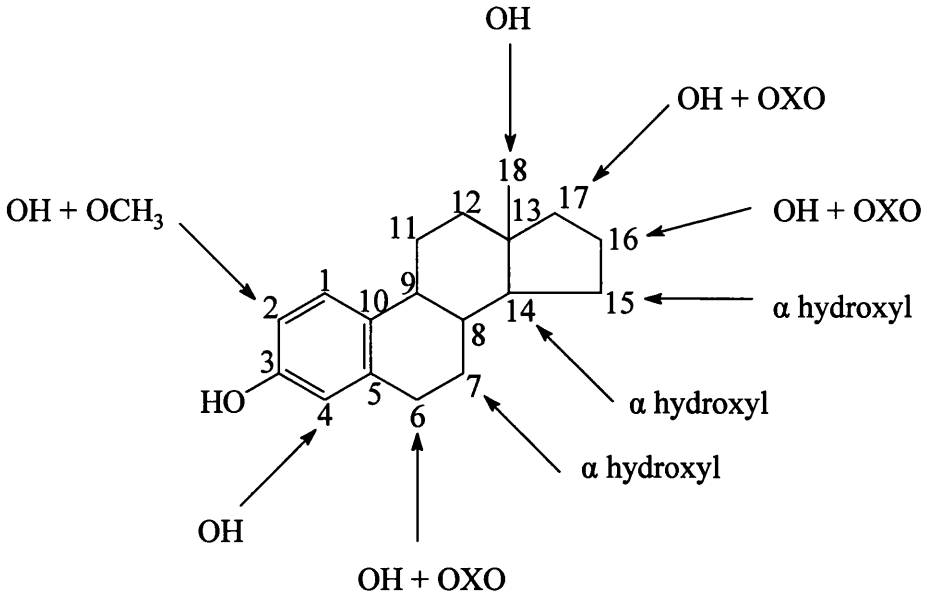
## Catabolism and Conjugation of Estrogen Steroids

Steroid hormones are secreted by the adrenals and the gonads into the peripheral circulation, subsequently numerous reactions occur in the liver and to some extent in the kidneys, which inactivate the physiologically active steroids. These reactions are mainly reductive in nature and render the molecules more polar. The inactive metabolites are then converted by the process of conjugation to sulphate esters or glucuronides. In this way the lipophilic steroids are converted into more water soluble conjugates, which can be excreted in the urine.

There are four main pathways in the catabolism of steroid hormones:

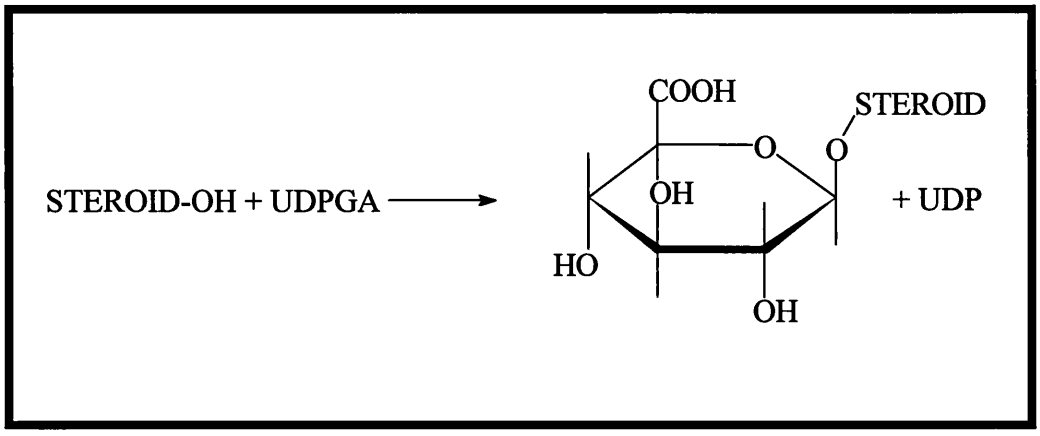
1. Reduction of the double bond at C-4 and the oxo group at C-3 making a secondary alcohol.
2. Reduction of the oxo group at C-20 to a secondary alcohol.
3. Oxidation of a 17β-hydroxyl group.
4. Hydroxylation at various points in the steroid nucleus.

Hydroxylation or ketone formation at various positions in the molecule result in loss of estrogenicity. The most important reaction is that of 16α-hydroxylation leading to estriol but hydroxylation can also occur at C-2, C-4, C-6α and β, C-7α, C-14α, C-15α, C-16β and C-18β. Some twenty estrogens can thus be found in human urine conjugated as glucuronides and sulphates (1).




**Glucuronides**

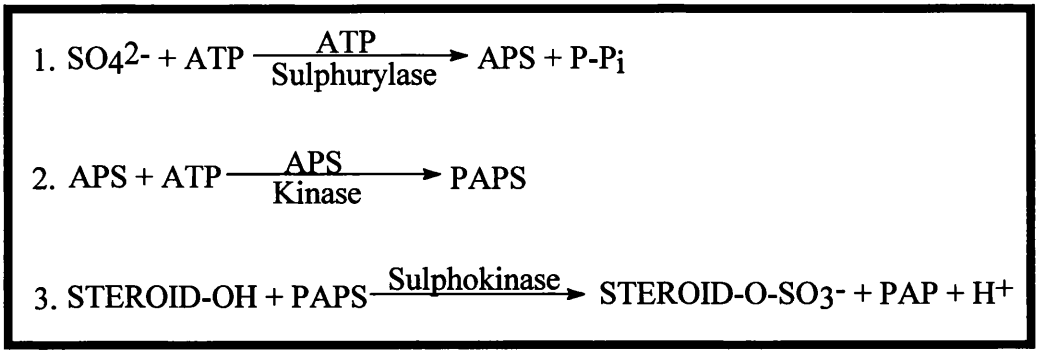
Glucuronide conjugates are formed by the reaction of the steroid metabolite with uridine diphosphoglucuronic acid (UDPGA) in the presence of glucuronic transferases that are found in the liver (1).




The kidneys readily excrete glucuronides.

## Sulphates

Sulphokinases occur in the cells of the liver, testis, adrenals and foetal tissues. These enzymes catalyse the reaction of steroids with sulphates, the presence of magnesium ions is necessary; the reaction takes place in three steps:




**Key**

ATP → Adenosine Triphosphate

APS → Adenosine-S-Phosphosulphate

P-$P_i$ → Pyrophosphate

PAPS → Phosphoadenosine-S-Phosphosulphate

PAP → 3,5-Phosphoadenosine

The hydroxyl group at C-3 is preferentially sulphated. The renal clearance of steroid sulphates is approximately 10% of that of glucuronides (1).

## Reason for analysis

Endocrine disruptors, chemicals that mimic steroid hormones have increased in our environment. Scientific research suggests a link between human development, in particular reproductive abnormalities, and endocrine disruptors. The environment agency reports that they were aware of hormonal changes in wildlife attributable to

endocrine disruptors 40 years ago. It is only in the last 10 years the issue has entered the public domain. A medical journal, The Lancet (3), published an article linking low sperm counts to endocrine disruptors in 1992. Since then research, publication and interest has increased significantly.

In light of this there has been an increase in the necessity to monitor the levels of estrogen steroids as well as the endocrine disruptors that mimic them in our rivers and drinking water.

Work carried out by CEFAS for the environment agency a few years ago showed that most of the estrogenicity in treated sewage effluent was due to the presence of 3 steroids; estrone, estradiol and ethynyl estradiol. As river waters receiving treated sewage effluents are used as sources of drinking water it is necessary to have a method to measure the levels of these 3 steroids in drinking water. As these steroids are present in the sewage effluent as a result of human excretion they are primarily present as conjugates.

It has been shown that commonly used drinking water treatment processes effectively remove the free steroids (4). There is concern however regarding the possibility of conjugated steroids getting through drinking water treatment. At the labs of WRc plc (Buckinghamshire, U.K) a method for the analysis of free steroids in drinking water using GC/MS/MS was successfully developed. It was attempted to apply their existing methodology to the analysis of conjugated steroids by using solvolytic methods to convert the conjugates to their free steroid counterparts; however when this approach was taken it was found that although some success was achieved with

regard to the sulphate conjugates the glucuronides required more forcing solvolytic methods which resulted in the decomposition of the liberated steroids. In light of this we were requested by WRc to develop an LC/MS/MS method to allow for the analysis of the steroid conjugates of interest directly.

## LC-UV For the Separation of 4 Conjugated Estrogen Steroids

The structures of the conjugated estrogen steroids can be seen in figure 3.5.

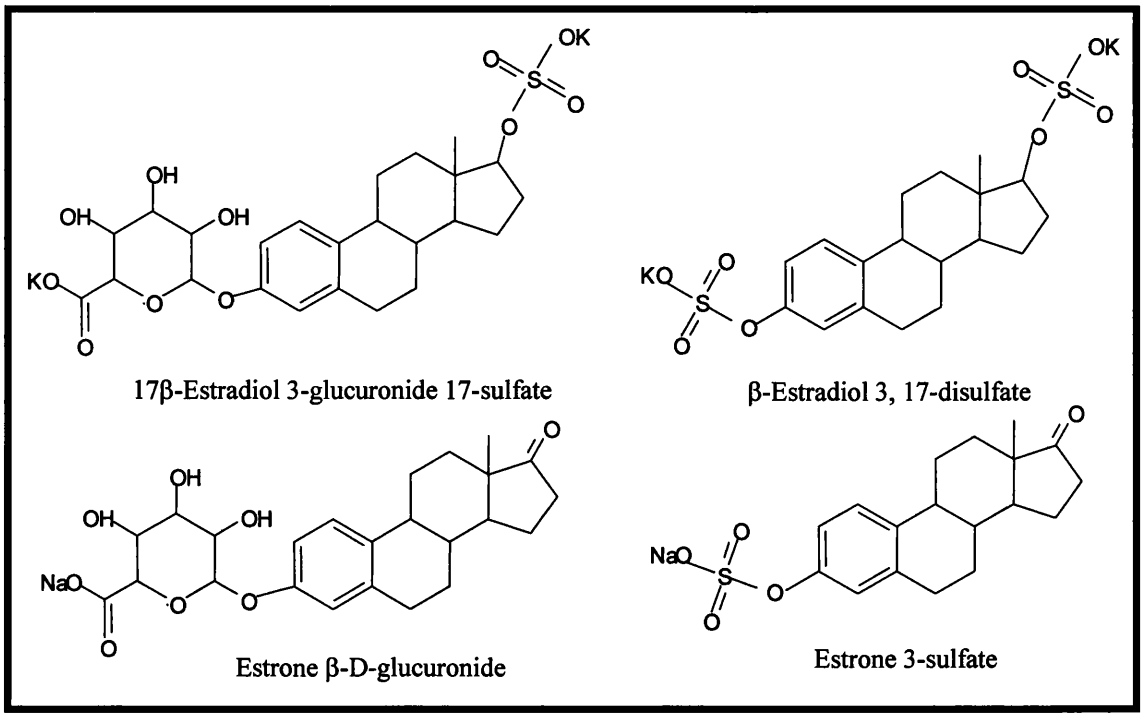



Figure 3.5 Structures of the conjugated Estrogen steroids investigated.

### Experimental

### Chemicals

HPLC grade solvents were purchased from Fisher Scientific (Loughborough, U.K.), gases from BOC Limited (Surrey, U.K.) and the following 4 steroid conjugates prepared in the labs of WRc plc (Buckinghamshire, U.K).

| | |
|---|---|
| 17β-Estradiol 3-glucuronide 17-sulfate | Estrone β-D-glucuronide |
| β-Estradiol 3, 17-disulfate | Estrone 3-sulfate |

## Sample Preparation

Standard solutions ranging from 0.1 to 0.5 mg/ml were made up in 50/50 methanol/water for each of the steroids; solutions for analysis were prepared and diluted from these as necessary.

## Instrumentation

A Hewlett Packard 1100 HPLC fitted with a diode array detector (DAD) was used to perform the chromatography with a Phenomenex Luna 4.6x100mm 3μ C18 (2) column. The mobile phase used for analysis was made up of acetonitrile and 7mM ammonium acetate pH 4.5 with acetic acid run in a gradient system.

## Results

Initial experiments were conducted with mobile phase combinations made up of methanol and ammonium acetate at various strengths as derived from references on the analysis of sulphate conjugates supplied by WRc (5, 6, 7). However it was found that using these conditions we were unable to gain sufficient resolution of the conjugate steroids; a switch was made to acetonitrile and a lower concentration of ammonium acetate combined with acidified conditions, which gave us sufficient retention and resolution of the 4 steroids.

The mixture of the 4 estrogen steroids was separated on the Phenomenex C18 column with the following chromatographic conditions.

| Time in minutes | %$CH_3CN$ | %7mM $NH_4Oac$ pH4.5 |
|---|---|---|
| 0.00 | 10 | 90 |
| 10.00 | 100 | 0 |

| | |
|---|---|
| Column Thermostat | Room Temperature |
| Flow Rate | 0.75mL/min |
| DAD | 220nm |

The following table gives the retention times for the compounds.

| Compound name | Peak label | RT minutes |
|---|---|---|
| 17β-Estradiol 3-glucuronide 17-sulfate | A+B | 1.348 + 3.464 |
| β Estradiol 3, 17-disulfate | C | 4.359 |
| Estrone β-D-glucuronide | D | 4.753 |
| Estrone 3-sulfate | E | 5.907 |

The 2 peaks occurring in the chromatogram corresponding to 17β-Estradiol 3-glucuronide 17-sulfate are possibly due to the presence of 2 isomers of the steroid conjugate.

Figure 3.6 on the following page shows the UV chromatogram of the separation of the 4 steroids.

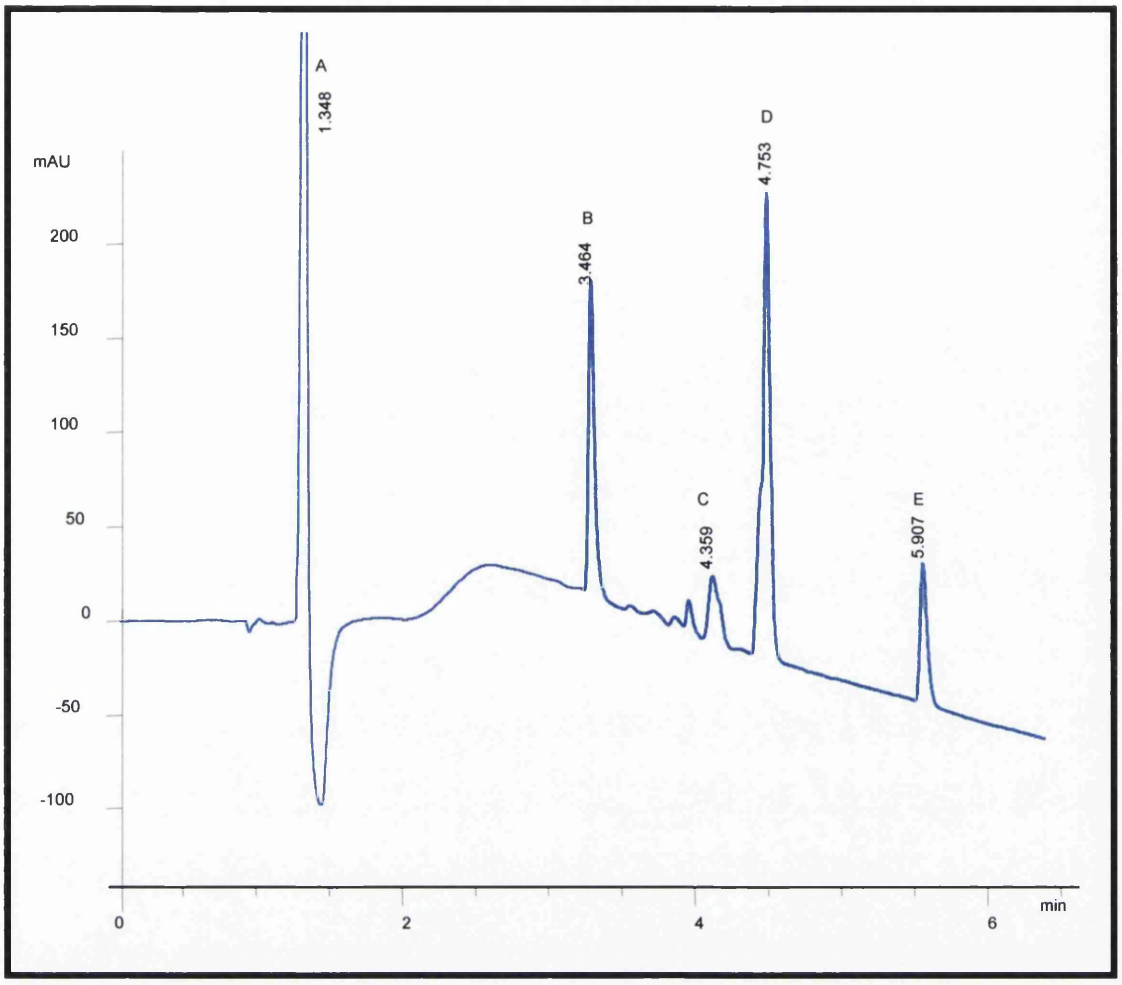



Figure 3.6 HPLC chromatogram of the separation of the 4 Estrogen steroids. 10 µL injection of a solution containing 10 µg/mL of each steroid conjugate.

The table below gives the corresponding resolution values (Rs) for the separation shown previously.

| Peaks | Rs Value |
|---|---|
| A+B | 10.57 |
| B+C | 2.89 |
| C+D | 2.50 |
| D+E | 10.67 |

## LC-MS For the Separation of 4 Conjugated Estrogen Steroids

The instrumentation and chromatographic conditions used for LC-MS analysis were the same as those used for LC-UV analysis. Mass spectrometric analysis was carried out using a Finnigan Mat LCQ ion trap. Steroid sulphate conjugates are known to show a good response on LC-MS analysis in the negative ion electrospray ionisation (ESI) mode (6).

Initial mass spectrometric analysis was carried out by using an infusion pump set to a flow rate of 5 to 10μL per minute to introduce standard solutions of each steroid into the source. From this analysis we were able to establish initial tune files for the compounds and identify m/z values associated with ionisation of the individual steroids. The conditions were adjusted for LC-MS to take account of the higher flow rates involved, analysis was performed with ESI in the negative ion-scanning mode, below are the conditions used for analysis.

**ESI**

Polarity: Negative.

Heated Capillary Temperature: 250°C.

Electron Multiplier Voltage: -1230V.

Needle Spray Voltage: 3.5kV.

Sheath Gas Flow: 90psi.

Auxiliary Gas Flow: 10psi.

Scan Range: 50-700amu.

The flow from the HPLC system was split to allow approximately 200μL per minute into the ion source (a split of 27%). Figure 3.7 shows the total ion chromatogram (TIC) for the 4 separated estrogen steroids.

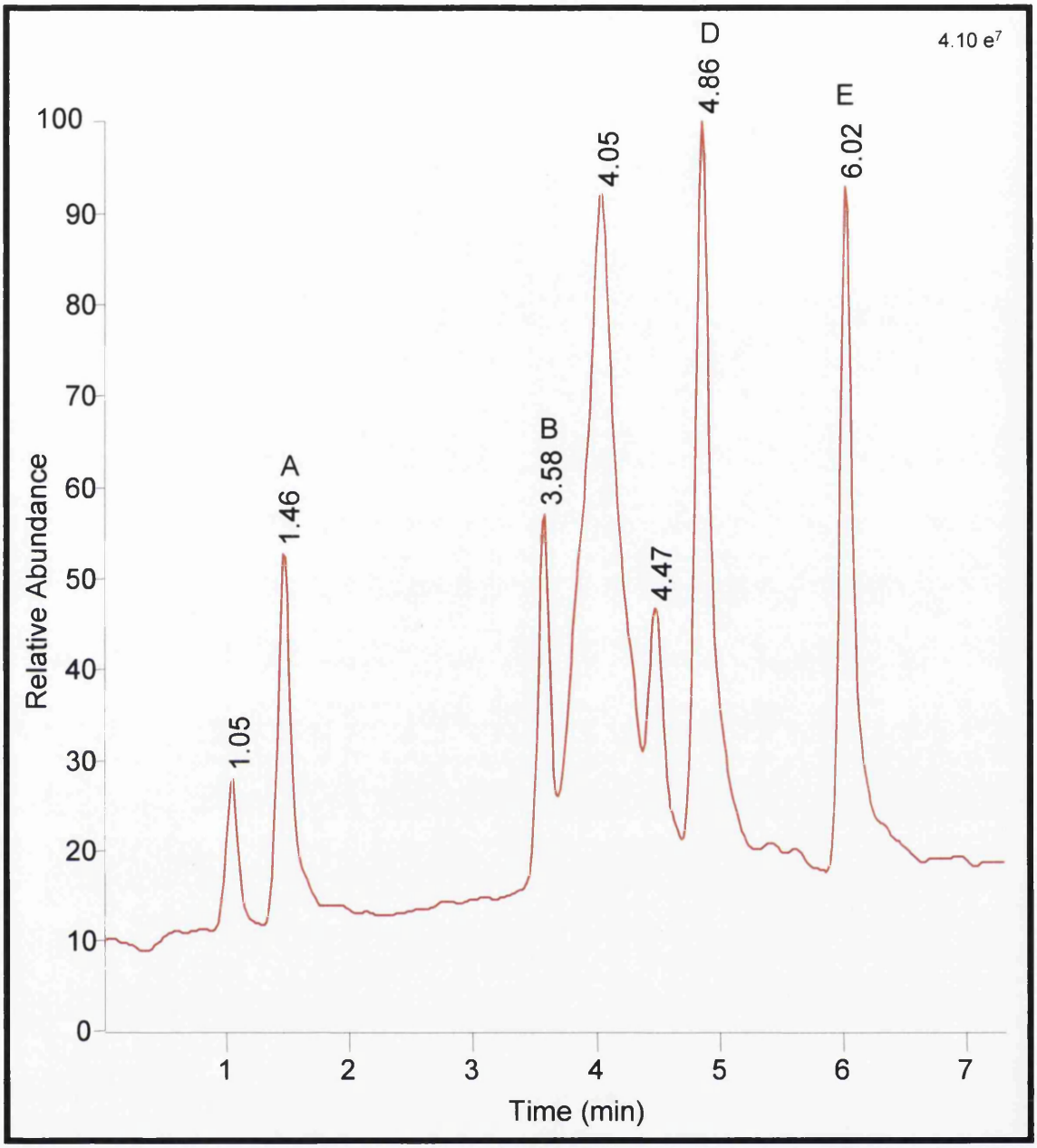



Figure 3.7 Negative LC-ESI-MS total ion chromatogram for the separation of the 4 estrogen steroids. 10μL injection of a 1μg/mL solution of each steroid conjugate; equal to 10ng on column of each.

Peak C, the peak corresponding to β-estradiol 3, 17-disulfate, is hidden in the TIC by the peak occurring at 4.05 minutes, which is part of the background. The following figure 3.8 shows the extracted ion chromatograms for the 4 steroids; as can be seen the peak for β-estradiol 3, 17-disulfate is easily distinguished from the background.

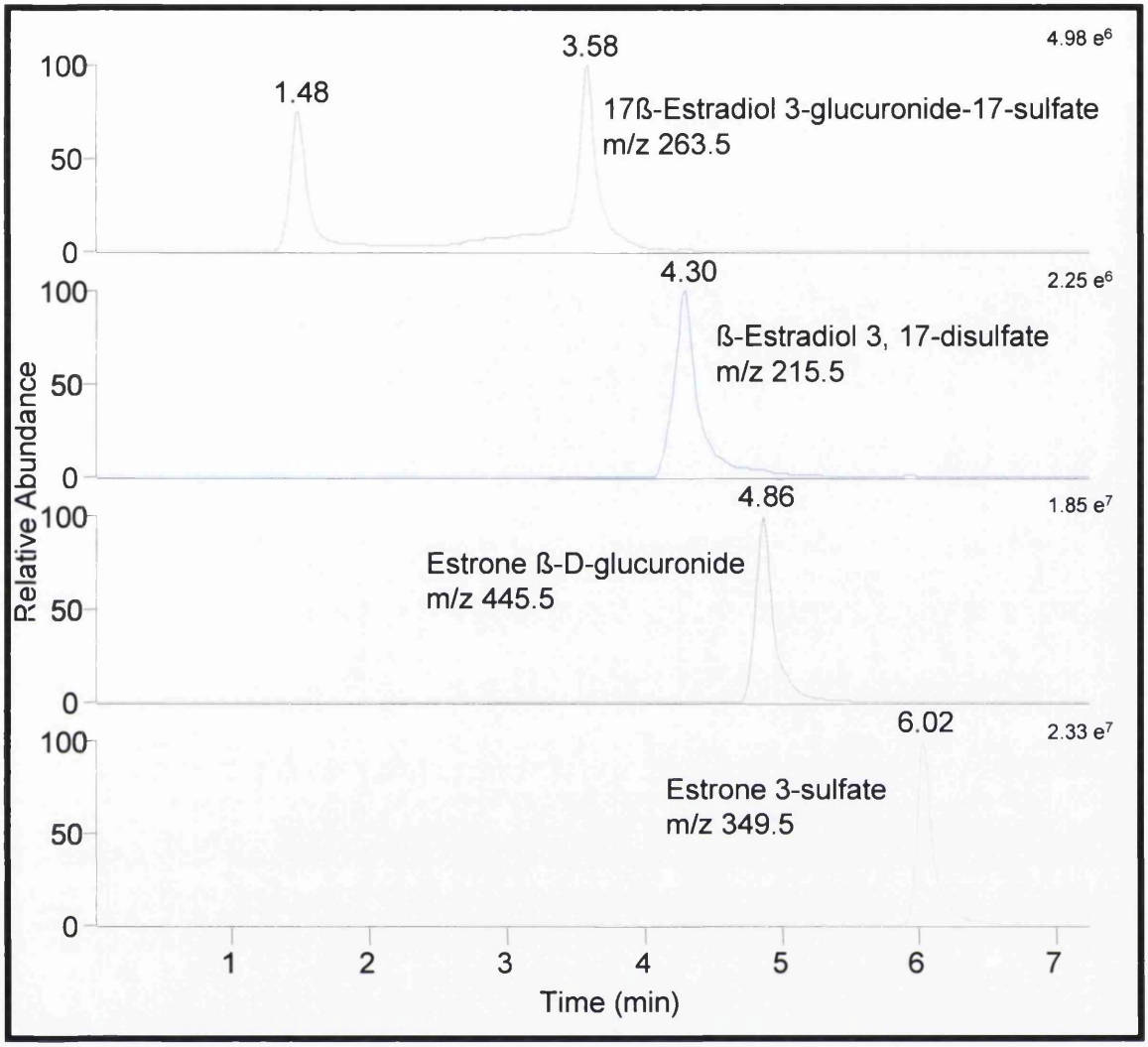



Figure 3.8 Extracted ion chromatograms for each of the 4 estrogen steroids. 10µL injection of a 1µg/mL solution of each steroid conjugate; equal to 10ng on column of each.

The table below lists the m/z values used for the extracted ion chromatograms.

| Compound name | m/z |
|---|---|
| 17β-Estradiol 3-glucuronide 17-sulfate | 263.5 |
| β Estradiol 3, 17-disulfate | 215.5 |
| Estrone β-D-glucuronide | 445.5 |
| Estrone 3-sulfate | 349.5 |

The following figures (3.9-3.13) show the mass spectrum for each of the steroid conjugates and the proposed mass spectrum interpretation; the m/z values associated with ions other than the parent ion are assumed to be due to in source fragmentation although this is not definitive and they may in fact be due to the presence of impurities in the standard materials. In the mass spectrum of β-estradiol 3, 17-disulfate there are two mass charges that we were unable to interpret, at m/z 511.4 and 678.0, we believe that these may be due to the formation of some kind of adduct. As we were unable to deduce the structure of these mass charges it was decided to use the less abundant m/z at 215.5, due to the doubly charged molecular ion, to monitor for the presence of the steroid.

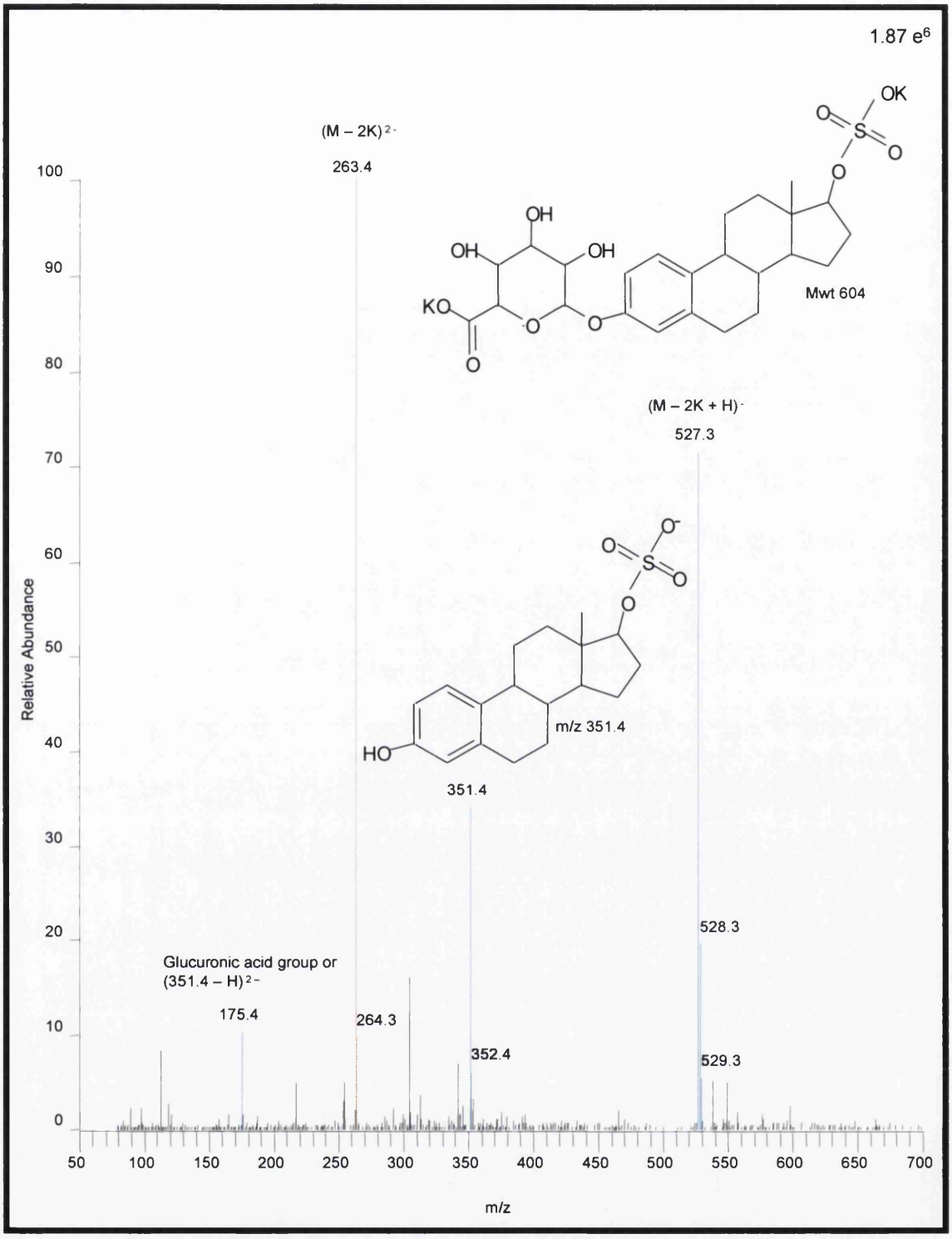



Figure 3.9 Negative LC-ESI-MS mass spectrum of 17ß-Estradiol 3-glucuronide 17-sulfate, 1.48 minutes. 10µL injection of a 1µg/mL solution; equal to 10ng on column.

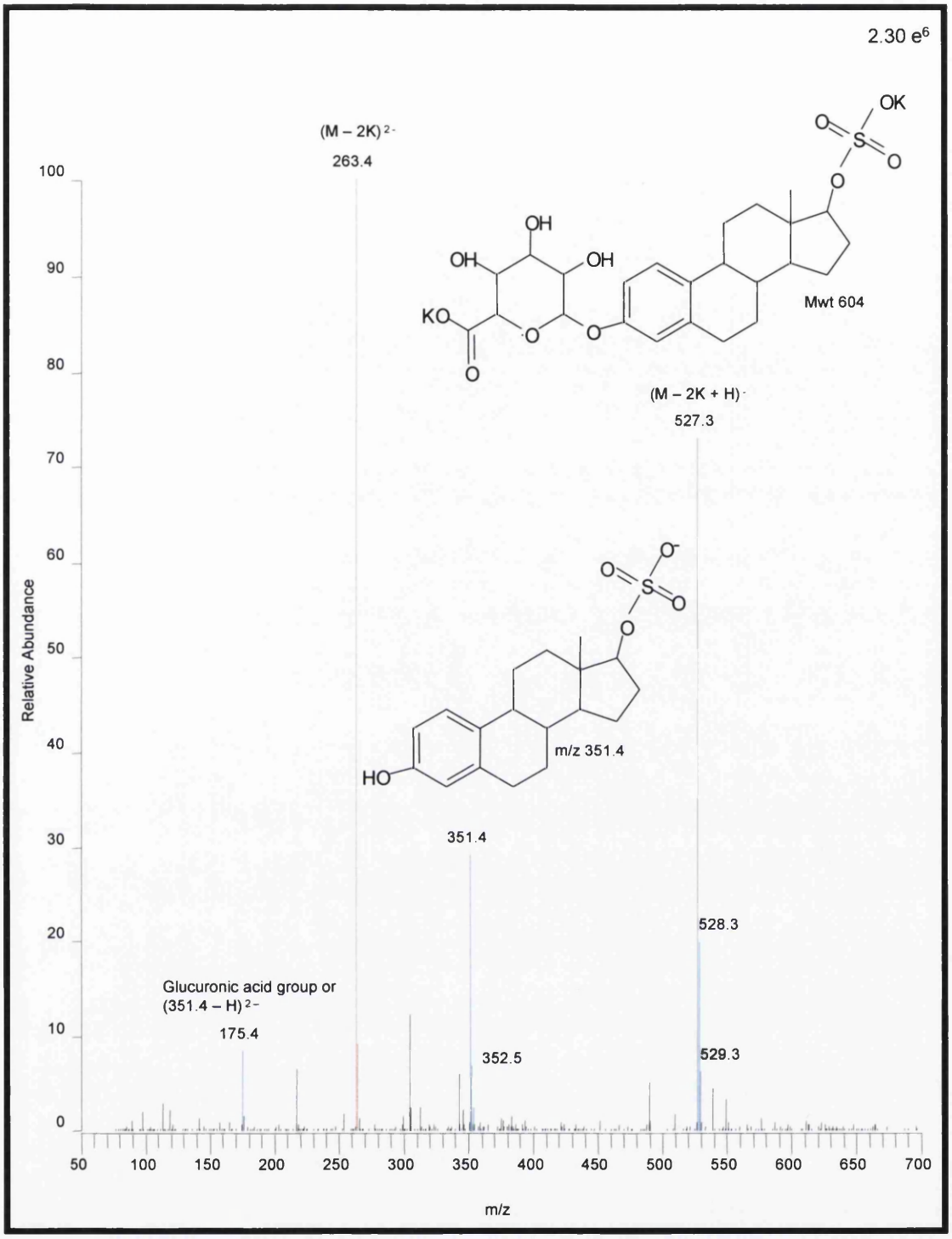



Figure 3.10 Negative LC-ESI-MS mass spectrum of 17ß-Estradiol 3-glucuronide 17-sulfate, 3.58 minutes. 10µL injection of a 1µg/mL solution; equal to 10ng on column.

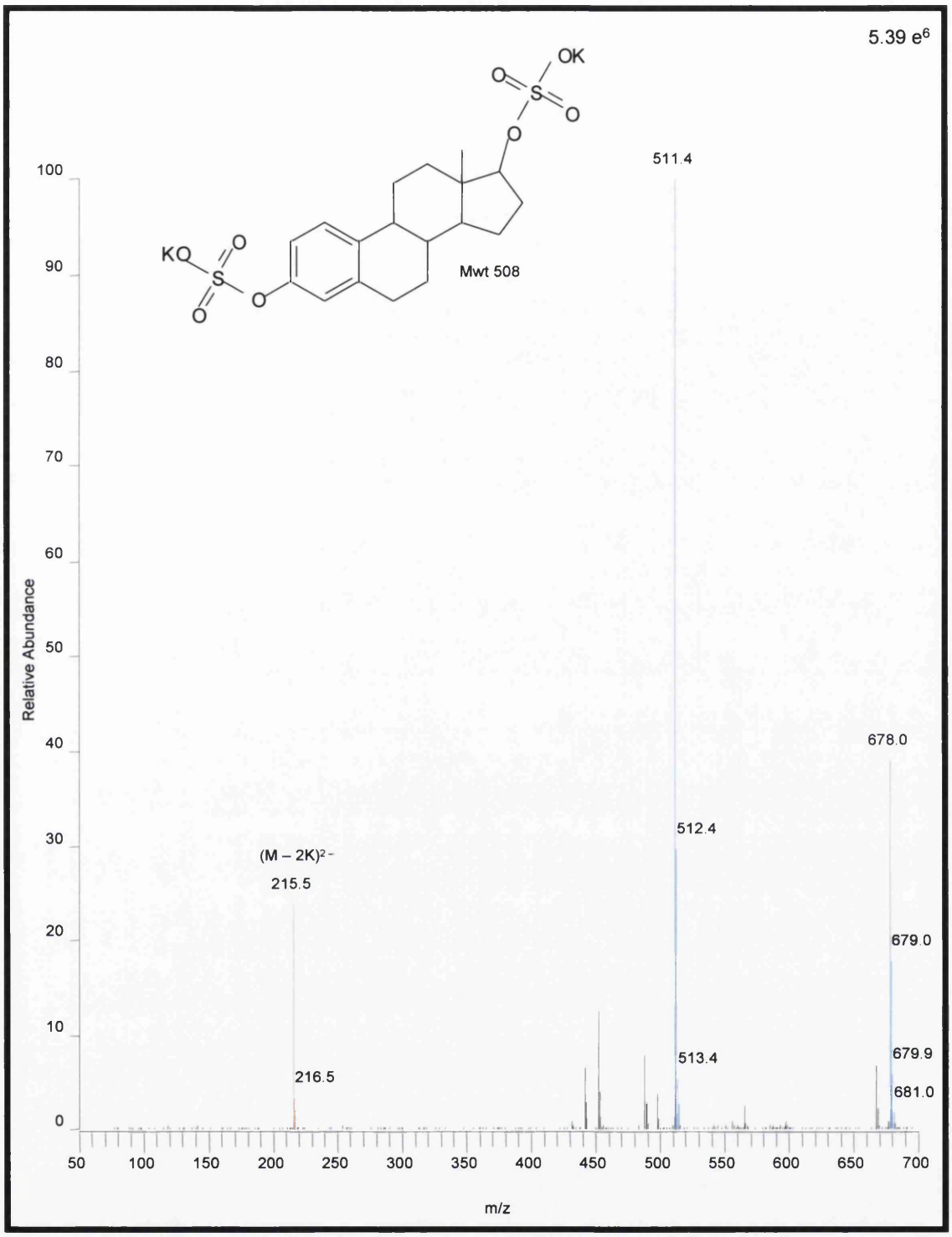



Figure 3.11 Negative LC-ESI-MS mass spectrum of ß-Estradiol 3, 17-disulfate. 10µL injection of a 1µg/mL solution; equal to 10ng on column.

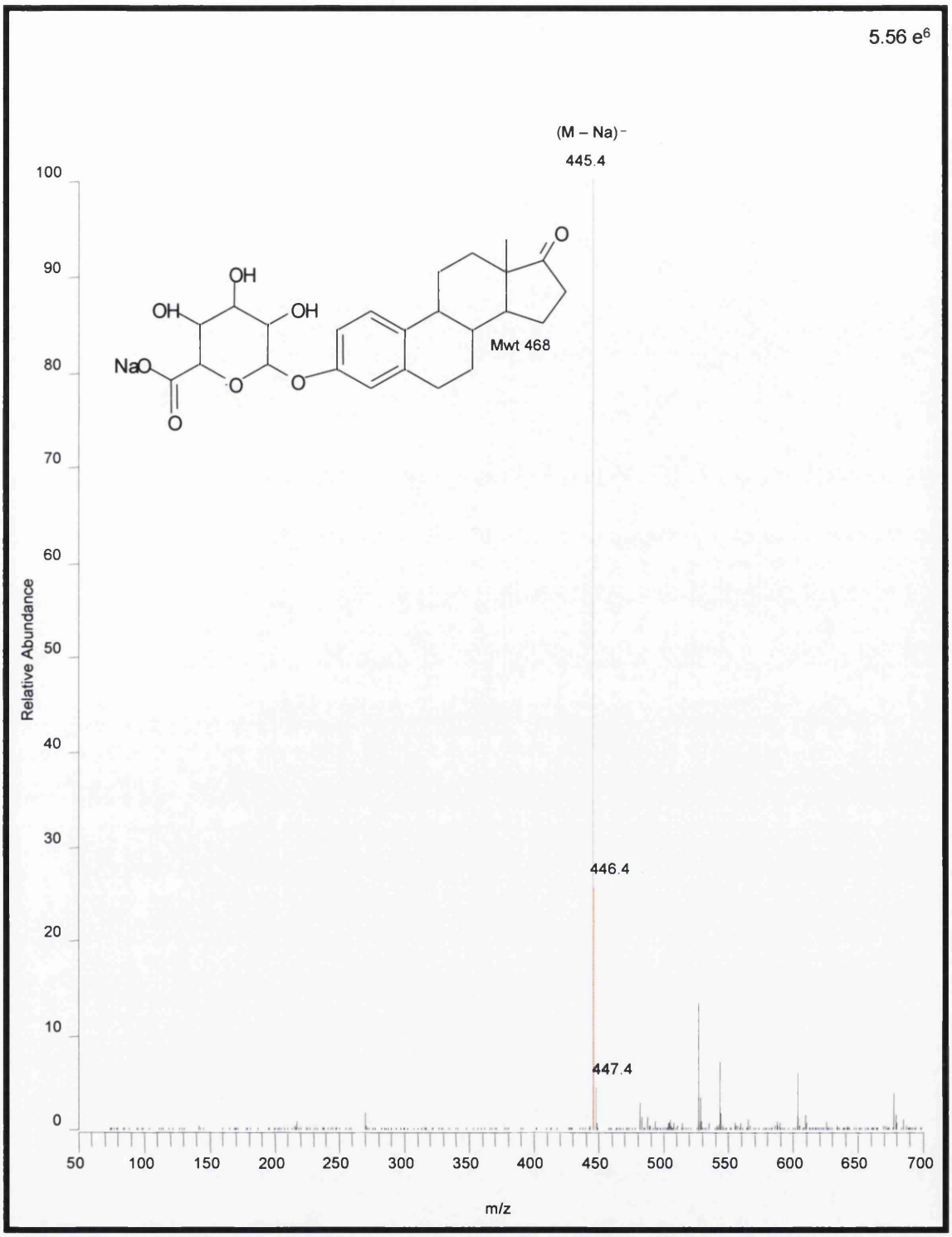



Figure 3.12 Negative LC-ESI-MS mass spectrum of Estrone ß-D-glucuronide. 10µL injection of a 1µg/mL solution; equal to 10ng on column.

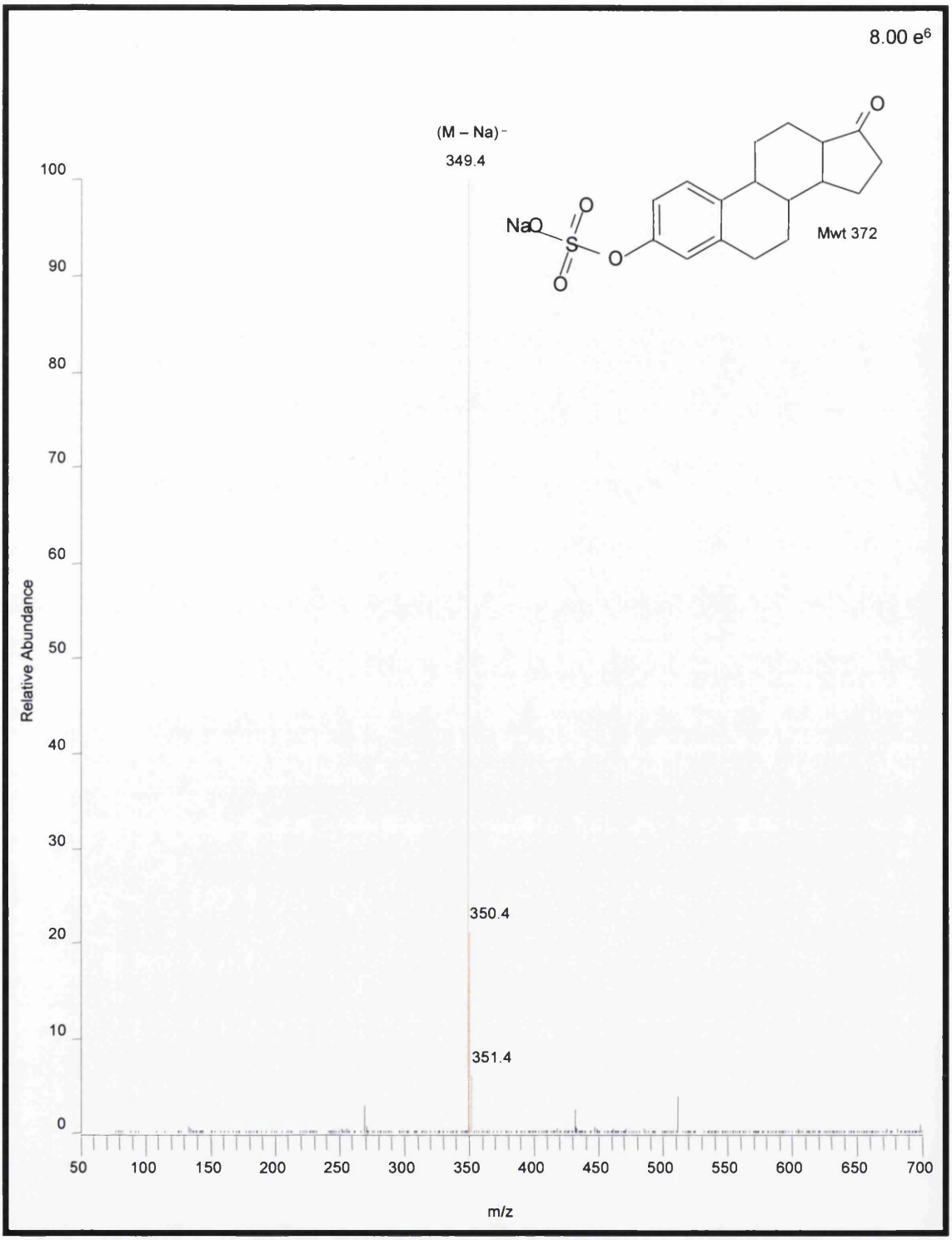



Figure 3.13 Negative LC-ESI-MS mass spectrum of Estrone 3-sulfate. 10µL injection of a 1µg/mL solution; equal to 10ng on column.

