COMPLEX MIXTURE ANALYSIS BY LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY

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By

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ABSTRACT

The combination of liquid chromatography with mass spectrometry is a valuable tool for the identification and quantification of compounds in crude biological matrices. This thesis describes the application of a variety of mass spectrometric techniques to three different analytical problems:

The metabolic fate of Hoe 127490, a compound which shows potential as a sulfonyl urea herbicide, was investigated. Rats were dosed with Hoe 127490 and liquid chromatography-tandem mass spectrometry (LC/MS/MS) with electrospray (ES) ionisation was employed to analyse a sample of urine from the treated animals. No pretreatment of the urine sample was performed prior to injection. Numerous metabolites of Hoe 127490 were identified and a metabolic pathway is proposed.

A semi-automated LC/MS/MS method using atmospheric pressure chemical ionisation (APCI) was developed which allows the rapid identification of dipeptides in complex mixtures, in particular in protein hydrolysates. Protein hydrolysates which are used for nutritional purposes are complex mixtures of peptides and free amino acids and their analysis poses a considerable challenge. The LC/MS/MS method was applied to a sample of a casein hydrolysate and fifty-two dipeptides were identified. Suggestions were also made into extending the method to allow the identification of larger peptides.

A rapid assay for the quantification of a potential insecticide, SN 609369, in plasma samples by thermospray liquid chromatography- mass spectrometry (LC/MS TSF) was developed. Sheep were dosed with SN 609369 and the level of the compound remaining in the animal's blood was monitored over a three week period. An earlier method of quantification had involved a lengthy extraction procedure followed by LC with ultraviolet detection. Sample analysis times were reduced from hours with this earlier method to minutes using LC/MS with selected ion recording. The necessity for a complex
extraction procedure was also eliminated. The detection limits for SN 609369 using TSP, ES and APCI ionisation methods were compared.
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For my Parents
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CHAPTER 1: THE COMBINATION OF CHROMATOGRAPHY WITH MASS SPECTROMETRY (MS)
1.1 INTRODUCTION
Mass spectrometry is routinely used in the study of complex mixtures, including urine, blood samples and synthetic mixtures. In order to obtain readily interpretable data, it is often necessary to separate the components present prior to mass spectrometric analysis. Separation methods employed include gas chromatography (GC), high performance liquid chromatography (HPLC), supercritical fluid chromatography (SFC) and capillary electrophoresis (CE). The separation power of the mass spectrometer using tandem mass spectrometry (MS/MS) may also be used. The latter method will be described more extensively in a later chapter (see Section 2.2).

As will be described in later chapters, the coupling of chromatography with mass spectrometry can be used in both the qualitative and quantitative modes, hence creating a powerful analytical tool with uses in many fields. The direct combination of chromatography and MS is desirable but the practicalities of doing so, as will be discussed, can be troublesome in some cases. This is because the requirements for the optimum operation of the chromatographic technique may be incompatible with the optimum operation of the mass spectrometer. Despite these difficulties, interfaces have been successfully developed to allow the coupling of mass spectrometry with each of the separative techniques mentioned above.

1.2 GAS CHROMATOGRAPHY-MASS SPECTROMETRY (GC-MS)
GC-MS using capillary GC columns is routinely employed for the analysis of mixtures of relatively volatile and thermally stable components, or for compounds which can be chemically derivatised to form such species\(^1\). Helium is used as the carrier gas at flow rates of 0.5 - 3 ml/min. The pumping systems of modern mass spectrometers are capable of handling these gas flow rates whilst maintaining a sufficiently high vacuum within the ion source, therefore allowing the exit end of the column to be placed directly into the source of the mass spectrometer. All the eluent from the column passes into the source and therefore no sample is lost, thus ensuring optimum sensitivity.

Packed columns may also be employed and were used universally on older instrumentation before capillary columns became readily available. Here, the helium flow rates are greater
(typically 25 - 30 ml/min) than when capillary columns are used and it is necessary to have an interface between the GC and the mass spectrometer to maintain the source at an optimum vacuum for ionisation. The most popular interface, which may still be found occasionally, is the jet separator\(^2\). The GC eluent is expanded into a region of low pressure through a small orifice. The higher mass of the analyte particles compared to the helium carrier gas results in the latter having relatively less momentum and therefore being dispersed further from the core of the expansion jet. The lighter helium molecules can therefore be skimmed off and pumped away. A small skimmer at the entrance to the mass spectrometer is placed on the axis of the GC line a short distance from the GC outlet. The GC eluent passing through the skimmer is enriched in analyte vapour. The disadvantage of this interface compared with the use of capillary columns is that a proportion of the analyte is lost with the carrier gas.