## Analysis of Spiked River Water

River water collected from the river Thames in south east England was spiked with the four estrogen conjugate steroids at levels ranging from 10 to 1000ng per litre and extracted in the labs of WRc plc (Buckinghamshire, U.K). The sample volumes used for the extraction ranged from 5 litres for the 10ng per litre spiked sample to 2 litres for the 1000ng per litre sample, all extracts were delivered to our labs in a final volume of 100μL of methanol. For commercial reasons the extraction procedure used by WRc was not divulged. Due to the nature of the final extracts it was necessary to dilute the samples to allow for analysis with LC-MS, see table below.

| Step | Procedure |
|---|---|
| 1 | 100μL of sample made up to 1000μL with 900μL Methanol. |
| 2 | Diluted sample filtered through fine pore disc filter. |
| 3 | Dry down under nitrogen and reconstitute in 50:50 (v/v) methanol/water to a volume of 200μL. |

Using this method we were able to detect the presence of all four of the steroids at the 1000ng per litre level and the presence of β-estradiol 3, 17-disulfate and estrone 3-sulfate down to the 100ng per litre level. We were unable to detect any of the steroids at the 10ng per litre level; selected ion monitoring (SIM) was used for the analysis. The table below shows the m/z values that were used for SIM and the retention time corresponding to each conjugate. Figure 3.14 on the following page shows an example SIM chromatogram of a 1000ng per litre sample.

| Compound name | m/z | RT minutes |
|---|---|---|
| 17β-Estradiol 3-glucuronide 17-sulfate | 263.5 | 1.54 |
| β Estradiol 3, 17-disulfate | 215.5 | 4.36 |
| Estrone β-D-glucuronide | 445.5 | 4.93 |
| Estrone 3-sulfate | 349.5 | 6.11 |

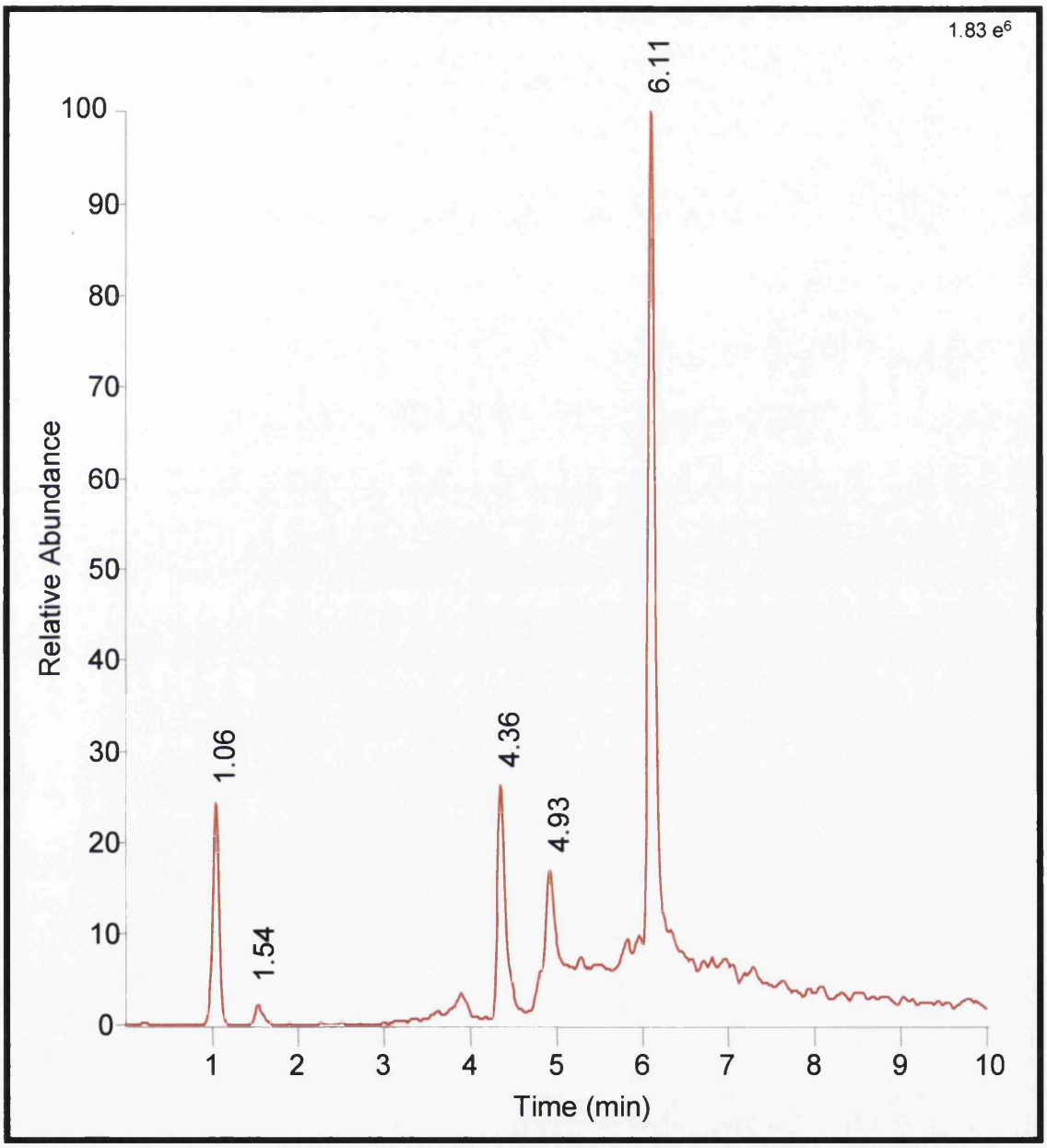



Figure 3.14 Negative LC-ESI-MS selected ion monitoring chromatogram for the 1000ng/L spiked extract.

As can be seen from the figure shown above only the first peak corresponding to 17β-estradiol 3-glucuronide 17-sulfate was detected in the extracted spiked river water samples.

## LC-UV For the Separation of a Mixture of 4 Estrogen Free Steroids and 4 Conjugated Steroids

The structures for the free steroids and conjugates are shown in figure 3.15.

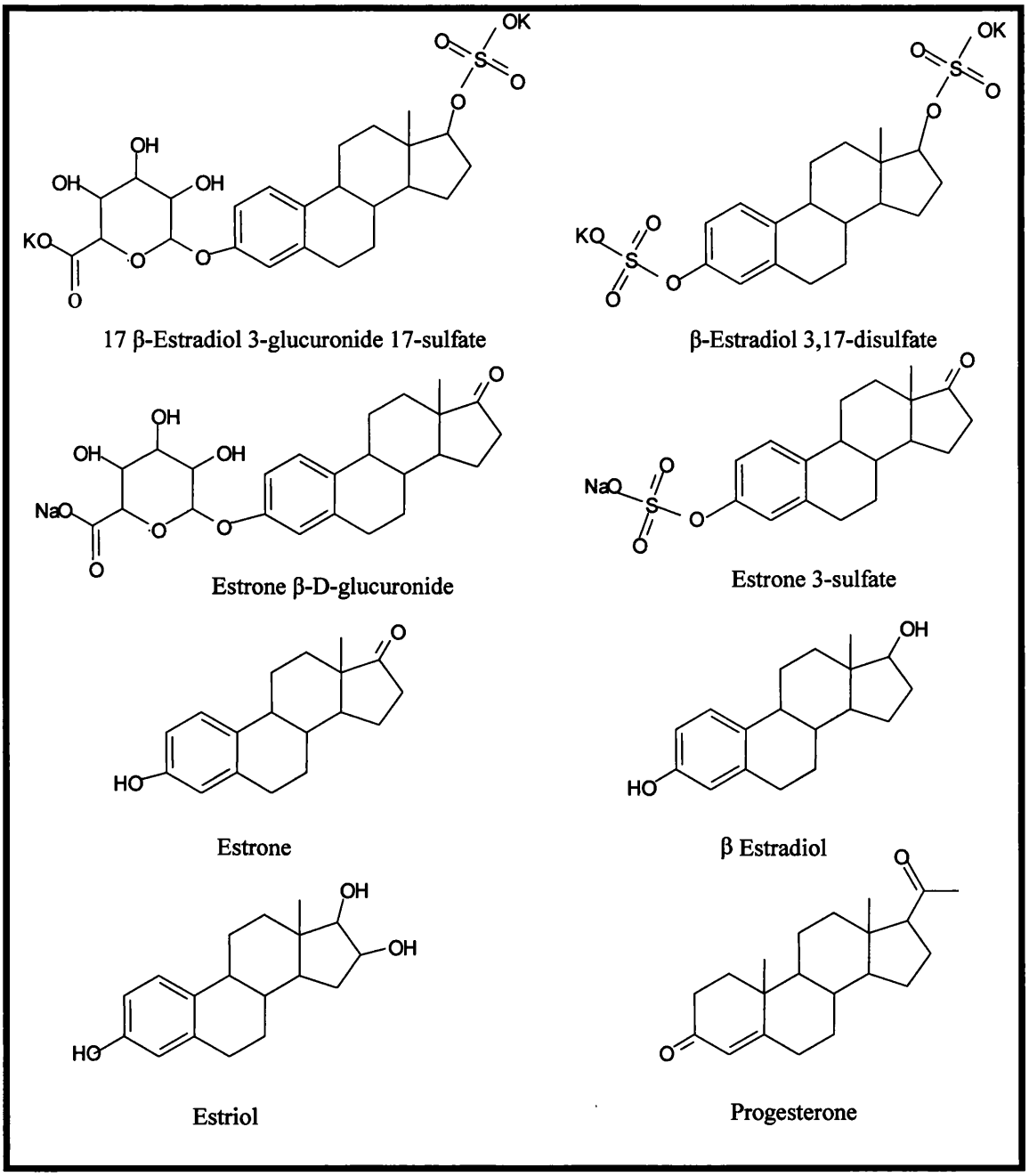



Figure 3.15 Structures of the Estrogen free and conjugated steroids investigated.

## Experimental

### Chemicals

HPLC grade solvents were purchased from Fisher Scientific (Loughborough, U.K.), gases from BOC Limited (Surrey, U.K.) and the following 8 steroids from Sigma-Aldrich Limited (Dorset, U.K.) or prepared in the labs of WRc plc (Buckinghamshire, U.K).

| | |
|---|---|
| 17β-Estradiol 3-glucuronide 17-sulfate | Estriol |
| β Estradiol 3, 17-disulfate | β-Estradiol |
| Estrone β-D-glucuronide | Estrone |
| Estrone 3-sulfate | Progesterone |

### Sample Preparation

Standard solutions ranging from 0.1 to 0.5 mg/ml were made up in 50/50 methanol/water for each of the steroids; solutions for analysis were prepared and diluted from these as necessary.

### Instrumentation

A Hewlett Packard 1100 HPLC fitted with a diode array detector (DAD) was used to perform the chromatography with a Phenomenex Luna 4.6x100mm 3μ phenyl-hexyl column. The mobile phase used for analysis was made up of methanol and water run in a gradient system.

**Results**

We initially attempted to adapt the chromatographic conditions we had used for the analysis of the conjugated steroids to allow for the analysis of the mixture of free and conjugated steroids. This however was unsuccessful and it was decided to switch to the Phenyl-Hexyl column as we had previously found it to perform well in the analysis of aromatic compounds (see chapter 2). Experiments were carried out using various mobile phases and it was found that the best results were achieved using methanol with an unbuffered aqueous phase. The mixture of the 8 estrogen steroids was separated on the Phenomenex Phenyl-Hexyl column with the following chromatographic conditions.

| Time in minutes | %MeOH | $\%H_2O$ |
|---|---|---|
| 0.00 | 20 | 80 |
| 1.00 | 20 | 80 |
| 2.50 | 40 | 60 |
| 5.00 | 40 | 60 |
| 12.50 | 90 | 10 |

| | |
|---|---|
| Column Thermostat | 40°C |
| Flow Rate | 0.75mL/min |
| DAD | 220nm |

The following table gives the retention times for the compounds. Figure 3.16 shows the UV chromatogram of the separation of the 8 steroids.

| Compound name | Peak label | RT minutes |
|---|---|---|
| 17β-Estradiol 3-glucuronide 17-sulfate | A | 1.003 |
| β Estradiol 3, 17-disulfate | B | 1.357 |
| Estrone β-D-glucuronide | C | 5.450 |
| Estrone 3-sulfate | D | 6.313 |
| Estriol | E | 10.931 |
| β-Estradiol | F | 13.404 |
| Estrone | G | 14.068 |
| Progesterone | H | 15.467 |

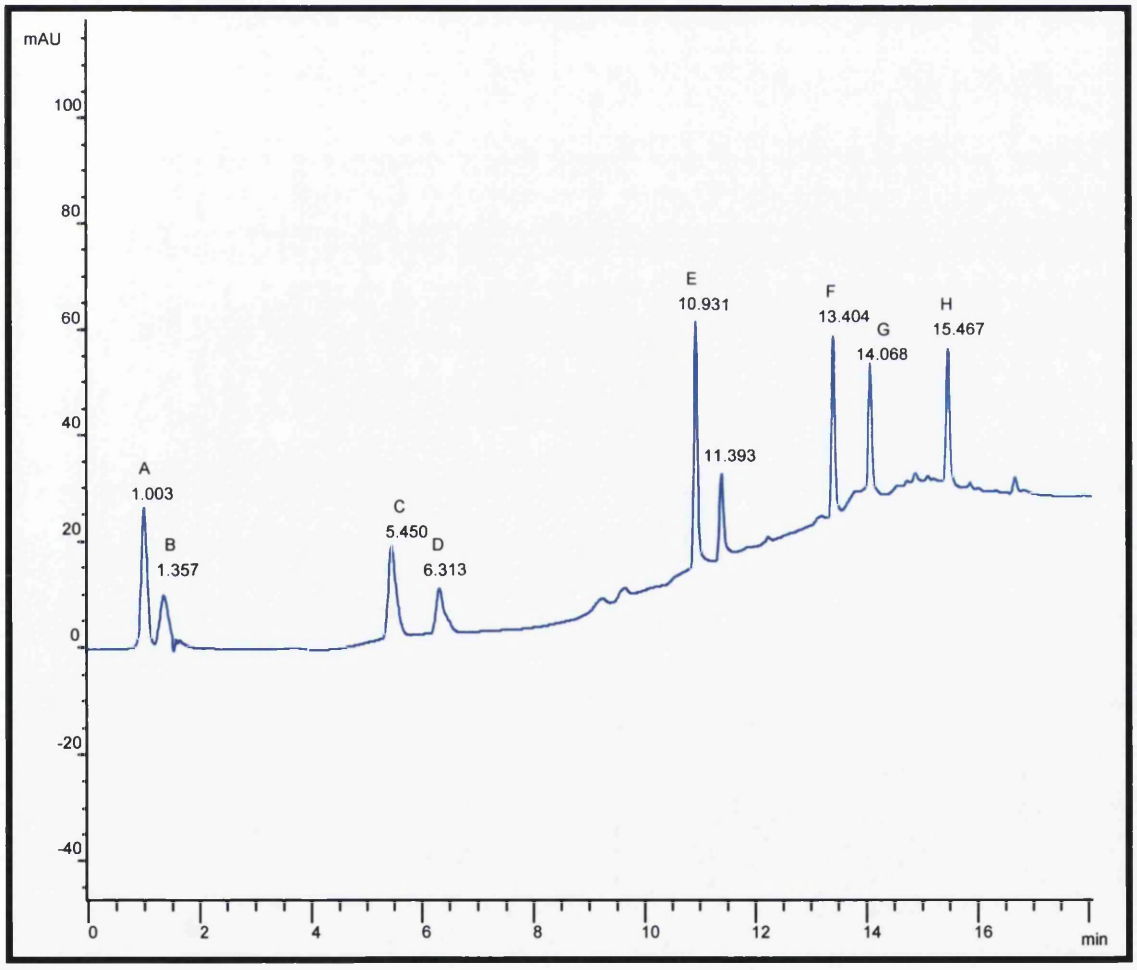



Figure 3.16 HPLC chromatogram of the separation of the 8 Estrogen steroids.The peak occurring at 11.393 is an unknown. 10 µL injection of a solution containing 1µg/mL of each steroid conjugate.

The table below gives the corresponding resolution values (Rs) for the separation shown on the previous page.

| Peaks | Rs Value |
|---|---|
| A+B | 0.89 |
| B+C | 9.60 |
| C+D | 1.82 |
| D+E | 13.50 |
| E+F | 14.50 |
| F+G | 4.00 |
| G+H | 6.40 |

## LC-MS For the Separation of the Mixture of 4 Estrogen Free Steroids and 4 Conjugated Steroids

The instrumentation and chromatographic conditions used for LC-MS analysis were the same as those used for LC-UV analysis. Mass spectrometric analysis was carried out using a Finnigan Mat LCQ ion trap; data was acquired in the negative ion-scanning mode.

Initial mass spectrometric analysis was carried out by using an infusion pump set to a flow rate of 5 to 10μL per minute to introduce standard solutions of each steroid into the source; electrospray ionisation (ESI) was used to perform the analysis due to its suitability to low flow rates. From this analysis we were able to establish initial tune files for the compounds and identify m/z values associated with ionisation of the individual steroids. LC-MS analysis was performed with both atmospheric pressure chemical ionisation (APCI) and ESI with the source conditions adjusted to suit the higher flow rates, below are the conditions used for both forms of ionisation.

**APCI**

| | |
|---|---|
| Polarity: Negative. | Sheath Gas Flow: 90psi. |
| Heated Capillary Temperature: 250°C. | Auxiliary Gas Flow: 50psi. |
| Electron Multiplier Voltage: -1230V. | Scan Range: 50-900amu. |
| Vaporiser Temperature: 300°C. | Corona Discharge Current: 5μ amps. |

**ESI**

Polarity: Negative.

Heated Capillary Temperature: 250°C.

Electron Multiplier Voltage: -1230V.

Needle Spray Voltage: 3.5kV.

Sheath Gas Flow: 90psi.

Auxiliary Gas Flow: 10psi.

Scan Range: 50-900amu.

For ESI analysis the flow from the HPLC system was split to allow approximately 200μL per minute into the ion source (a split of 27%).

It was found that both forms of ionisation yielded the same ions and that although ion ratios varied the base ion remained the same for the free steroids and the sulfate conjugates with each technique; however in the case of the glucuronide conjugates the base ion for ESI was found to have the glucuronic acid group intact whereas with APCI the base ion resulted from loss of the glucuronic acid group. Figures 3.17 and 3.18 show comparisons of the ESI and APCI spectra obtained for Estrone β-D-glucuronide and Estrone 3-sulfate.

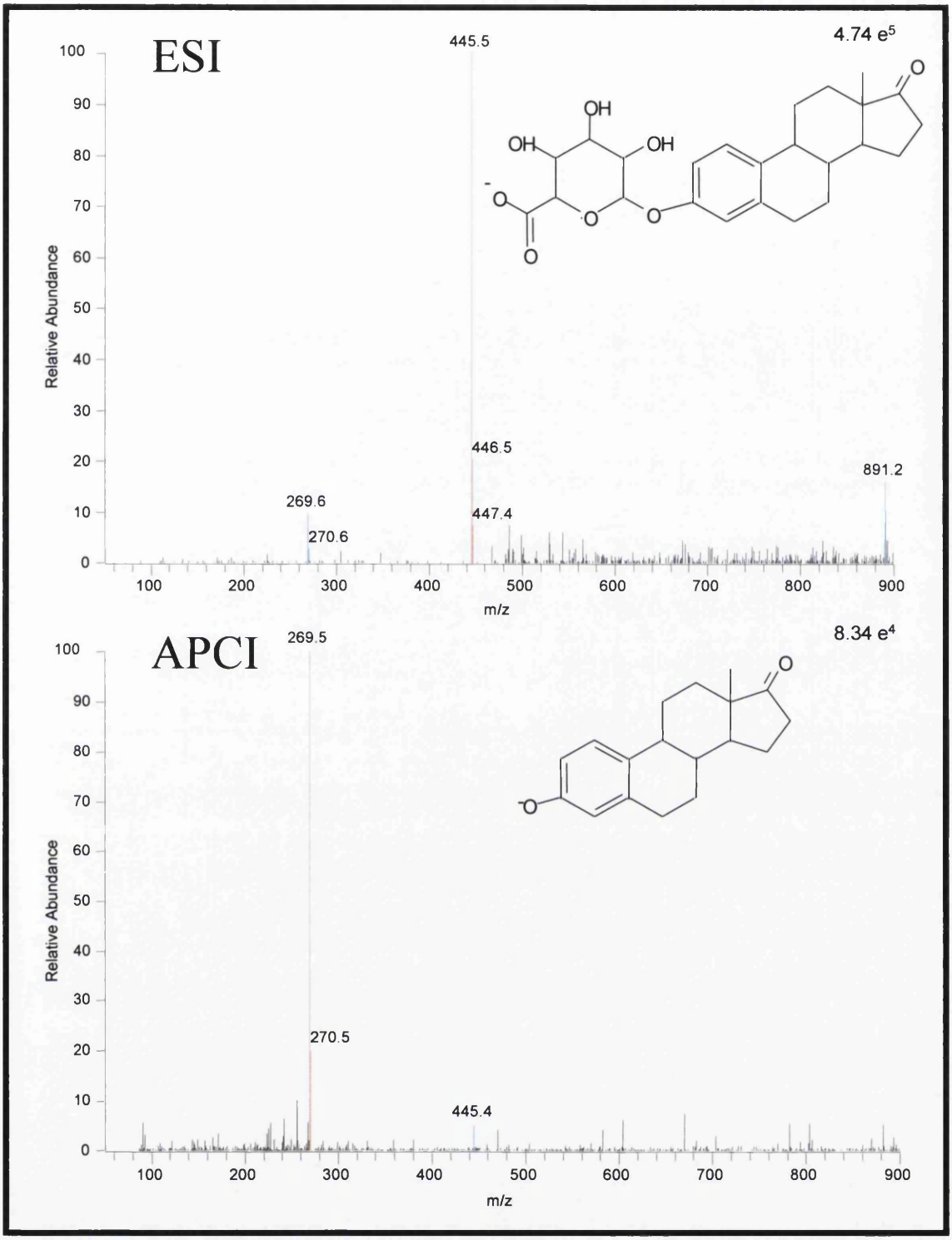



Figure 3.17 Negative ESI and APCI mass spectrum comparison of Estrone ß-D-glucuronide. 10µL injection of a 1µg/mL solution; equal to 10ng on column.

The spectra of estrone β-D-glucuronide both contain m/z at 269.5 and 445.5; in the ESI spectrum 445.5 is the base ion, however in the APCI spectrum although 445.5 is present the base ion is 269.5 which results from the loss of the glucuronic acid group.

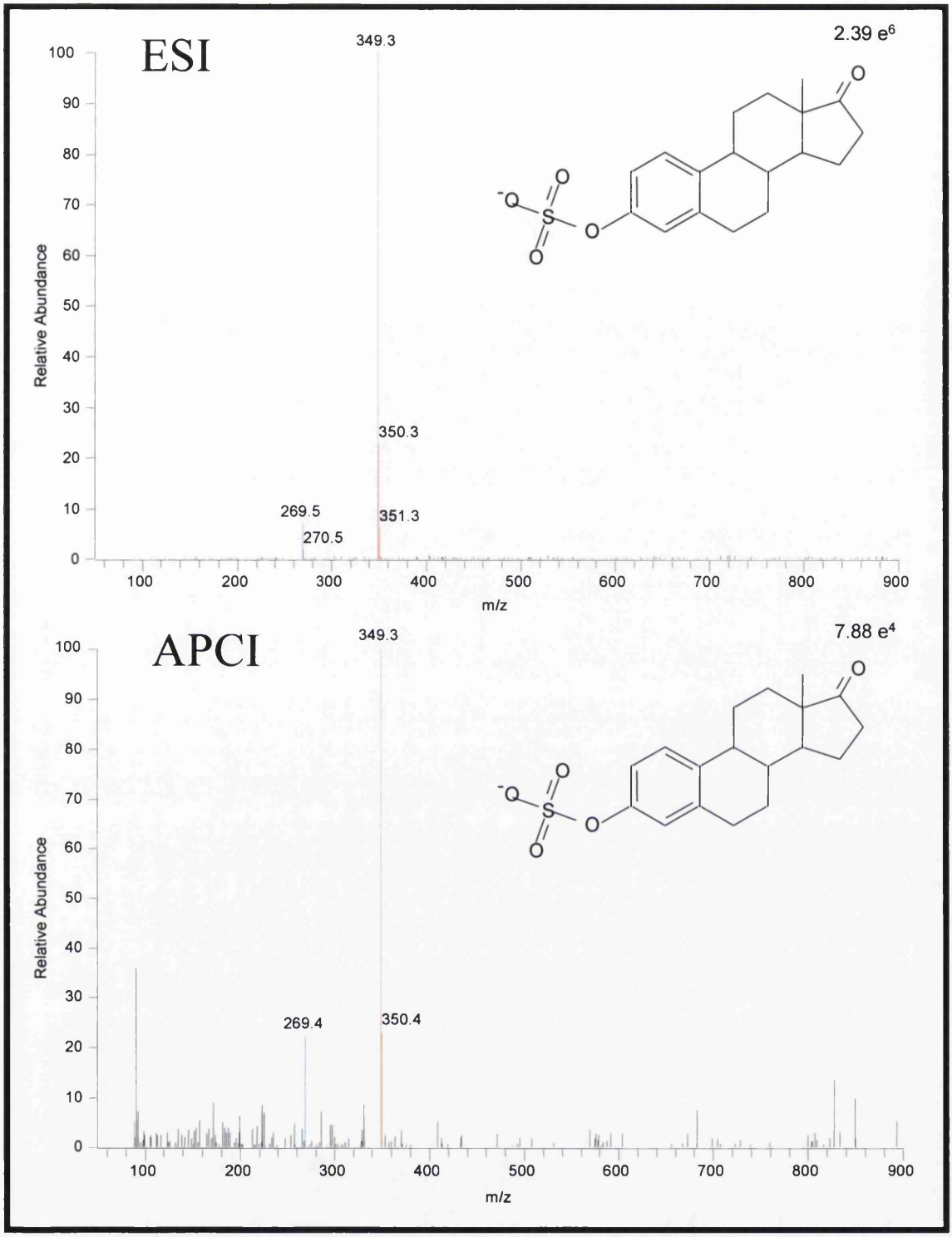



Figure 3.18 Negative ESI and APCI mass spectrum comparison of Estrone 3-sulfate. 10µL injection of a 1µg/mL solution; equal to 10ng on column.

The spectra of estrone 3-sulfate both contain m/z at 269.5 and 349.5; in both the ESI and APCI spectra the base ion occurs at 349.5, however in the case of APCI the relative intensity of the 269.5 ion is approximately 3 times higher than in ESI.

Figure 3.19 shows the total ion chromatogram (TIC) for the 8 separated estrogen steroids using ESI and figure 3.20 the extracted ion chromatograms of the most abundant ion of each steroid.

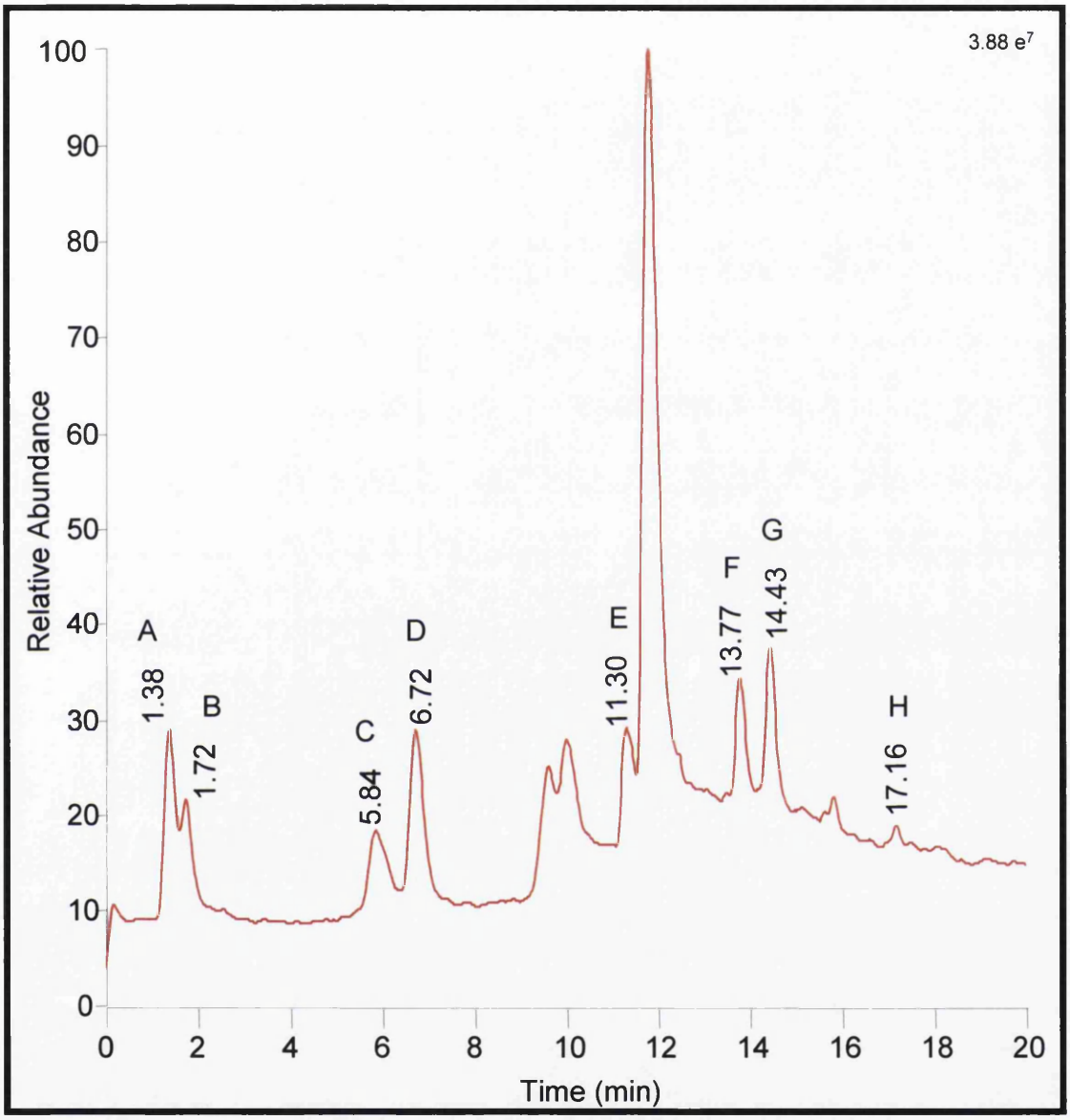



Figure 3.19 Negative LC-ESI-MS total ion chromatogram for the separation of the 8 estrogen steroids. 10μL injection of a 1μg/mL solution of each steroid conjugate; equal to 10ng on column of each.

The peak occurring at 6.72 minutes in the extracted ion chromatogram of estrone is due to loss of the sulfate group during the ionisation of estrone 3-sulfate.

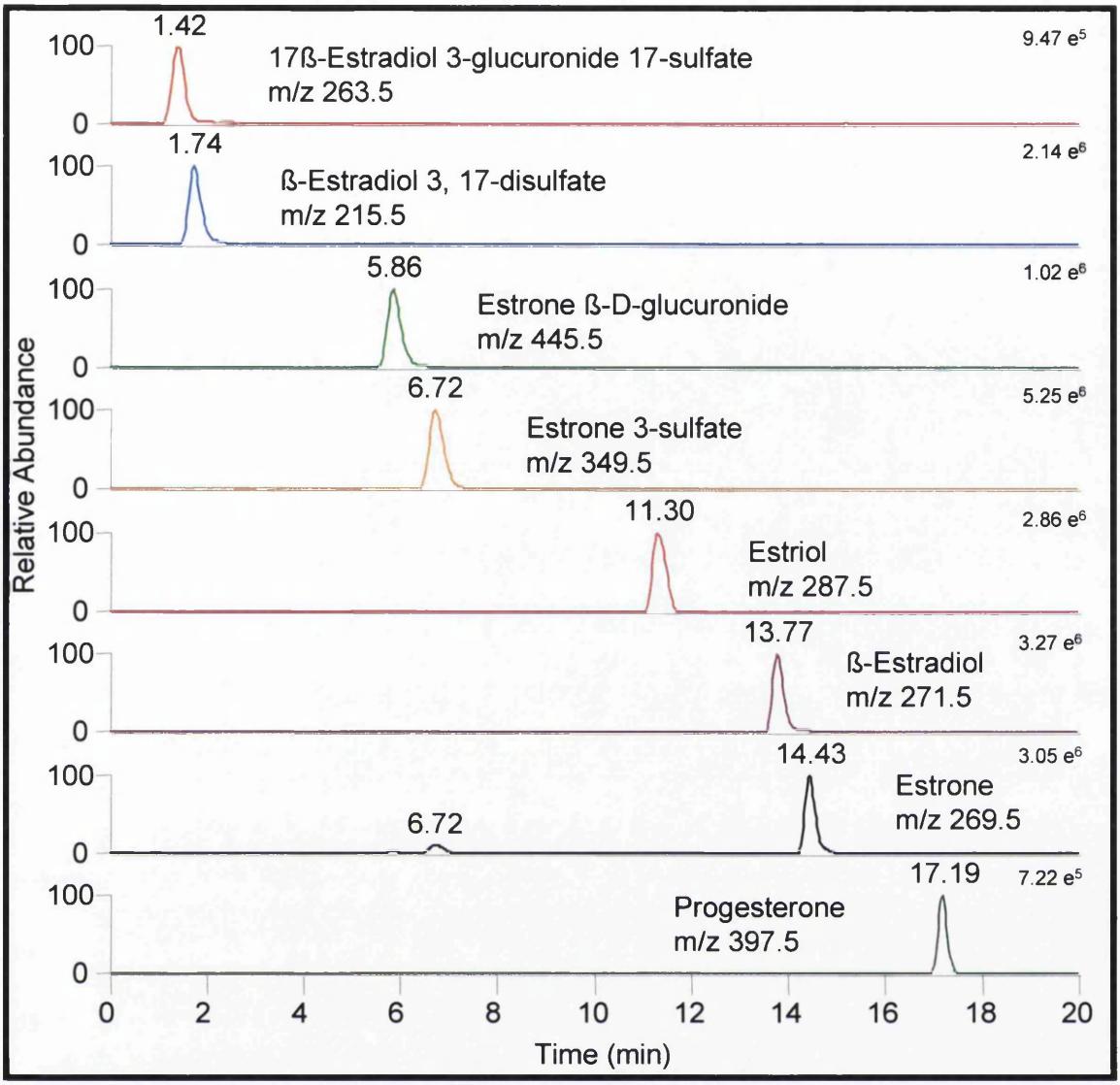



Figure 3.20 Extracted ion chromatograms of the base ions for each of the 8 estrogen steroids. 10µL injection of a 1µg/mL solution of each steroid conjugate; equal to 10ng on column of each.

The table below lists the m/z values used for the extracted ion chromatograms.

| Compound name | TIC Peak label | m/z |
|---|---|---|
| 17β-Estradiol 3-glucuronide 17-sulfate | A | 263.5 |
| β Estradiol 3, 17-disulfate | B | 215.5 |
| Estrone β-D-glucuronide | C | 445.5 |
| Estrone 3-sulfate | D | 349.5 |
| Estriol | E | 287.5 |
| β-Estradiol | F | 271.5 |
| Estrone | G | 269.5 |
| Progesterone | H | 397.5 |

The following figures (3.22-3.29) show the mass spectrum, with ESI, for each of the steroids and the proposed mass spectrum interpretation; the m/z values associated with ions other than the parent ion are assumed to be due to in source fragmentation although this is not definitive and they may in fact be due to the presence of impurities in the standard materials.

We were unable to interpret the mass spectrum of progesterone in negative ionisation; but we were able to interpret the spectrum attained with positive ionisation, see figure 3.21 below. $[M+H]^+$ was the most abundant ion and MS-MS analysis yielded only the fragments shown in the interpretation. However as the sensitivity of the method for the other steroids was found to be far greater with negative ionisation (in some cases there was no signal with positive ionisation) it was attempted to run the method using polarity switching however the "jump" in the background noise reduced sensitivity to an extent that it was decided to perform analysis using solely negative ionisation for all steroids.

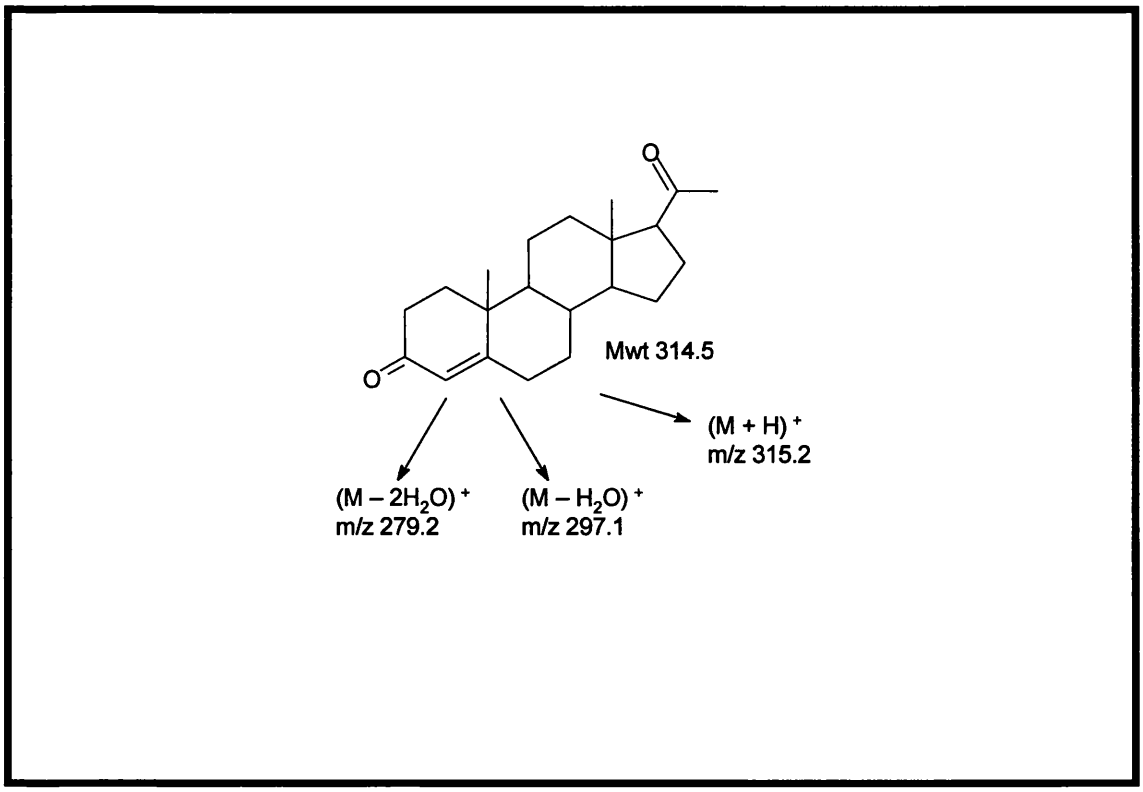



Figure 3.21 Positive ESI-MS fragmentation pattern interpretation for Progesterone.

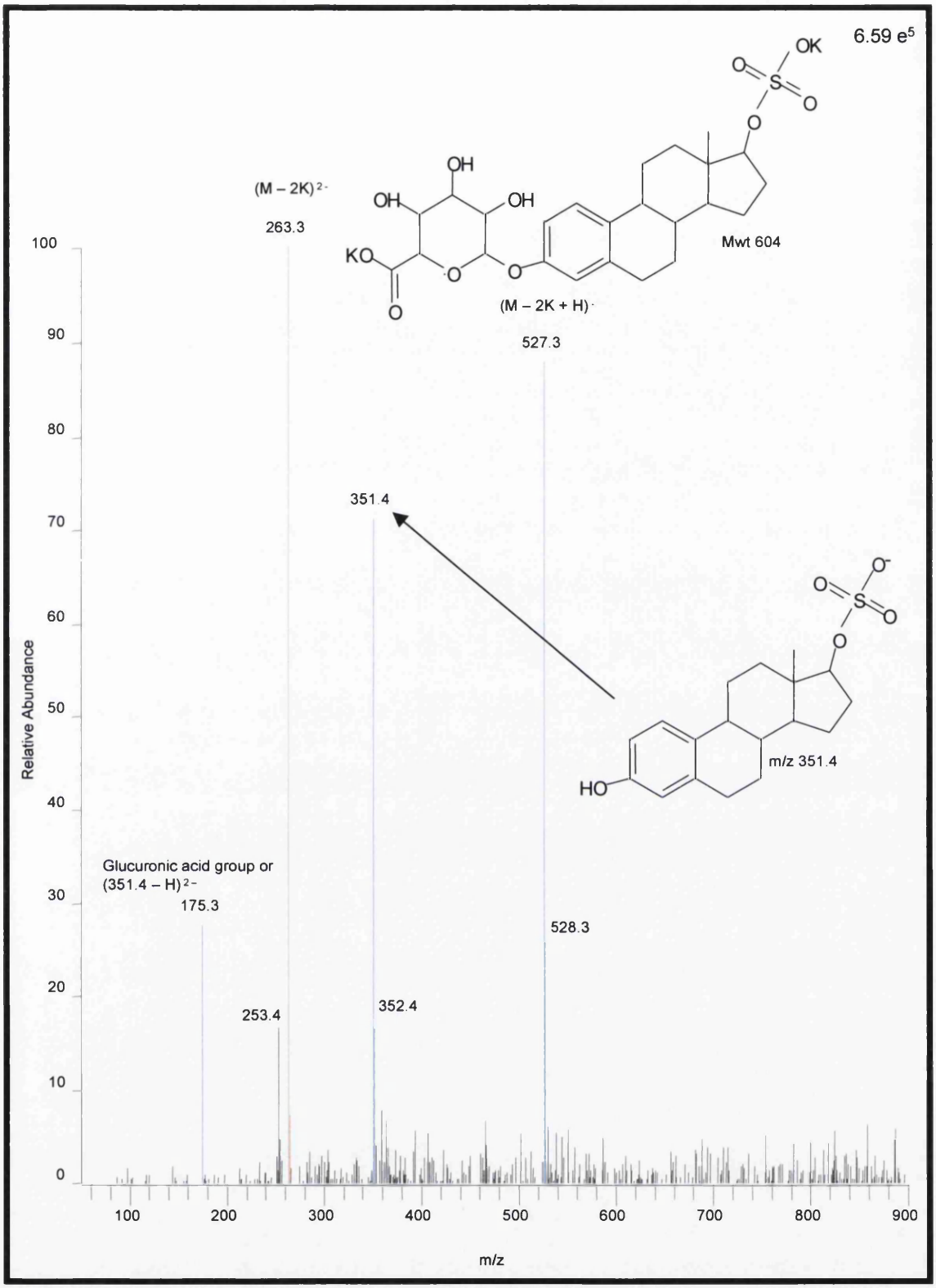



Figure 3.22 Negative LC-ESI-MS mass spectrum of 17ß-Estradiol 3-glucuronide 17-sulfate. 10µL injection of a 1µg/mL solution; equal to 10ng on column.

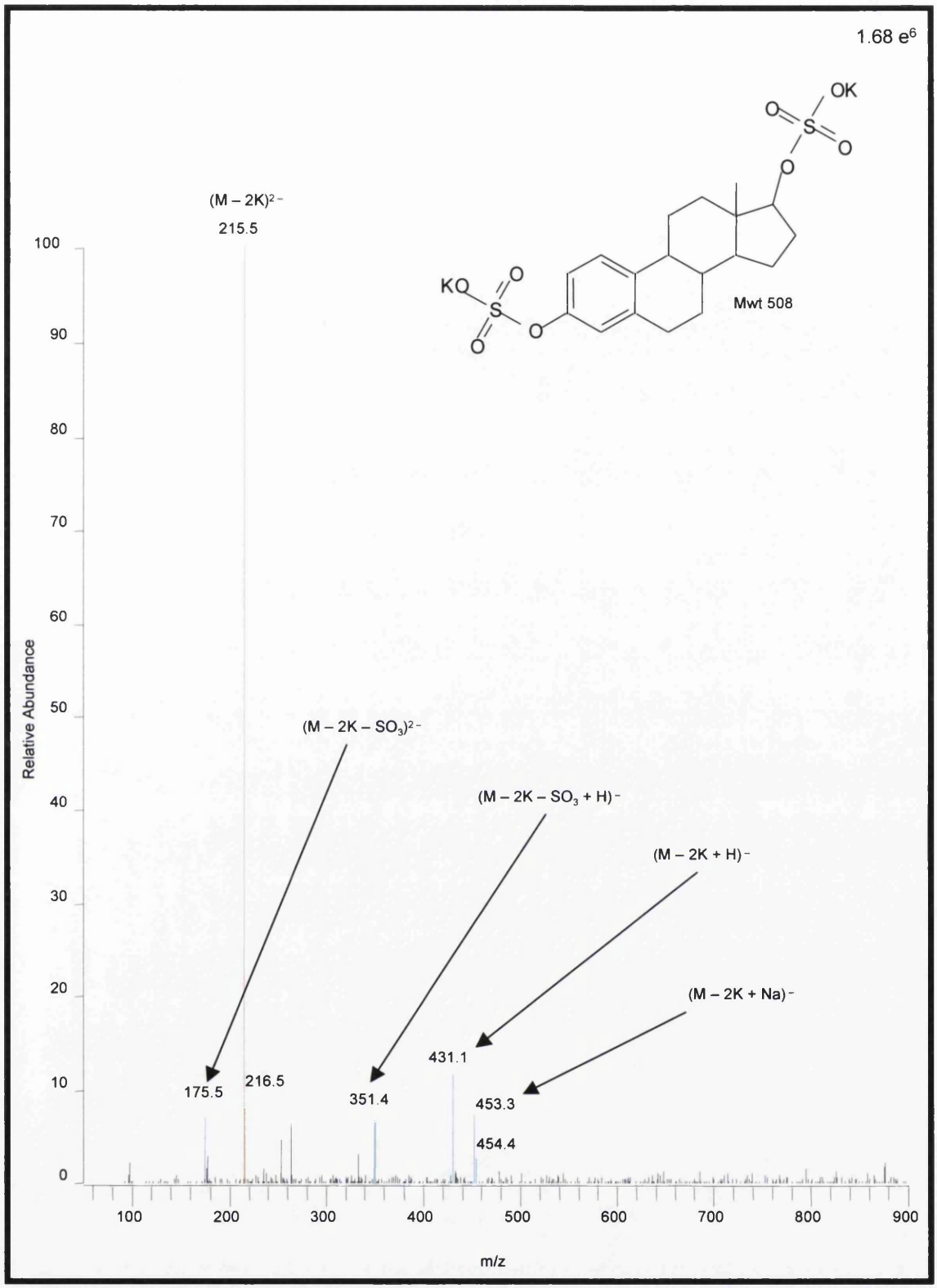



Figure 3.23 Negative LC-ESI-MS mass spectrum of ß-Estradiol 3, 17-disulfate. 10µL injection of a 1µg/mL solution; equal to 10ng on column.

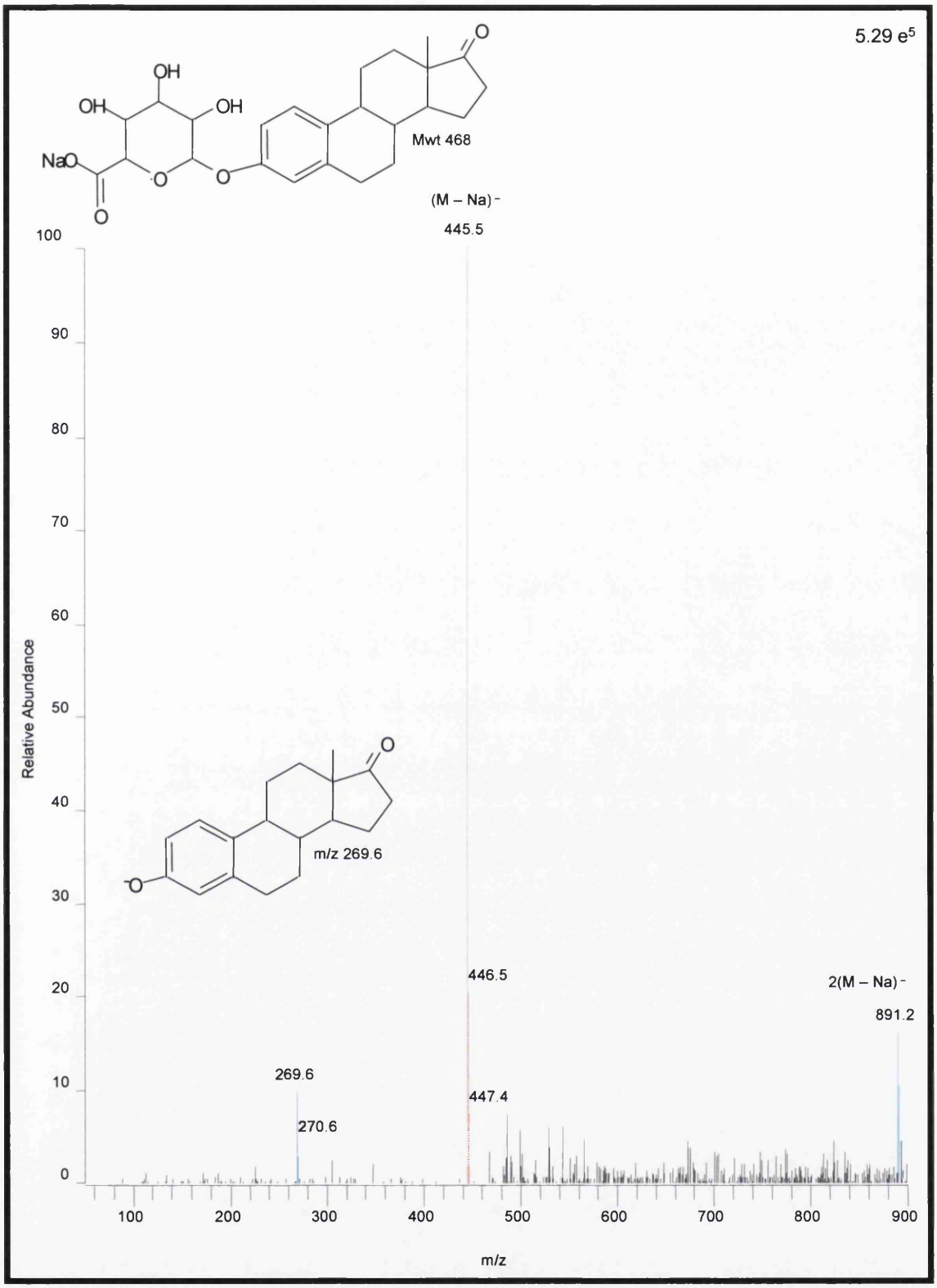



Figure 3.24 Negative LC-ESI-MS mass spectrum of Estrone ß-D-glucuronide. 10µL injection of a 1µg/mL solution; equal to 10ng on column.