GC-MS can be performed in both the electron and chemical ionisation modes. In the electron ionisation (El) mode, sometimes referred to as electron impact, the molecule (M) eluting from the GC column is bombarded by electrons with typical energies of 70 eV. This causes an electron to be ejected from the molecule to produce a positively charged species, the molecular ion (M\(^+\)):

\[
M + e^- \rightarrow M^+ + 2 e^-
\]

The molecular ion has excess energy and therefore fragments to produce lower mass ions, radicals and neutral species. The resulting ions may fragment further until the excess energy is exhausted, as shown below:

\[
M^+ \rightarrow A^+ + N_1
\]

or
\[
M^+ \rightarrow B^+ + R^-
\]

then
\[
A^+ \rightarrow C^+ + N_2\text{ etc.}
\]

(Where A, B and C are ions, N\(_1\) and N\(_2\) are neutral molecules and R\(^-\) is a radical species)

The fragmentation process is related to the structure of the molecule and the resulting mass spectrum is a fingerprint of the compound ionised. Libraries of reference spectra are readily available to aid in the identification of unknown species. El is commonly used for the identification of compounds but it has the disadvantage that some molecules have so much energy that they fragment too much and the molecular ion is not observed.
Chemical ionisation (CI) is a milder method of ionisation and therefore is more likely to produce information on the molecular mass of a compound. A reagent gas (commonly ammonia, isobutane or methane) is introduced into the ion source so that the pressure in the source is much higher than it is for El (typically $10^{-3}$ mbar compared to $10^{-6}$ mbar). The reagent gas is ionised by the high energy electrons, e.g., for ammonia:

$$\text{NH}_3 + e^- \rightarrow \text{NH}_3^+ + 2e^-$$

The resulting ions then collide with neutral ammonia molecules to produce ammonium ions:

$$\text{NH}_3^+ + \text{NH}_3 \rightarrow \text{NH}_4^+ + \text{NH}_2$$

The gaseous analyte molecules (M) collide with the ammonium ions, resulting in ionisation either by proton transfer or by addition depending on the basicity of the analyte molecules with respect to the ammonia:

$$\text{NH}_4^+ + M \rightarrow \text{MH}^+ + \text{NH}_3 \quad \text{(if M is more basic than NH}_3)$$

$$\text{NH}_4^+ + M \rightarrow \text{MNH}_4^+ \quad \text{(if M is less basic than NH}_3)$$

Little or no fragmentation of the resulting pseudo-molecular ions occur and CI spectra are generally much simpler than the corresponding El spectrum. CI and El are therefore complimentary techniques with El producing fragmentation (structural) information and CI confirming the molecular mass of the analyte.

As stated previously, GC-MS can be used in both the El and CI modes but unfortunately, the technique has the major drawback that the analytes must be of relatively high volatility and thermal stability.

1.3 SUPERCRITICAL FLUID CHROMATOGRAPHY-MASS SPECTROMETRY (SFC-MS)

SFC became popular in the eighties as an alternative to GC and HPLC. A supercritical fluid arises when a gas is heated to above its critical temperature whilst being simultaneously compressed to a pressure above its critical pressure. Under these conditions, the gas is converted into a single phase, dense fluid whose solvent properties differ from both gas and liquid. Carbon dioxide is commonly used in SFC and, in its supercritical state, has solvating powers similar to normal phase LC solvents. The
solubilizing properties of the supercritical fluid can be influenced by varying its density. This can be achieved by controlling the temperature and pressure which allows the chromatographic separation of non-polar and moderately polar compounds. For the analysis of more polar compounds, higher fluid densities are required. The addition of polar modifiers, for example methanol, increases the polarity of the supercritical fluid and therefore allows the elution of polar species. Both capillary and packed columns have been employed in SFC.

The volatility of SFC mobile phases provides a significant advantage for the design of an SFC-MS interface compared to an LC-MS system. An essential requirement for SFC is that the supercritical conditions are maintained throughout the chromatographic process. A back-pressure restrictor therefore has to be placed immediately prior to the low pressure region of the mass spectrometer. Various interfaces have been employed for coupling SFC to a mass spectrometer including the moving-belt, thermospray, particle beam and electrospray interfaces (see Section 1.5 for further details on these interfaces).

SFC-MS has an advantage over GC-MS in that it can be applied to the analysis of species which are too involatile or thermally labile to be separated under GC conditions. However, it is less flexible than HPLC for the separation of a wider variety of compounds, and analytes must show sufficient thermal stability to withstand the elevated temperatures used.