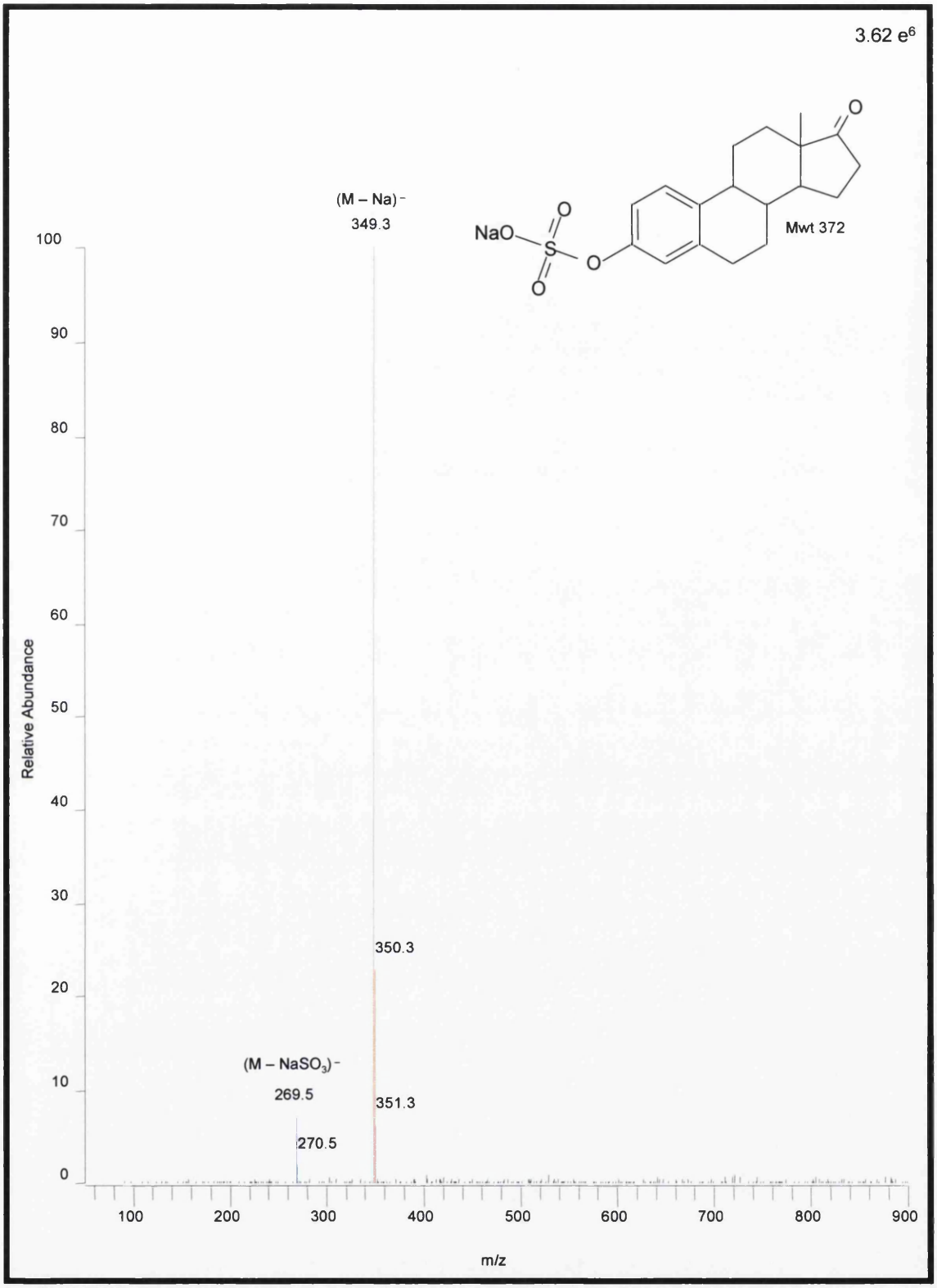



Figure 3.25 Negative LC-ESI-MS mass spectrum of Estrone 3-sulfate. 10µL injection of a 1µg/mL solution; equal to 10ng on column.

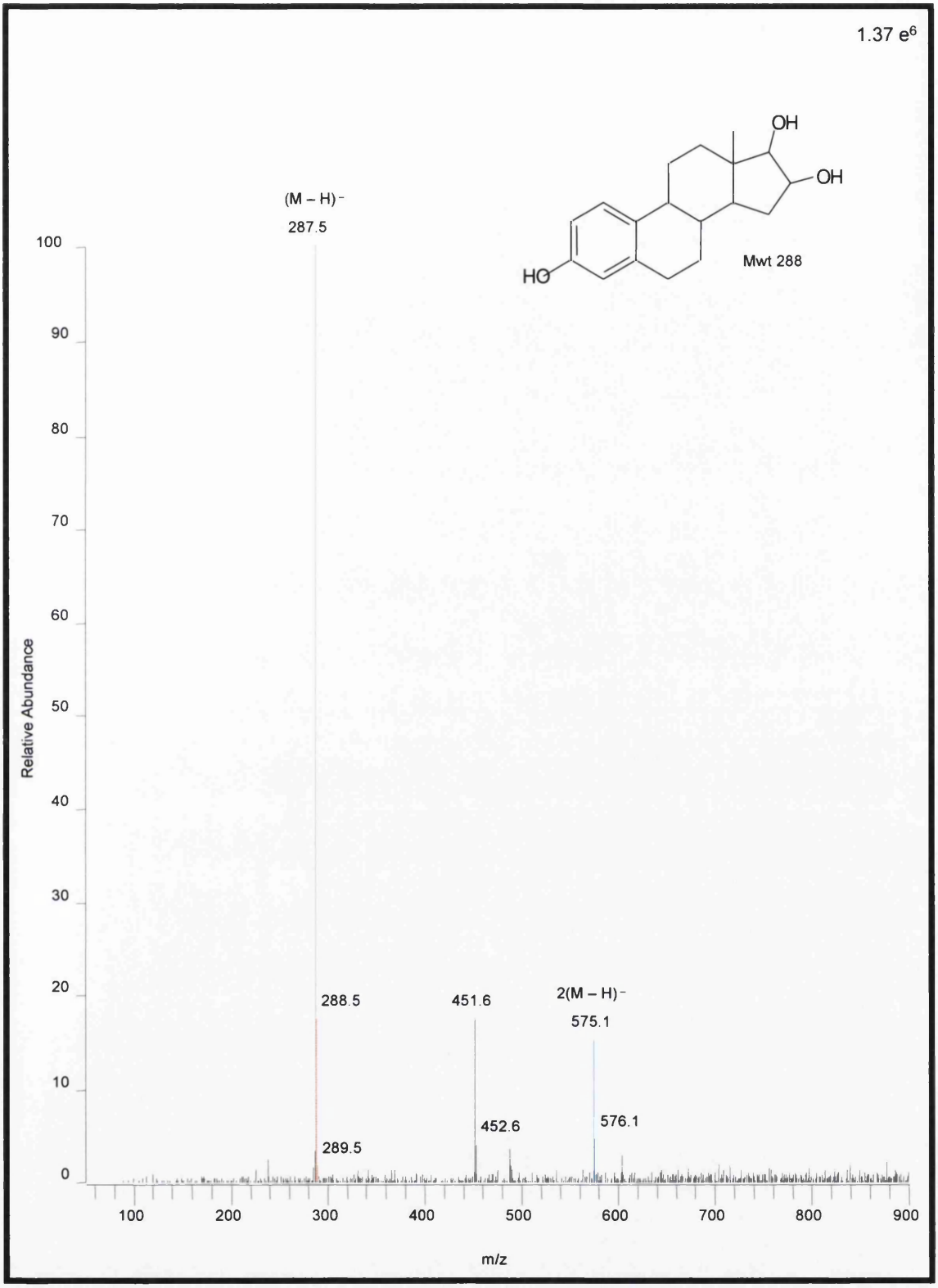



Figure 3.26 Negative LC-ESI-MS mass spectrum of Estriol. 10µL injection of a 1µg/mL solution; equal to 10ng on column.

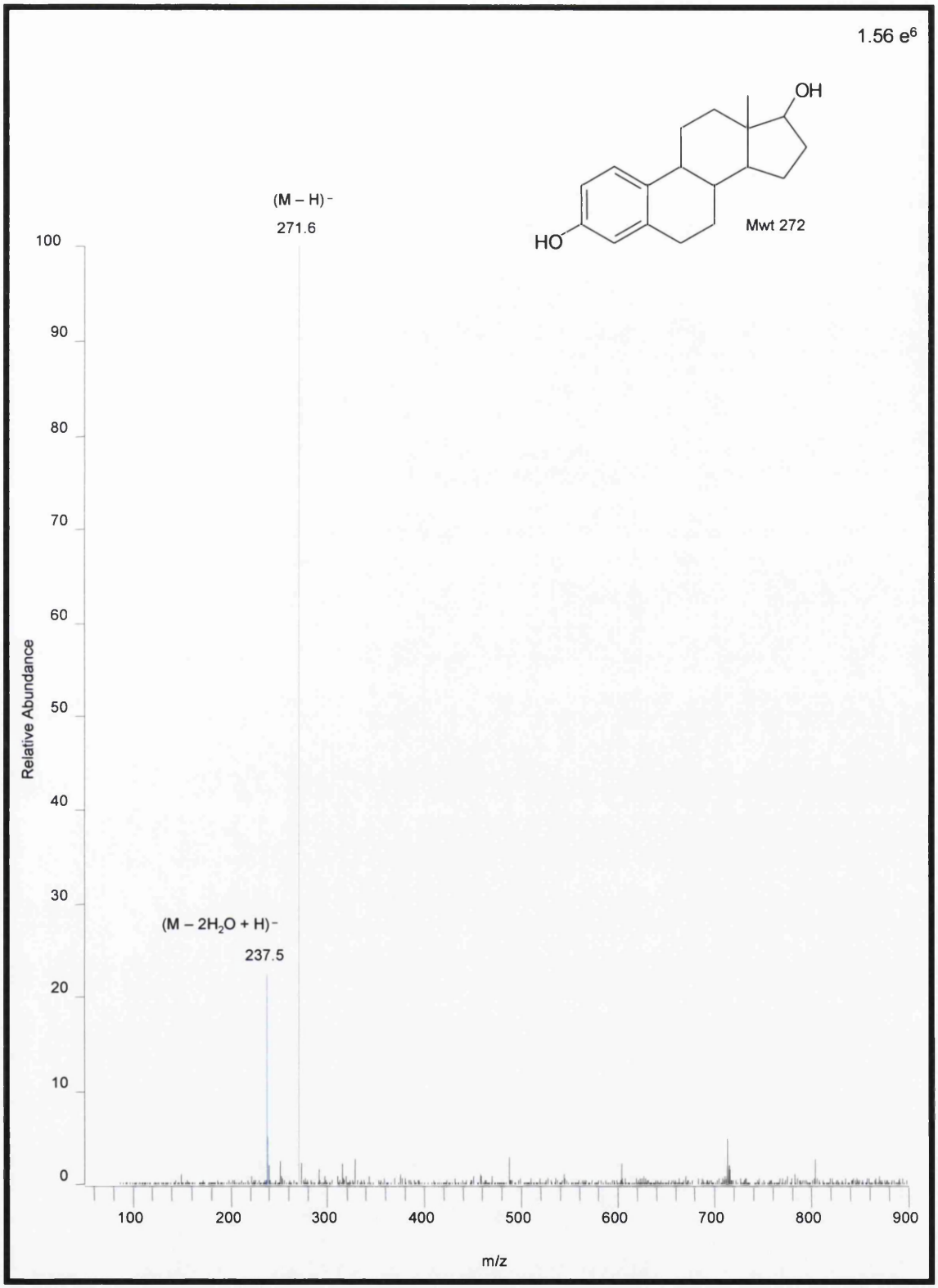



Figure 3.27 Negative LC-ESI-MS mass spectrum of ß-Estradiol. 10µL injection of a 1µg/mL solution; equal to 10ng on column.

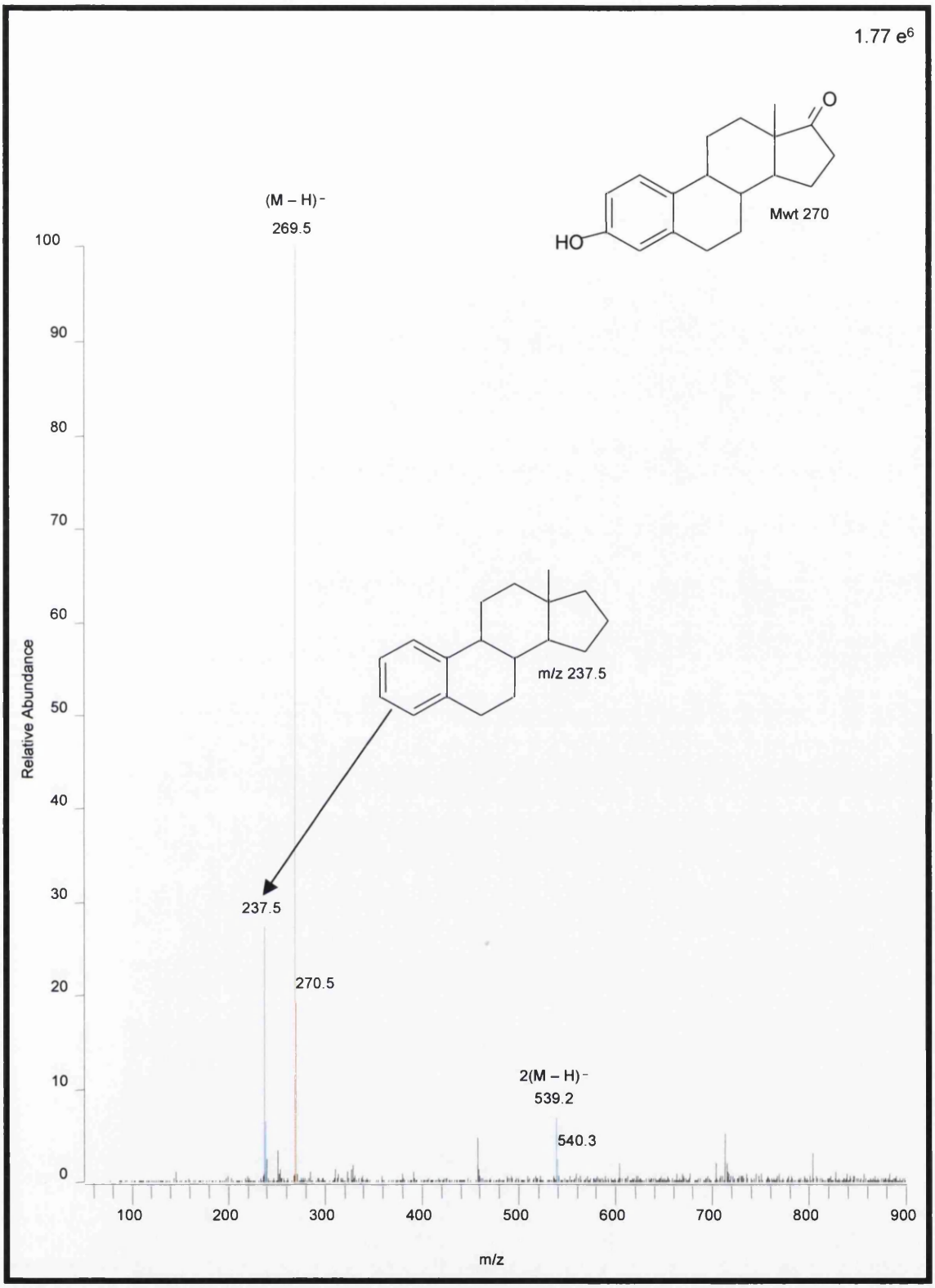



Figure 3.28 Negative LC-ESI-MS mass spectrum of Estrone. 10µL injection of a 1µg/mL solution; equal to 10ng on column.

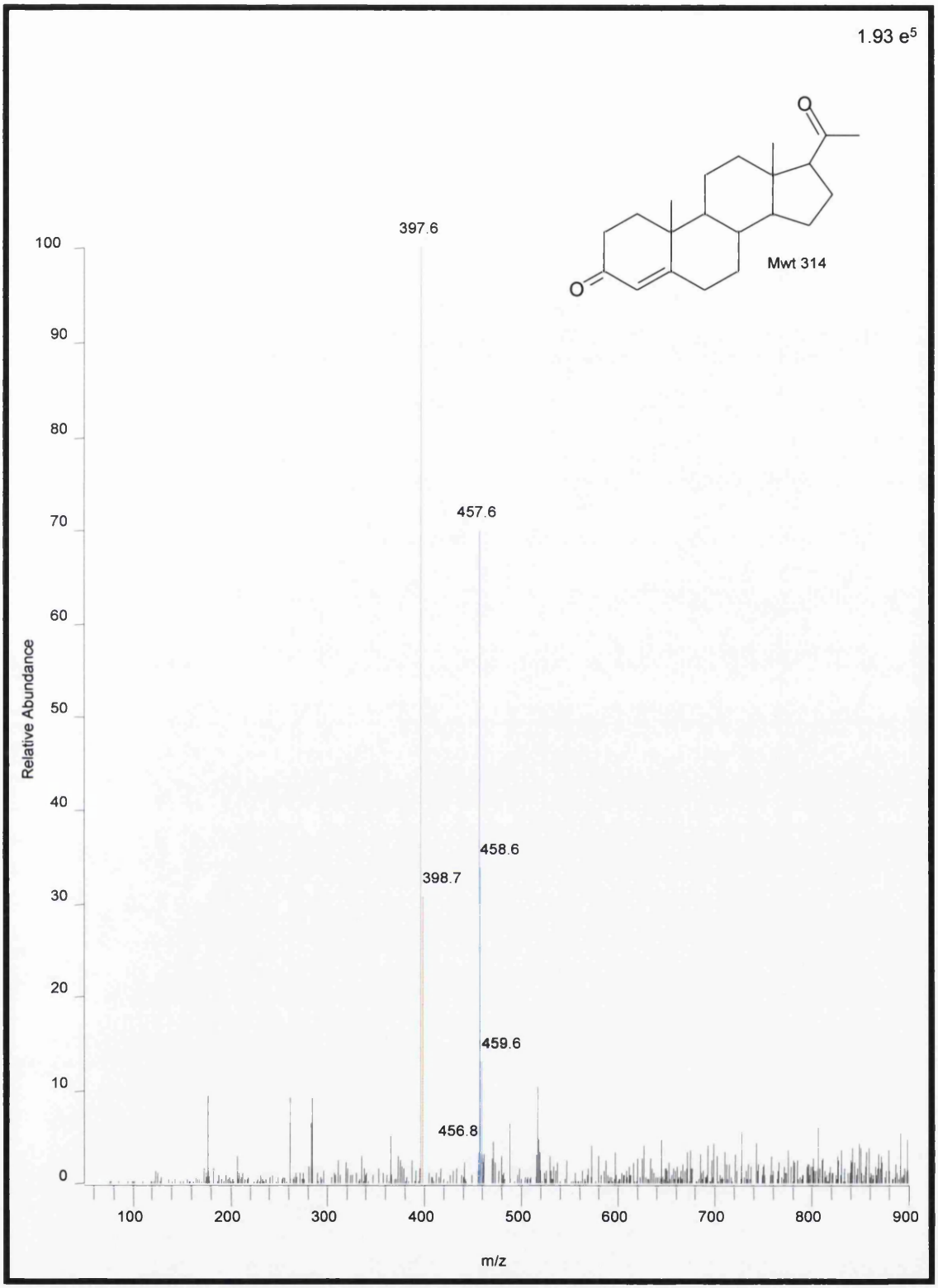



Figure 3.29 Negative LC-ESI-MS mass spectrum of Progesterone. 10μL injection of a 1μg/mL solution; equal to 10ng on column.

## Limits of Detection for the 8 Estrogen Steroids

Detection limits were obtained by taking extracted ion chromatograms of the m/z of the most abundant ion for each steroid from the TIC. Limits of detection (LOD) were calculated from analysis of a set of solutions at the following concentrations; 0.1ng/mL, 1ng/mL, 10ng/mL and 100g/mL, the LOD was regarded as the concentration at which a signal to noise ratio of at least 5 to 1 was achievable. The tables below show the LOD achieved with ESI and APCI; the amount of compound injected on column is also given.

**ESI**

| Compound name | m/z Monitored | LOD ESI | Amount on column |
|---|---|---|---|
| 17β-Estradiol 3-glucuronide 17-sulfate | 263.5 | 1ng/mL | 10pg |
| β Estradiol 3, 17-disulfate | 215.5 | 1ng/mL | 10pg |
| Estrone β-D-glucuronide | 445.5 | 1ng/mL | 10pg |
| Estrone 3-sulfate | 349.5 | 1ng/mL | 10pg |
| Estriol | 287.5 | 1ng/mL | 10pg |
| β-Estradiol | 271.5 | 1ng/mL | 10pg |
| Estrone | 269.5 | 1ng/mL | 10pg |
| Progesterone | 397.5 | 1ng/mL | 10pg |

On column injection volume of 10μL. LOD is calculated as the concentration of the solution being injected.

**APCI**

| Compound name | m/z Monitored | LOD APCI | Amount on column |
|---|---|---|---|
| 17β-Estradiol 3-glucuronide 17-sulfate | 351.5 | 10ng/mL | 100pg |
| β Estradiol 3, 17-disulfate | 215.5 | 10ng/mL | 100pg |
| Estrone β-D-glucuronide | 269.5 | 10ng/mL | 100pg |
| Estrone 3-sulfate | 349.5 | 10ng/mL | 100pg |
| Estriol | 287.5 | 1ng/mL | 10pg |
| β-Estradiol | 271.5 | 1ng/mL | 10pg |
| Estrone | 269.5 | 1ng/mL | 10pg |
| Progesterone | 397.5 | 10ng/mL | 100pg |

On column injection volume of 10μL. LOD is calculated as the concentration of the solution being injected.

As can be seen from the tables ESI was found to be more sensitive than APCI in the case of the majority of the 8 steroids and as such was the chosen form of ionisation for MS-MS analysis

**Instrument Response**

The table below lists the instrument response in ESI-MS mode to a 10μL injection of a solution made up of a mixture of each of the steroids at a concentration of 1μg/mL (the raw data has been presented earlier in this chapter in figures 3.22-3.29). From this data the response per nanogram on column of each steroid has been calculated.

| Compound name | Instrument response | Response per ng on column |
|---|---|---|
| 17β-Estradiol 3-glucuronide 17-sulfate | $6.59\ e^6$ | $6.59\ e^5$ |
| β Estradiol 3, 17-disulfate | $1.68\ e^6$ | $1.68\ e^5$ |
| Estrone β-D-glucuronide | $5.29\ e^5$ | $5.29\ e^4$ |
| Estrone 3-sulfate | $3.62\ e^6$ | $3.62\ e^5$ |
| Estriol | $1.37\ e^6$ | $1.37\ e^5$ |
| β-Estradiol | $1.56\ e^6$ | $1.56\ e^5$ |
| Estrone | $1.77\ e^6$ | $1.77\ e^5$ |
| Progesterone | $1.93\ e^5$ | $1.93\ e^4$ |

The response per nanogram on column could be used to give an indication of the amount of a steroid present in an unknown sample; however without the use of an internal standard its value is limited.

## LC-ESI-MS-MS For the Separation of the Mixture of 4 Estrogen Free Steroids and 4 Conjugated Steroids

Structural information was gained by the fragmentation of each of the most abundant ions obtained from LC-ESI-MS. This gives a specific method of identification for each of the steroids for on-line analysis. The following table shows the precursor and product ions and the relative collision energies required to induce fragmentation.

| Compound name | Precursor ion | CE % | Product ions |
|---|---|---|---|
| 17β-Estradiol 3-glucuronide 17-sulfate | 263.5 | 22.0 | 351.5 |
| β Estradiol 3, 17-disulfate | 215.5 | 22.0 | 80.1, 97.2, 175.3, 333.3, 350.3 |
| Estrone β-D-glucuronide | 445.5 | 24.5 | 174.9, 269.5, 427.3 |
| Estrone 3-sulfate | 349.5 | 24.0 | 269.4 |
| Estriol | 287.5 | 27.5 | 145.2, 159.3, 171.3, 211.2, 269.4, 287.5 |
| β-Estradiol | 271.5 | 27.1 | 145.3, 183.4, 253.4 |
| Estrone | 269.5 | 26.1 | 123.1, 145.3, 183.4, 253.3 |
| Progesterone | 397.5 | 24.1 | 177.3, 207.1, 365.3 |

The following figures (3.30-3.45) show the LC-ESI-MS-MS mass spectrum for each steroid and the proposed fragmentation pattern interpretation.

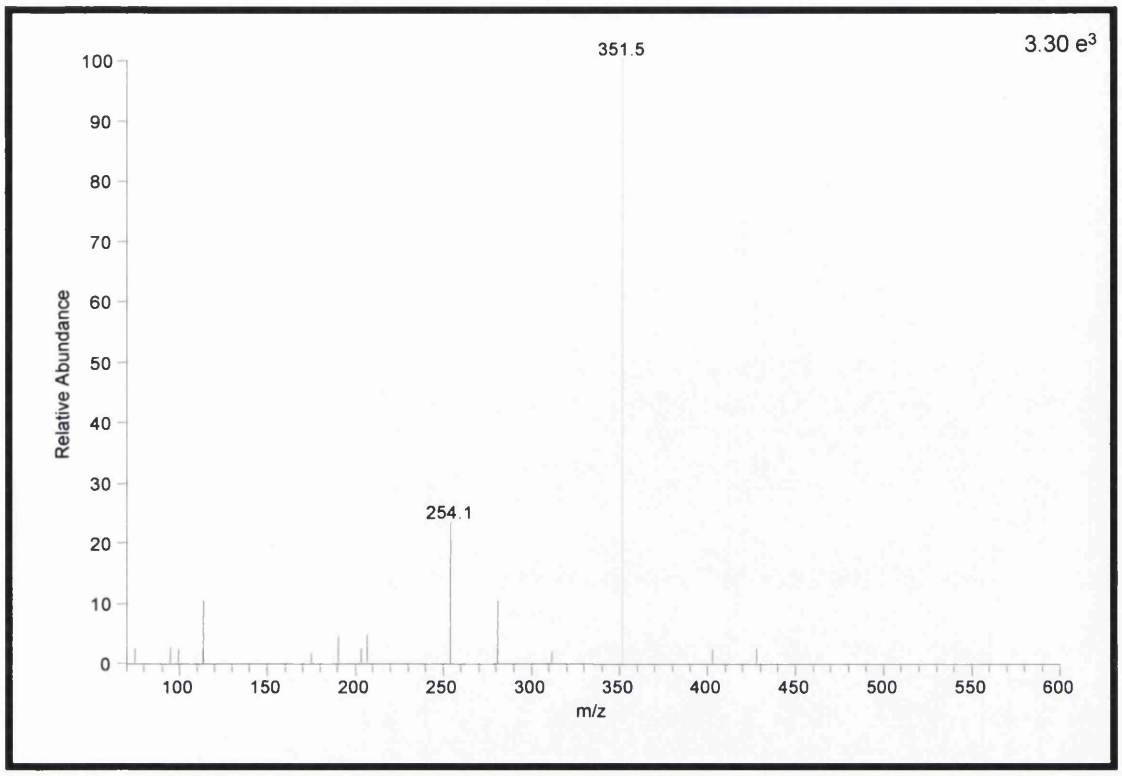



Figure 3.30 Negative LC-ESI-MS-MS spectrum of 17ß-Estradiol 3-glucuronide 17-sulfate. 10µL injection of a 1µg/mL solution; equal to 10ng on column.

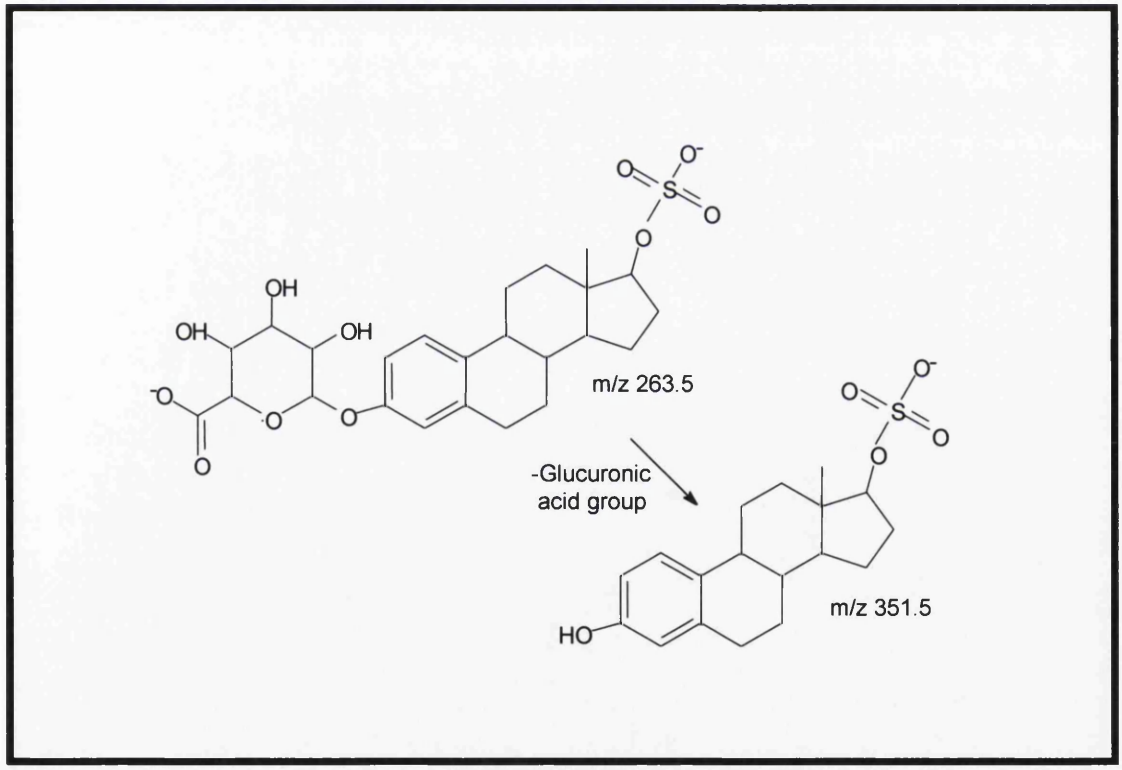



Figure 3.31 Proposed 17ß-Estradiol 3-glucuronide 17-sulfate fragmentation pattern interpretation.

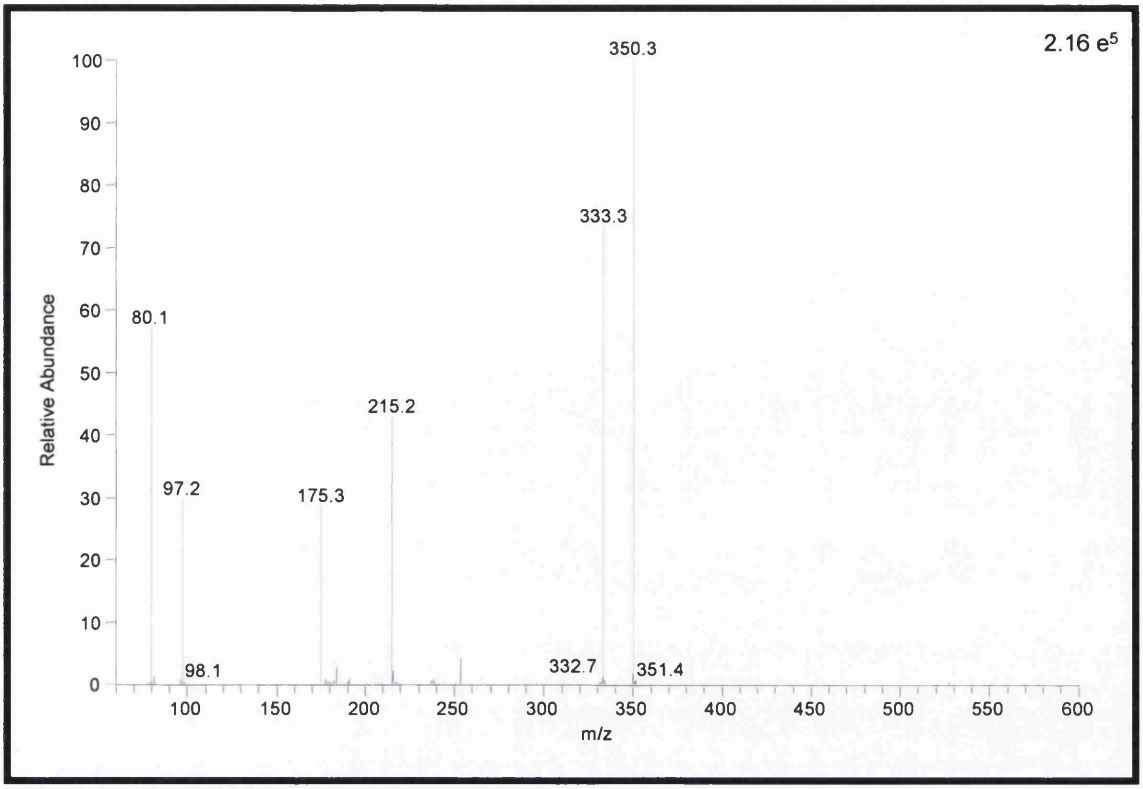



Figure 3.32 Negative LC-ESI-MS-MS spectrum of ß-Estradiol 3, 17-disulfate. 10µL injection of a 1µg/mL solution; equal to 10ng on column.

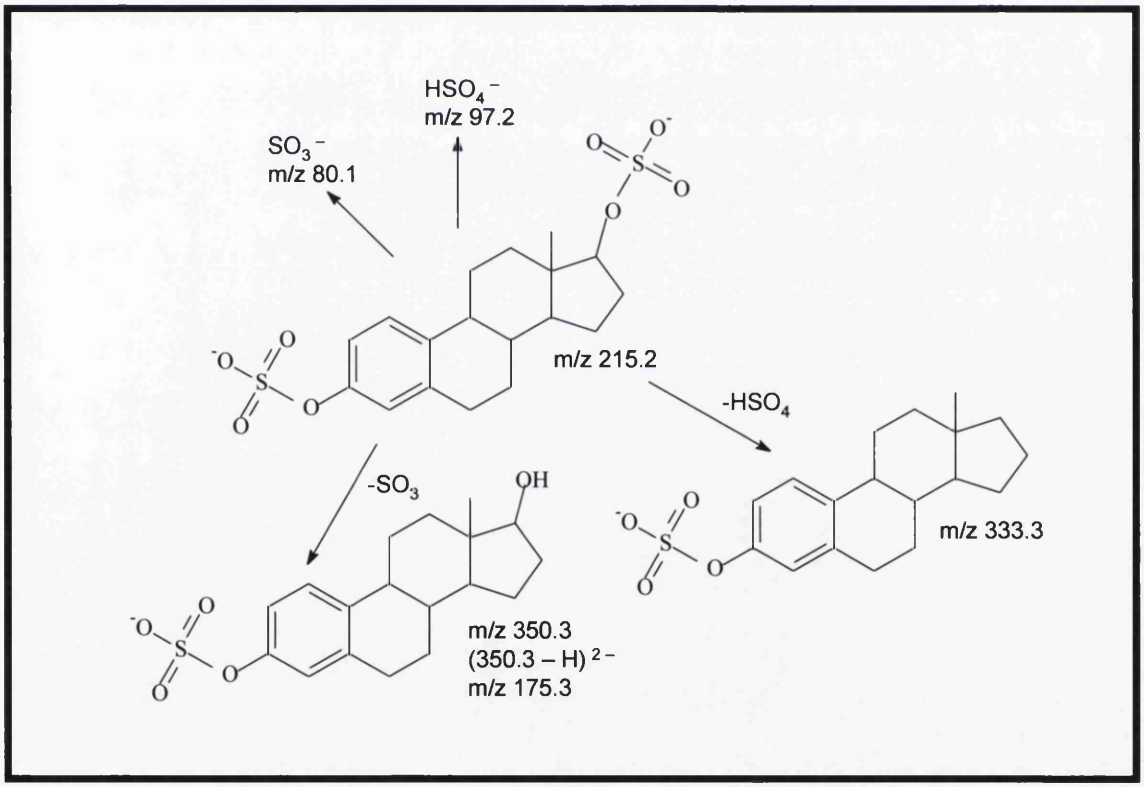



Figure 3.33 Proposed ß-Estradiol 3, 17-disulfate fragmentation pattern interpretation.

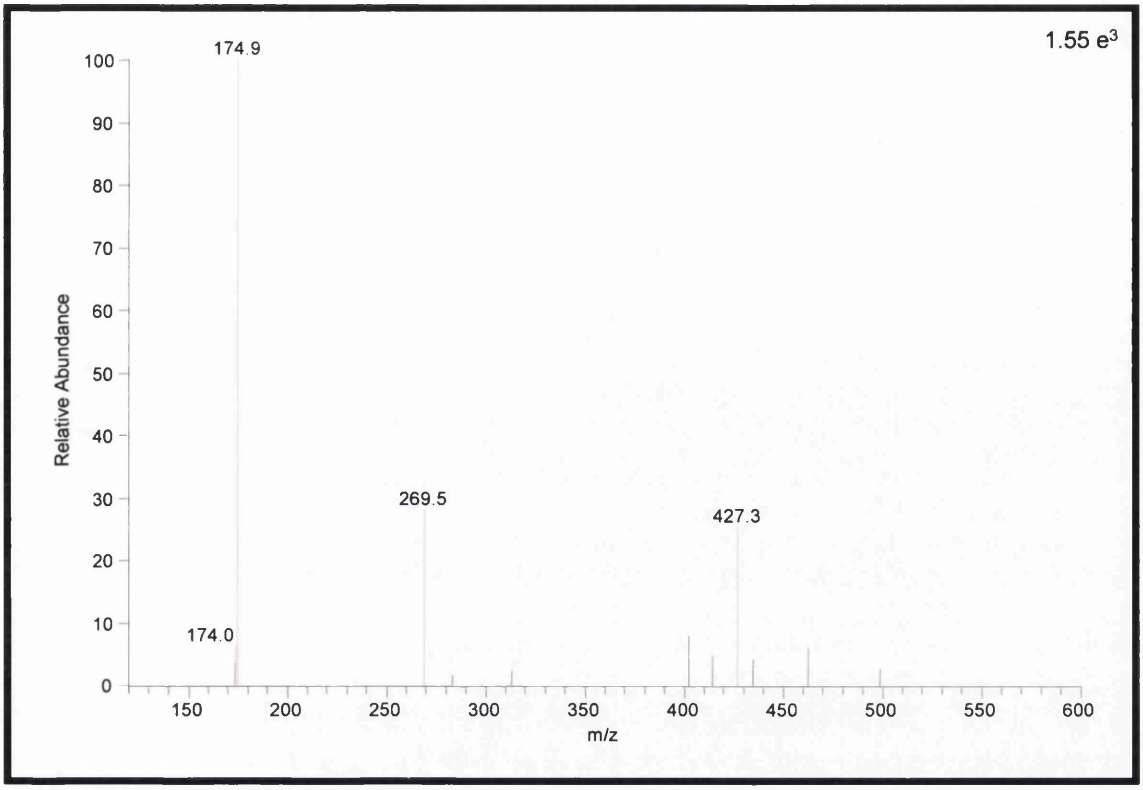



Figure 3.34 Negative LC-ESI-MS-MS spectrum of Estrone ß-D-glucuronide. 10µL injection of a 1µg/mL solution; equal to 10ng on column.

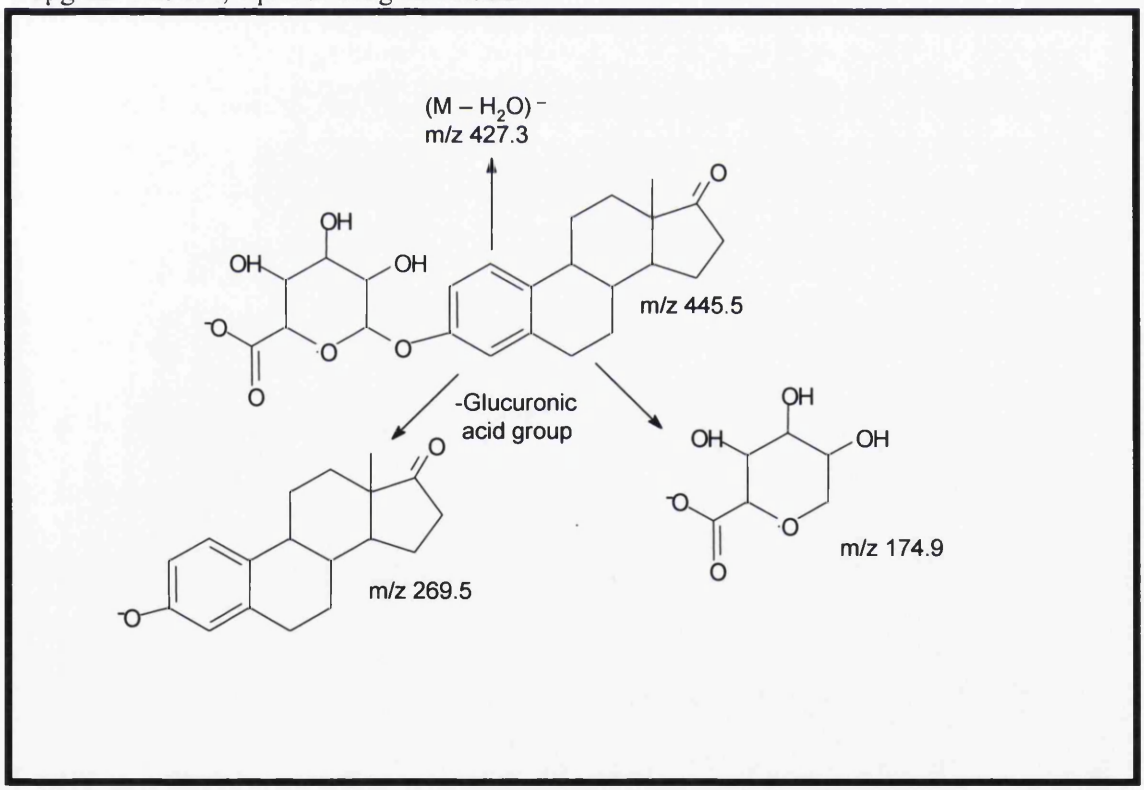



Figure 3.35 Proposed Estrone ß-D-glucuronide fragmentation pattern interpretation.

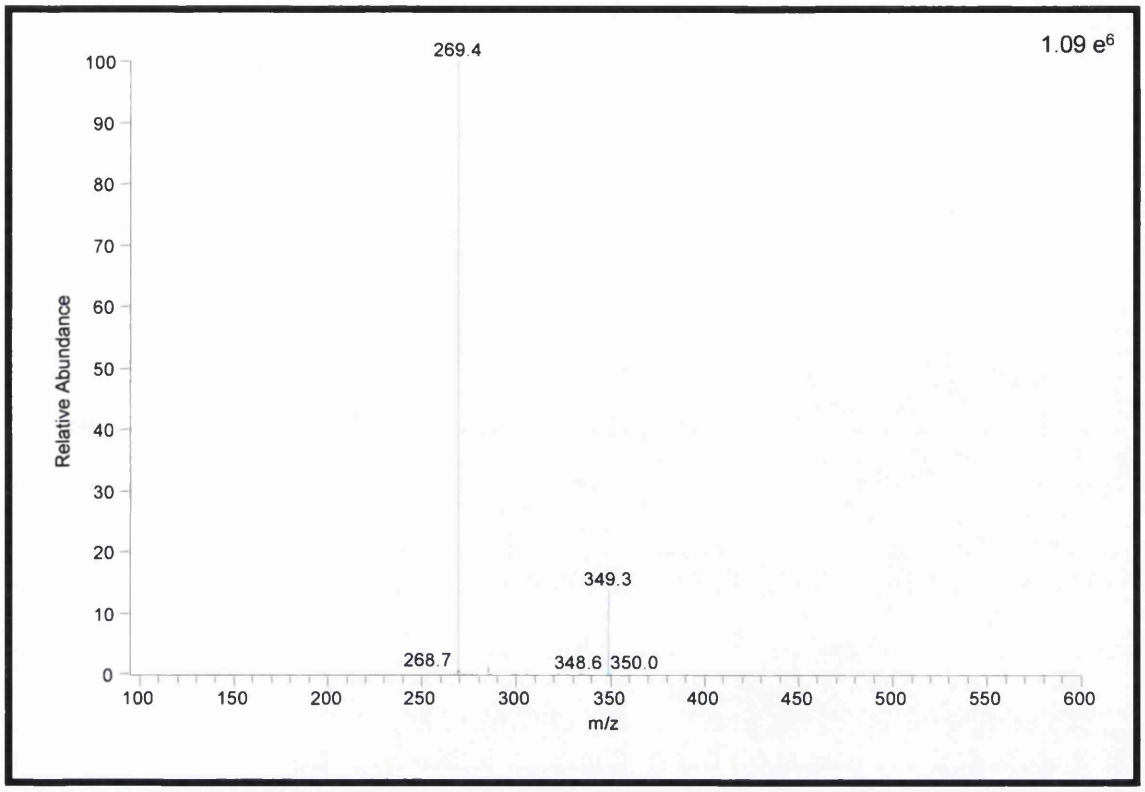



Figure 3.36 Negative LC-ESI-MS-MS spectrum of Estrone 3-sulfate. 10µL injection of a 1µg/mL solution; equal to 10ng on column.

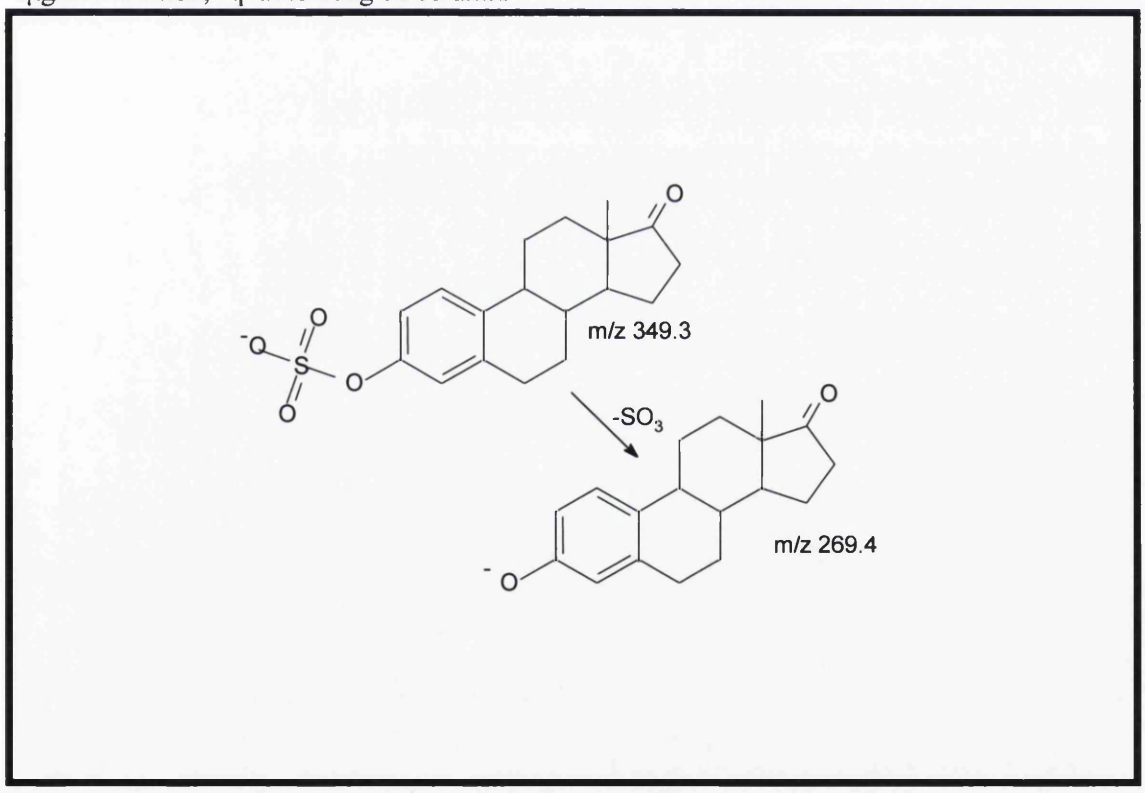



Figure 3.37 Proposed Estrone 3-sulfate fragmentation pattern interpretation.

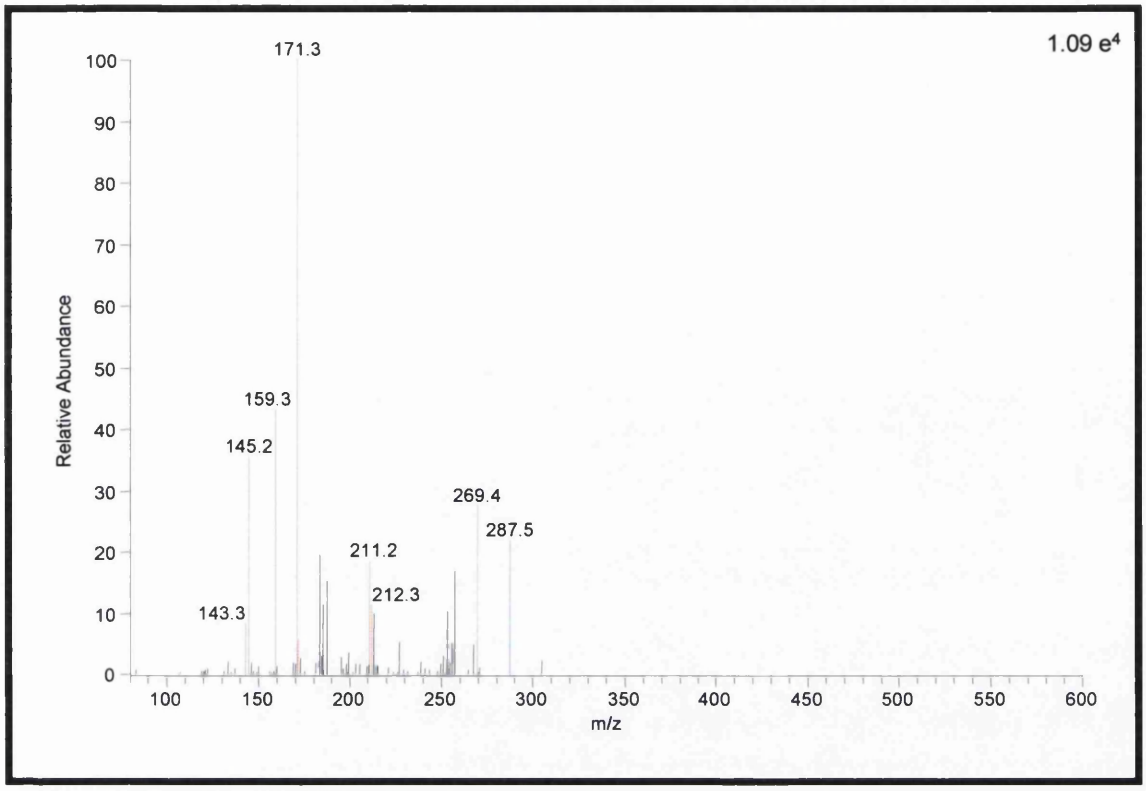



Figure 3.38 Negative LC-ESI-MS-MS spectrum of Estriol. 10µL injection of a 1µg/mL solution; equal to 10ng on column.

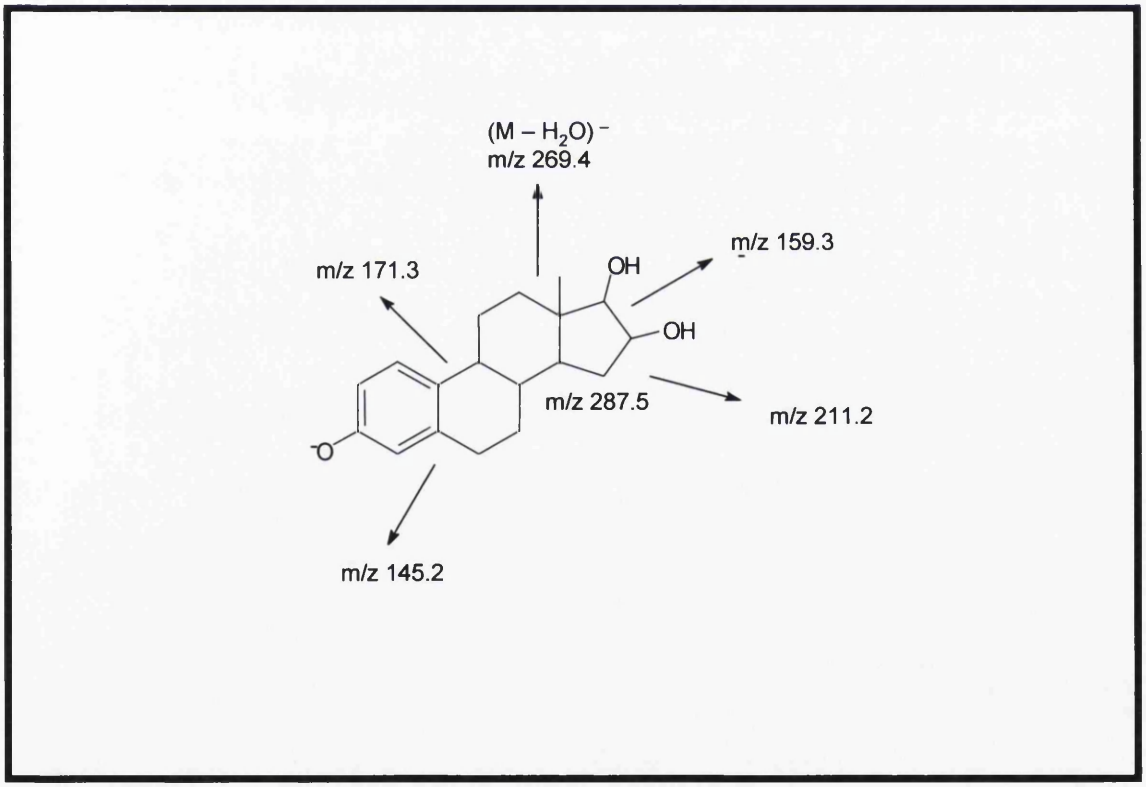



Figure 3.39 Proposed Estriol fragmentation pattern interpretation.

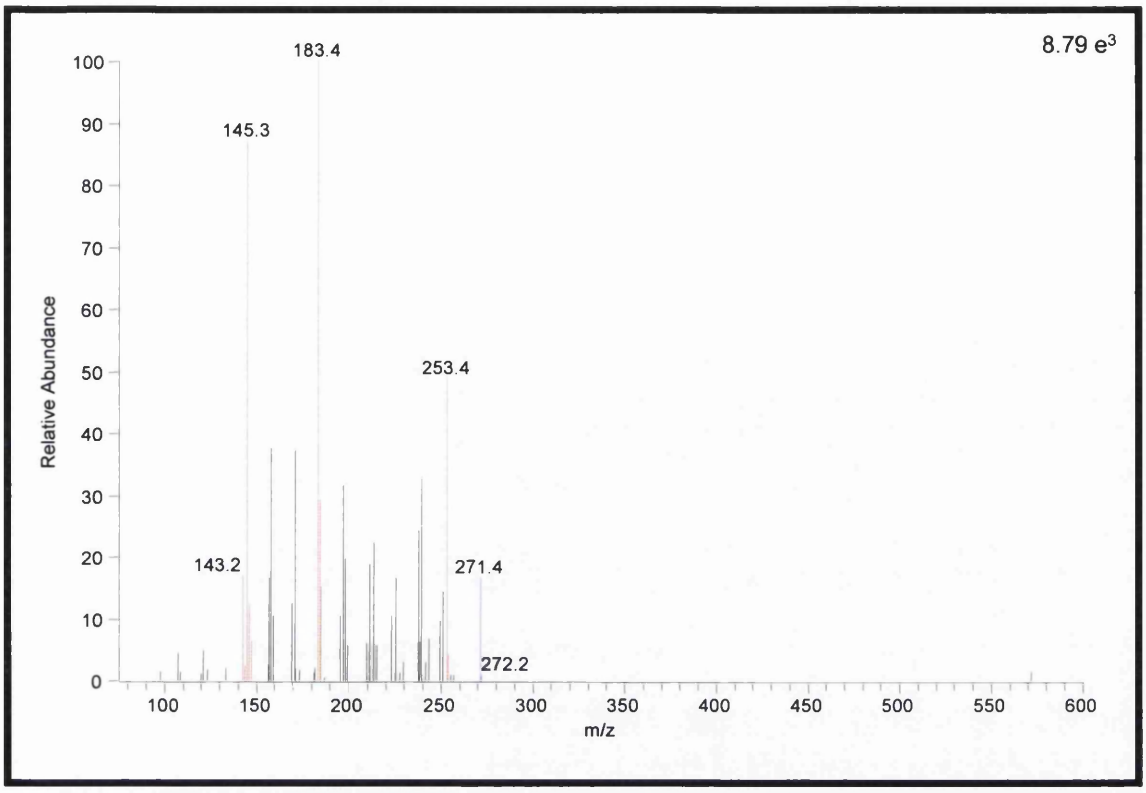



Figure 3.40 Negative LC-ESI-MS-MS spectrum of ß-Estradiol. 10µL injection of a 1µg/mL solution; equal to 10ng on column.

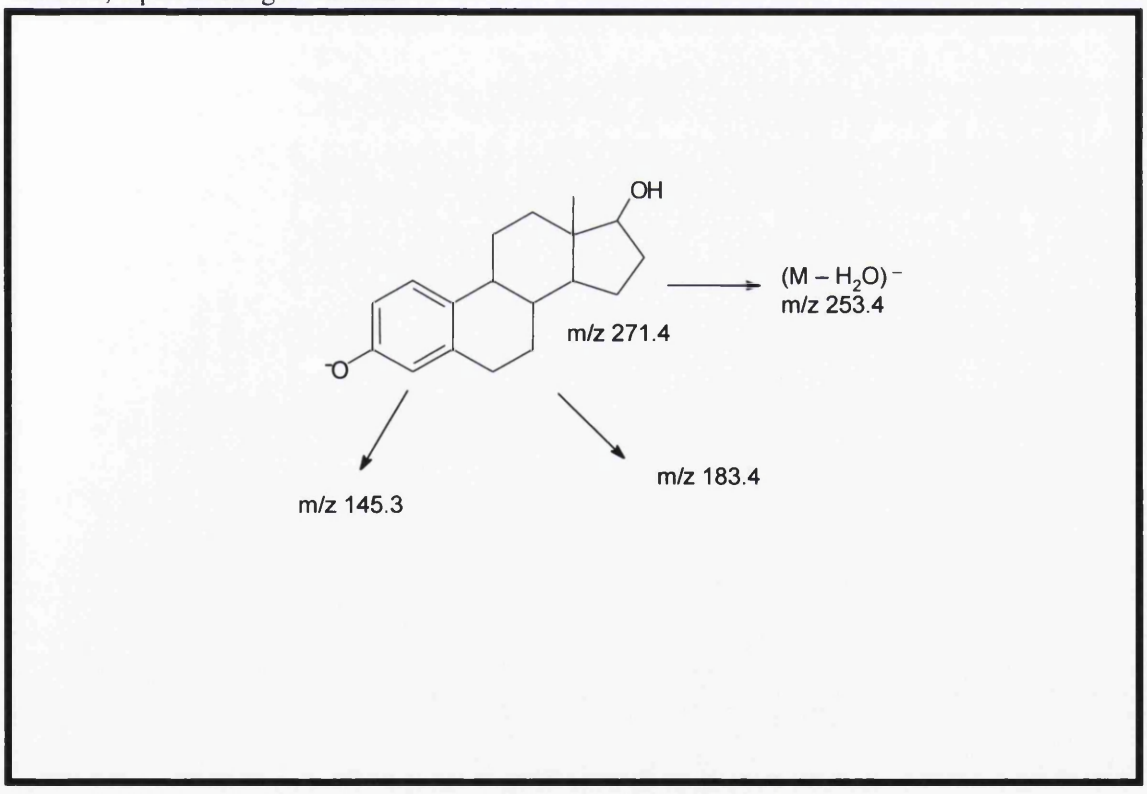



Figure 3.41 Proposed ß-Estradiol fragmentation pattern interpretation.

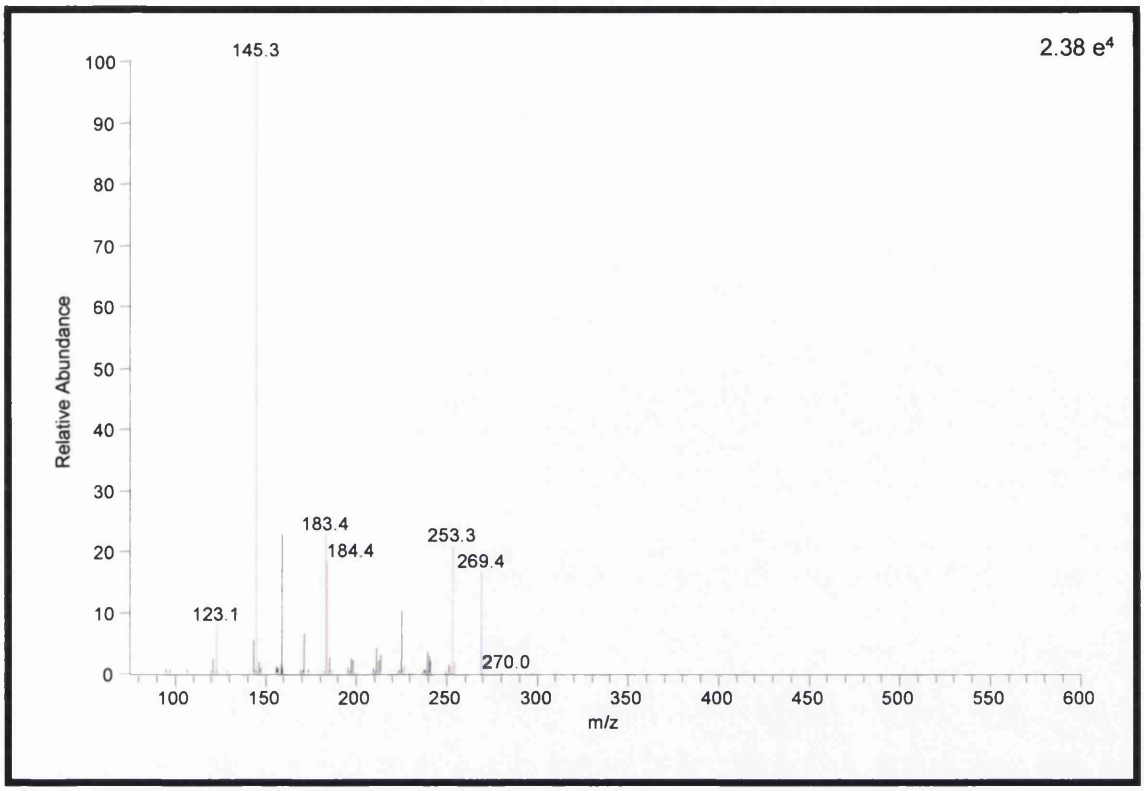



Figure 3.42 Negative LC-ESI-MS-MS spectrum of Estrone. 10µL injection of a 1µg/mL solution; equal to 10ng on column.

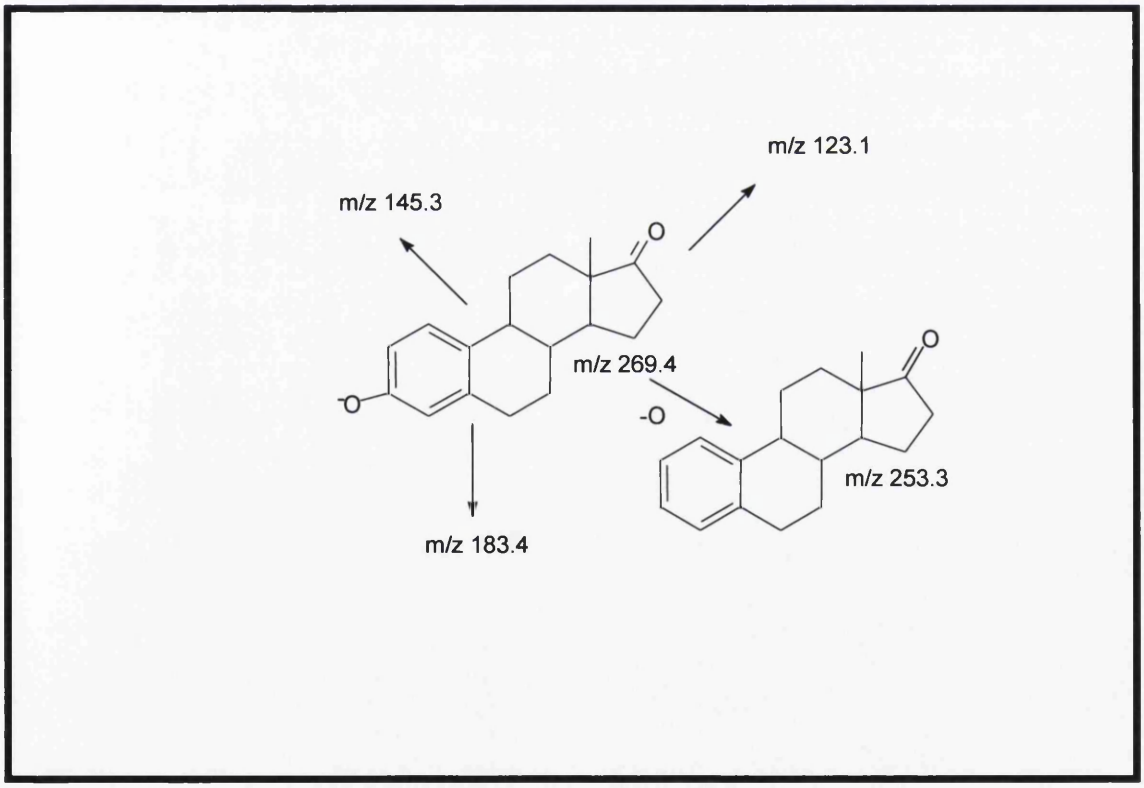



Figure 3.43 Proposed Estrone fragmentation pattern interpretation.

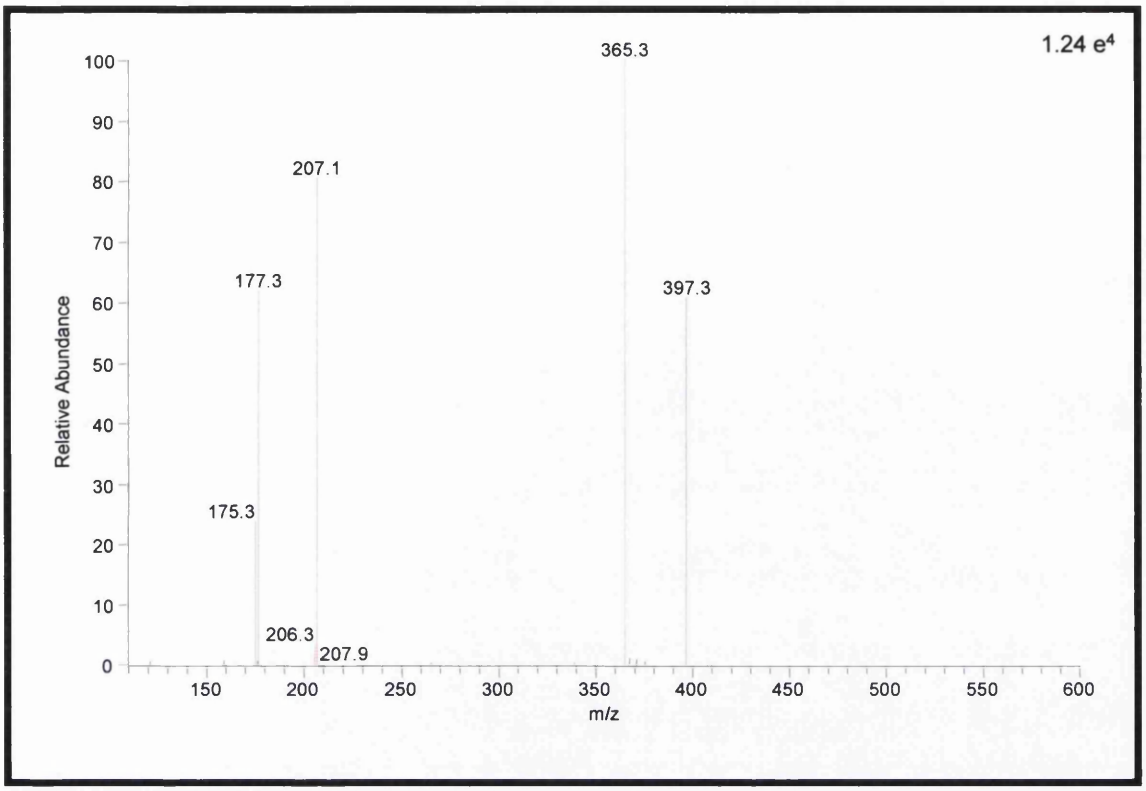



Figure 3.44 Negative LC-ESI-MS-MS spectrum of Progesterone. 10µL injection of a 1µg/mL solution; equal to 10ng on column.

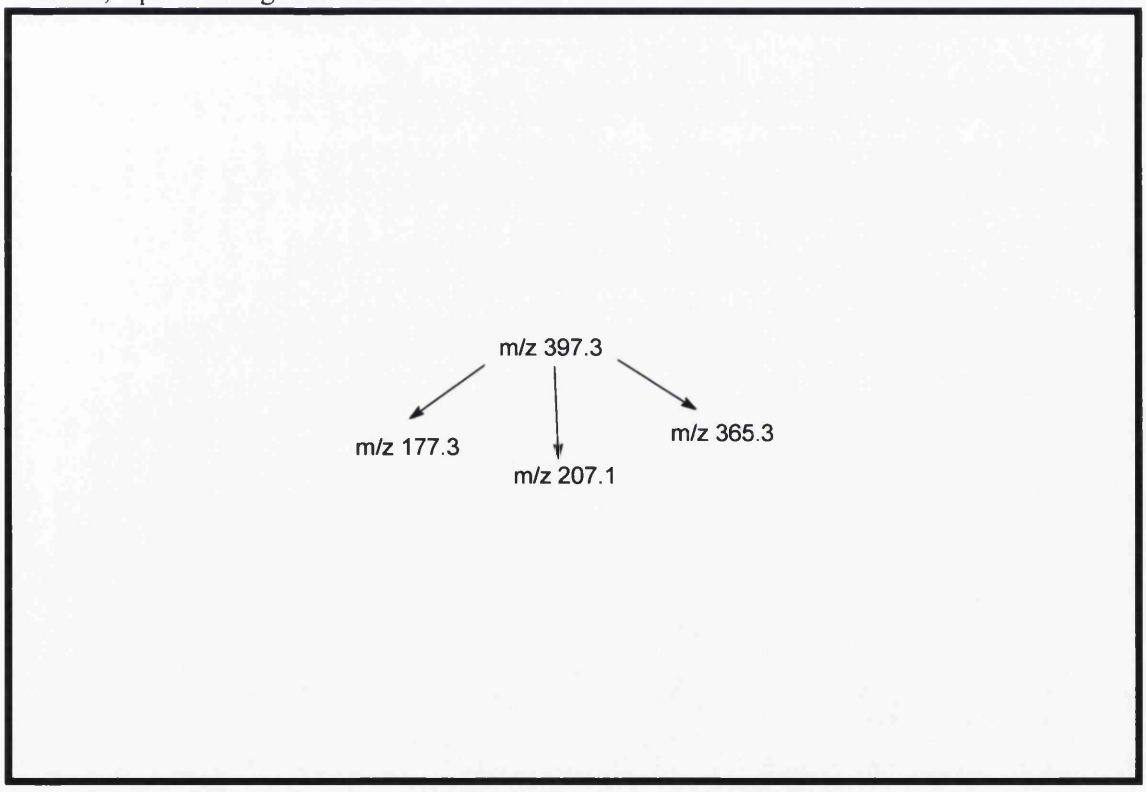



Figure 3.45 Proposed Progesterone fragmentation pattern interpretation.

# Solid Phase Extraction of the Estrogen Steroids

## Experimental

### Chemicals

HPLC grade solvents were purchased from Fisher Scientific (Loughborough, U.K.), gases from BOC Limited (Surrey, U.K.) and solid phase extraction (SPE) columns from Jones Chromatography Limited (Mid Glamorgan, U.K.).

### Instrumentation

SPE was performed on an International Sorbent Technology VacMaster extraction system using Isolute C8 1g/6mL SPE columns.

### Results

It was attempted to develop an extraction method for the 8 free and conjugated estrogen steroids to allow for the LC-ESI-MS analysis of river water samples. We were only able to develop a method that allowed for the analysis of 6 of the 8 steroids; various attempts were made using different phases and mixed mode columns but we were unable to achieve better than 6 out of 8 steroids. Below is the extraction method used for the analysis of the 6 steroids.

**Extraction Procedure:**

| Step | Procedure |
|---|---|
| Sample Pre-treatment | 5% by volume methanol added to sample. |
| Column Solvation | Column conditioned with 6mL methanol followed by 6mL water at a flow rate of 5mL/min. |
| Sample Application | Sample applied to column at a flow rate of 10mL/min. |
| Interference Elution | Interferences eluted with 6mL of water at a flow rate of 5mL/min. |
| Column Drying | Dry column under full vacuum for 30 seconds. |
| Analyte Elution | Steroids eluted with 3mL of methanol at a flow rate of 5mL/min. |
| Reconstitution | Dry down under nitrogen and reconstitute in 50:50 (v/v) methanol/water to a volume of 1mL. |

The following table shows the m/z values used for the extracted ion chromatograms and the LOD achieved for each of the steroids in spiked river water. The volume of river water extracted for analysis was 2 litres; to ensure that there would be no interference from steroids already present in the water used for determining the LOD it was collected from the source of the river Lougher in South Wales.

| Compound name | m/z | LOD |
|---|---|---|
| Estrone β-D-glucuronide | 445.5 | 1ng/L |
| Estrone 3-sulfate | 349.5 | 0.1ng/L |
| Estriol | 287.5 | 0.1ng/L |
| β-Estradiol | 271.5 | 0.1ng/L |
| Estrone | 269.5 | 0.1ng/L |
| Progesterone | 397.5 | 1ng/L |

On column injection volume of 50μL. LOD is calculated as the concentration of the steroid spiked into the blank river water.

Figure 3.46 below is an example of a extracted ion chromatogram of an extraction of 2 litres of river water spiked at 1ng/L for each of the 6 steroids.

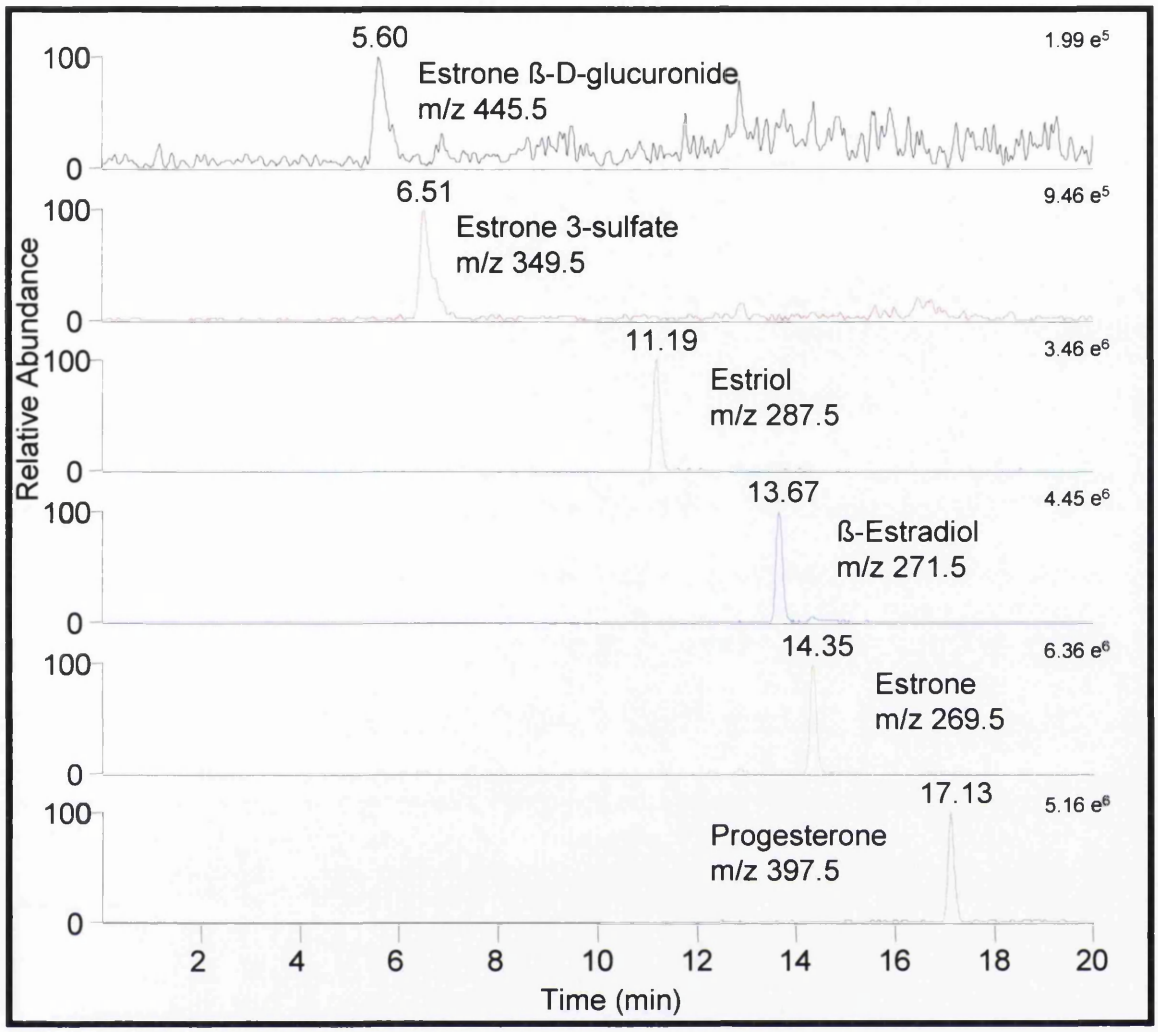



Figure 3.46 Negative LC-ESI-MS extracted ion chromatograms of the 6 estrogen steroids. SPE of river water spiked with 1ng/L of each steroid

## Analysis of River Water Samples

River Water samples were collected from various points along the rivers Lougher and Cennen in South Wales; at each site 4 litres of river water was collected. 2 litres of river water was extracted using the technique described previously from each site and in the case of positive results the second 2 litres were then extracted and also analysed as confirmation of the positive result. Estrogen steroids were only detected in samples

from two sites; one from each river and both samples having been collected from sites in close proximity to dairy farms. Figures 3.47 to 3.50 show extracted ion chromatograms and example mass spectra from the positive river water extraction results, estrone 3-sulfate from the river Cennen and estrone from the river Lougher.

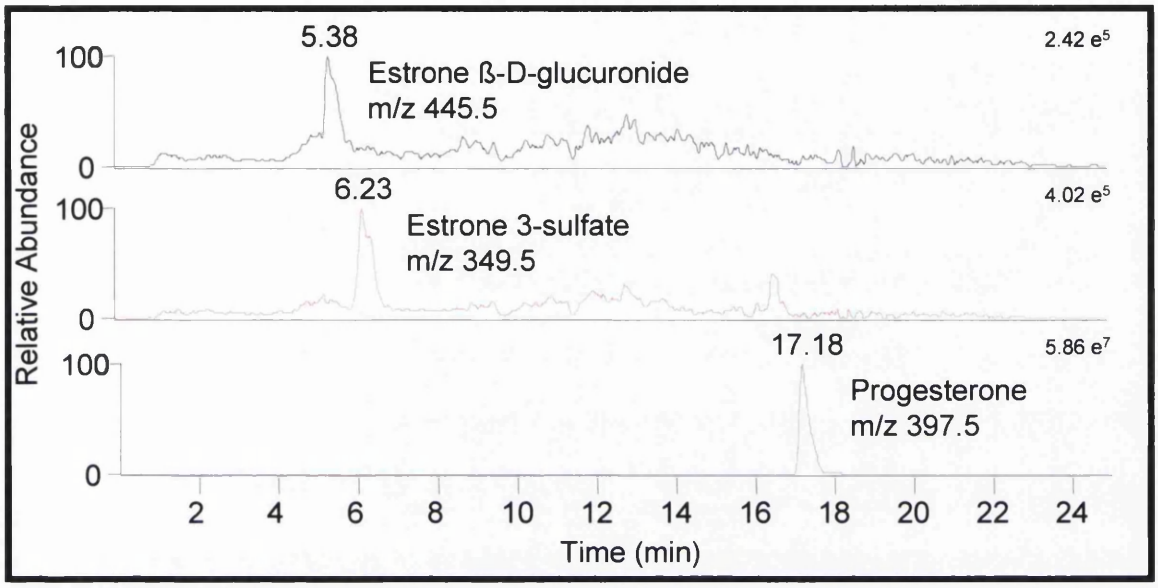



Figure 3.47 Extracted ion chromatogram of the estrogen steroid positive results from the river Cennen Site.

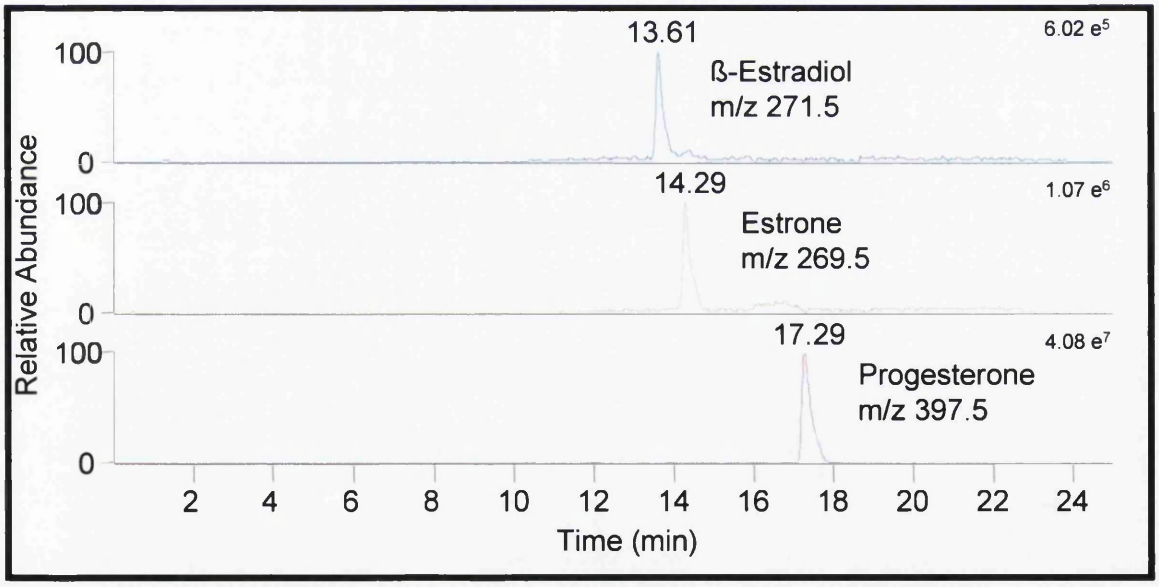



Figure 3.48 Extracted ion chromatogram of the estrogen steroid positive results from the river Lougher Site.

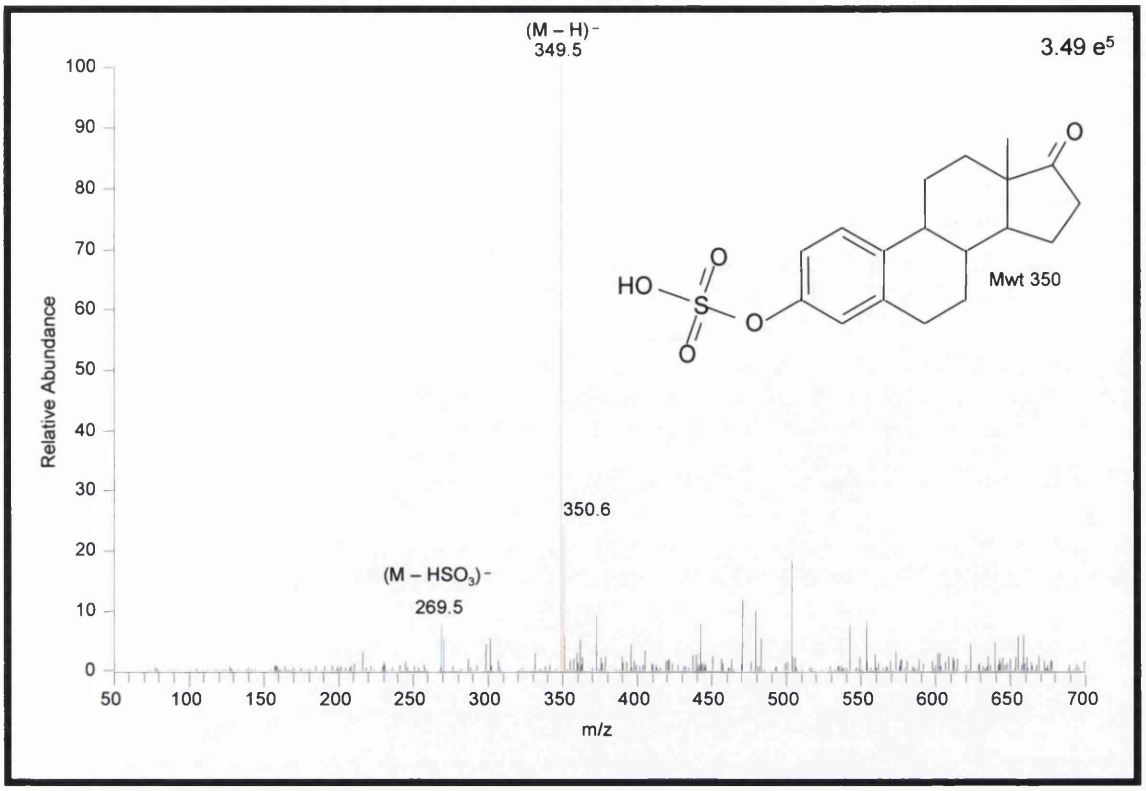



Figure 3.49 Negative LC-ESI-MS mass spectrum of Estrone 3-sulfate from extraction of river Cennen sample.

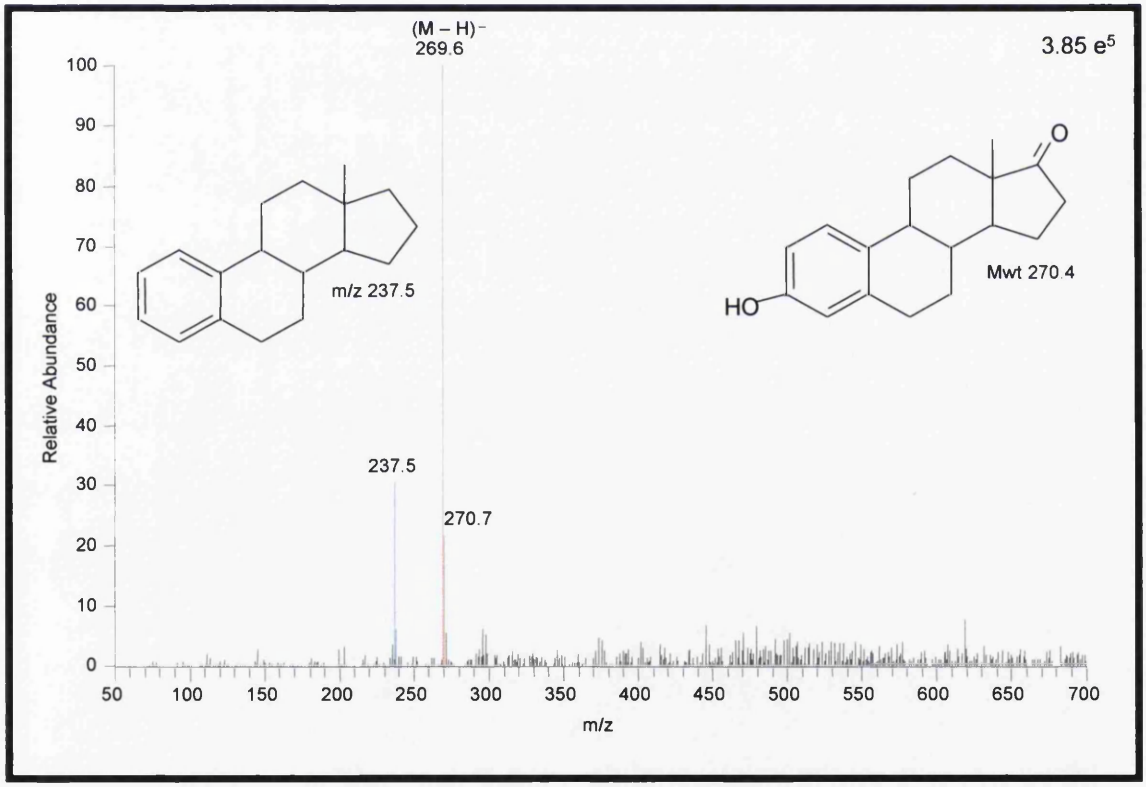



Figure 3.50 Negative LC-ESI-MS mass spectrum of Estrone from extraction of river Lougher sample.

**Discussion**

The main goal of this study was to develop a reliable and sensitive determination method for free and conjugated estrogen steroids in river water using LC-MS analysis.

GC-MS has been used successfully for the determination of estrogen free steroids (4); to apply this form of analysis to the conjugated steroids requires that they first be converted to free steroids. The conversion of the conjugated steroids to free steroids can be achieved by hydrolysing them either enzymatically or by solvolysis. Using solvolytic methods WRc had some success with the sulfate conjugates but found that the glucuronide conjugates were more difficult to convert and that the use of more forcing solvolytic conditions resulted in the decomposition of the liberated steroids. The enzymatic methods for converting glucuronide conjugates to their free steroid counterparts are not quantitative and may result in the rearrangement of some steroids (4).

The use of LC-MS analysis allows for the direct analysis of the steroid conjugates without the need for time consuming derivatization methods or conversion to free steroids. LC-MS should also be amenable to quantitative analysis via the use of an internal standard (6).

The river water samples spiked with the 4 steroid conjugates and extracted in the labs of WRc were difficult to analyse due to the nature of the final extracts. We found that even after dilution and filtration in our own labs the blown down and reconstituted samples were still very "dirty" to the extent that it was not possible to run an analysis

of more than one extract without suffering a substantial loss in sensitivity thus requiring that we clean the ion source after each analysis. This obviously made the analysis process time consuming as it was necessary to then also perform a test run to ensure that the system was functioning correctly and a blank run to guard against carry over from the test run between each sample analysis.

When we developed the methodology of the chromatographic separation of the mixture of the free and conjugated steroids we initially started our experiments using the C18 column and mobile phase conditions with which we had performed the analysis of the conjugated steroids alone. However with this approach we found that the conditions required to retain and resolve the more polar conjugates resulted in the free steroids being retained for too long and having a poor peak shape. Attempts to resolve these problems by altering the gradient set up resulted in co-elution of the free steroids. We decided to switch to the phenyl-hexyl column as the nature of this packing makes it amenable towards relatively fast separations of polar aromatic compounds (see chapter 2). With the phenyl-hexyl column we conducted experiments using various mobile phase combinations, including the mobile phase originally used with the C18 column, and found that a simple unbuffered methanol/water system gave the best results.

The LC-MS analysis performed on the mixture of the 8 steroids proved to be more sensitive in the negative ESI mode than in the negative APCI mode, this is probably due to ESI being a "softer" ionisation technique than APCI thus causing less degradation of the molecule to take place during the ionisation process. This can be seen in the mass spectra of the glucuronide conjugates, where the base ions obtained

with ESI were due to $[M-H]^-$ whereas with APCI they resulted from loss of the glucuronic acid group, and the sulfate conjugates, where although the base ions obtained for both ESI and APCI were due to $[M-H]^-$ the relative intensity of the m/z signal due to the loss of the sulfate group was greater with APCI.

In our studies we found that negative ionisation was substantially more sensitive than positive, in some cases there was no signal with positive ionisation. The pH of the mobile phase has a strong influence on the ESI detection sensitivities of steroids; at neutral pH, as used in our chromatography of the 8 free and conjugated steroids, negative ionisation gives a strong m/z signal corresponding to $[M-H]^-$ for steroids as shown by the work of Volmer and Hui (7). The anionic nature of the steroid conjugates also favours the use of negative ionisation over positive (6, 7).

Our initial SPE method was developed with advice from Jones Chromatography Limited (Mid Glamorgan, UK). Using a C8 extraction sorbent we were able to create a relatively simple extraction procedure to allow for the successful extraction of 6 out of the 8 steroids of interest. In our SPE method the sample is pre-treated with 5% by volume methanol; this is because at the volumes of aqueous sample that we were extracting it is possible for the sorbent bed to become de-solvated making it less likely to retain the isolates of interest and the results to be less reproducible. Attempts were made at developing an extraction procedure that would allow for the extraction of all 8 steroids. Experiments were conducted with cation exchange phases and mixed mode columns run with neutral and acidic conditions but we were unsuccessful in developing a method to allow for the extraction of 17β-Estradiol 3-glucuronide 17-sulfate and β Estradiol 3, 17-disulfate from water samples. We believe that this may

be due to the anionic nature of steroid conjugates and that the use of an anion exchange phase may be successful in isolating them from aqueous matrices.

## Conclusion

GC-MS analysis is currently the most popular technique in use for the analysis of estrogen steroids in environmental matrices and offers picogram per litre levels of sensitivity for free steroids (8, 9,) with analysis run times of between 20 and 30 minutes. The main disadvantage of GC-MS when compared to LC-MS is the requirement for steroids to undergo derivitization prior to analysis with gas chromatography. Derivitization processes are not only costly and time consuming but also make it difficult to measure the levels of conjugated steroids present as the conjugates must first be converted to their free steroid counterparts by the use of some form of hydrolysis. The processes used for the hydrolysis of the glucuronide conjugates in particular have been found to be unreliable and are not considered to be amenable to quantification (4, 10).

LC-MS is ideally suited to the analysis of low volatility polar conjugated steroids without the need for either a hydrolysis or a derivitization step and as our experiments have shown can be used for the simultaneous analysis of free and conjugated steroids present in environmental water samples. The levels of sensitivity we achieved with LC-MS while not as low as those which can be achieved with GC-MS could be improved upon by the use of lower reconstitution volumes; solid phase micro extraction could also provide a means of lowering detection limits with LC-MS (7). The ability of LC-MS to allow for the direct analysis of both free and conjugated

steroids should make it an attractive alternative option to GC-MS in the analysis of environmental and effluent water samples and it is already viewed as the most promising technique for the analysis of these compounds in biological matrices due to its sensitivity, specificity and versatility (10).

Immunoassay techniques are another means of monitoring for the presence of estrogen steroids in the environment having long been established as a technique used for the analysis of estrogens in biological matrices.

The advantage of immunoassay techniques is that they are cost effective and have the ability to screen for all members of a class of steroids in a very short period of time (30 seconds per analysis has been quoted in the literature for estrogen steroids (11)) they also achieve limits of detection similar to those that we have found with LC-MS. However a secondary analysis is required to allow for the definitive identification of estrogen steroids which is not possible with immunoassay alone and as such immunoassay can be used as a first stage screening process in conjunction with LC-MS and can be used to cut down on the amount of LC-MS analyses required to be performed by quickly eliminating those samples which give only negative responses with the immunoassay (12).

LC-ESI-MS was found on the whole to be more sensitive than LC-APCI-MS for the analysis of the estrogen steroids investigated. The levels of detection achieved with LC-ESI-MS were sufficient to allow for analysis of the estrogen steroids to within the requirements of WRc. MS-MS data was acquired which allows for more definitive

identification of the steroids although we were unable to interpret the MS or MS-MS data acquired for progesterone in the negative ionisation mode.

The solid phase extraction method we developed allowed for the sensitive analysis of river water samples containing sub nanogram per litre levels of estrogen steroids thus proving the value of the method; although ideally we would have liked to have been able to analyse for the presence of all eight of the steroids of interest.

The results of this chapter show that LC-MS can be used as a reliable and sensitive technique for the low level analysis of estrogen free and conjugated steroids.

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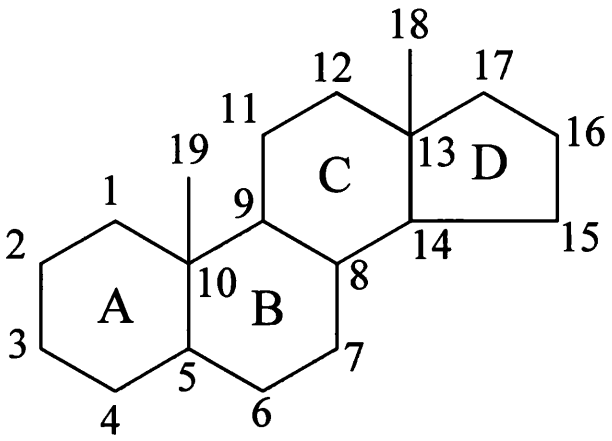
# Chapter 4

# LC-MS Analysis of Steroid Conjugates in Colt Urine

# Chapter 4 LC-MS Analysis of Steroid Conjugates in Colt Urine

## Introduction to Steroids in Equine Urine




The figure shown above gives the basic structure and nomenclature of steroids. The steroids of interest in this study are the C19 steroids which are known as the androgens, and form the male sex hormones and the C18 steroids which are the estrogens and form the female sex hormones. The estrogens have the same basic structure as the androgens but with the absence of the C19 methyl group.

Some typical examples of steroids naturally occurring in horses are shown in figure 4.1 on the following page.