1.4 CAPILLARY ELECTROPHORESIS-MASS SPECTROMETRY (CE-MS)

Capillary electrophoresis (CE) is the general term for a variety of separation techniques including capillary zone electrophoresis (CZE), micellar electrokinetic chromatography (MEKC), capillary isotachophoresis (CITP), capillary isoelectric focussing (CIEF) and capillary gel electrophoresis (CGE). Of these techniques, CZE is most commonly coupled with MS.

CZE separations are based on the different migration rates of charged analytes that occur in an electric field. A buffer-filled fused-silica capillary (typically 20-200 μm internal diameter) is employed in CZE to perform highly efficient separations of both large and small molecules. The ends of the capillary are placed in two buffer reservoirs, each
containing an electrode connected to a high voltage power supply. One buffer solution is kept at ground potential and the other is held at a high voltage (typically 10-30 kV). After filling the capillary with buffer, the sample is introduced in a narrow plug, either by dipping the capillary inlet into the sample solution and applying a voltage, or by hydrostatic methods which include raising the level of the sample vial, pressurising the inlet vial, or applying a vacuum to the outlet vial. When the high voltage is applied, the sample components migrate through the capillary by electrophoresis and electroosmosis under the influence of the electric field.

Electrophoresis is based on the differences in solute velocity in an electric field and is dependent upon the charge and size of the analyte. The ionic species in the sample plug migrate with a velocity and in a direction which is determined by the charge and mass of the species. This is known as the electrophoretic mobility. Small, highly charged species have high electrophoretic mobilities whereas large, minimally charged species have low mobilities.

Electroosmosis is the bulk flow of liquid which occurs when an electric potential is applied across the liquid filled capillary and is caused by the charge on the interior surface of the capillary wall. In fused-silica capillaries, the silanol groups exist in their anionic form. Cations build up near the surface to maintain a charge balance. The cations move towards the cathode when the voltage is applied and, because they are solvated, their movement pulls the bulk solution in the capillary towards the cathode. The electroosmotic flow sweeps all analytes through the capillary, including negatively charged analytes which are swept towards the cathode rather than the anode. Electroosmosis has a flat flow profile unlike the parabolic flow profile observed in a pressure-driven system (for example in HPLC) and therefore does not result in band broadening. The electroosmotic flow enables cations, neutrals and anions to be analysed in a single run. Under normal analysis conditions (i.e. when a fused-silica column is employed with the flow from the anode to the cathode), cations will migrate fastest because the electrophoretic mobility and electroosmotic flow are in the same direction, neutrals will flow at the same velocity as the electroosmotic flow but will not be separated from each other, and anions will migrate
slowest because they are attracted to the anode but are still being carried by the electoosmotic flow towards the cathode.

CZE has been coupled to a mass spectrometer using both electrospray and continuous-flow fast atom bombardment interfaces (see Sections 1.5.1.3 and 1.5.1.4 for details of these interfaces)*9. Low flow rates (typically 0 - 100 nl/min) are observed from the capillary column in CZE and these interfaces cannot be operated efficiently at these very low flow rates. Post-capillary addition of a make-up solvent is therefore required. The make-up solvent can be added using either a coaxial tube or a T-piece with a very low dead volume. In both cases, care is needed to ensure that peak broadening and the loss of resolution do not occur. The newly developed nanoelectrospray source10 which operates at nanolitre/minute flow rates may offer an advantage for coupling MS with CZE but little work has been performed to date in this area.

Applications of CZE-MS include the analysis of peptides, proteins, drugs, herbicides and detergents8,11.

1.5 LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY (LC-MS)

The combination of high performance liquid chromatography (HPLC) and mass spectrometry (MS) has been described as a difficult courtship because it involves the coupling of two seemingly incompatible techniques12. Conventional HPLC requires solvent flow rates of typically 1 ml/min resulting in gas volumes at atmospheric pressure of between 170 and 1200 ml/min for hexane and water respectively, whereas a typical mass spectrometer pumping system will only maintain a high vacuum if gas flow into the ion source remains below about 10 ml/min13. Other difficulties in coupling the two techniques include the frequent use of involatile inorganic buffers and/or gradient elutions in routine LC separations and the fact that the samples are frequently involatile or thermally labile and are therefore unsuitable for ionisation under electron impact (EI) or chemical ionisation (CI) conditions.

Despite these difficulties, several LC-MS interfaces have been developed using a range of ionisation techniques. These allow different types of compound to be analysed and different types of information to be generated. The interfaces fall into two broad categories
- those in which the LC eluent is introduced directly into the mass spectrometer ion source and is required for solute ionisation, and those in which the solvent is removed before the solute reaches the ion source. Unfortunately there is no universal interface.

1.5.1 Direct Introduction of Eluent into Ion Source

1.5.1.1 Direct Liquid Introduction (DLI)

This was one of the earliest types of interface which was developed by Tal'roze\textsuperscript{14} and McLafferty\textsuperscript{15}. A typical DLI interface is shown in Figure 1.1. Part of the LC eluent is introduced directly into the chemical ionisation (CI) source of a mass spectrometer through a 1 - 5 μm diameter orifice in a nickel or stainless-steel diaphragm\textsuperscript{16}. The amount of solvent entering the ion source is regulated by a needle valve placed between the UV detector and the probe. Solvent molecules are vaporised in a heated desolvation chamber and the solute molecules are analysed under CI conditions with the solvent molecules acting as the reagent gas (see Section 1.2). The probe is water-cooled to prevent evaporation of the LC eluent within the probe.

![Figure 1.1: A Typical DLI interface](image)

DLI has proved to be extremely effective for some classes of compound including nucleosides, drugs, pesticides and peptides but there are a number of reasons for its decline in use\textsuperscript{16-19}. A major drawback of the interface is the narrow aperture of the sampling orifice which can result in partial or complete blockage of the probe and therefore erratic
performance. A further disadvantage of the DLI interface is that only approximately 10 - 50 μl/min of eluent typically enters the ion source which is equivalent to about 1 - 5% of the flow-rate used in conventional LC. It is, therefore, necessary to either split the eluent or to use microbore LC columns. The latter would allow all of the eluent to enter the ion source, thereby increasing overall sensitivity. This interface is unsuitable for use with species which are thermally labile or involatile.

1.5.1.2 Thermospray (TSP)

The thermospray interface, developed by Blakely and Vestal, is a valuable analytical tool. Thermospray LC-MS is routinely used in many laboratories for both qualitative and quantitative analyses. An advantage of this technique over the DLI probe is that both involatile and thermally labile species may be detected. A typical commercial thermospray source is shown in Figure 1.2.

![Figure 1.2: Schematic diagram of a thermospray interface](image)

The LC eluent is pumped through a vaporiser which consists of a heated stainless steel capillary. The eluent emerges from the capillary into the first thermospray chamber in the form of an expanding supersonic jet of vapour and droplets. This aerosol jet passes through two independently heated thermospray chambers into a pumping region where excess solvent is removed from the system. Ions in the jet pass into the mass analyser.
through an orifice in the sampling cone which is placed at right angles to the vapour flow. A repeller electrode opposite the sampling cone optimises this process\textsuperscript{26}.

Thermospray ionisation in the ‘filament off’ mode relies on the presence of a buffer in the mobile phase to produce ions which interact with the sample molecules to cause their ionisation. Ammonium acetate, at a typical concentration of 0.1M, is the most commonly used buffer. The ionisation process is represented in Figure 1.3.

![Liquid droplet, electric field increases as solvent evaporates, ions are emitted from the droplet](image)

**Figure 1.3**: Schematic representation of the thermospray process

The eluent is vaporised at such a rate as to produce a superheated mist of droplets in a supersonic jet\textsuperscript{27}. These droplets consist of a core of the solute molecule surrounded by the solvent and buffer ions. Droplets can have a positive or negative charge although the overall aerosol remains neutral. The solvent molecules evaporate and the radii of the droplets decrease until the coulombic repulsion between the charged species in the droplet exceeds the surface tension of the droplet. Ions are then ejected into the gas phase. These ions are called primary ions and consist of ammonium adducts of solvent and analyte\textsuperscript{28}. The primary ions subsequently undergo gas phase ion-molecule interactions with gaseous analyte molecules to produce CI type spectra which are dependant upon the interface temperature and pressure, the repeller potential and the position of the repeller electrode\textsuperscript{26,29-31}.