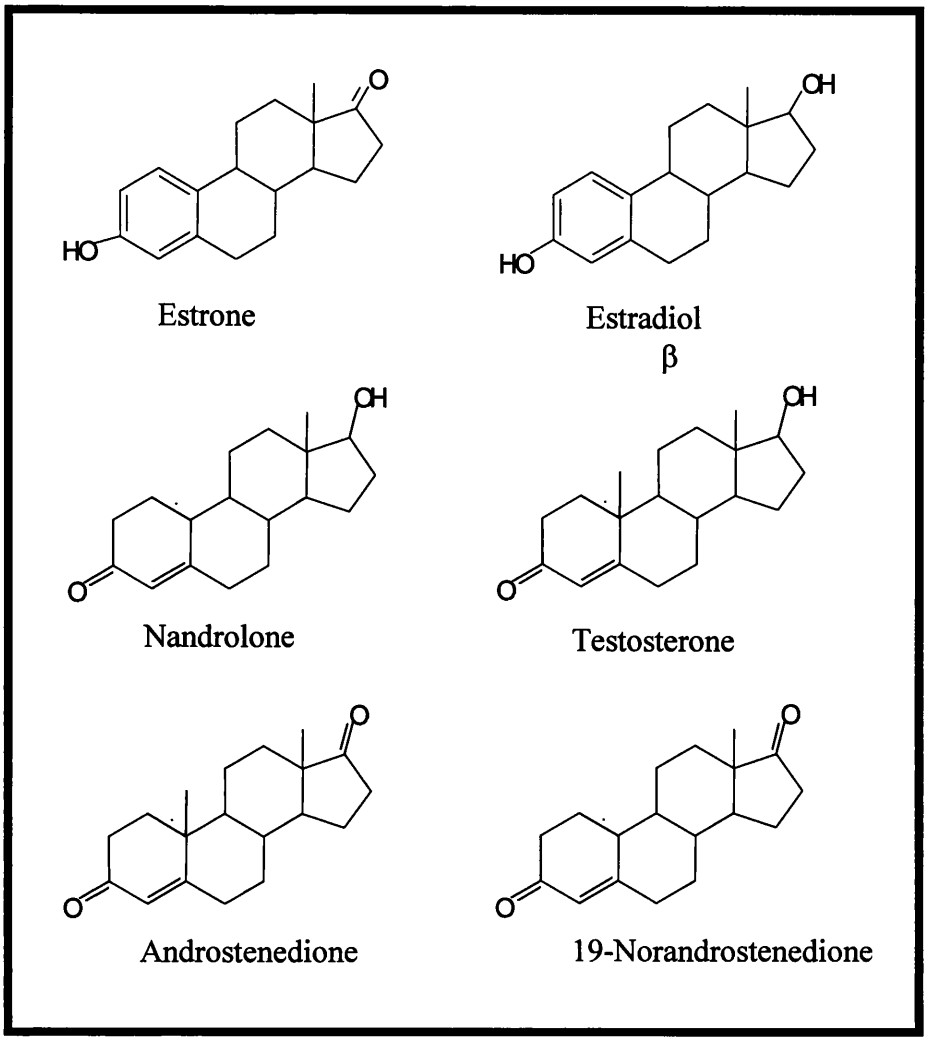



Figure 4.1 Typical equine steroids.

The steroid hormones in horses undergo extensive metabolism involving conjugation with both glucuronic acid and sulfate and are excreted in urine predominantly in their conjugate forms. Figure 4.2 below shows example glucuronide and sulfate conjugates.

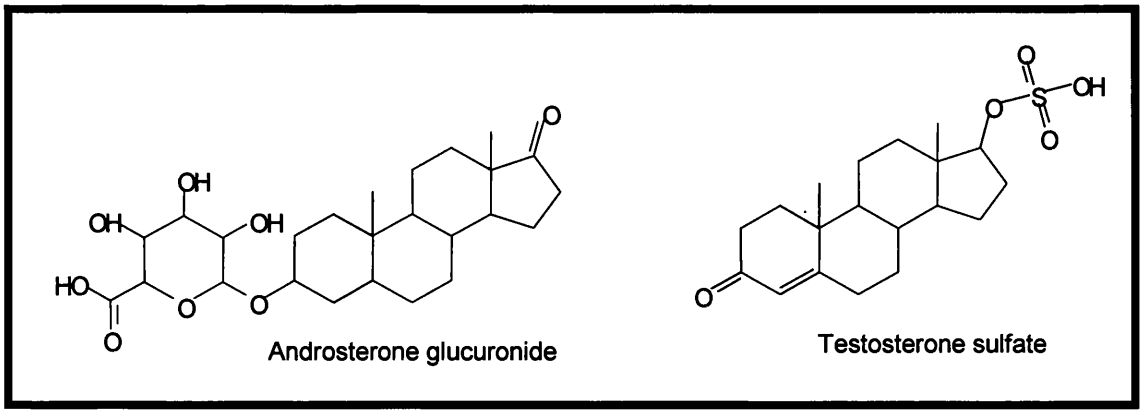



Figure 4.2 Example steroid conjugates found in equine urine.

Colt urine contains large amounts of several steroids including testosterone, estrone, estradiol, 19-norandrostenediol and nandrolone. The presence of estrogenic steroids in high quantities in the male is unusual in mammalian species. The situation, by contrast, with fillies, mares and gelded animals is somewhat different; these animals excrete low levels of steroids and no nandrolone. The studies in this chapter are concerned with the analysis of steroid conjugates in colt urine and in particular nandrolone (1).

## Nandrolone

Nandrolone is manufactured and sold as an anabolic agent but is also found in male equine urine. The situation with nandrolone in gelded animals, fillies and mares is straight forward as these animals do not posses endogenous nandrolone so if the presence of nandrolone is detected in their urine it can only be from an exogenous source and therefore administration. The colt is somewhat more complicated as its urine does contain endogenous nandrolone and it is therefore necessary to have a means of distinguishing between when an animal has been doped and when the levels of nandrolone found in its urine are occurring naturally. In studies conducted by HFL

(Cambridgeshire, UK) into the levels of endogenous nandrolone present in blank colt urine it was found that the levels varied extensively from 8ng/mL to 434ng/mL the latter of which is approaching the levels found in post administration urine samples (1).

Following administration of nandrolone and its esters the parent compound is excreted in the urine predominantly as a sulfate conjugate. For analysis of steroid conjugates HFL employ the use of GC-MS and as such it is necessary to convert the conjugated steroids to their free steroid counterparts to allow for GC-MS analysis. Following solvolysis or methanolysis of normal colt urine and GC-MS analysis HFL found that nandrolone and the related steroid 19-norandrostenedione were detected. During the course of a variety of experiments it became apparent to HFL that the nandrolone detected in normal colt urine was not derived from the 17β-sulfate conjugate that post administration nandrolone was derived from.

Sulfate conjugates can be readily separated from glucuronic acid conjugates using specific cleavage methods or by chromatographic means. When HFL separates conjugate group fractions chromatographically the unconjugated steroids appear in the same fraction as the glucuronic acids. Usually sulfate conjugates are fairly difficult to hydrolyse requiring reasonably aggressive treatment with acid. Glucuronic acid conjugates can be readily cleaved using specific enzymes. It was assumed that normally occurring nandrolone was simply present as a sulfate conjugate as was the case following administration. However it was suggested by a co-worker of HFL that in fact it was present as glucuronic acid conjugate as it appeared in the free/glucuronic acid fraction when the conjugate groups were separated chromatographically. This

was inconsistent with the normal findings of HFL. At this point HFL made the following supposition...Was endogenous nandrolone generated from a 19-norandrostenedione enol-sulfate conjugate? (see figure 4.3 below)

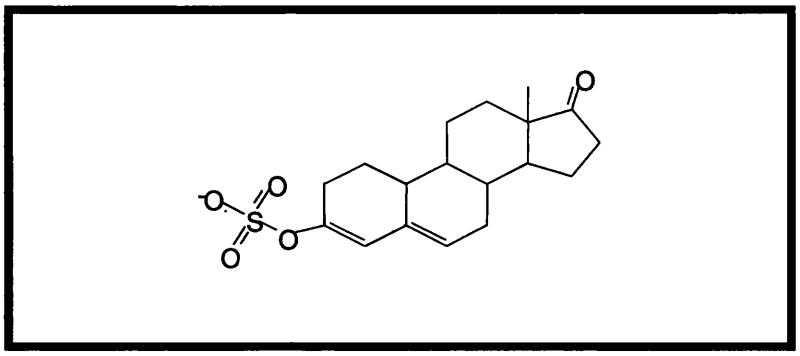


Figure 4.3 Proposed 19-norandrostenedione enol-sulfate.

HFL's supposition was that in such a compound the enol sulfate would be readily cleaved by even mild acid treatment hence the variability with the fraction in which this compound is located. The presence of a large amount of an enol-sulfate in urine is unheard of and it could also be indicative of the metabolic pathway by which estrogens are produced in the colt. However HFL only has indirect proof supporting the theory of the presence of an enol sulfate:

1. Mild acid treatment of normal colt urine leads to the appearance of nandrolone in the free/glucuronic acid fraction.
2. Mild acid treatment of post administration nandrolone colt urine does not affect the presence of this material in the sulfate conjugate group.

This methodology shows that the nandrolone appearing in the free/glucuronic acid fraction results from a source other than nandrolone 17β-sulfate. It does not however provide proof that endogenous nandrolone is due to an enol-sulfate (1).

Direct analysis of the steroid conjugates without the need for their conversion to free steroids could potentially provide more information towards identifying the structure of the unknown precursor of endogenous nandrolone as well as allowing for the detection of the presence of other unknown steroid conjugates.

LC-MS allows for the direct analysis of conjugated steroids and with this in mind HFL sponsored us to develop an LC-MS method to analyse for the presence of a set of known conjugated steroids with a view to then also applying it to the analysis of unknowns.

## LC-MS For the Separation of 6 Conjugated Steroids

The structures of the conjugated steroids studied are shown in figure 4.4 below. Dehydroepiandrosterone is abbreviated to DHA.

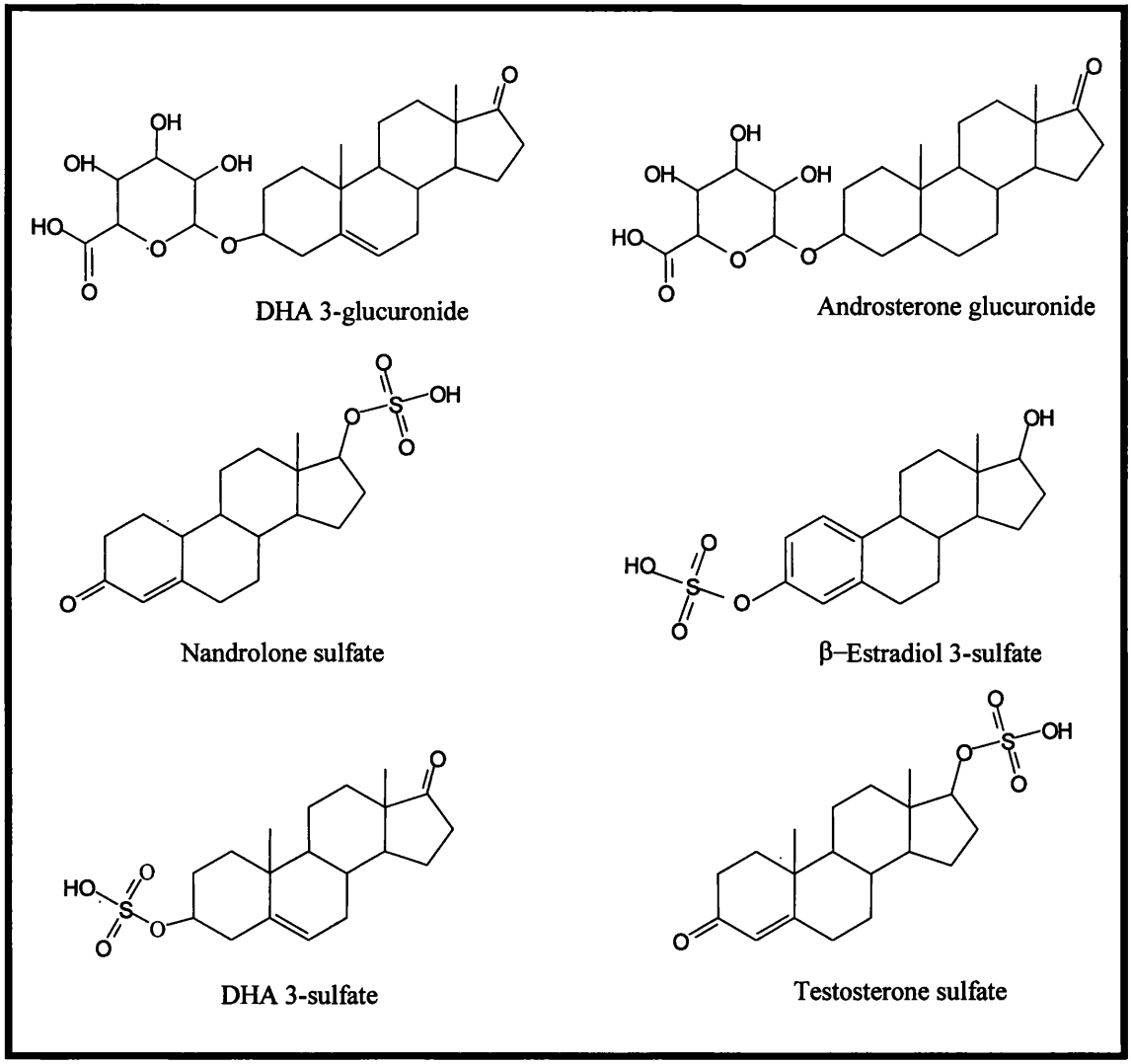



Figure 4.4 Structures of the conjugated steroids investigated.

## Experimental

### Chemicals

HPLC grade solvents were purchased from Fisher Scientific (Loughborough, U.K.), gases from BOC Limited (Surrey, U.K.) and the following 6 steroids from Sigma-Aldrich Limited (Dorset, U.K.) or prepared in the labs of HFL (Cambridgeshire, U.K).

| | |
|---|---|
| DHA 3-glucuronide | Androsterone glucuronide |
| Nandrolone sulfate | β Estradiol 3-sulfate |
| DHA 3-sulfate | Testosterone sulfate |

Dehydroepiandrosterone is abbreviated to DHA.

### Sample Preparation

Standard solutions of 0.1 mg/ml were made up in 50/50 methanol/water for each of the steroids; solutions for analysis were prepared and diluted from these as necessary.

### Instrumentation

A Hewlett Packard 1100 HPLC was used to perform the chromatography with a Phenomenex Luna 4.6x100mm 3μ Phenyl-Hexyl column; the Finnigan Mat LCQ ion trap was used as the detector. The mobile phase used for analysis was made up of acetonitrile and 10mM ammonium acetate run in an isocratic system.

## Results

Whereas in previous chapters our chromatography has been developed with the use of a UV detector in the case of the 6 steroid conjugates of interest in this study we had very little sample to work with and found that there was not sufficient sensitivity with UV to allow for our method development to be performed with this technique. It was therefore decided to use the Finnigan Mat LCQ ion trap as the detector for chromatographic development. The 6 steroid conjugates were first infused with ESI to set up a tune file on the mass spectrometer which would allow for detection of all 6; the sheath and auxiliary gas flows were then adjusted to take account of the higher flow rates entering the ion source when coupled to the HPLC. Below are the conditions used for ESI analysis.

**ESI**

Polarity: Negative.

Heated Capillary Temperature: 200°C.

Electron Multiplier Voltage: -1230V.

Needle Spray Voltage: 3.5kV.

Sheath Gas Flow: 50psi.

Auxiliary Gas Flow: 20psi.

Scan Range: 50-700amu.

The flow from the HPLC system was split to allow approximately 200μL per minute into the ion source (a split of 27%).

Initial chromatographic experiments were conducted using the conditions described previously in chapter 3 for the separation of the 8 free and conjugated estrogen steroids. Using these conditions it was found that we were unable to achieve sufficient

retention or resolution to allow for adequate analysis of the 6 conjugates of interest. As we had previously had success with the separation of conjugated steroids using acetonitrile and ammonium acetate with a C18 column (see chapter 3) it was decided to perform experiments with various combinations of these 2 mobile phases. We found that the most promising results were achieved with a mobile phase consisting of acetonitrile and 10mM ammonium acetate, although it was necessary to cool the column to gain a reasonable amount of resolution. Experiments were conducted with various gradient systems but it was found that the best results were achieved by using a simple isocratic set up. The mixture of the 6 conjugate steroids was separated on the Phenomenex Phenyl-Hexyl column with the following chromatographic conditions.

| Time in minutes | %$CH_3CN$ | %10mM $NH_4Oac$ |
|---|---|---|
| 0.00 | 30 | 70 |
| 8.00 | 30 | 70 |

Column Thermostat 10°C

Flow Rate 0.75mL/min

The following table gives the retention times for the compounds.

| Compound name | TIC Peak label | RT minutes |
|---|---|---|
| DHA 3-glucuronide | A | 2.70 |
| Androsterone glucuronide | B | 3.29 |
| Nandrolone sulfate | C | 3.52 |
| β-Estradiol 3-sulfate | D | 3.88 |
| DHA 3-sulfate | E | 4.31 |
| Testosterone sulfate | F | 6.66 |

Figure 4.5 shows the total ion chromatogram (TIC) of the separation of the 6 steroids.

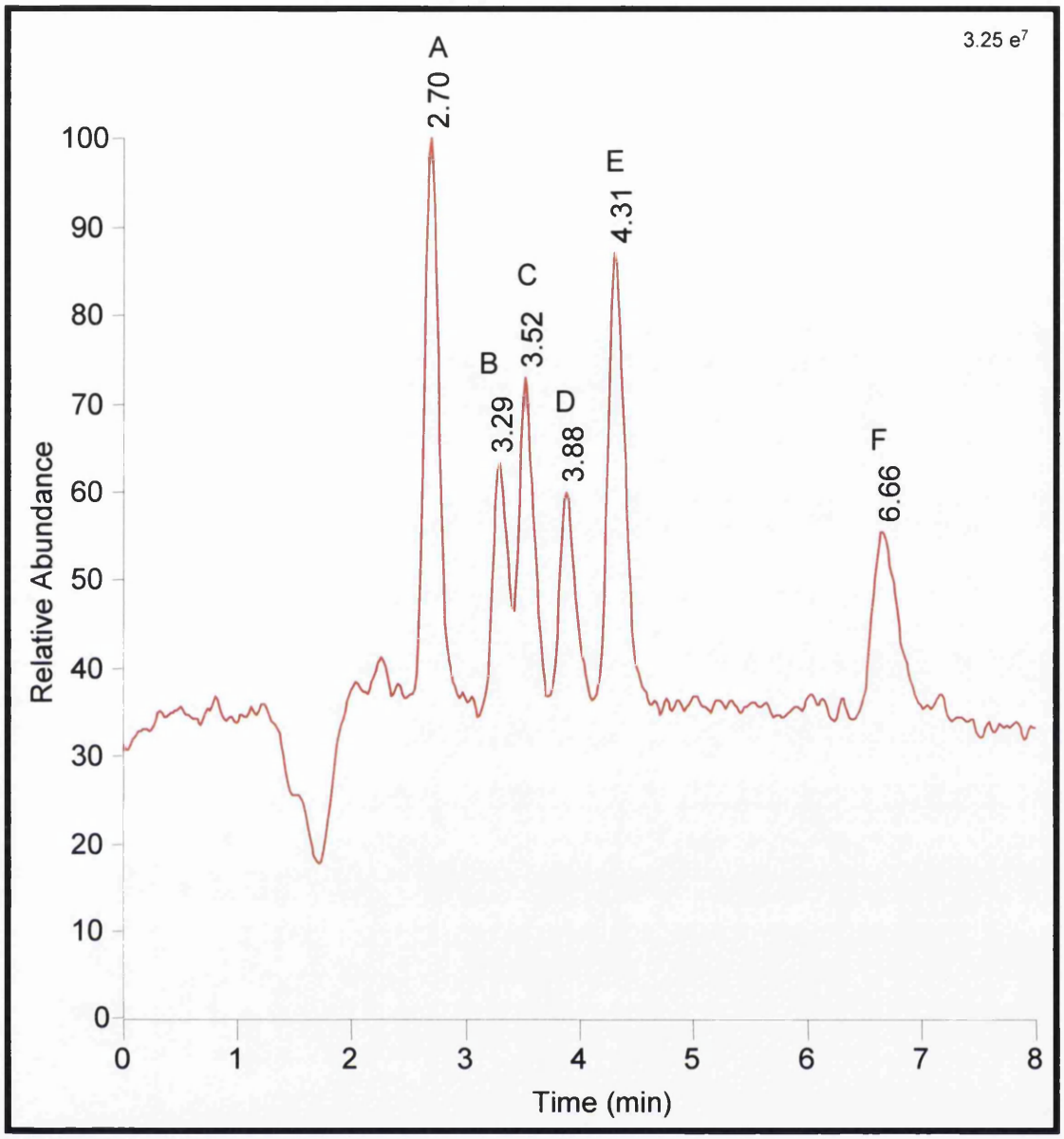



Figure 4.5 Negative LC-ESI-MS total ion chromatogram for the separation of the 6 conjugate steroids. 10μL injection of a 100ng/mL solution of each steroid conjugate; equal to 1ng on column of each.

The table below gives the corresponding resolution values (Rs) for the separation shown above.

| Peaks | Rs Value |
|---|---|
| A+B | 1.78 |
| B+C | 0.69 |
| C+D | 0.97 |
| D+E | 1.24 |
| E+F | 5.33 |

Figure 4.6 shows the extracted ion chromatograms of the most abundant ion of each steroid conjugate.

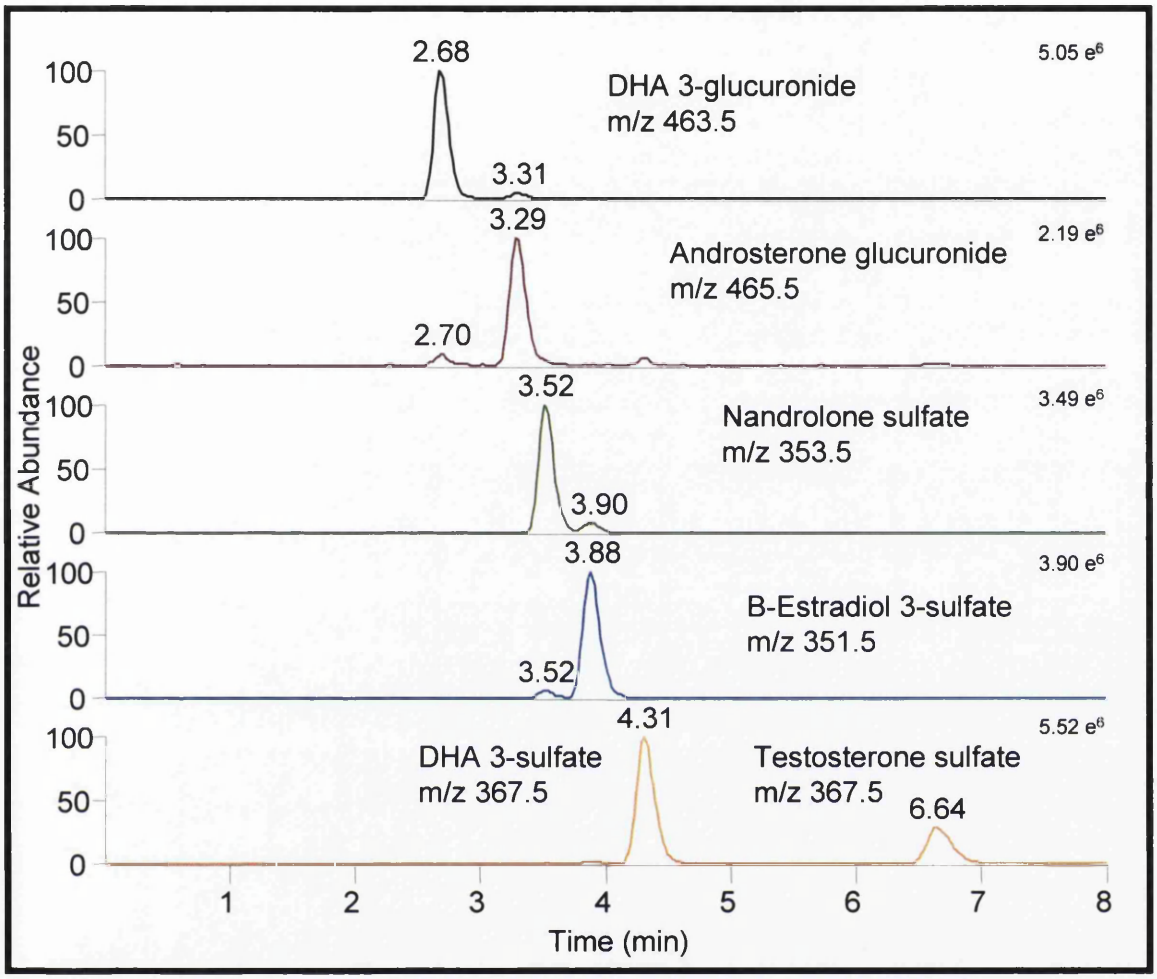



Figure 4.6 Extracted ion chromatograms of the base ions for each of the 6 conjugate steroids. 10µL injection of a 100ng/mL solution of each steroid conjugate; equal to 1ng on column of each.

The peaks occurring at 3.31 minutes in the extracted ion chromatogram of DHA 3-glucuronide and 2.70 minutes in the extracted ion chromatogram of androsterone glucuronide are due to the presence of isotopes of each steroid corresponding to the base ion m/z of the other steroid. As is the case with the peaks occurring at 3.90 minutes and 3.52 minutes in the extracted ion chromatograms of nandrolone sulfate and β-estradiol 3-sulfate; this occurs because both pairs of conjugates are only 2 mass units apart in molecular weight and their base ion m/z are due to $[M-H]^-$ ions.

The table below lists the m/z values used for the extracted ion chromatograms.

| Compound name | m/z |
|---|---|
| DHA 3-glucuronide | 463.5 |
| Androsterone glucuronide | 465.5 |
| Nandrolone sulfate | 353.5 |
| β-Estradiol 3-sulfate | 351.5 |
| DHA 3-sulfate | 367.5 |
| Testosterone sulfate | 367.5 |

The amount of acetonitrile in the mobile phase system was found to be critical to the ability of the system to achieve a separation in a reasonable amount of time and with sufficient resolution; in fact an increase of 10%, greater than the final conditions used, results in co-elution of all 6 conjugates whereas a decrease of 10% causes a major increase in retention time and hence broadening of the peaks to the extent that there is very little sensitivity (see figures 4.7-4.10).

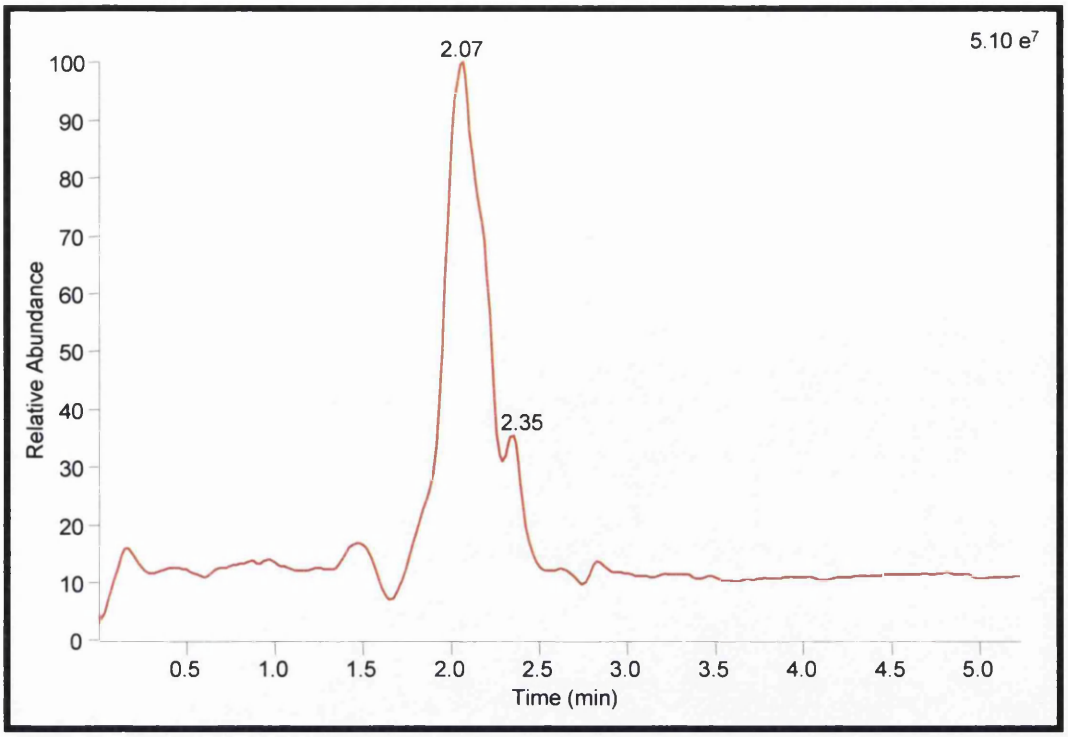



Figure 4.7 Negative LC-ESI-MS TIC of the separation of the 6 conjugates with 10% more acetonitrile. 10µL injection of a 100ng/mL solution of each steroid conjugate; equal to 1ng on column of each.

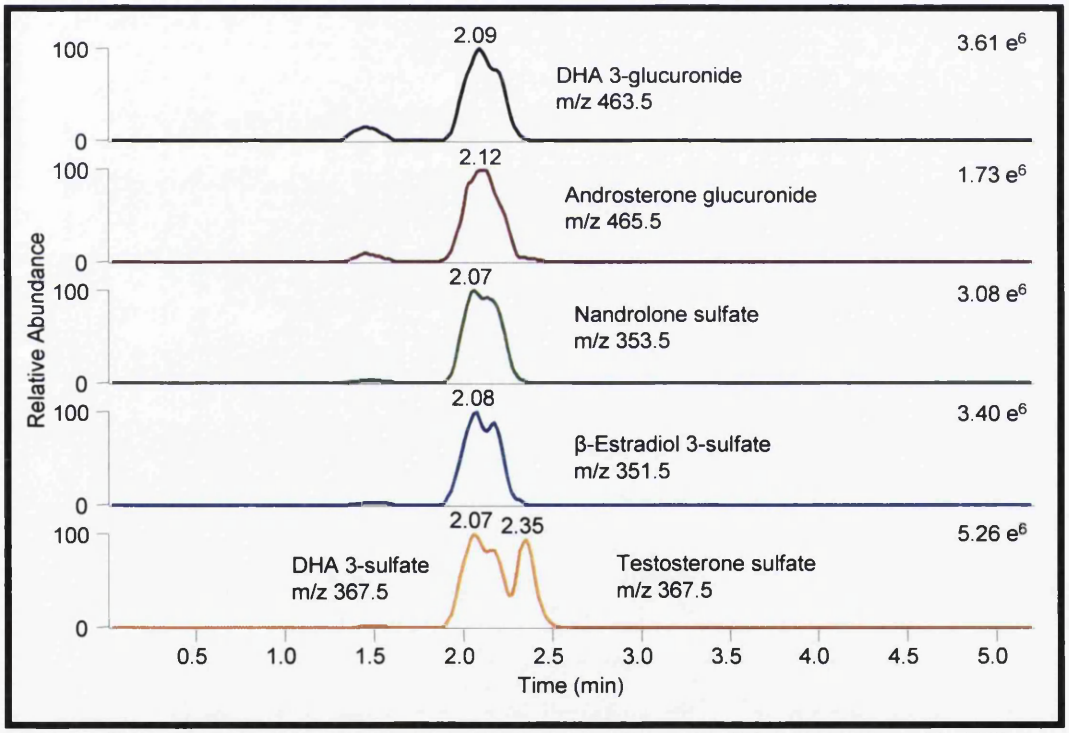



Figure 4.8 Extracted ion chromatograms of the base ions for each of the conjugates with 10% more acetonitrile. 10µL injection of a 100ng/mL solution of each steroid conjugate; equal to 1ng on column of each.

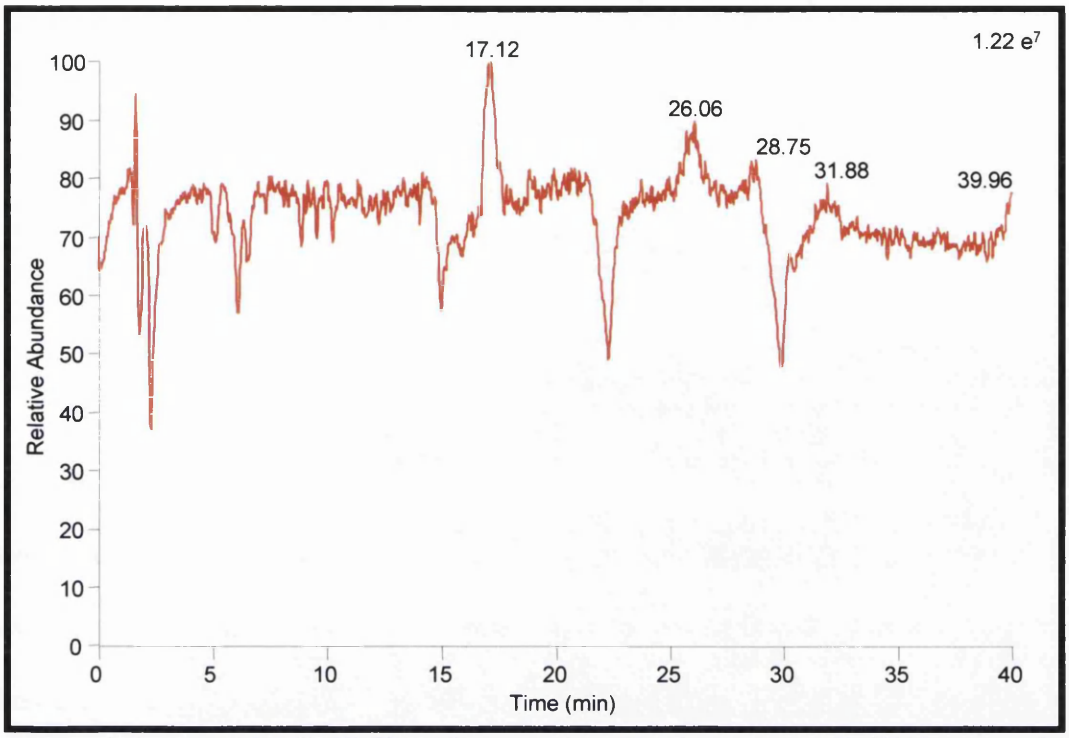



Figure 4.9 Negative LC-ESI-MS TIC of the separation of the 6 conjugates with 10% less acetonitrile. 10µL injection of a 100ng/mL solution of each steroid conjugate; equal to 1ng on column of each.

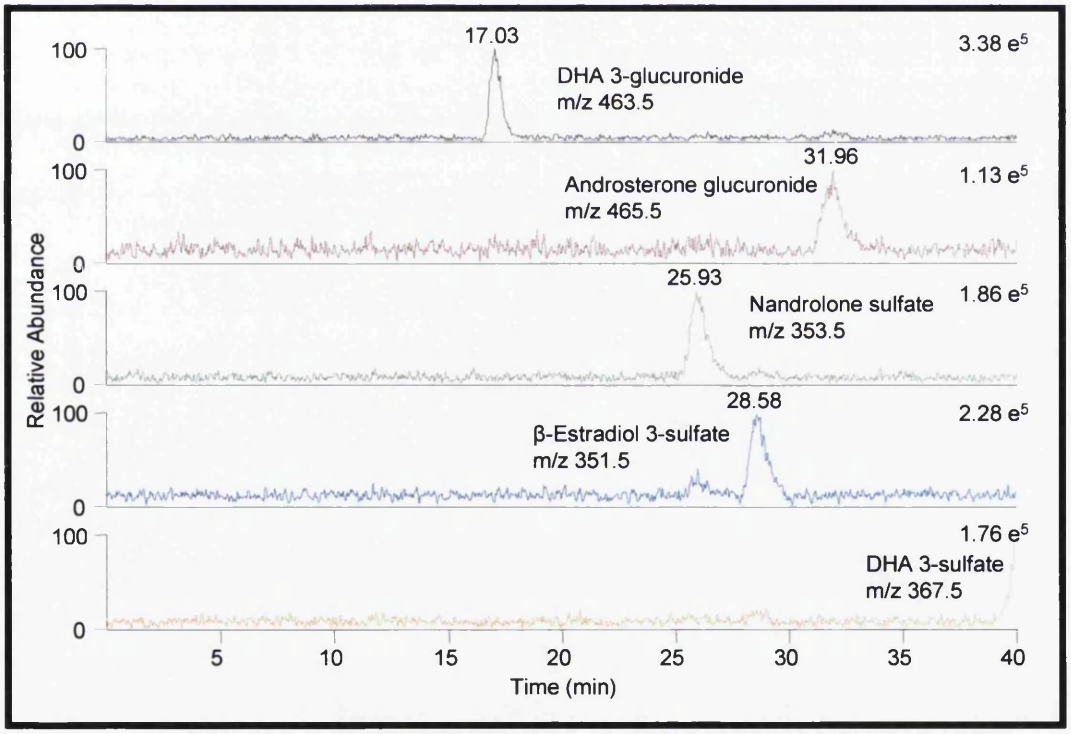



Figure 4.10 Extracted ion chromatograms of the base ions for each of the conjugates with 10% less acetonitrile. 10µL injection of a 100ng/mL solution of each steroid conjugate; equal to 1ng on column of each.

In figure 4.10 on the previous page the analysis was stopped at 40 minutes, as can be seen from the extracted ion chromatograms DHA 3-sulfate has just started to elute from the column when the run has been stopped. This shows that to perform analysis under these conditions would require run times well in excess of 40 minutes. It should also be noted that the elution order changes when the amount of acetonitrile is decreased; androsterone glucuronide elutes after both nandrolone sulfate and β estradiol 3-sulfate when the acetonitrile content of the mobile phase is decreased sufficiently. All of the chromatograms shown for comparison were obtained from analysis of 100ng/mL solutions. In the example analysis run with conditions of 10% less acetonitrile the response is approximately 10 fold lower, this is due to the much wider peak shape that occurs under these conditions.

Mass spectrometric analysis was also carried out using atmospheric pressure chemical ionisation (APCI), below are the conditions used for LC-APCI-MS analysis.

**APCI**

| | |
|---|---|
| Polarity: Negative. | Sheath Gas Flow: 80psi. |
| Heated Capillary Temperature: 300°C. | Auxiliary Gas Flow: 40psi. |
| Electron Multiplier Voltage: -1230V. | Scan Range: 50-700amu. |
| Vaporiser Temperature: 300°C. | Corona Discharge Current: 7μ amps. |

The flow from the HPLC was not split for APCI analysis, however it was necessary to increase the gas flow rates and the heated capillary temperature to improve the sensitivity of the technique.

It was found that both forms of ionisation yielded the same ions and that the spectra obtained were very "clean" with only β-estradiol 3-sulfate having a m/z present resulting from the loss of the sulfate group. This was unexpected as our previous studies of conjugated steroids (see chapter 3) had shown that the base ions resulting from glucuronide conjugates ionised with APCI were due to loss of the glucuronic acid group and that all the conjugated steroids studied previously displayed m/z values resulting from the loss of their conjugate groups with this occurring to a greater degree in the case of APCI when compared to ESI. Figures 11 and 12 show comparisons of the ESI and APCI spectra obtained for DHA 3-glucuronide and β-estradiol 3-sulfate.

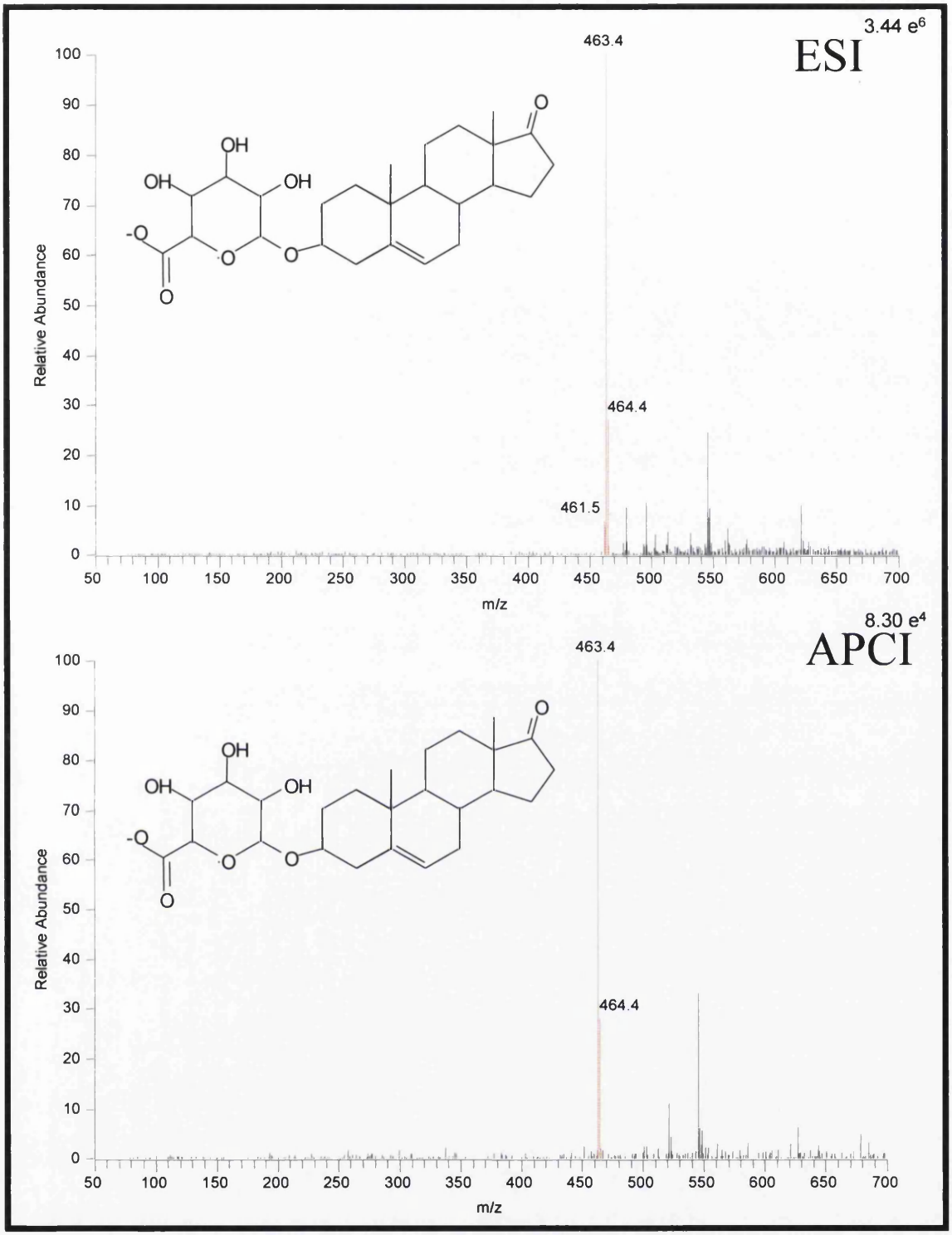



Figure 4.11 Negative ESI and APCI mass spectrum comparison of DHA 3-glucuronide. 10µL injection of a 100ng/mL solution of each steroid conjugate; equal to 1ng on column of each.

As can be seen from the above spectra comparison there is no sign of a m/z due to loss of the glucuronide in the APCI spectra, this is in contrast to the results obtained in our analysis of the estrogen glucuronide conjugates.

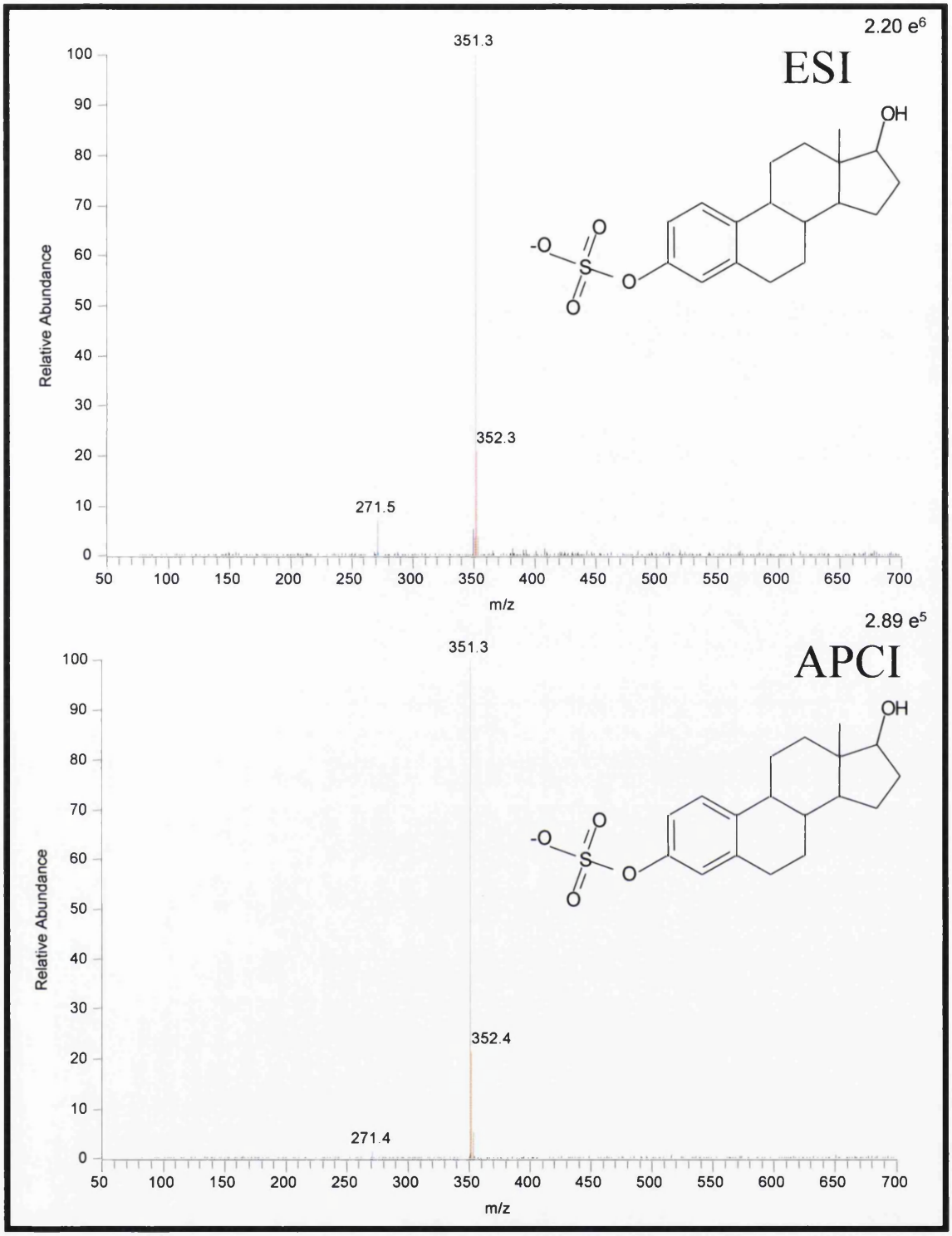



Figure 4.12 Negative ESI and APCI mass spectrum comparison of ß Estradiol 3-sulfate. 10µL injection of a 100ng/mL solution of each steroid conjugate; equal to 1ng on column of each.

The spectra of β-estradiol 3-sulfate do show a m/z at the value expected for loss of the sulfate group. However there appears to be a decrease in the relative intensity of the

ion at m/z 271.5 when APCI is the chosen form of ionisation, this is again in contrast to the results we obtained for our analysis of the estrogen conjugates in chapter 3.

Figure 4.13 shows the TIC for the 6 separated conjugate steroids using APCI.

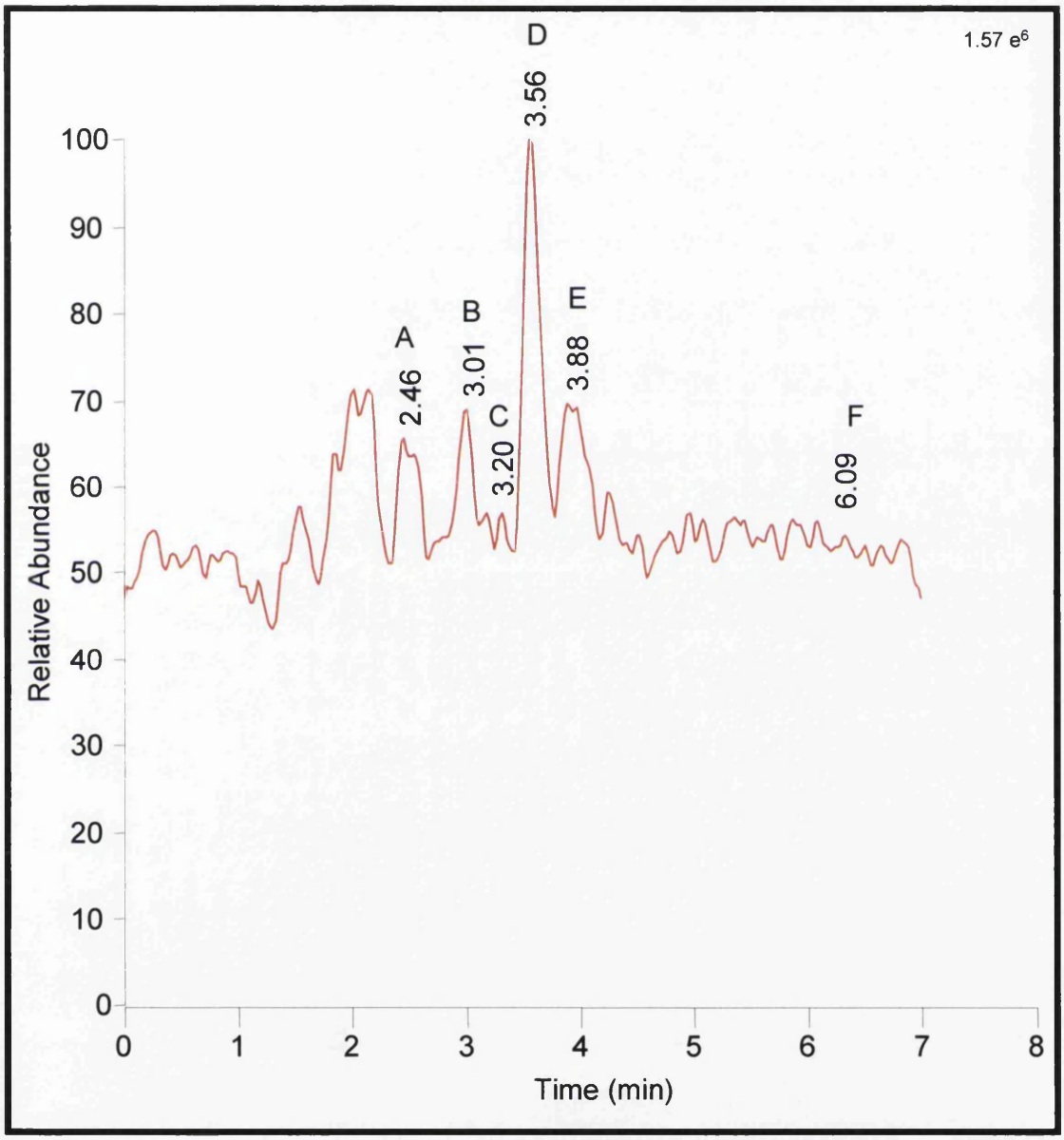



Figure 4.13 Negative LC-APCI-MS total ion chromatogram for the separation of the 6 conjugate steroids. 10µL injection of a 100ng/mL solution of each steroid conjugate; equal to 1ng on column of each.

As can be seen from the LC-APCI-MS TIC the peaks representing the 6 steroid conjugates are less distinctive from the background than when ESI is used for the analysis, this is due to a higher level of background noise occurring when APCI is used to perform ionisation. However when extracted ion chromatograms of the most abundant ion of each conjugate are taken from the TIC the conjugate peaks are easily identified. Figure 4.14 below shows the extracted ion chromatograms of each steroid conjugate taken from the TIC shown on the previous page.

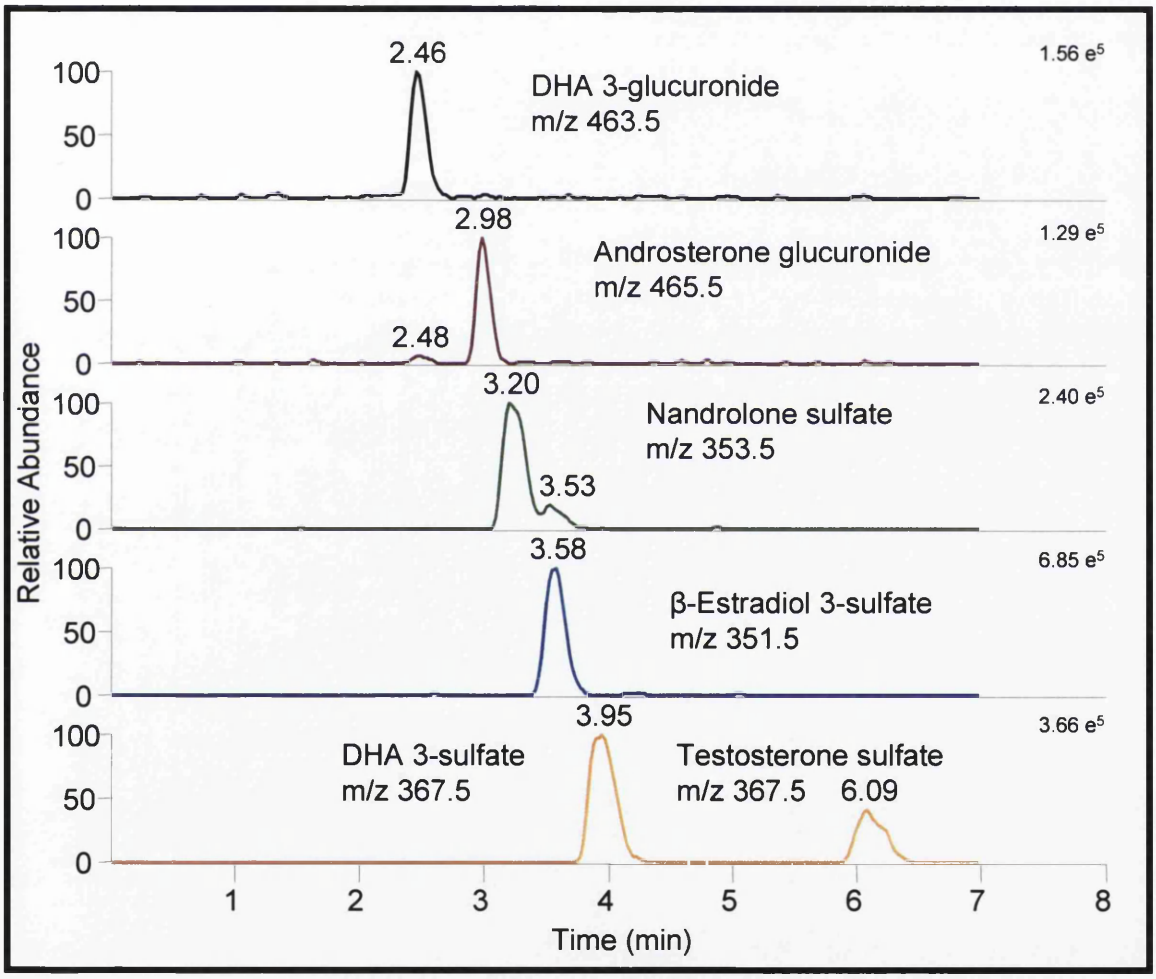



Figure 4.14 Extracted ion chromatograms of the base ions for each of the 6 conjugate steroids. 10µL injection of a 100ng/mL solution of each steroid conjugate; equal to 1ng on column of each.

The following figures (4.15-4.20) show the mass spectrum, with APCI, for each of the steroids and the proposed mass spectrum interpretation; the m/z values associated

with ions other than the parent ion are assumed to be due to in source fragmentation although this is not definitive and they may in fact be due to the presence of impurities in the standard materials.

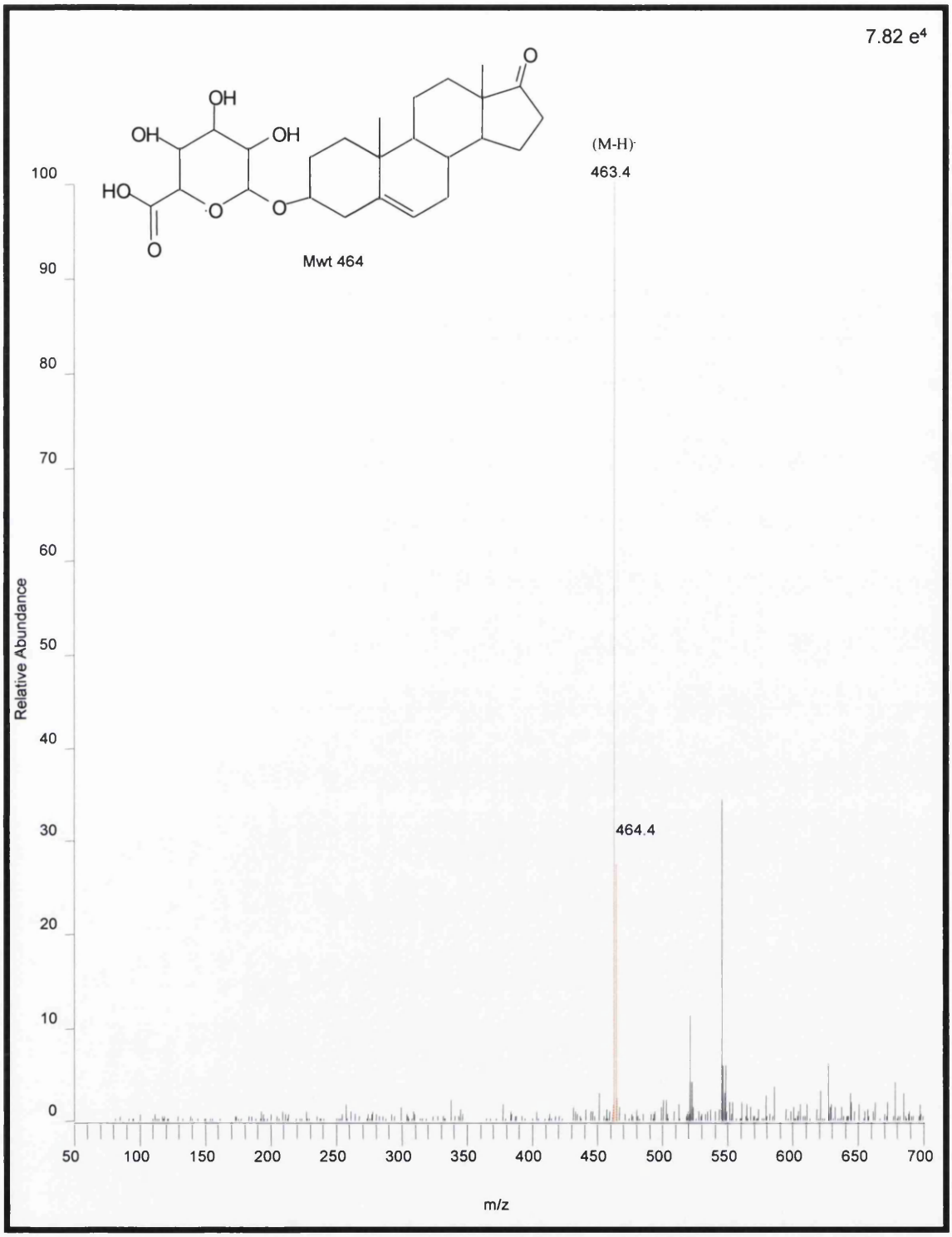



Figure 4.15 Negative LC-APCI-MS mass spectrum of DHA 3-glucuronide. 10µL injection of a 100ng/mL solution; equal to 1ng on column.

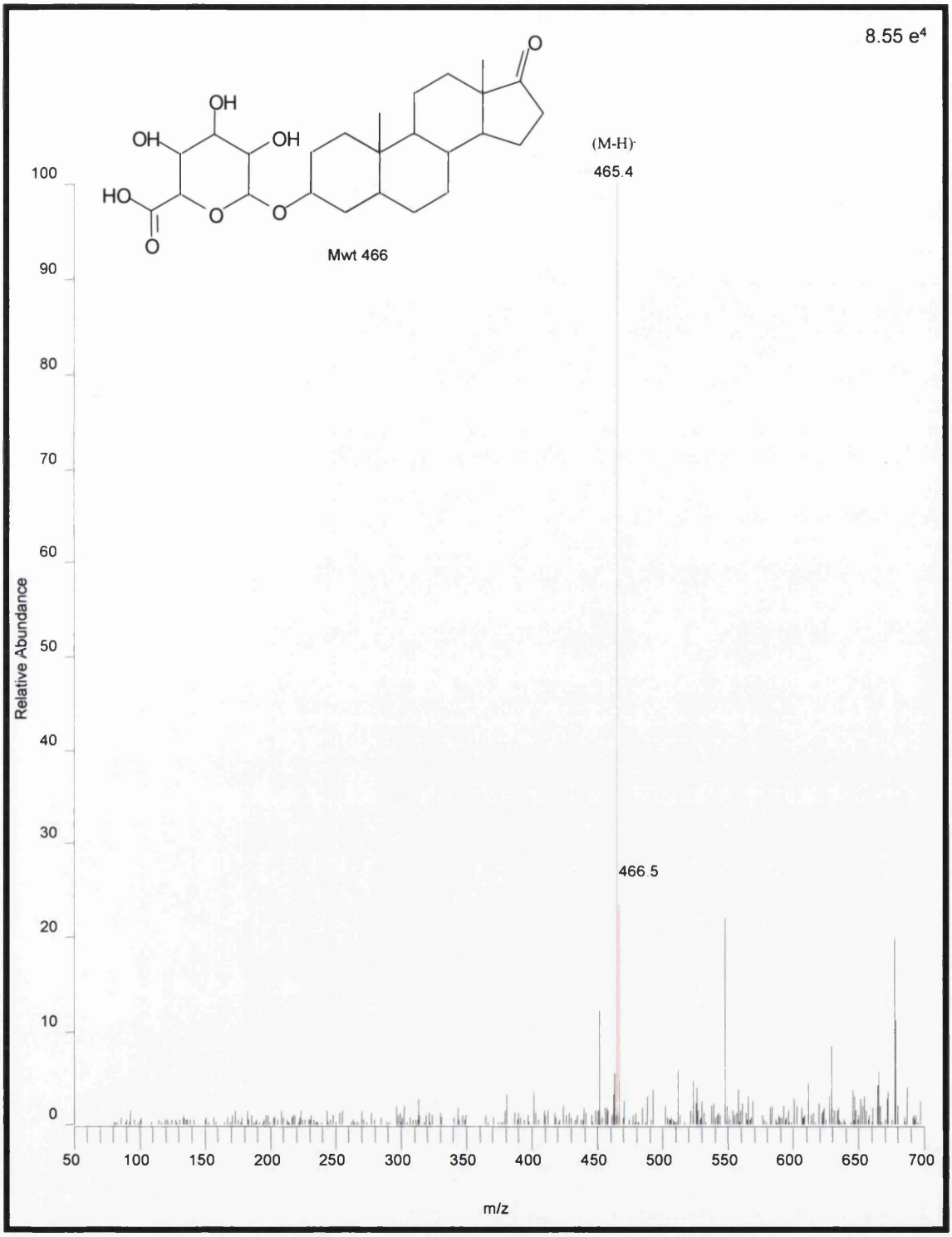



Figure 4.16 Negative LC-APCI-MS mass spectrum of Androsterone glucuronide. 10µL injection of a 100ng/mL solution; equal to 1ng on column.

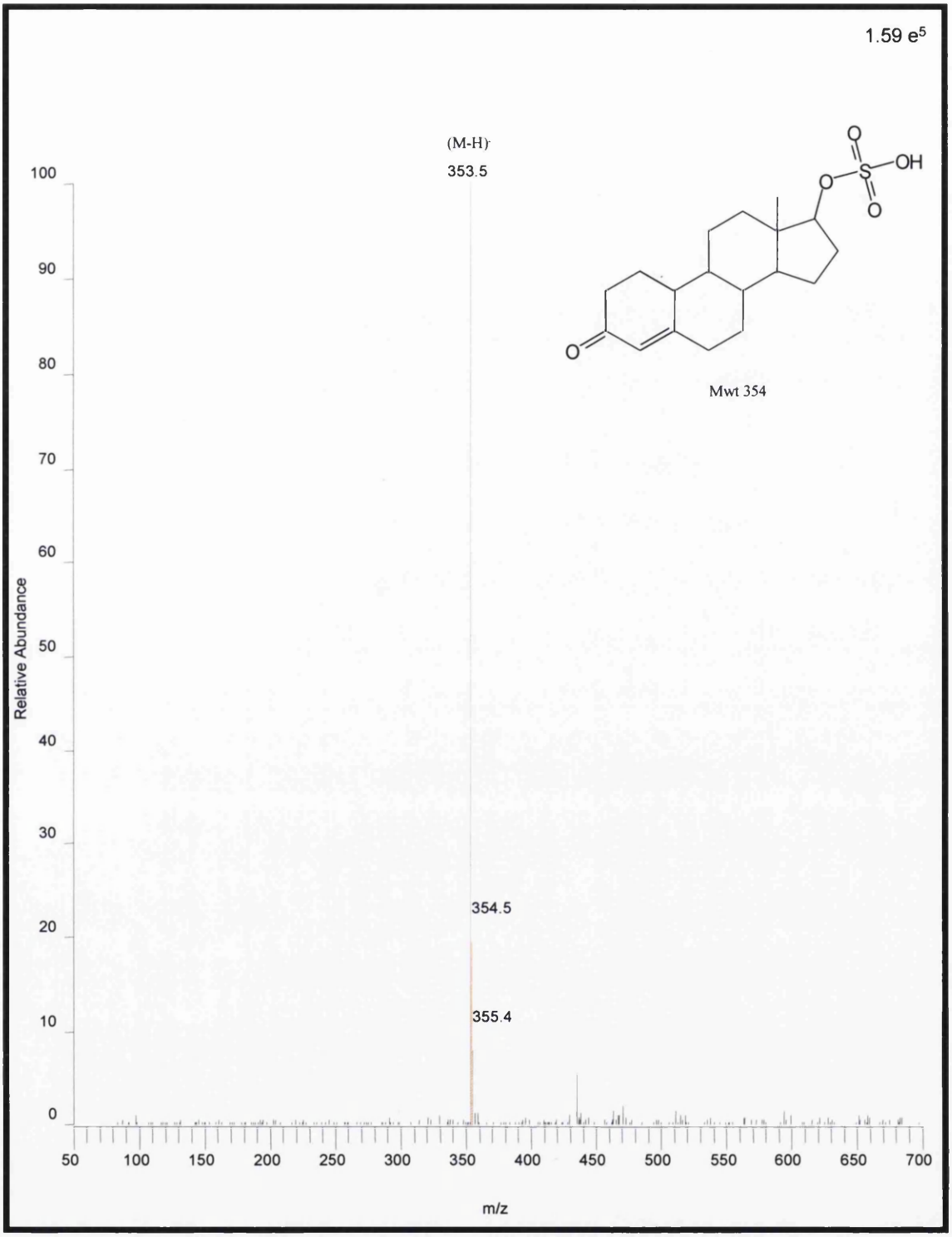



Figure 4.17 Negative LC-APCI-MS mass spectrum of Nandrolone sulfate. 10µL injection of a 100ng/mL solution; equal to 1ng on column.

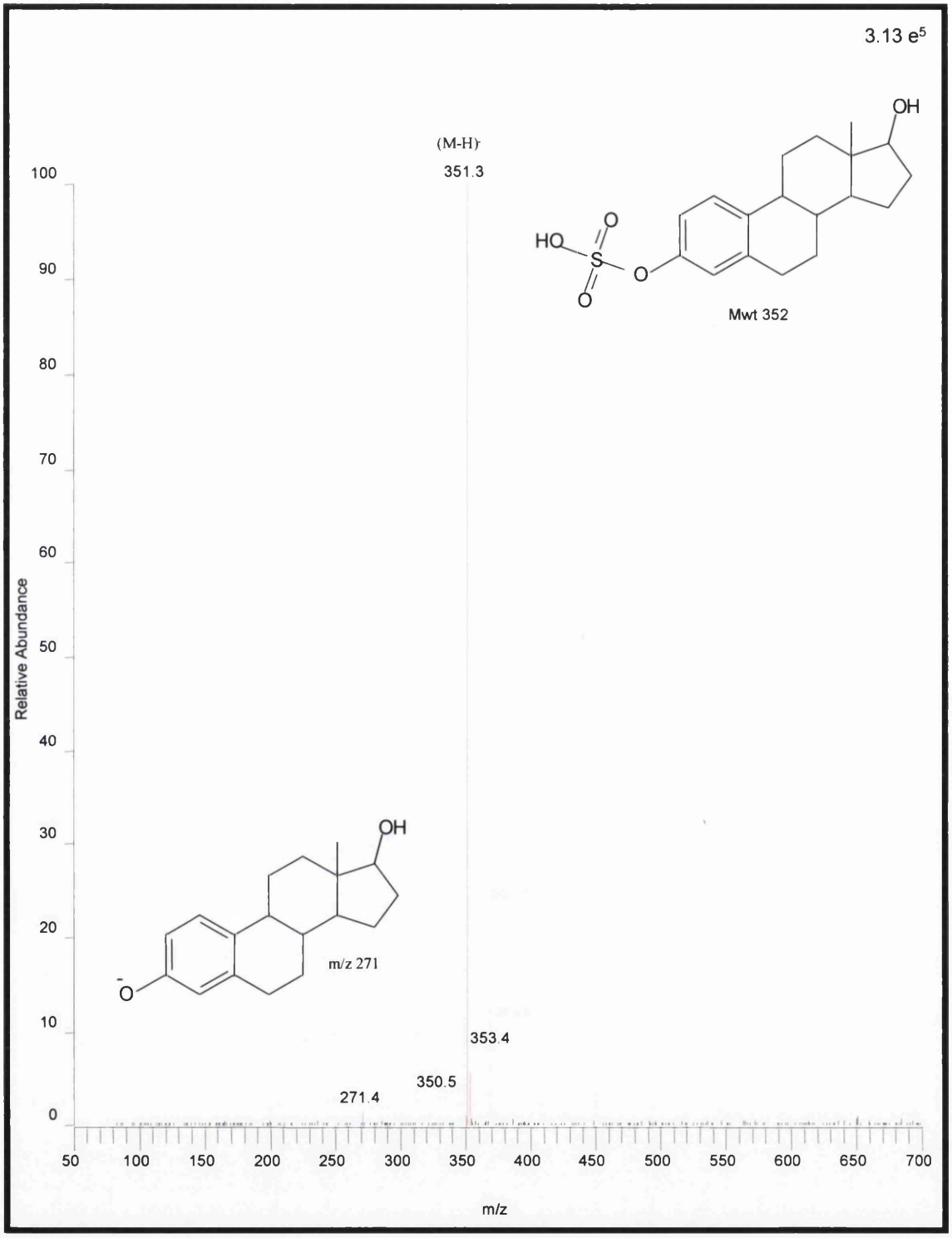



Figure 4.18 Negative LC-APCI-MS mass spectrum of β-Estradiol 3-sulfate. 10µL injection of a 100ng/mL solution; equal to 1ng on column.

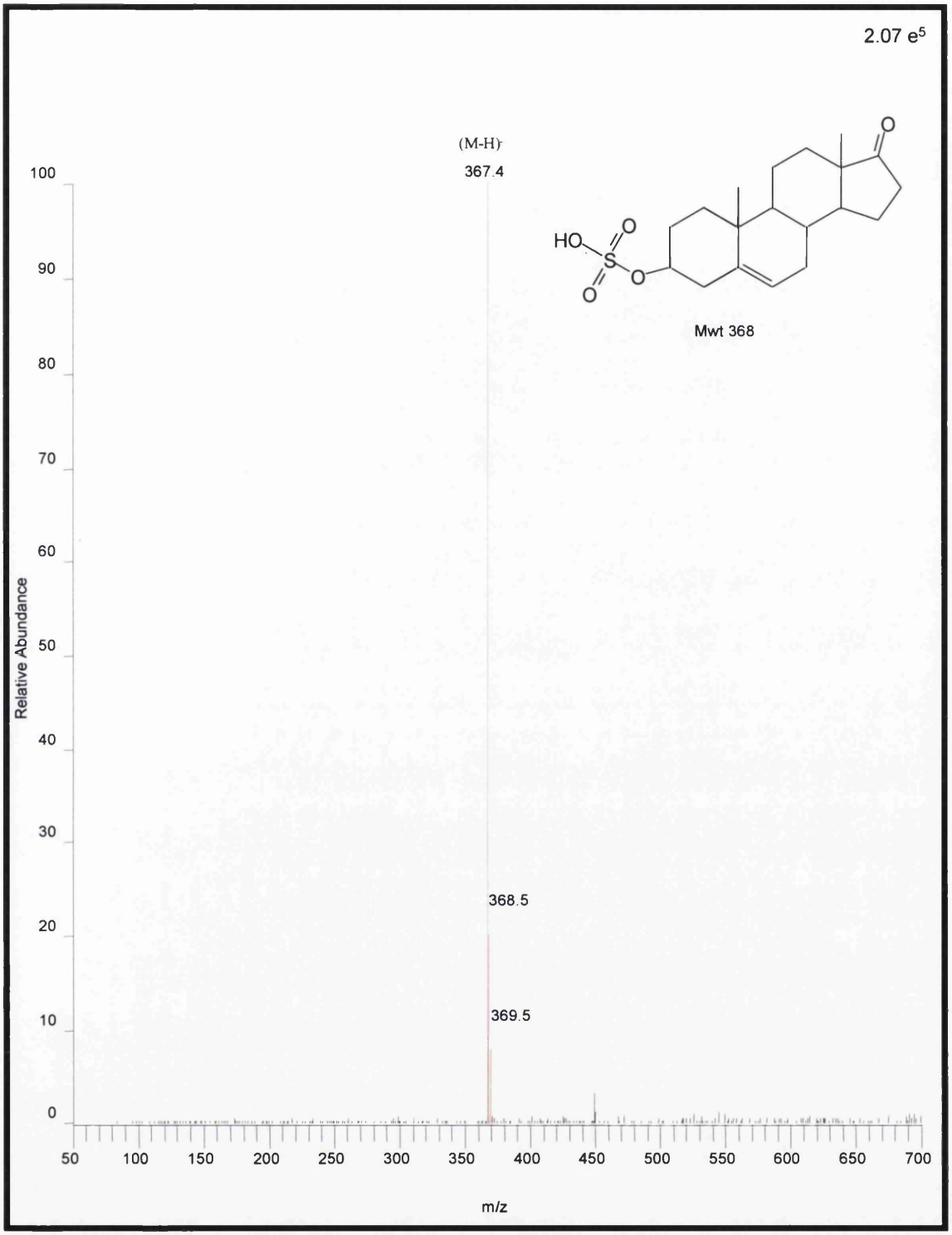



Figure 4.19 Negative LC-APCI-MS mass spectrum of DHA 3-sulfate. 10µL injection of a 100ng/mL solution; equal to 1ng on column.

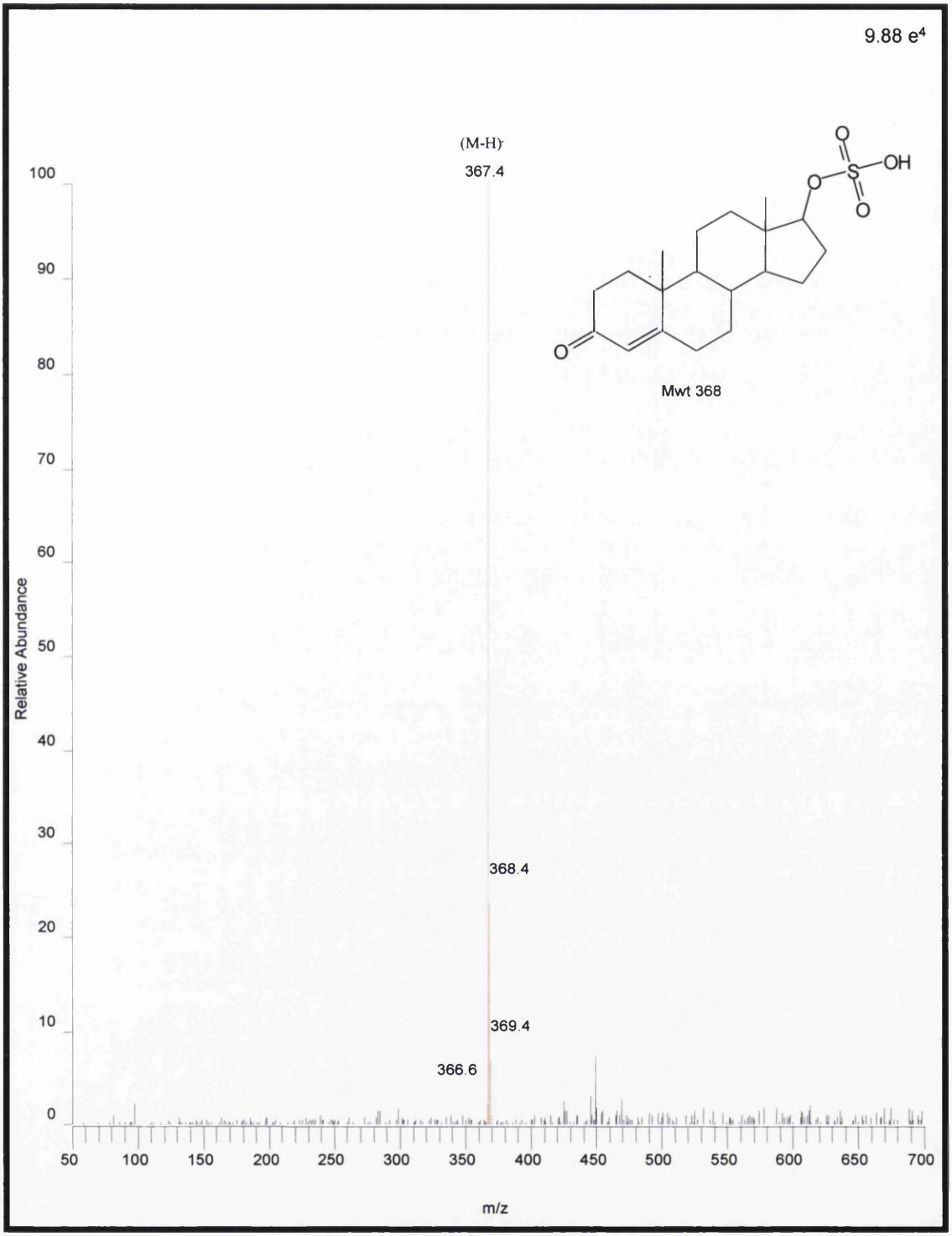



Figure 4.20 Negative LC-APCI-MS mass spectrum of Testosterone sulfate. 10µL injection of a 100ng/mL solution; equal to 1ng on column.

## Limits of Detection for the 6 Conjugated Steroids

Detection limits were obtained by taking extracted ion chromatograms of the m/z of the most abundant ion for each steroid from the TIC. Limits of detection (LOD) were calculated from the analysis of a series of solutions at the following concentrations; 0.1ng/mL, 1ng/mL, 10ng/mL, 100ng/mL, 1000ng/mL, the LOD was regarded as the concentration from this series at which a signal to noise ratio of at least 5 to 1 was achievable. The tables below show the LOD achieved with ESI and APCI; the amount of compound injected on column is also given.

**ESI**

| Compound name | m/z Monitored | LOD ESI | Amount on column |
|---|---|---|---|
| DHA 3-glucuronide | 463.5 | 10ng/mL | 100pg |
| Androsterone glucuronide | 465.5 | 10ng/mL | 100pg |
| Nandrolone sulfate | 353.5 | 10ng/mL | 100pg |
| β-Estradiol 3-sulfate | 351.5 | 1ng/mL | 10pg |
| DHA 3-sulfate | 367.5 | 10ng/mL | 100pg |
| Testosterone sulfate | 367.5 | 10ng/mL | 100pg |

On column injection volume of 10μL. LOD is calculated as the concentration of the solution being injected.

**APCI**

| Compound name | m/z Monitored | LOD APCI | Amount on column |
|---|---|---|---|
| DHA 3-glucuronide | 463.5 | 100ng/mL | 1ng |
| Androsterone glucuronide | 465.5 | 100ng/mL | 1ng |
| Nandrolone sulfate | 353.5 | 10ng/mL | 100pg |
| β-Estradiol 3-sulfate | 351.5 | 10ng/mL | 100pg |
| DHA 3-sulfate | 367.5 | 1ng/mL | 10pg |
| Testosterone sulfate | 367.5 | 100ng/mL | 1ng |

On column injection volume of 10μL. LOD is calculated as the concentration of the solution being injected.

As can be seen from the tables ESI was found to be more sensitive than APCI in the case of the majority of the 6 steroids and as such was the chosen form of ionisation for MS-MS analysis.

**Instrument Response**

The table below lists the instrument response in APCI-MS mode to a 10μL injection of a solution made up of a mixture of each of the steroids at a concentration of 100ng/mL (the raw data has been presented earlier in this chapter in figures 4.15-4.20). From this data the response per nanogram on column of each steroid has been calculated.

| Compound name | Instrument response | Response per ng on column |
|---|---|---|
| DHA 3-glucuronide | $7.82\ e^4$ | $7.82\ e^4$ |
| Androsterone glucuronide | $8.55\ e^4$ | $8.55\ e^4$ |
| Nandrolone sulfate | $1.59\ e^5$ | $1.59\ e^5$ |
| β-Estradiol 3-sulfate | $3.13\ e^5$ | $3.13\ e^5$ |
| DHA 3-sulfate | $2.07\ e^5$ | $2.07\ e^5$ |
| Testosterone sulfate | $9.88\ e^4$ | $9.88\ e^4$ |

The response per nanogram on column could be used to give an indication of the amount of a steroid present in an unknown sample; however without the use of an internal standard its value is limited.

## LC-ESI-MS-MS For the Separation of the 6 Conjugated Steroids

Structural information was gained by the fragmentation of each of the most abundant ions obtained from LC-ESI-MS. This gives a specific method of identification for the steroids for on-line analysis. The following table shows the precursor and product ions and the relative collision energies required to induce fragmentation.

| Compound name | Precursor ion | CE % | Product ions |
|---|---|---|---|
| DHA 3-glucuronide | 463.5 | 21 | 445.2, 403.2, 175.0, 157.0 |
| Androsterone glucuronide | 465.5 | 21 | 447.3, 405.0, 175.1, 156.9 |
| Nandrolone sulfate | 353.5 | | |
| β-Estradiol 3-sulfate | 351.5 | 22 | 271.4 |
| DHA 3-sulfate | 367.5 | 27 | 352.2, 337.3 |
| Testosterone sulfate | 367.5 | | |

We were unable to perform MS-MS analysis of nandrolone sulfate or testosterone sulfate, in the case of both these conjugates experiments were conducted whereby we increased the collision energy incrementally with the aim of finding a value that would induce fragmentation. Unfortunately we found that no fragmentation appeared to occur although a drop in signal of the precursor ion was observed as the collision energy was increased until there no longer was a signal. The following figures (4.21-4.28) show the LC-ESI-MS-MS mass spectrum for each of the other 4 steroids and the proposed fragmentation pattern interpretation.

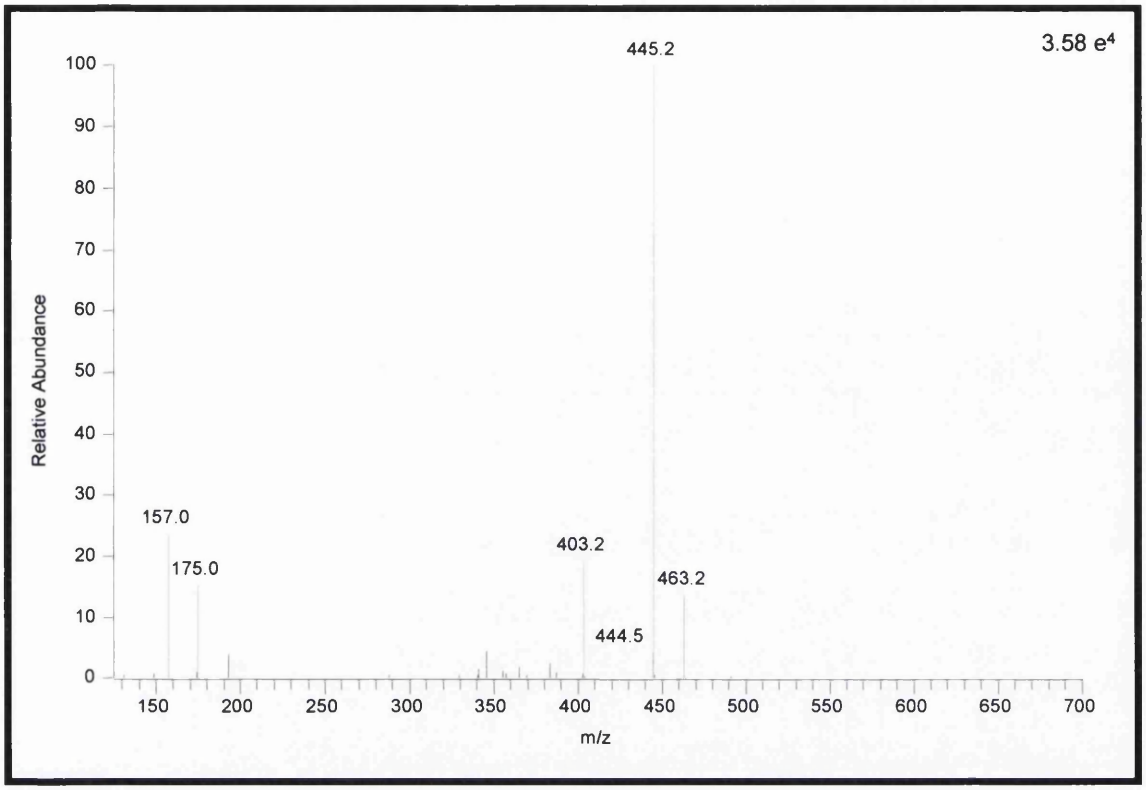



Figure 4.21 Negative LC-ESI-MS-MS spectrum of DHA 3-glucuronide. 10µL injection of a 100ng/mL solution; equal to 1ng on column.

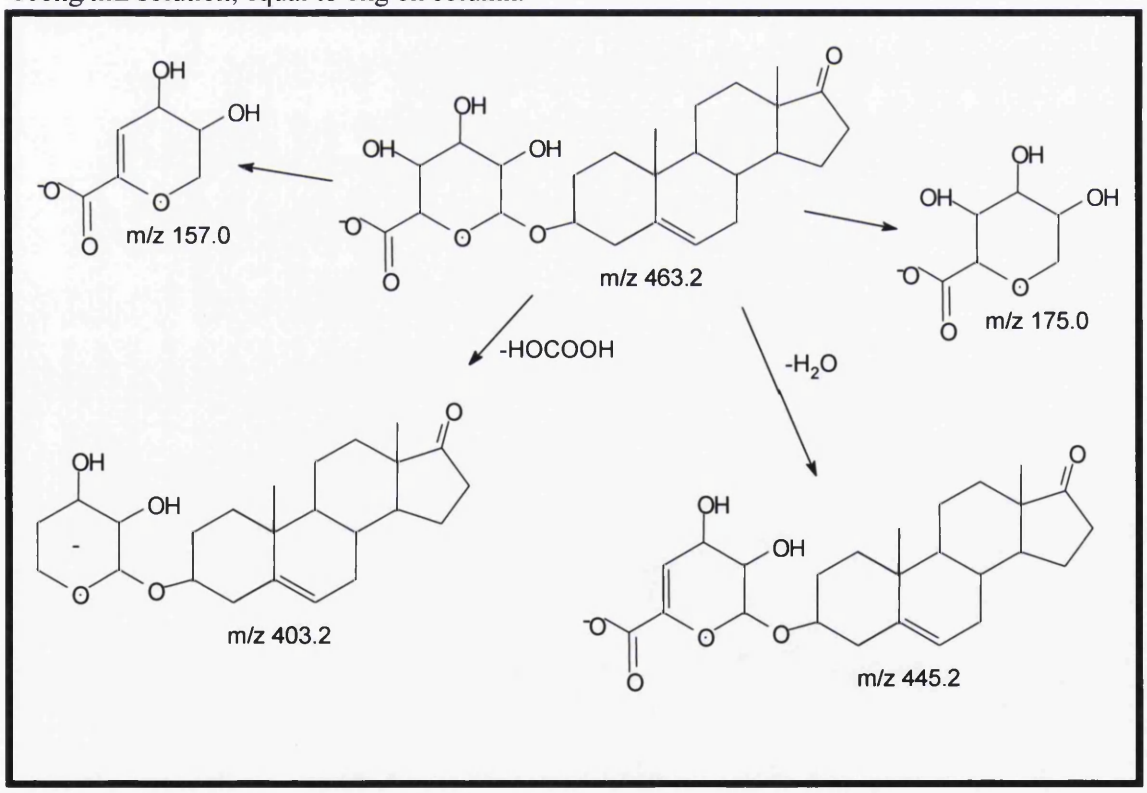



Figure 4.22 Proposed DHA 3-glucuronide fragmentation pattern interpretation.

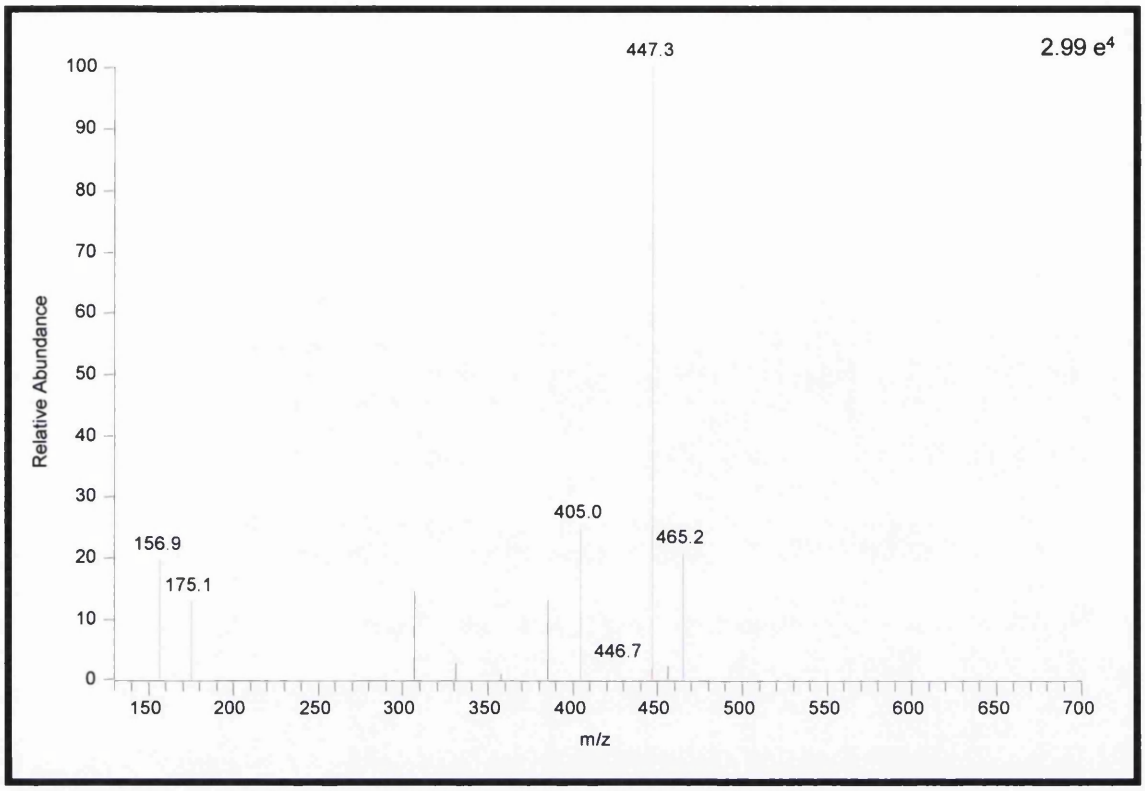



Figure 4.23 Negative LC-ESI-MS-MS spectrum of Androsterone glucuronide. 10µL injection of a 100ng/mL solution; equal to 1ng on column.

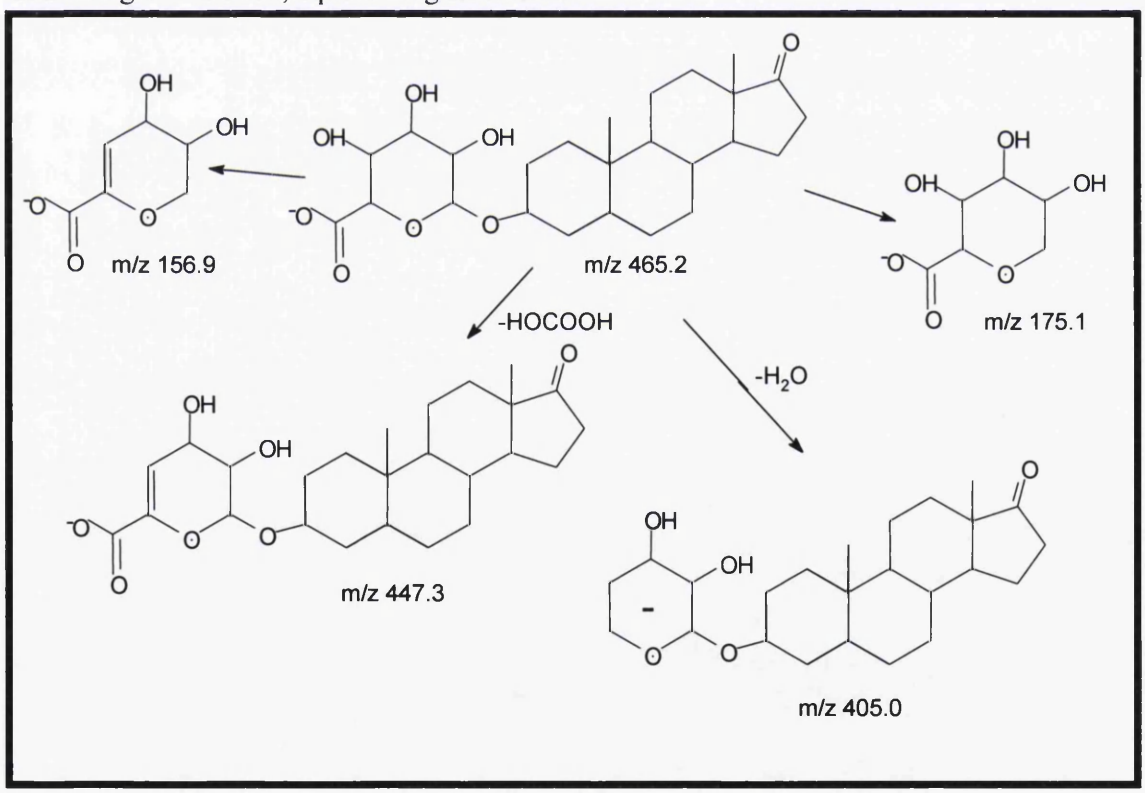



Figure 4.24 Proposed Androsterone glucuronide fragmentation pattern interpretation.

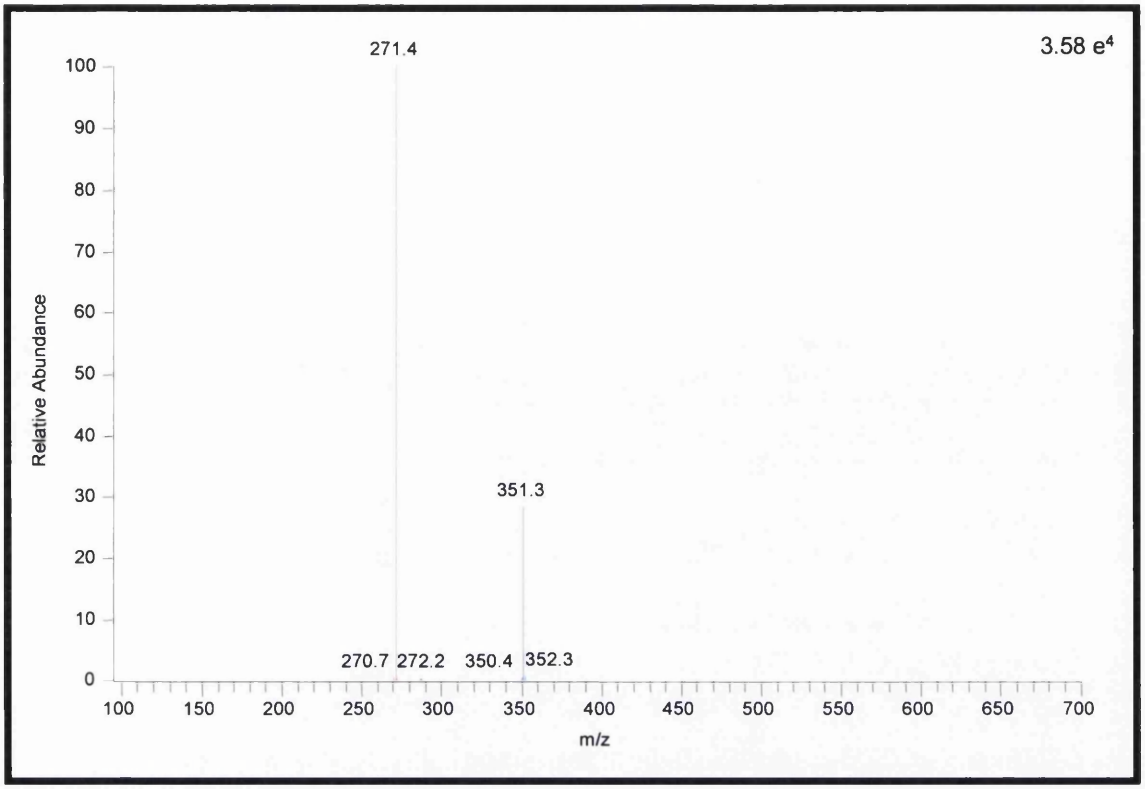



Figure 4.25 Negative LC-ESI-MS-MS spectrum of ß-Estradiol 3-sulfate. 10µL injection of a 100ng/mL solution; equal to 1ng on column.

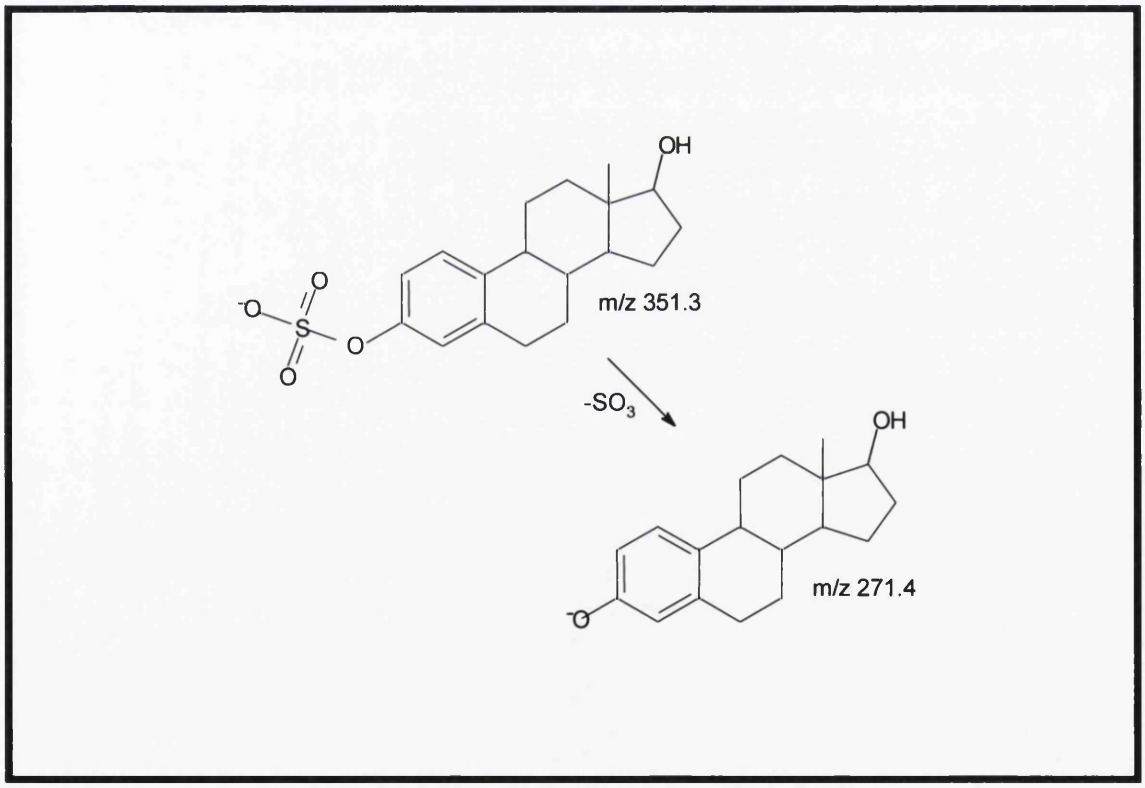



Figure 4.26 Proposed ß-Estradiol 3-sulfate fragmentation pattern interpretation.

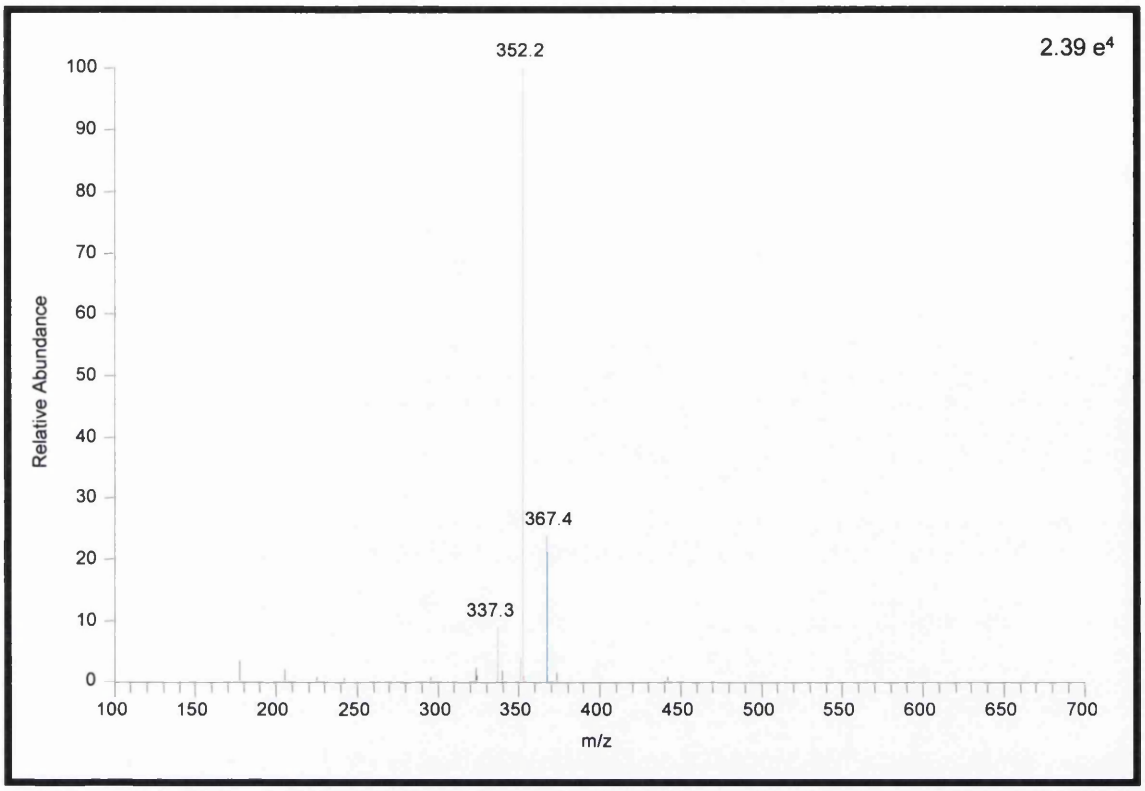



Figure 4.27 Negative LC-ESI-MS-MS spectrum of DHA 3-sulfate. 10µL injection of a 100ng/mL solution; equal to 1ng on column.

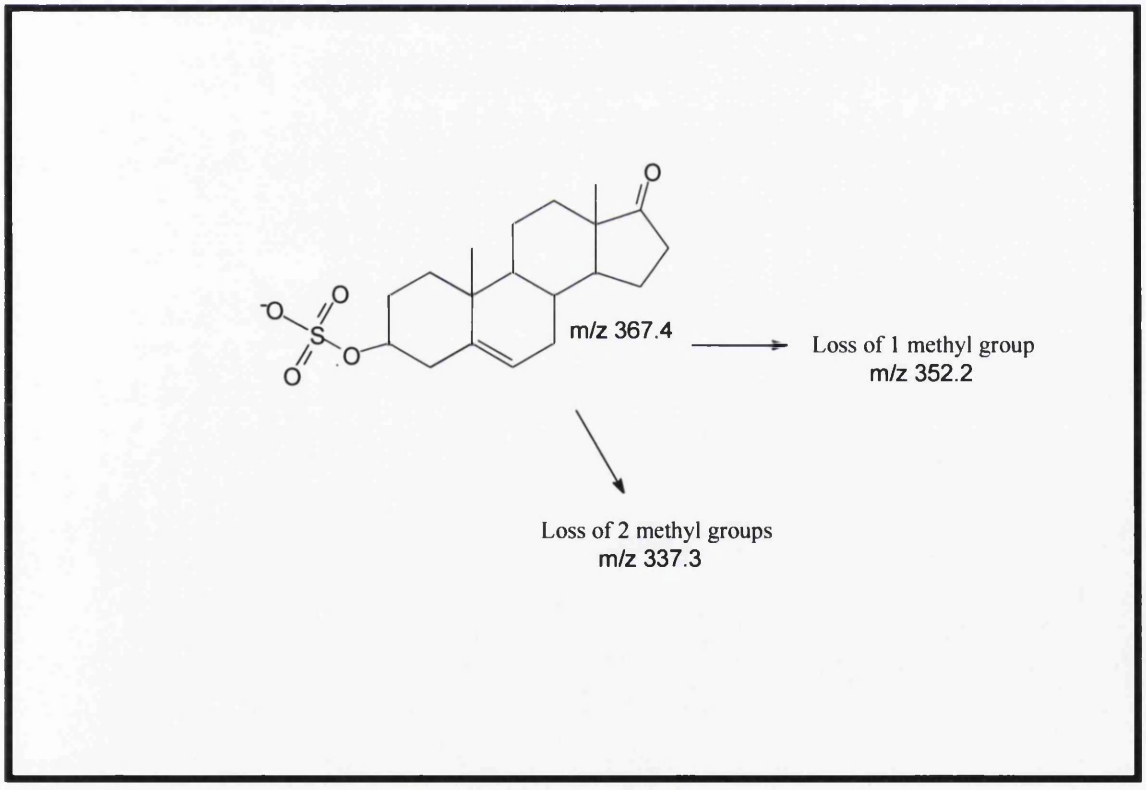



Figure 4.28 Proposed DHA 3-sulfate fragmentation pattern interpretation.

The loss of the methyl groups in the fragmentation of DHA 3-sulfate leads to the formation of a diradical. This is unexpected as free radical ions are very energetic and therefore tend not to have a great deal of stability. As such the formation of a diradical is very unusual.

## Analysis of Equine Urine Samples

Colt urine samples were extracted in the labs of HFL (Cambridgeshire, UK) using SPE. The extraction was performed with Bond Elut C18 LRC 200mg SPE cartridges and the following extraction method was used.

**Extraction Procedure:**

| Step | Procedure |
|---|---|
| Sample Pre-treatment | Urine mixed with an equal volume of ammonium acetate-acetic acid buffer; 0.1mol/L, pH4.5. |
| Column Solvation | Column conditioned with 1mL methanol, followed by 1mL water and finally 2mL ammonium acetate-acetic acid buffer; 15mmol/L, pH4.5. |
| Sample Application | Sample applied to column. |
| Interference Elution | Interferences eluted with 2mL ammonium acetate-acetic acid buffer; 15mmol/L, pH4.5, followed by 2mL methanol/water 2:3 v/v with 15mmol/L ammonium acetate and the same concentration of acetic acid as the previous wash and finally 2mL of water. |
| Analyte Elution | Analytes eluted with 5mL of methanol/water 1:1 v/v. |

HFL supplied us with five colt urine samples that had been extracted using the procedure described above. We received the samples in the form of the 5mL of methanol/water 1:1 v/v solution that had been eluted from the SPE cartridges.

Analysis was performed directly from these with no pre-treatment in our labs. Below is the list of the five samples:

| | |
|---|---|
| Blank one | A blank colt urine sample. |
| Blank two | A blank colt urine sample. |
| Blank three | A blank colt urine sample. |
| U 1 | The first urine voided after a bolus administration of nandrolone to a colt. |
| U 16 | A urine sample voided seven days after an administration of nandrolone phenyl propionate to a colt. |

## Results

Analysis of the samples was performed with negative LC-ESI-MS using the conditions described previously in this chapter with a view to determining the presence of the steroid conjugates of interest and also with the methodology we developed previously for the analysis of free and conjugated estrogen steroids described in chapter 3. Unknown peaks in the total ion chromatograms we obtained from our analysis were investigated for the presence of losses associated with sulfate and glucuronide conjugated steroids.

The analysis we performed with the methodology developed in chapter 3 gave us positive results for the presence of estrone 3-sulfate in all the samples except Blank three. Figure 4.29 shows example extracted ion chromatograms for the base ion of estrone 3-sulfate and the m/z associated with loss of the sulfate group from this

conjugate and figure 4.30 the mass spectrum; both results were obtained from the analysis of Blank one.

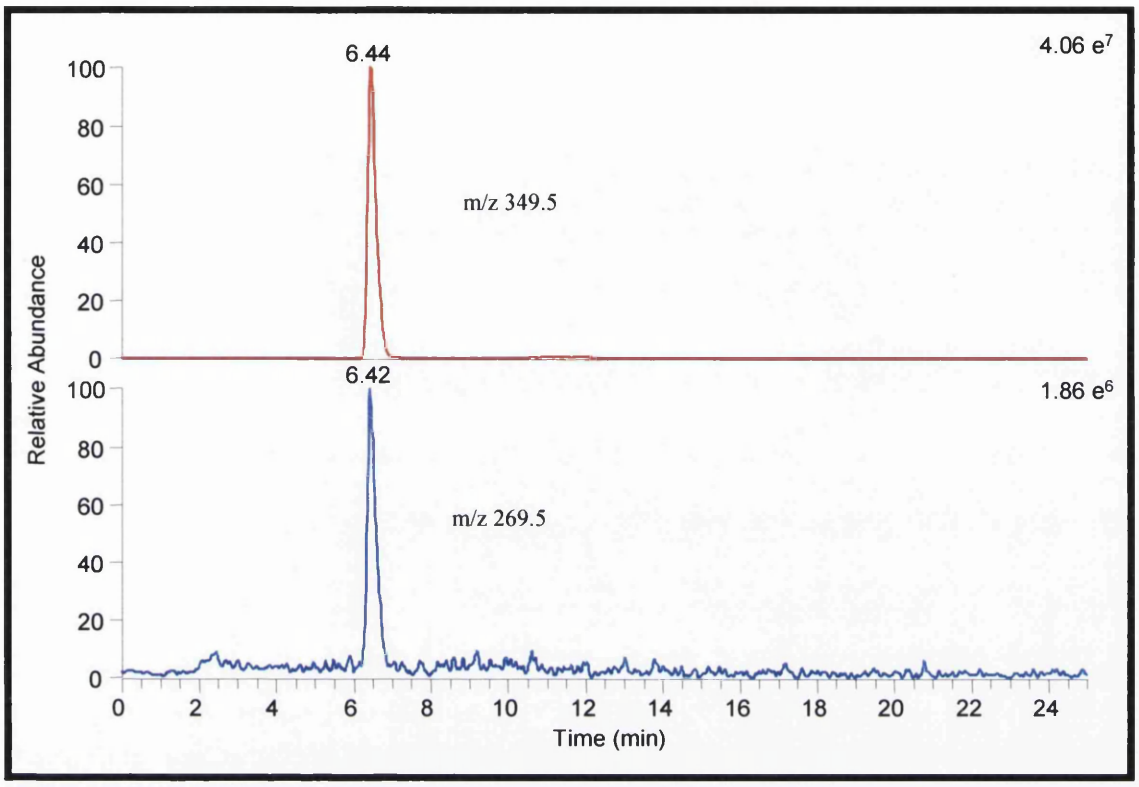



Figure 4.29 Negative LC-ESI-MS extracted ion chromatograms of the Estrone 3-sulfate result from Blank one.

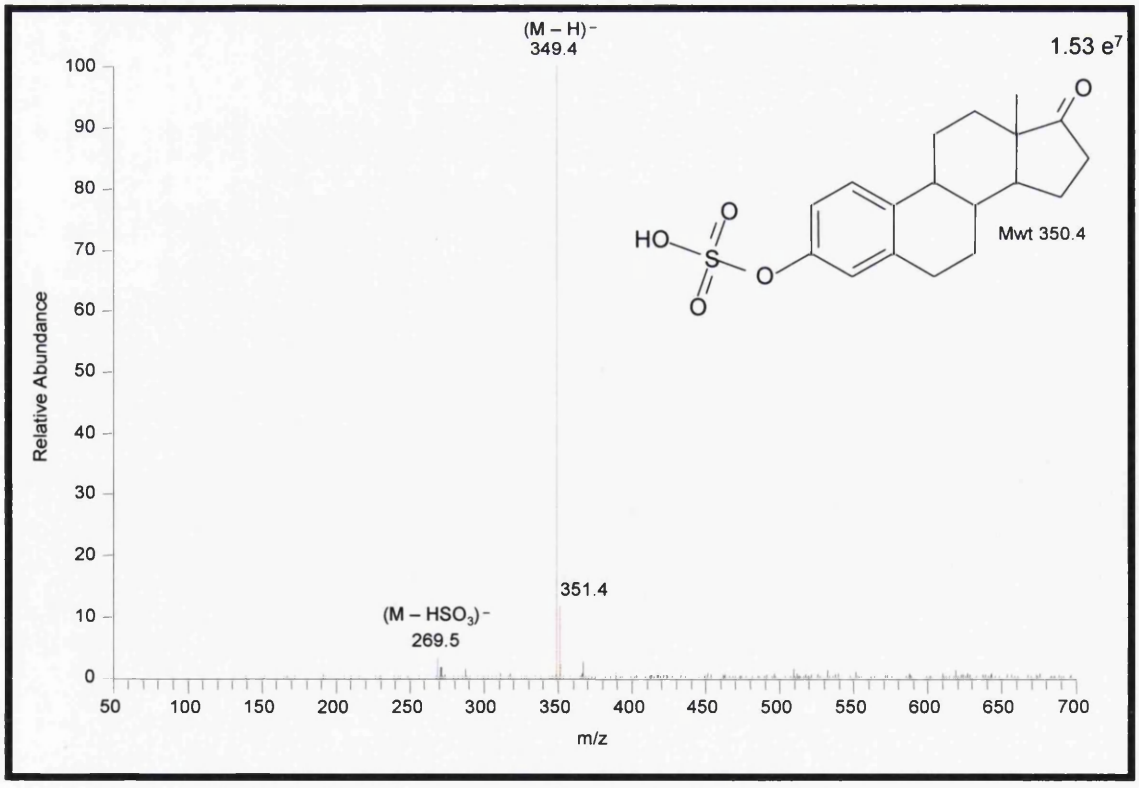



Figure 4.30 Negative LC-ESI-MS spectrum of Estrone 3-sulfate result from Blank one.

The results of the analysis of the five equine urine samples performed with the conditions described earlier in this chapter are presented over the following pages in the form of the total ion chromatograms obtained for each sample with tabulated results and example mass spectra.

**Blank one – urine sample from an undoped colt**

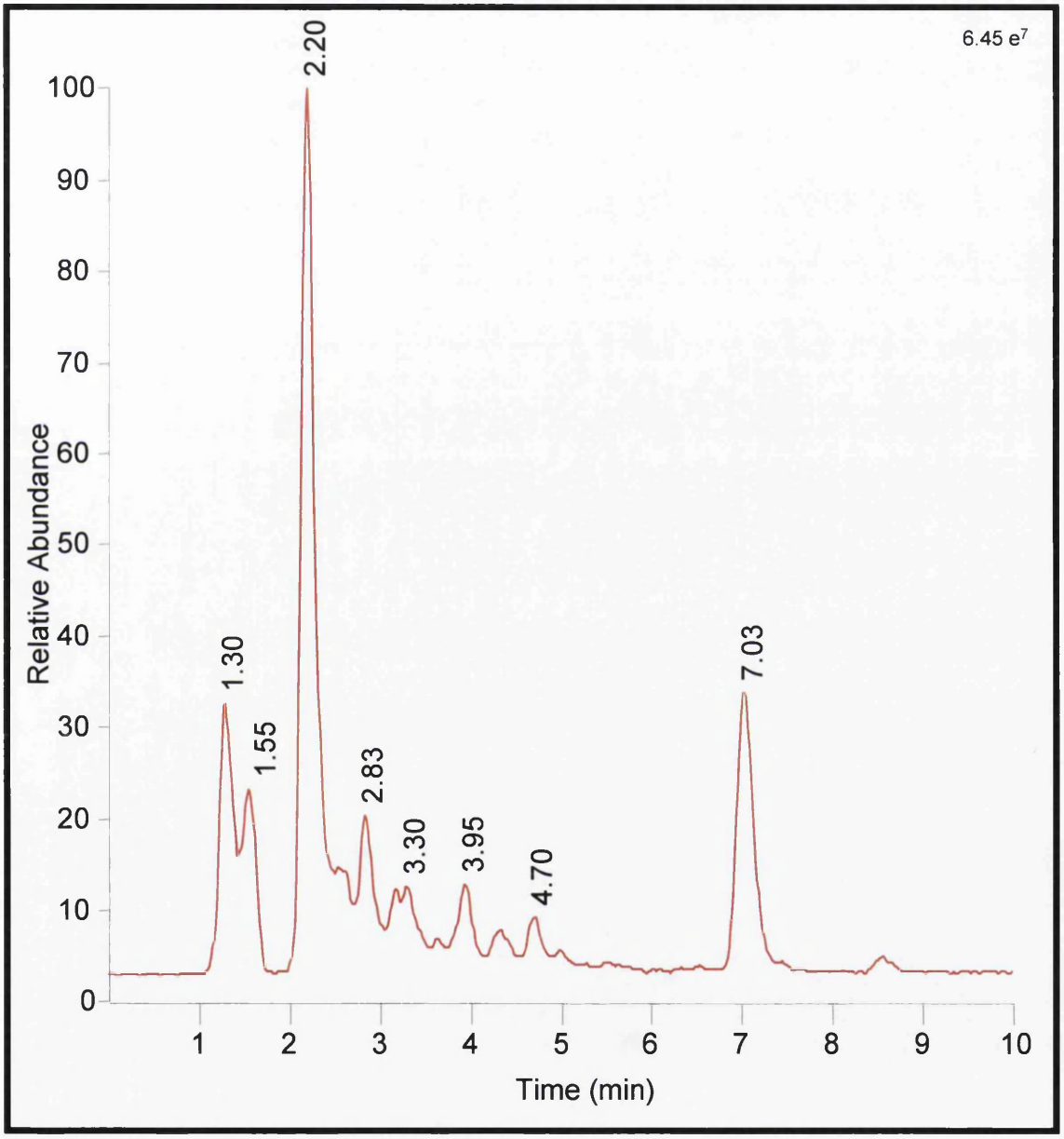



Figure 4.31 Negative LC-ESI-MS total ion chromatogram of sample Blank one.

| Retention Time in Minutes | m/z values | Identification |
|---|---|---|
| 2.83 | 575.4, 493.4, 317.6 | Losses associated with glucuronide sulfate conjugate. |
| 3.30 | 573.4, 491.4, 315.6 | Losses associated with glucuronide sulfate conjugate. |
| 3.95 | 351.4, 271.6 | β-Estradiol 3-sulfate |
| 3.95 | 369.3, 289.3 | Co-eluting with β Estradiol 3-sulfate. Loss associated with sulfate conjugate. |
| 4.70 | 351.4, 271.5 | Loss associated with sulfate conjugate. |
| 7.03 | 349.4, 269.5 | Loss associated with sulfate conjugate. |

Figures 4.32 and 4.33 show example mass spectra and proposed fragmentation pattern interpretations taken from the TIC of Blank one for the peaks occurring at 2.83 and 3.95 minutes.

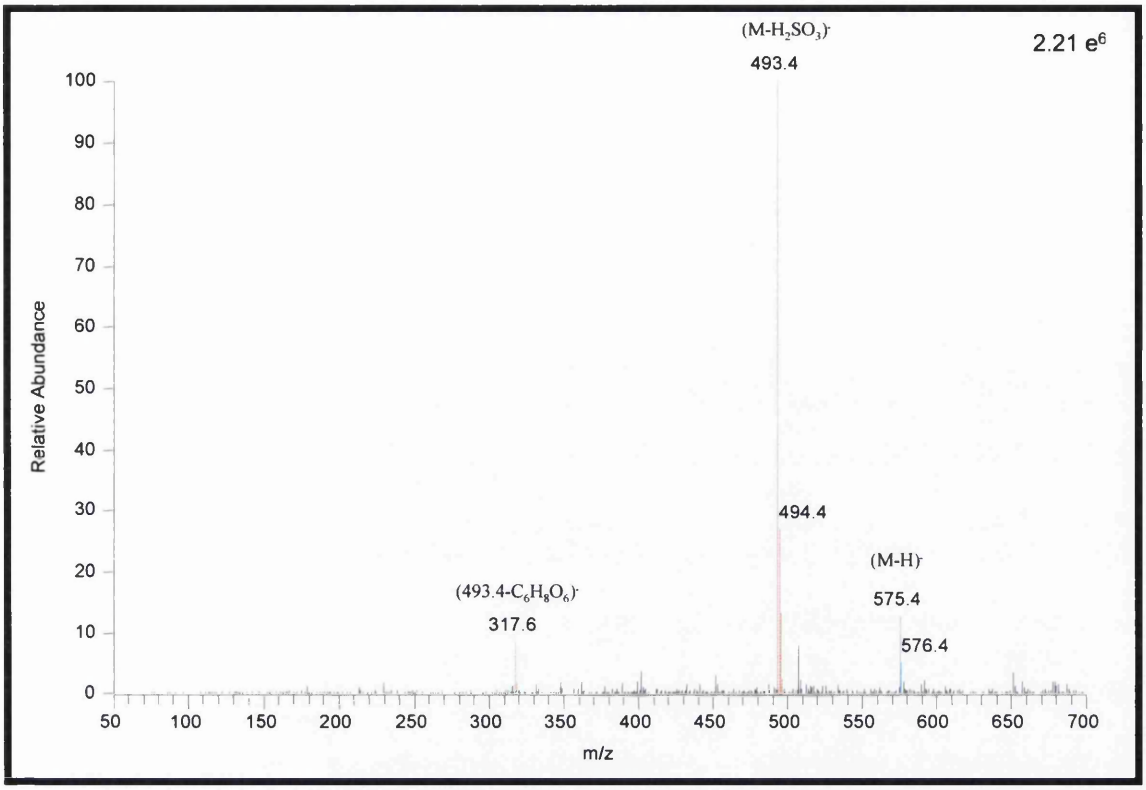



Figure 4.32 Negative LC-ESI-MS mass spectrum of peak at 2.83 minutes in TIC of Blank one.

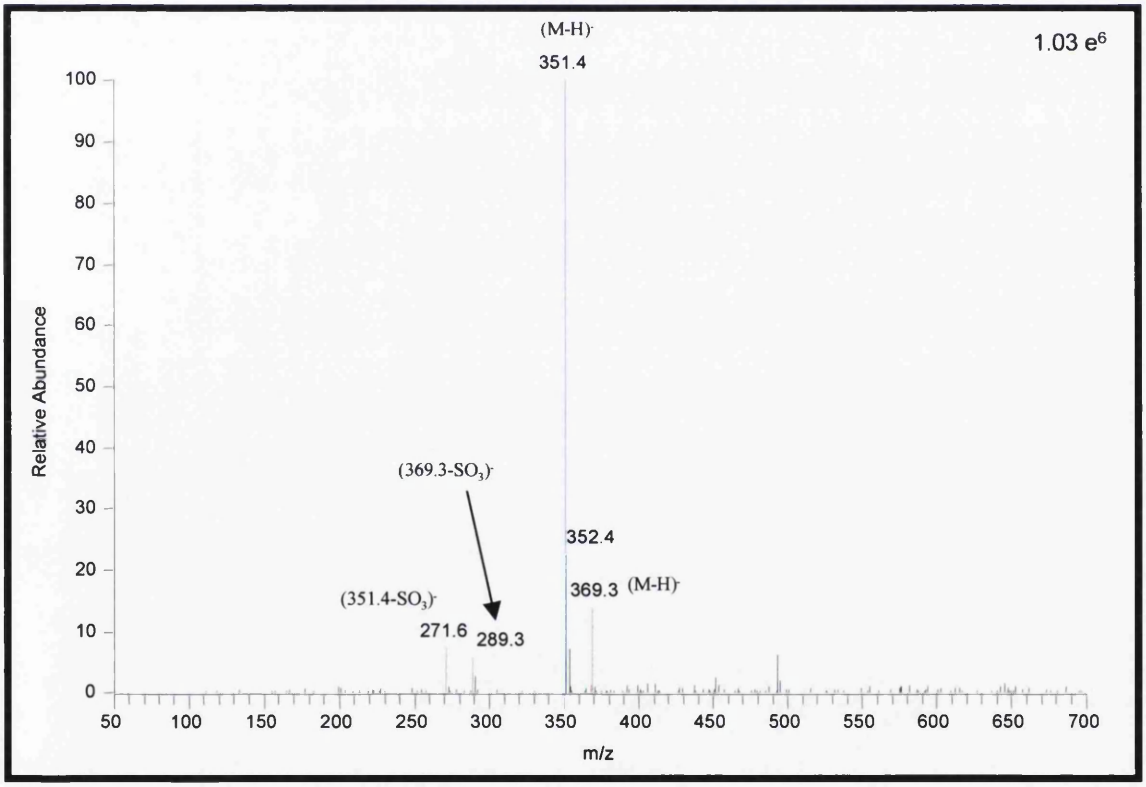



Figure 4.33 Negative LC-ESI-MS mass spectrum of peak at 3.95 minutes in TIC of Blank one.

**Blank two – urine sample from an undoped colt**

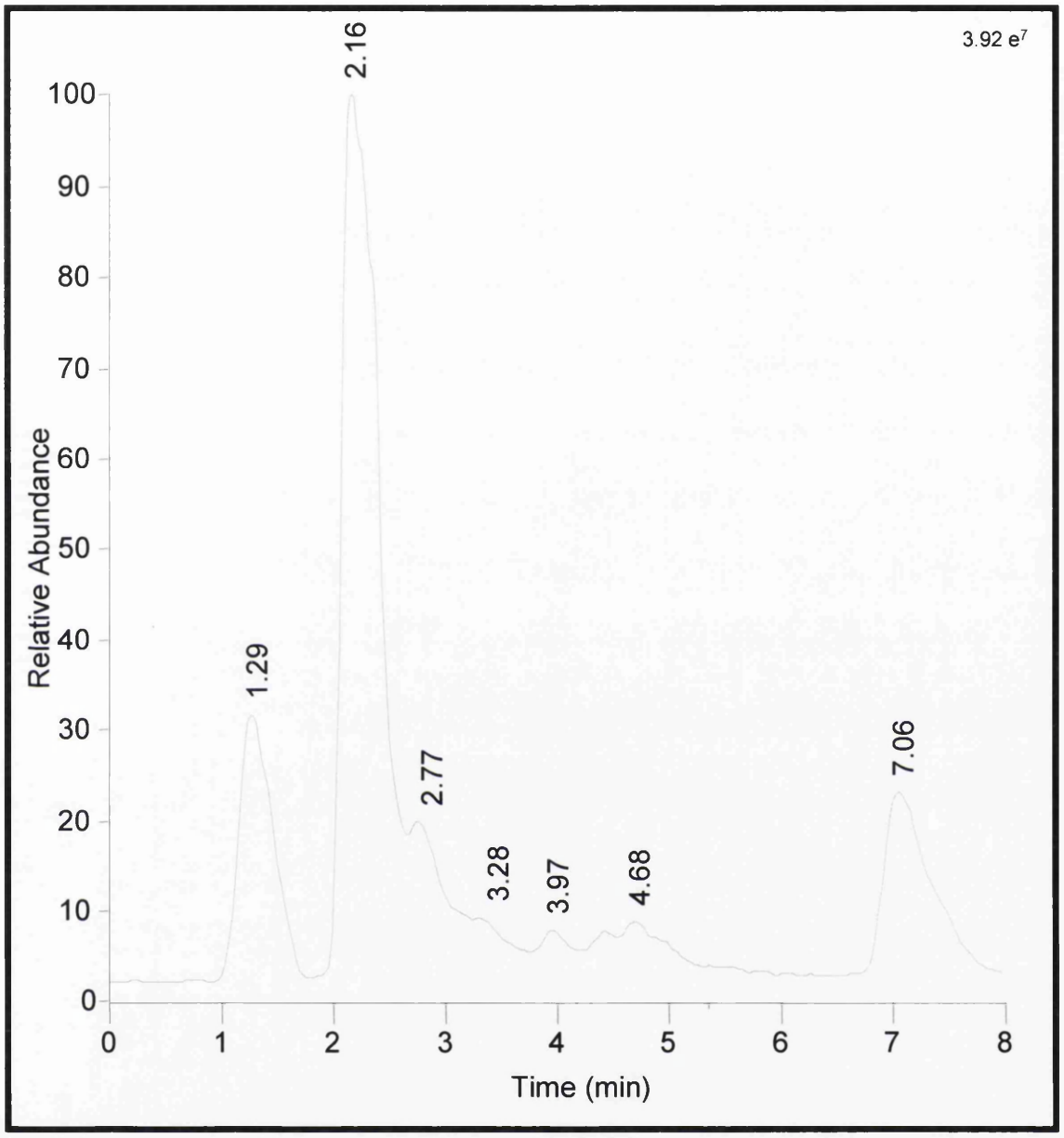



Figure 4.34 Negative LC-ESI-MS total ion chromatogram of sample Blank two.

| Retention Time in Minutes | m/z values | Identification |
|---|---|---|
| 3.28 | 573.2, 491.4, 315.7 | Losses associated with glucuronide sulfate conjugate. |
| 3.97 | 351.4, 271.6 | β-Estradiol 3-sulfate |
| 4.68 | 351.4, 271.6 | Loss associated with sulfate conjugate. |
| 4.68 | 377.4, 297.5 | Loss associated with sulfate conjugate. |
| 7.06 | 349.4, 269.6 | Loss associated with sulfate conjugate. |
| 7.06 | 365.3, 285.6 | Loss associated with sulfate conjugate. |

Figures 4.35 and 4.36 show example mass spectra and proposed fragmentation pattern interpretations taken from the TIC of Blank two for the peaks occurring at 3.97 and 7.06 minutes.

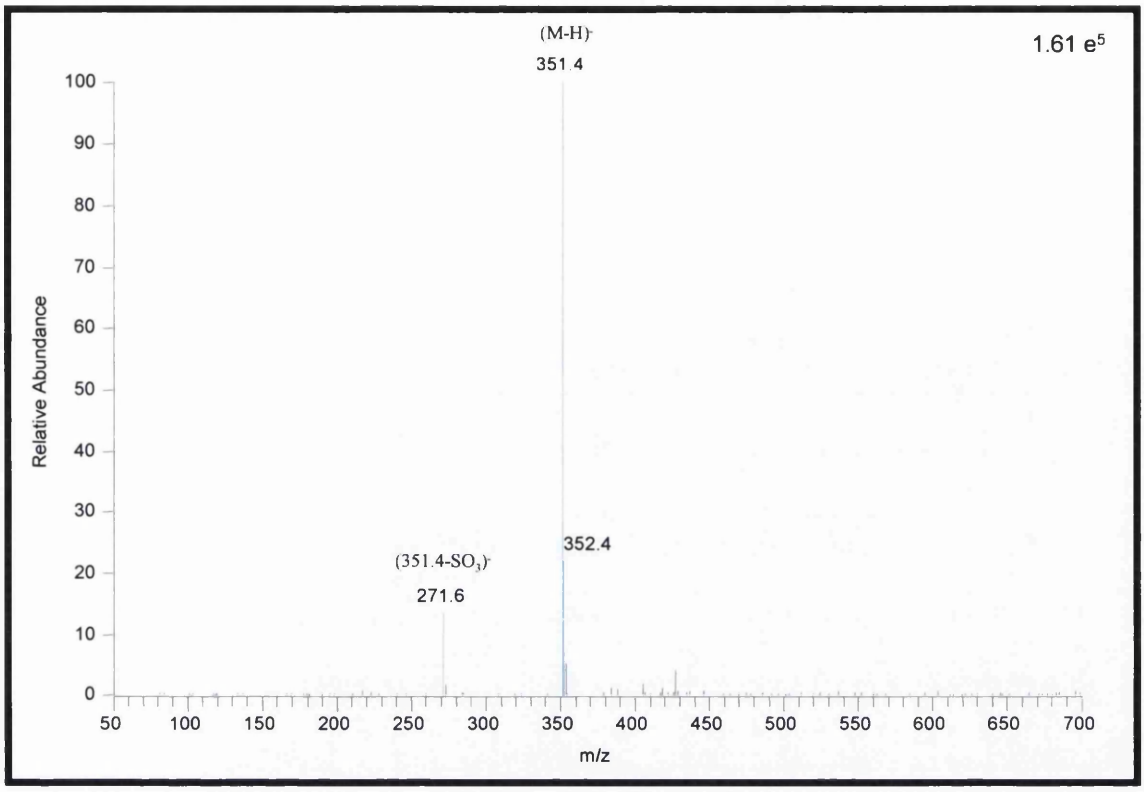



Figure 4.35 Negative LC-ESI-MS mass spectrum of peak at 3.97 minutes in TIC of Blank two.

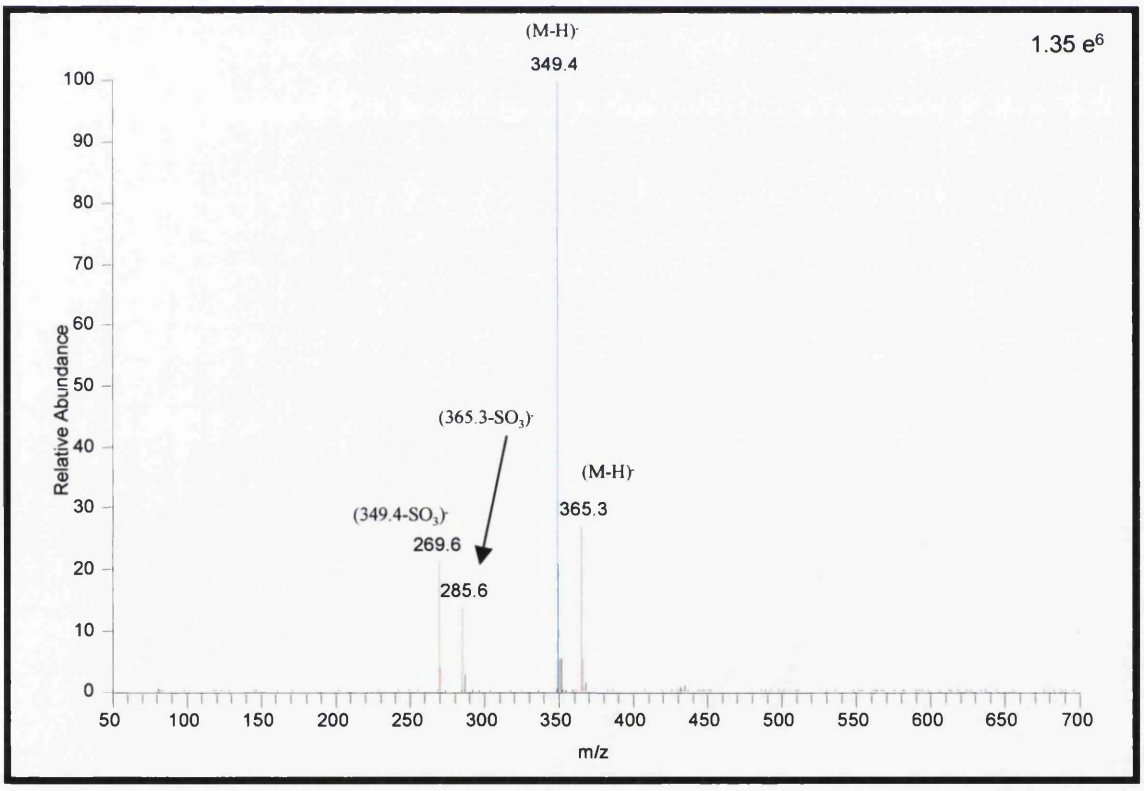



Figure 4.36 Negative LC-ESI-MS mass spectrum of peak at 7.06 minutes in TIC of Blank two.

**Blank three – urine sample from an undoped colt**

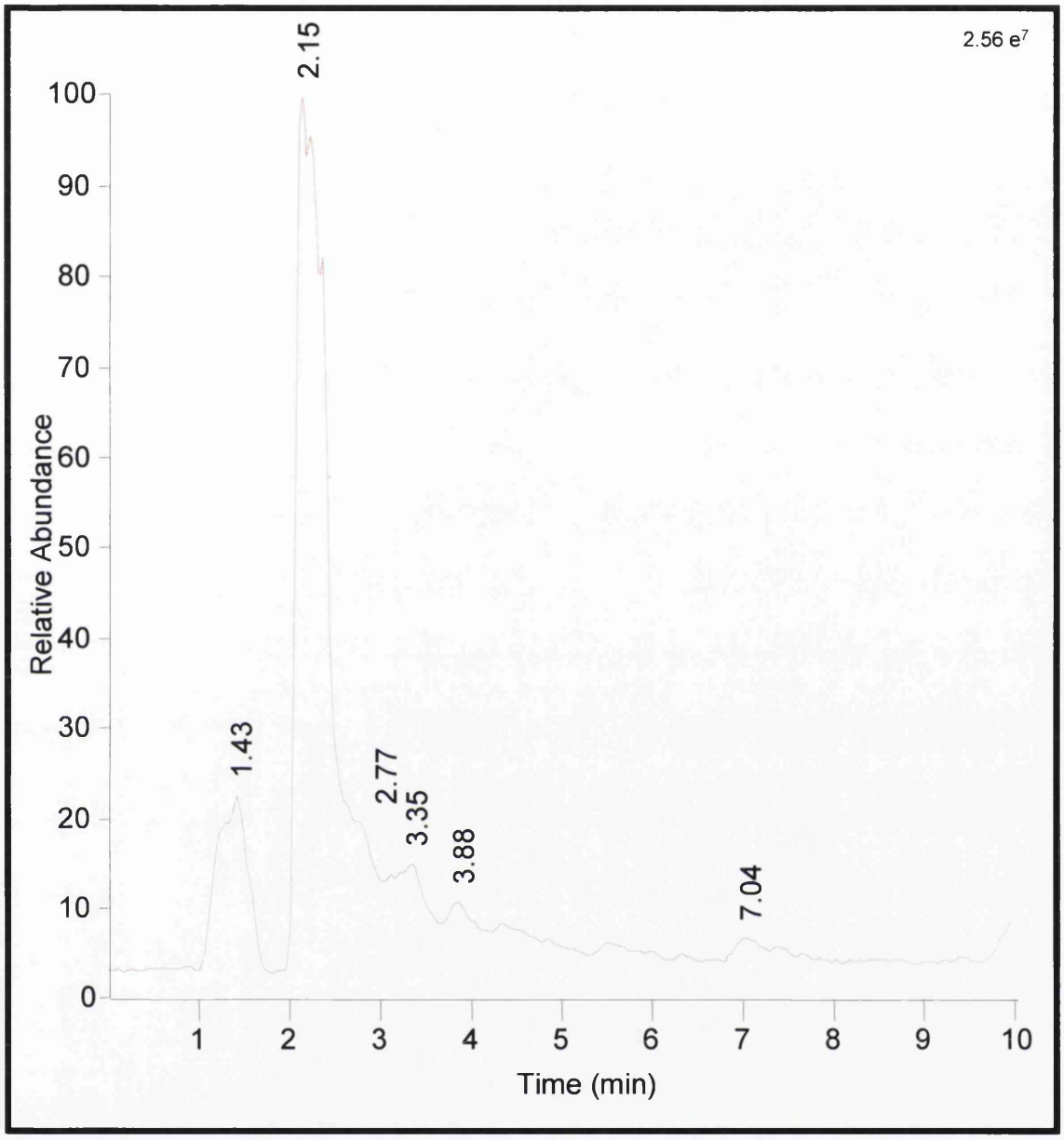



Figure 4.37 Negative LC-ESI-MS total ion chromatogram of sample Blank three.

| Retention Time in Minutes | m/z values | Identification |
|---|---|---|
| 2.77 | 575.2, 493.4, 317.6 | Losses associated with glucuronide sulfate conjugate. |
| 3.35 | 573.4, 491.3, 315.6 | Losses associated with glucuronide sulfate conjugate. |
| 3.88 | 369.3, 289.5 | Loss associated with sulfate conjugate. |
| 7.04 | 381.1, 301.3 | Loss associated with sulfate conjugate. |

Figures 4.38 and 4.39 show example mass spectra and proposed fragmentation pattern interpretations taken from the TIC of Blank three for the peaks occurring at 3.35 and 3.88 minutes.

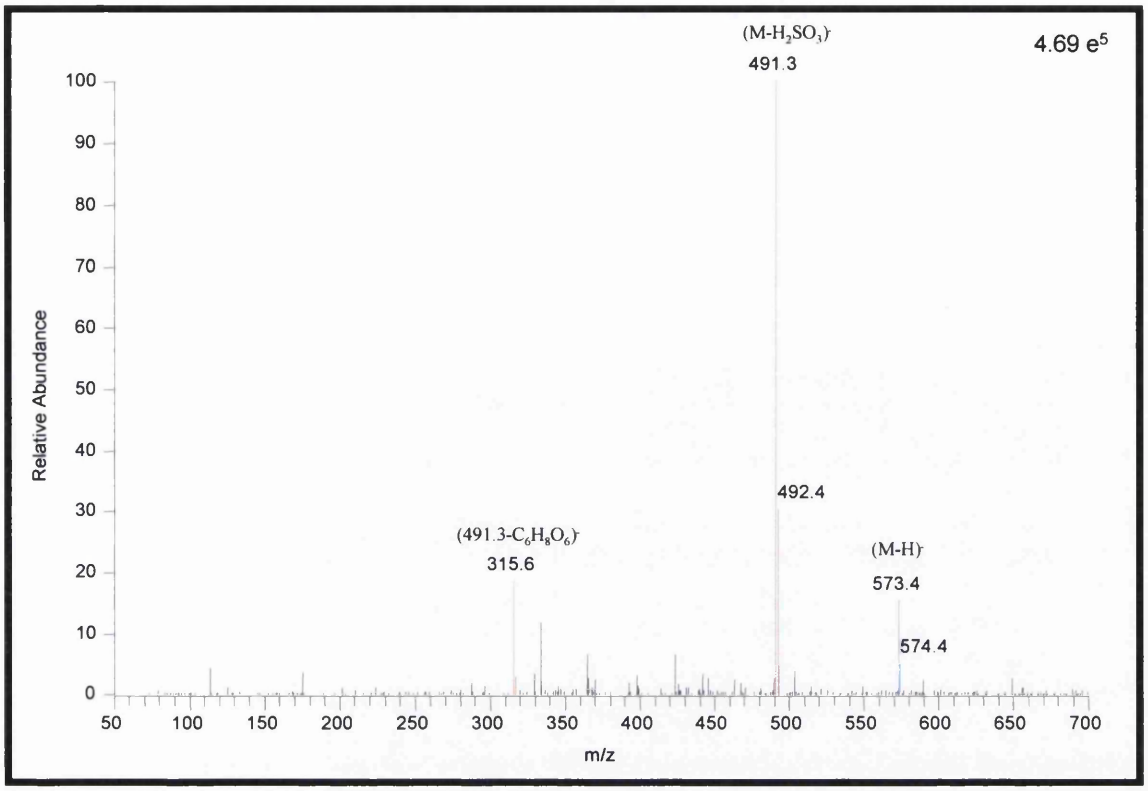



Figure 4.38 Negative LC-ESI-MS mass spectrum of peak at 3.35 minutes in TIC of Blank three.

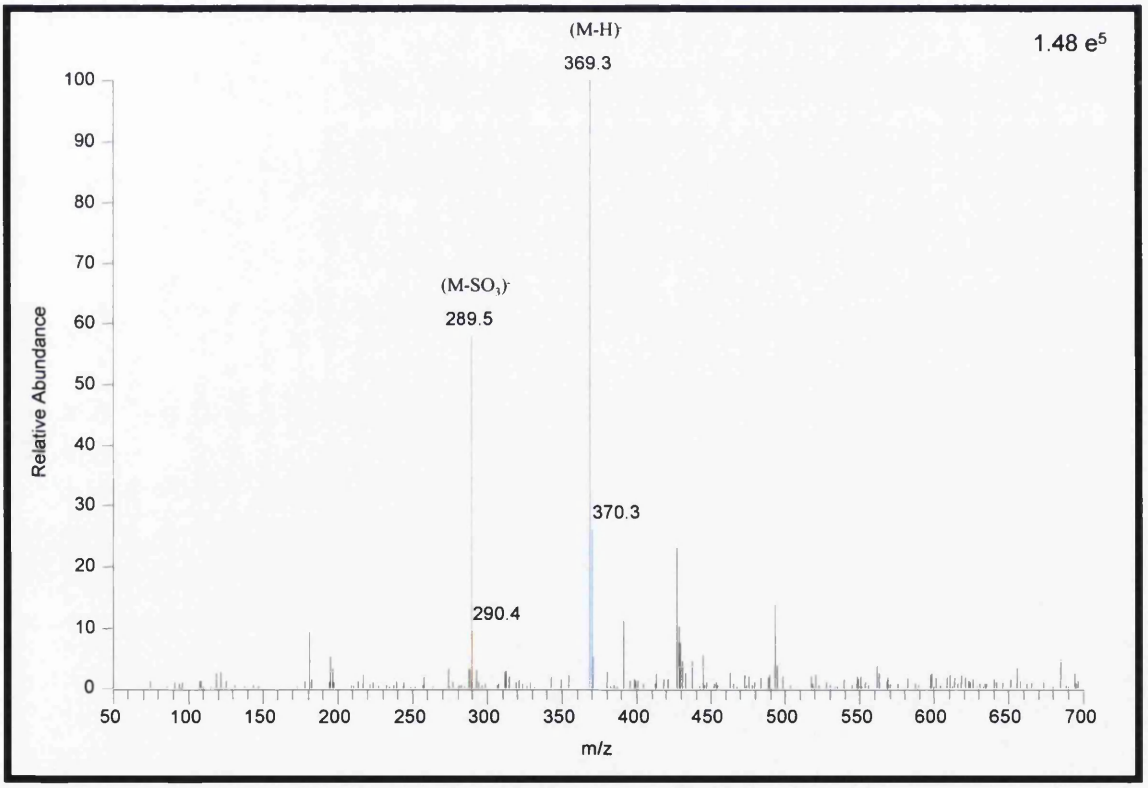



Figure 4.39 Negative LC-ESI-MS mass spectrum of peak at 3.88 minutes in TIC of Blank three.

**U 1 – first urine sample voided after administration of nandrolone to a colt**

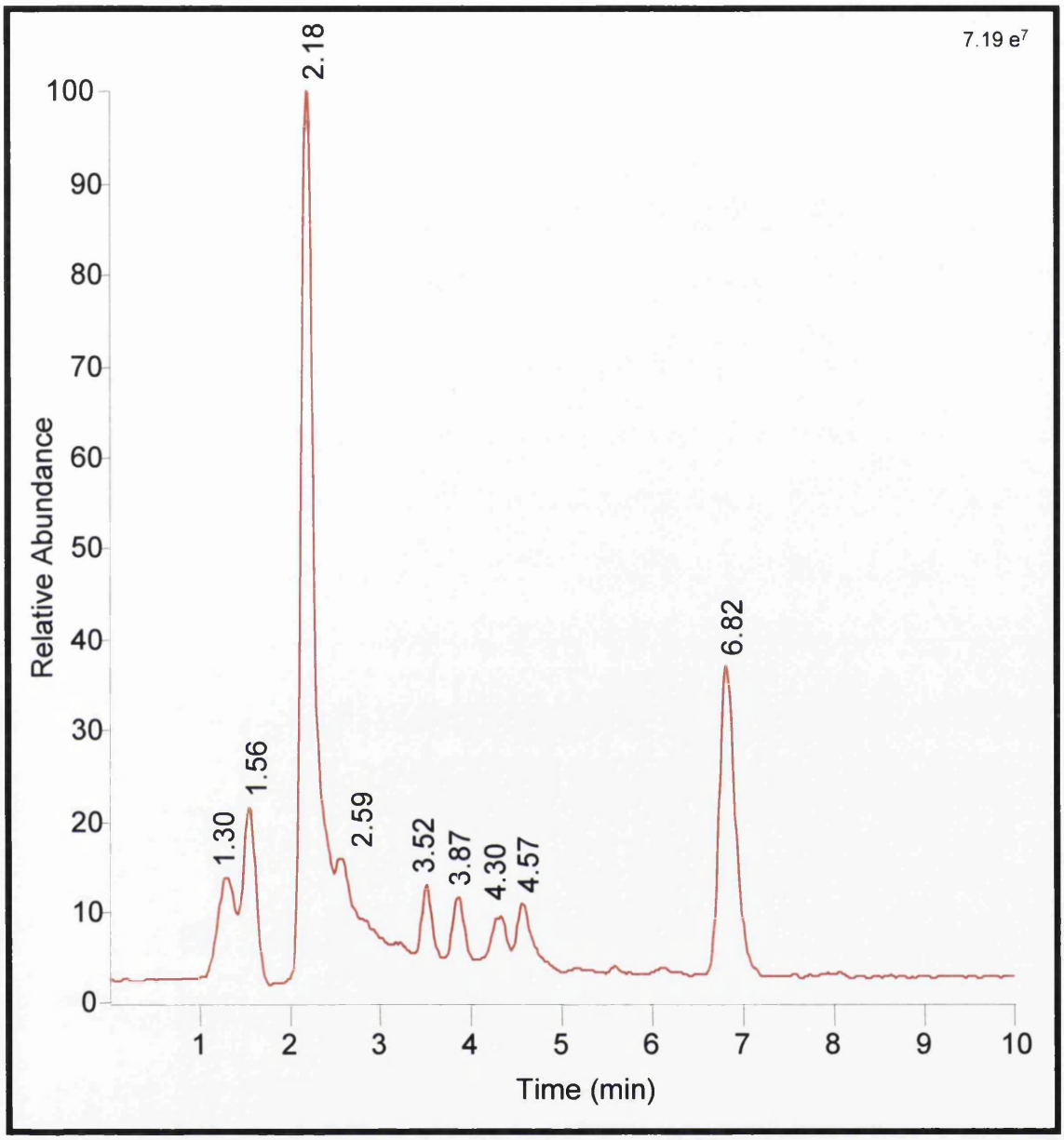



Figure 4.40 Negative LC-ESI-MS total ion chromatogram of sample U1.

| Retention Time in Minutes | m/z values | Identification |
|---|---|---|
| 3.52 | 353.6 | Nandrolone sulfate |
| 3.87 | 351.4, 271.6 | β-Estradiol 3-sulfate |
| 3.87 | 369.3, 289.5 | Co-eluting with β-Estradiol 3-sulfate. Loss associated with sulfate conjugate. |
| 4.30 | 367.6 | DHA 3-sulfate |
| 4.30 | 576.4, 494.7, (409.3, 233.5) | Co-eluting with DHA 3-sulfate. Loss associated with sulfate conjugate, (possible glucuronide loss). |
| 4.57 | 351.4, 271.6 | Loss associated with sulfate conjugate. |
| 6.82 | 349.4, 269.5 | Loss associated with sulfate conjugate. |

Figures 4.41 and 4.42 show example mass spectra and proposed fragmentation pattern interpretations taken from the TIC of U1 for the peaks occurring at 3.52 and 4.30 minutes.

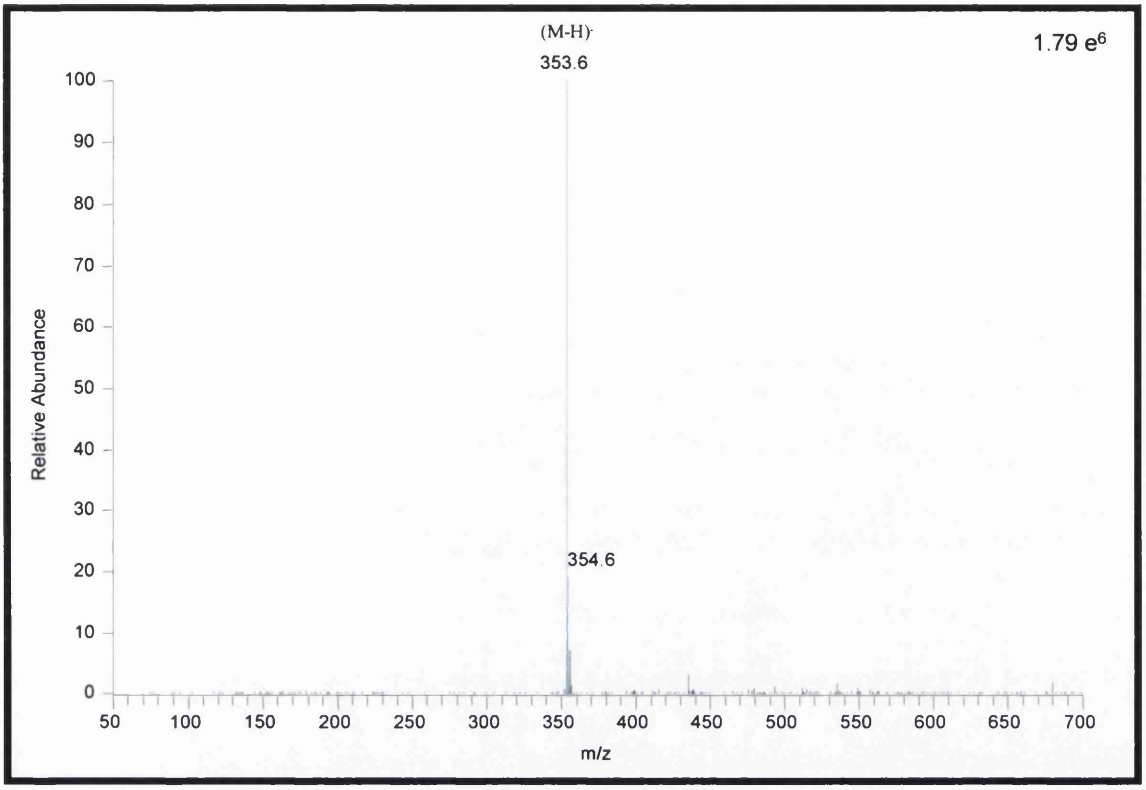



Figure 4.41 Negative LC-ESI-MS mass spectrum of peak at 3.52 minutes in TIC of U1.

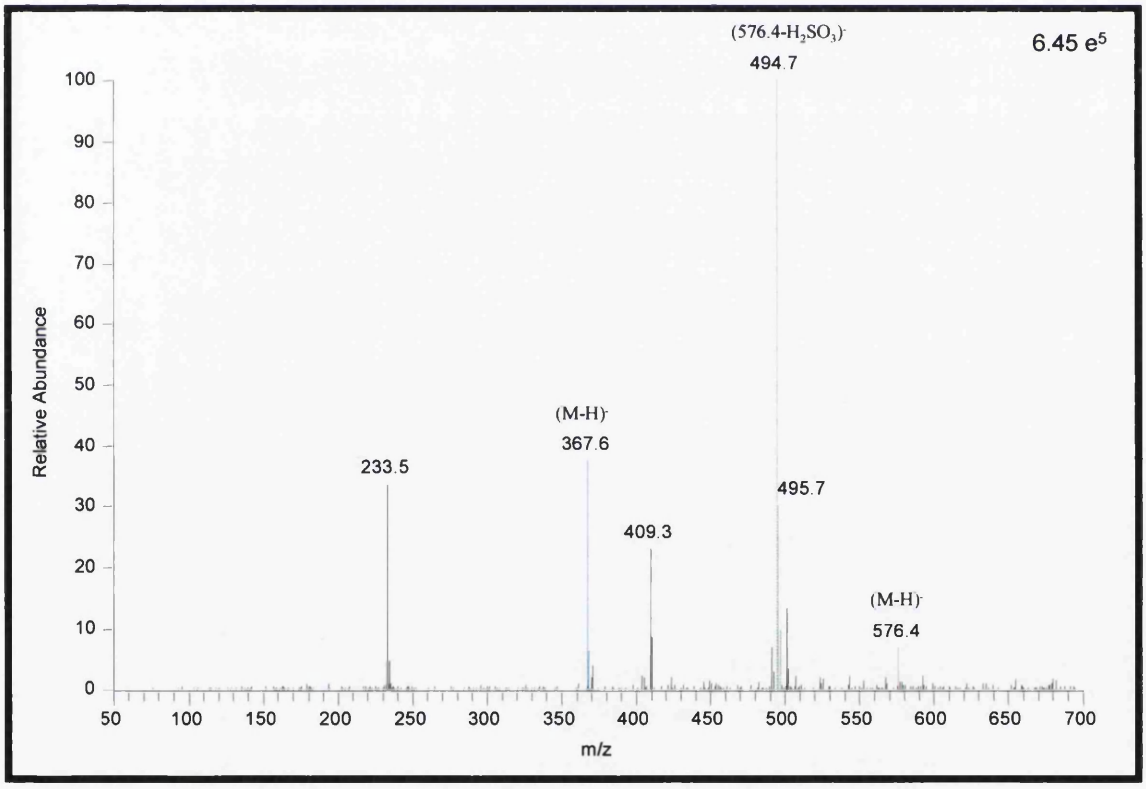



Figure 4.42 Negative LC-ESI-MS mass spectrum of peak at 4.30 minutes in TIC of U1.

**U 16 – urine sample voided seven days after administration of nandrolone phenyl propionate to a colt**

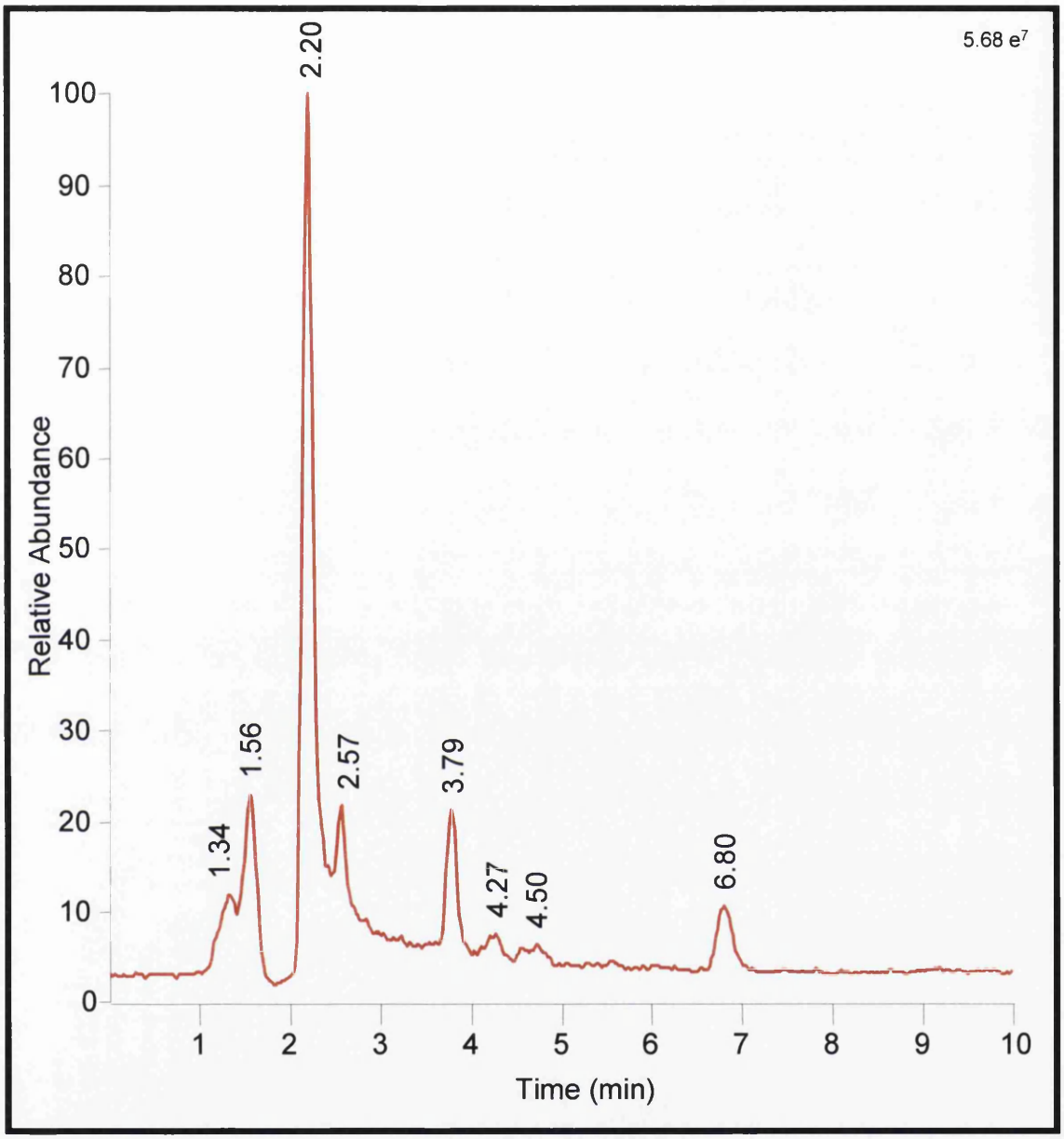



Figure 4.43 Negative LC-ESI-MS total ion chromatogram of sample U16.

| Retention Time in Minutes | m/z values | Identification |
|---|---|---|
| 3.50* | 353.5 | Nandrolone sulfate |
| 3.79 | 351.4 | β-Estradiol 3-sulfate |
| 3.79 | 369.3, 289.4 | Co-eluting with β-Estradiol 3-sulfate. Loss associated with sulfate conjugate. |
| 4.50 | 351.4, 271.6 | Loss associated with sulfate conjugate. |
| 6.80 | 349.4, 269.5 | Loss associated with sulfate conjugate. |

*The nandrolone sulfate peak cannot be seen in the total ion chromatogram as there is only a low response; however it does appear when an extracted ion chromatogram is taken from the TIC (see figure 4.44).

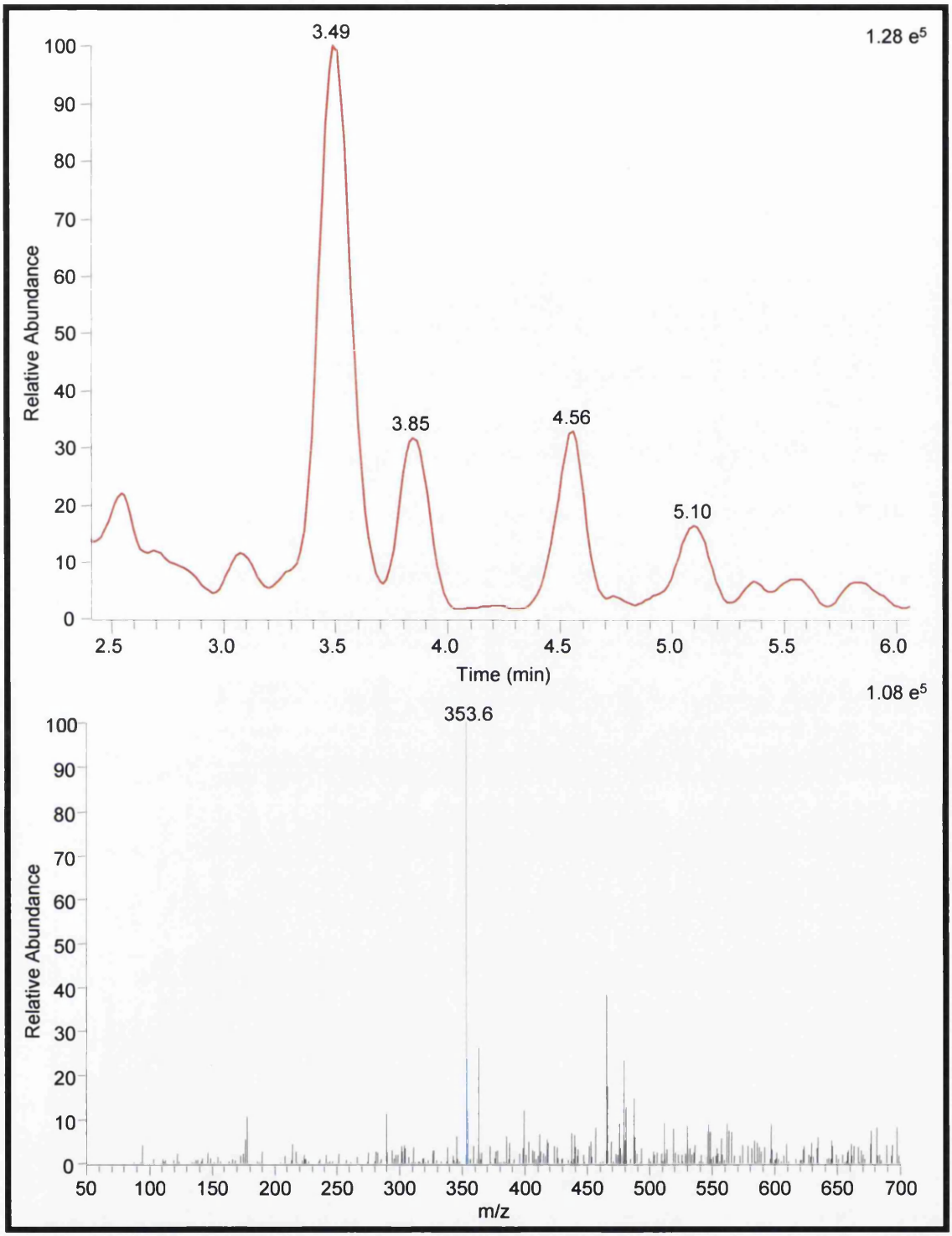



Figure 4.44 Negative LC-ESI-MS extracted ion chromatogram and mass spectrum of nandrolone sulfate taken from TIC of U16.

Figure 4.45 shows an example mass spectrum and proposed fragmentation pattern interpretation taken from the TIC of U16 for the peak occurring at 4.50 minutes.

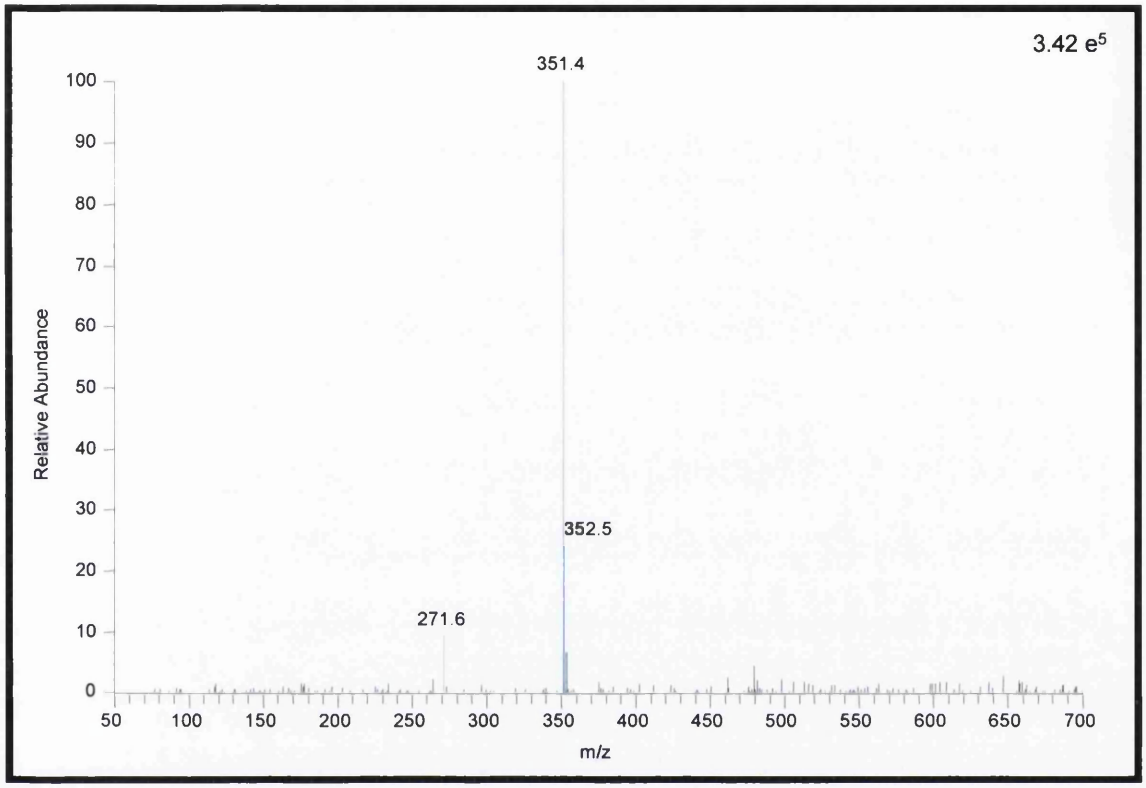



Figure 4.45 Negative LC-ESI-MS mass spectrum of peak at 4.50 minutes in TIC of U16.

## Interpretation of Unknown Responses Showing Possible Conjugate Losses

Without the use of known standards it is not possible to state the identity of the compounds that give rise to the unknown peaks in the equine urine analysis that we performed with any great degree of certainty. However by taking into account the predominant m/z values present in the peaks the following steroid conjugates have been proposed as potential candidates.

**Unknown Peak 1-2.8 minutes**

Figure 4.46 below shows the fragmentation pattern interpretation proposed for the m/z values resulting from the unknown peak occurring at 2.8 minutes.

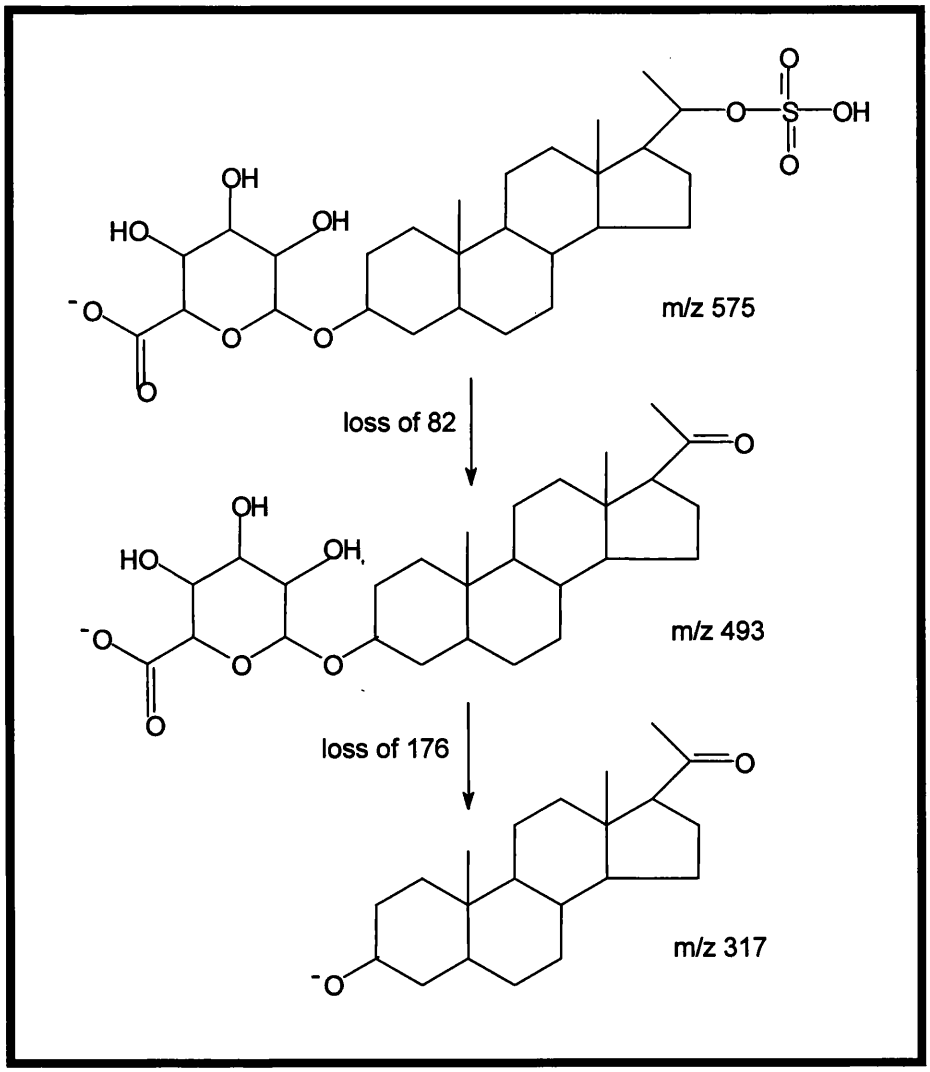



Figure 4.46 Proposed fragmentation pattern interpretation of peak occurring at 2.8 minutes.

This corresponds to the steroid conjugate pregnanediol 3-glucuronide 20-sulfate. Pregnanediol is known to be one of the major metabolites of progesterone in humans and is predominantly excreted in human urine as a glucuronide conjugate (2, 3).

**Unknown Peak 2-3.3 minutes**

Figure 4.47 below shows the fragmentation pattern interpretation proposed for the m/z values resulting from the unknown peak occurring at 3.3 minutes.

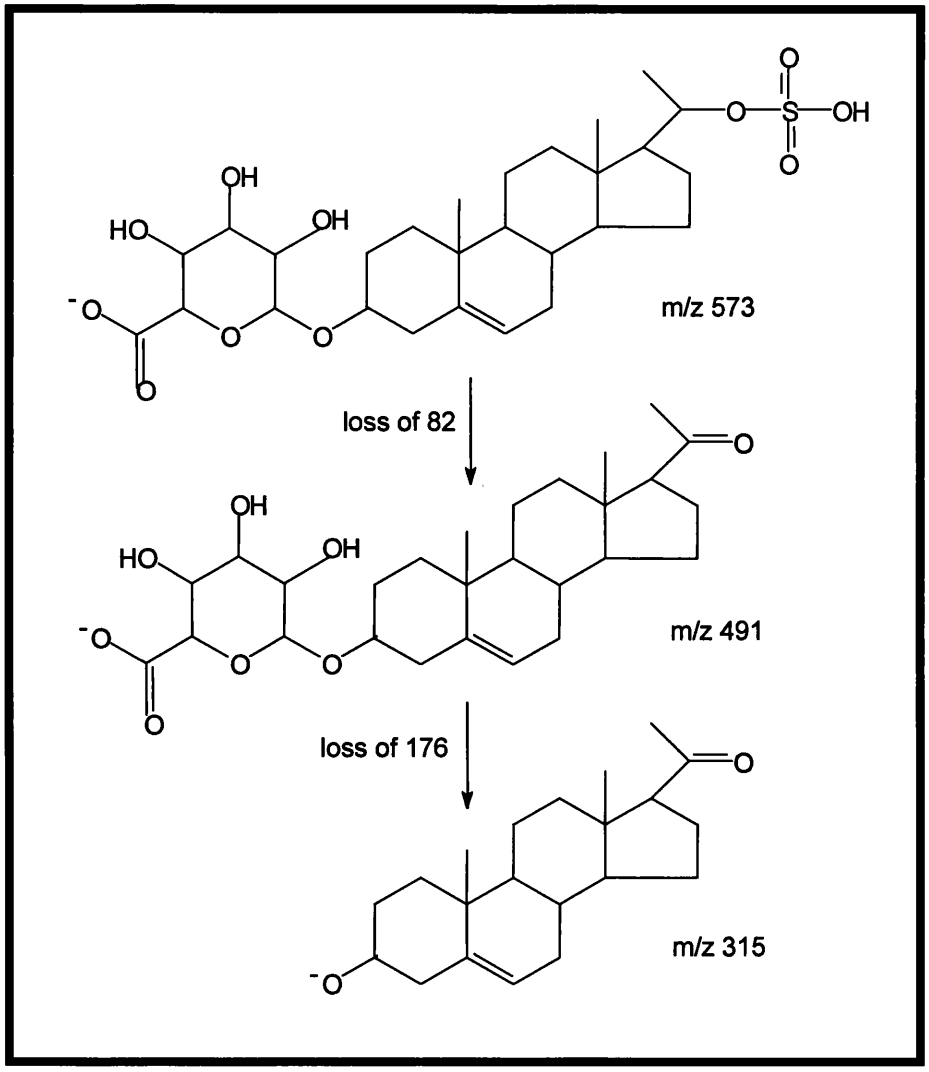



Figure 4.47 Proposed fragmentation pattern interpretation of peak occurring at 3.3 minutes.

This corresponds to the steroid conjugate pregnenediol 3-glucuronide 20-sulfate. Pregnenolone is involved in the biosynthetic pathway of the androgens in humans and its main metabolites are pregnenediol sulfate and pregnanediol glucuronide. 20β-hydroxy 4-pregnen-3-one is also a metabolite of progesterone in humans (2, 3).

**Unknown Peak 3-3.8 minutes**

Figure 4.48 below shows the fragmentation pattern interpretation proposed for the m/z values resulting from the unknown peak occurring at 3.8 minutes.

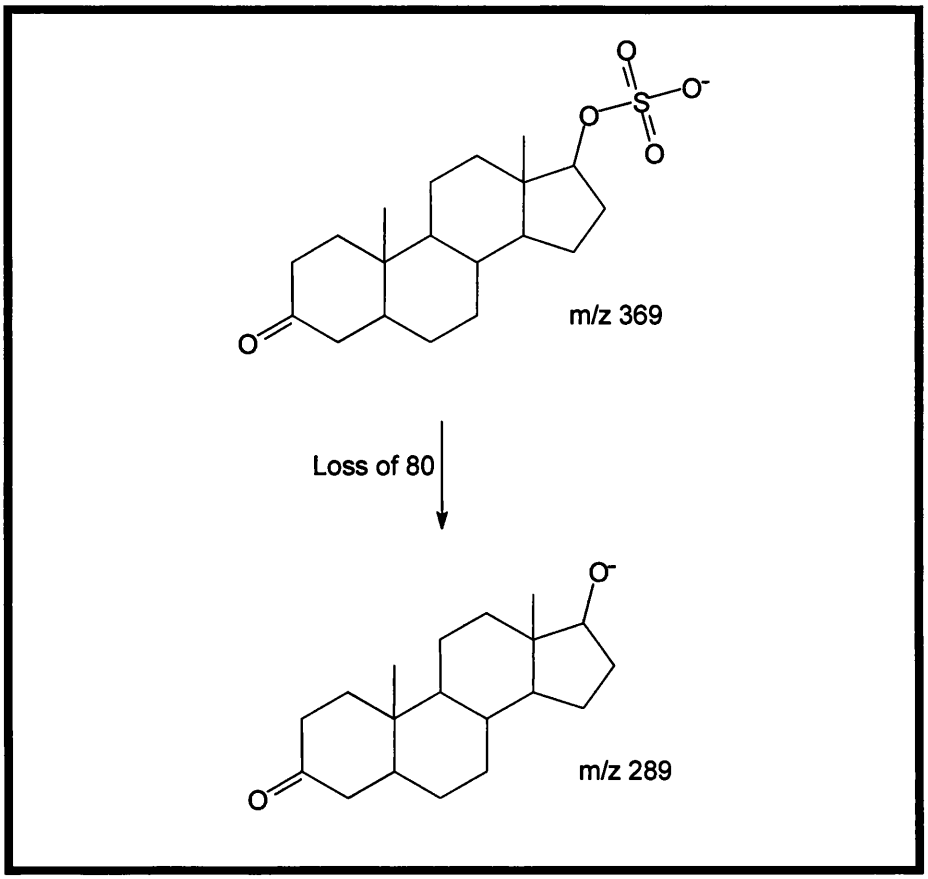



Figure 4.48 Proposed fragmentation pattern interpretation of peak occurring at 3.8 minutes.

This corresponds to the steroid conjugate androsterone sulfate. Our analysis was developed to allow for the analysis of androsterone glucuronide and in man this is the major form of androsterone found in urine, however approximately 10% of the androsterone found in human urine is in the form of the sulfate conjugate (2, 3) and in equine urine the sulfate conjugate predominates (1).

**Unknown Peak 4-4.5 minutes**

Figure 4.49 below shows one of the fragmentation pattern interpretations proposed for the m/z values resulting from the unknown peak occurring at 4.5 minutes and figure 4.50 the other.

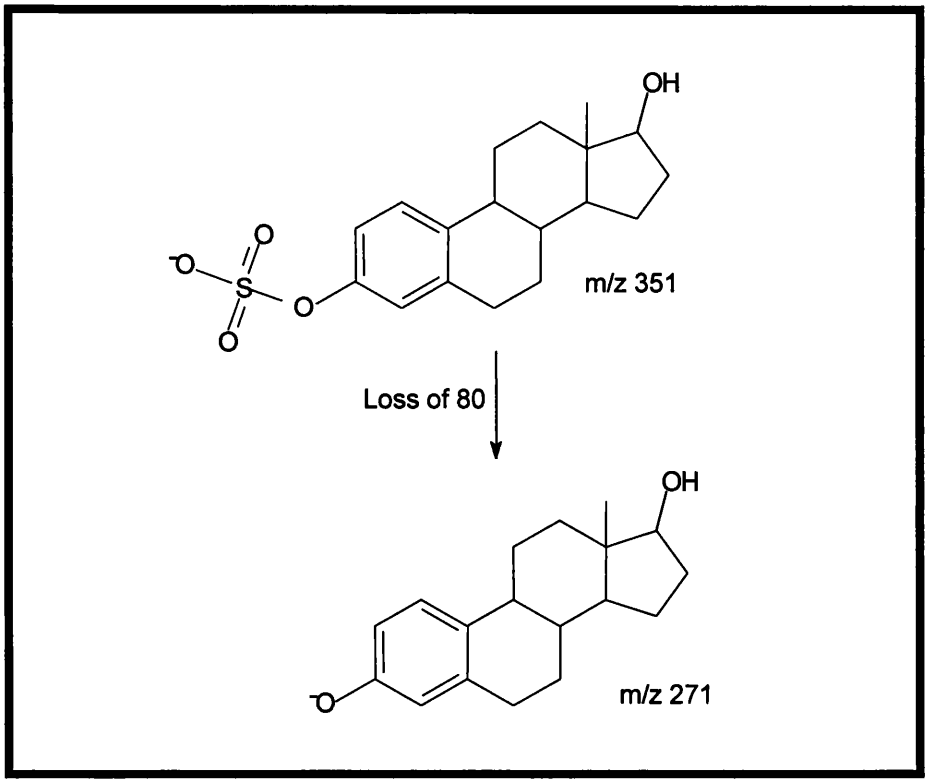



Figure 4.49 Proposed fragmentation pattern interpretation 1 of peak occurring at 4.5 minutes.

This corresponds to estradiol 3-sulfate. In all of the samples except Blank three there is a positive result for the presence of β-estradiol 3-sulfate; the peak at 4.5 minutes may be due to the presence of α-estradiol 3-sulfate.

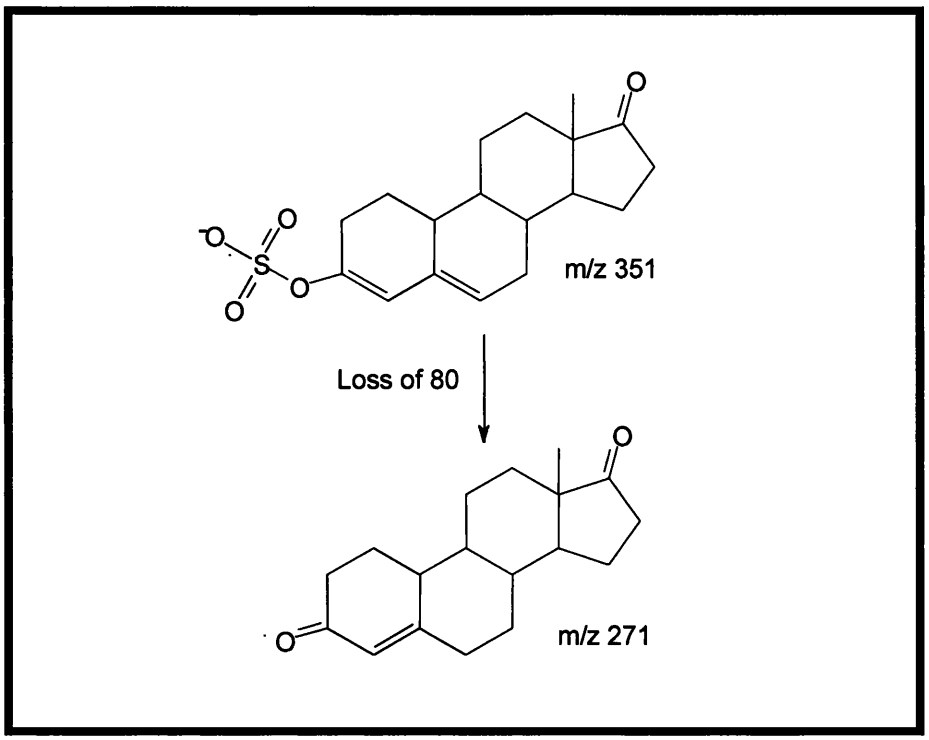



Figure 4.50Proposed fragmentation pattern interpretation 2 of peak occurring at 4.5 minutes.

This corresponds to the 19-norandrostenedione enol-sulfate conjugate that has been proposed as a possible source of endogenous nandrolone in colts (1).

**Unknown Peak 5-7.0 minutes**

Figure 4.51 on the following page shows the fragmentation pattern interpretation proposed for the m/z values 349 and 269 and figure 4.52 the fragmentation pattern interpretation proposed for the m/z values 365 and 285 resulting from the unknown peak occurring at 7.0 minutes.

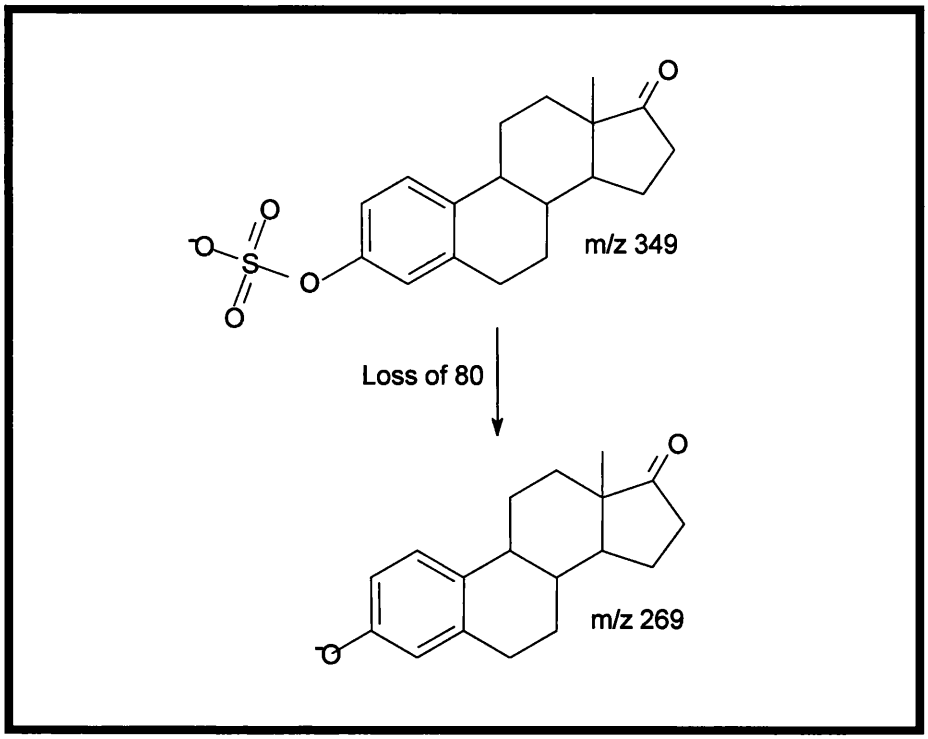



Figure 4.51 Proposed fragmentation pattern interpretation of peak occurring at 7.0 minutes.

This corresponds to estrone 3-sulfate. As our analysis using the methodology described previously in chapter 3 gave us positive results for the presence of estrone 3-sulfate in all the samples except Blank three and this result appears in all the samples except Blank three it seems likely that estrone 3-sulfate is the source of this result.

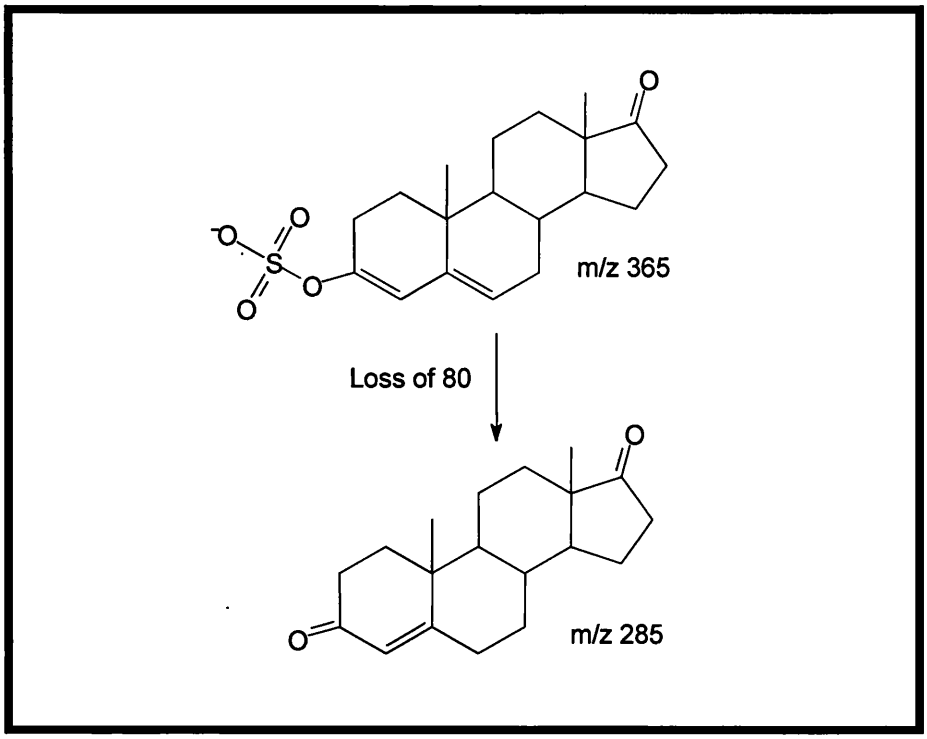



Figure 4.52 Proposed fragmentation pattern interpretation of peak occurring at 7.0 minutes.

This corresponds to the androstenedione version of the enol-sulfate conjugate proposed as a possible source of endogenous nandrolone.

## Discussion

The main aim of this study was to develop an LC-MS method to allow for the analysis of conjugated steroids in equine urine without the need for prior derivitization or conversion to free steroids.

GC-MS has been used successfully for the determination of steroids in equine urine however it is necessary to convert conjugated steroids to their free steroid counterparts to allow for analysis with GC-MS and as such it is not possible to analyse the conjugated steroids directly (1).

Nandrolone is manufactured and sold as an anabolic agent but is also found in large amounts in male equine urine. When nandrolone is administered to a horse the parent compound is predominantly excreted as a sulfate conjugate. Gelded animals, fillies and mares excrete low levels of steroids and no nandrolone; therefore if nandrolone is present in the urine of these animals its source must be from administration. However in colts large amounts of several steroids including nandrolone are found naturally occurring in the urine. During the course of a variety of experiments it became evident to HFL that nandrolone detected in normal colt urine samples using GC-MS methodologies was not derived from the 17β-sulfate conjugate that administered nandrolone was derived from. By use of LC-MS it should be possible to analyse for the unknown nandrolone precursor directly this would then provide a means of differentiating endogenous and exogenous nandrolone.

The development of our chromatographic separation was initially started with experiments involving the use of the conditions we had previously used in our analysis of free and conjugated estrogen steroids (chapter 3). The steroid conjugates of interest in this study, unlike the estrogen steroids previously studied, were not all of an aromatic nature and this may have had some bearing on the ability of the phenyl-hexyl packing to retain these compounds under the conditions previously used. We decided to continue our experiments with the phenyl-hexyl column but switched our mobile phase to the conditions that had previously been successful in the analysis of the conjugated estrogen steroids in chapter 3 when run with a C18 column. From these mobile phase conditions we derived our final conditions although it was necessary to cool the column to 10°C to gain a reasonable amount of resolution. No advantage was gained from the experiments we conducted to develop a gradient system so our final conditions consisted of a relatively simple isocratic system that allowed for analysis of the 6 steroid conjugates of interest in under 7 minutes.

The LC-MS analysis performed on the mixture of the 6 steroids proved to be more sensitive in the ESI mode than in the APCI mode. We believe this to be due to ESI having significantly less background noise than APCI as demonstrated by comparison of the total ion chromatograms achieved with each technique. Both forms of ionisation produced intense $[M-H]^-$ ions for all the conjugates of interest which is in agreement with results found in the literature (4, 5, 6).

LC-MS-MS analysis of the glucuronide conjugates produced fragmentation patterns which were consistent with those described by Kuuranne *et al* (4) in their work involving infusion of anabolic steroid glucuronides. The LC-MS-MS analysis of β

estradiol 3-sulfate gave results which were consistent with our previous findings (chapter 3) of analysis of sulfate conjugates; however the analysis we performed on the other 3 sulfate conjugates showed no losses of the sulfate group. We were unable to obtain any MS-MS results for testosterone or nandrolone sulfate and the MS-MS analysis of DHA 3-sulfate produced m/z values which resulted from loss of 15 and 30 from the $[M-H]^-$ ion and were presumably due to loss of the methyl groups; there was no sign of a fragment attributable to loss of the sulfate group. This was unexpected as we had predicted that the most abundant ion resulting from MS-MS analysis of the sulfate conjugates would be due to loss of the sulfate group from the parent ion.

Our studies were performed in the negative ionisation mode as it was found that the sulfate conjugates in particular gave very poor responses in the positive mode which is consistent with data found in the literature (5, 6).

The analysis of the equine urine samples proved that our LC-MS method could be used for detecting the presence of β estradiol 3-sulfate, nandrolone sulfate and DHA 3-sulfate in real samples. Without the extraction of spiked blank urine samples it is not possible to determine limits of detection that could be achieved in real samples with our methodology or how successful our analysis technique would be for the determination of the other 3 steroid conjugates studied in this chapter, although we had expected to be able to detect the presence of testosterone sulfate as it is known to be present in significant amounts in colt urine (1).

Using the methodology we developed in chapter 3 for the analysis of the free and conjugated estrogen steroids we were able to detect the presence of estrone 3-sulfate

in all of the colt urine samples with the exception of Blank three, as was the case with β estradiol 3-sulfate.

Analysis of the unknown peaks in the total ion chromatograms of the extracts indicated the presence of a significant number of other sulfate and glucuronide conjugates, which is as would be expected in the analysis of urines. The greatest responses were due to possible sulfate conjugates, which are the main form of steroid conjugate found in colt urine (1).

The unknown peak corresponding to approximately 4.5 minutes in the total ion chromatograms of the urine extracts may be due to the 19-norandrostenedione enol-sulfate conjugate which HFL proposed as a possible precursor to the endogenous nandrolone detected using GC-MS analysis. Isolation of the nandrolone precursor has been performed in the labs of HFL using semi-prep LC fraction collection with GC-MS analysis of the fractions giving positive results, after methanolysis, for nandrolone and 19-norandrostenedione; hence the proposed 19-norandrostenedione enol-sulfate precursor. To allow for confident LC-MS analysis of the precursor in urine extracts it would be necessary to first determine its fragmentation pattern from MS infusion of the isolated compound and then perform LC-MS analysis of the compound to determine its chromatographic retention time. LC-MS should then allow for direct analysis of the precursor without the need for prior derivitization.

## Conclusion

GC-MS is the technique that is generally used for the analysis of anabolic substances involved in doping; it provides a sensitive, accurate, reliable and specific method of analysis of urine and other biological fluids (7, 8, 9, 10). The negative side of GC-MS analysis is the requirement of lengthy derivitization processes and the inability to analyse metabolites of anabolic steroids directly. In the case of nandrolone, which is known to also occur naturally in male horses and appears to be present in equine urine via two separate metabolic pathways dependent on whether it comes from an exogenous or endogenous source, the inability of GC-MS to distinguish different metabolites of the steroid becomes a significant disadvantage (1).

LC-MS should allow for the direct analysis of the unknown nandrolone precursor believed to arise from endogenous nandrolone and could thus provide a means of easily distinguishing between doped and undoped horses. As the majority of steroids present in male equine urine are found in large amounts it should be possible to gain sufficient sensitivity with LC-MS to allow for their analysis to be performed.

LC-ESI-MS was found on the whole to be more sensitive than LC-APCI-MS for the analysis of the conjugated steroids investigated. The levels of detection achieved with LC-ESI-MS, while not as sensitive as GC-MS (1), were sufficient to allow for the requirements of HFL. MS-MS data was acquired for 4 of the steroid conjugates investigated; we were unable to achieve this for nandrolone and testosterone sulfate.

The results of this chapter show that LC-MS can be a useful and rapid method of analysis for the direct determination of conjugated steroids in equine urine. However without the use of standard materials it is difficult to state with any degree of certainty the identity of the unknown sulfate and glucuronide conjugates that were found in the urine samples we analysed.

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