The thermospray ionisation mechanism is ideally suited to reverse phase LC systems containing a volatile buffer. A typical thermospray interface will accept flow rates of up to
2 ml/min allowing conventional LC columns to be used. A conventional LC separation can, therefore, be easily transferred to the LC-MS system without modification. Buffer can be added post-column, prior to the thermospray interface, if it hinders the LC separation\textsuperscript{32}. If the proportion of water in the chromatographic eluent is low and non-polar solvents are used, thermospray ionisation is not as effective and sensitivity is low. Ionisation can be induced with an electron beam, resulting in a similar CI process to that observed in conventional EI/Cl ion sources (see Section 1.2). A filament (typically thoriated iridium), or a discharge from a high voltage electrode can be placed in the mist eluting from the capillary\textsuperscript{29}. The spray of droplets then pass through the negative discharge, where electrons are removed from the neutral molecules, resulting in ionised solvent molecules. Analyte molecules are then ionised by a similar mechanism to that observed in conventional CI. This technique is known as \textit{plasmaspray} or \textit{‘filament-on’} thermospray.

A drawback of the thermospray interface is the difficulty of maintaining a stable signal intensity because small changes in the instrumental parameters, especially interface temperatures, can produce significant changes in the resulting spectra. It is, therefore, necessary to optimise the instrument parameters for all compounds prior to analysis\textsuperscript{30}. The vaporiser orifice diameter is also critical and it may be necessary to crimp the capillary tip in some interface designs to produce an optimum and stable spray prior to use\textsuperscript{33}.

Thermospray has proved to be very successful for the analysis of a wide range of compounds, but there are examples where compounds can not be successfully analysed. These compounds include hydrocarbons, carboxylic acids and organometallics\textsuperscript{34}. TSP is more sensitive to nitrogen-containing compounds and less sensitive to compounds containing only carbon and hydrogen. T.R. Covey et al. have reported that in the analysis of a urinary extract for unknown metabolites, one major metabolite could not be detected by TSP LC/MS alone\textsuperscript{35}. Care is therefore required when using TSP because even if one compound can be analysed easily, it cannot be guaranteed that a related species will be detected. TSP is very compound and functional group specific and the conditions employed are very important - changes in buffer, buffer concentration, and interface temperature are critical for the analysis of certain species.
1.5.1.3 Atmospheric Pressure Ionisation (API)

API is the general term used for the interfaces in which, as the name implies, the ion source is kept at atmospheric pressure. The three interfaces which use this design are electrospray (ES), ionspray (IS) and atmospheric pressure chemical ionisation (APCI). A multiple-stage vacuum system is required to allow the combination of an API source with a mass analyser (see Figure 1.4).

![Figure 1.4: Schematic diagram of the vacuum stages of an API source coupled to a mass analyser](image)

Ions produced in the source pass through a sampling orifice into the first chamber which is evacuated to approximately 1 Torr by a rotary pump. Ions then pass into the second chamber which is pumped to approximately $10^{-3}$ Torr by a diffusion or turbomolecular pump. Most of the gas molecules are removed at this stage and ions are transmitted into the mass analyser, which is evacuated to approximately $10^{-5}$ Torr with another diffusion or turbomolecular pump.

Electrospray mass spectrometry was introduced by Yamashita and Fenn in 1984 but it was not until 1988, when Fenn and co-workers used the technique to generate a series of multiply-charged ions from protein solutions, that its importance was fully recognised by the mass spectrometry community.

The analyte, dissolved in a suitable solvent (typically 50% aqueous methanol or acetonitrile containing 1 - 5% acetic or formic acid), is admitted into the source via a
stainless steel capillary. An electric field is generated by applying a high positive or negative potential (typically 3-5kV) directly to the capillary with a counter electrode located a few millimetres away. Electrospray can be used in both the positive and negative ionisation modes, dependant upon the polarity of the potential applied to the capillary with respect to the counter electrode.

In positive ionisation, the high potential breaks up the liquid surface at the tip of the capillary when sufficient positive charge has been acquired\textsuperscript{37}. The coulombic repulsions thus generated overcome the cohesive forces within the liquid and small positively charged droplets are expelled, which move towards the counter electrode. In the Finnigan-MAT source, the counter electrode is the wall of the source, whereas the VG source uses a “pepperpot” as the counter electrode. Charge continues to build-up at the liquid front and the formation of charged droplets is repeated (see Figure 1.5). Negative ions are removed from the sample solution by electrochemical discharge against the wall of the metal spray capillary.

A negatively charged aerosol is formed by a similar process but in this case a negative electric field is applied to the tip of the capillary. The sample solution emerges as a mist of highly charged droplets with an excess of positive or negative electrical charge on their surface, dependant on the polarity of the capillary. The diameter of the droplets (typically approximately 1 μm) is dependant on the solvent and solutes.

Two different mechanisms have been proposed to describe the formation of gas-phase ions from the charged droplets. The ion evaporation model\textsuperscript{38} assumes that solvent evaporates...
rapidly from the highly charged droplets. As the droplets decrease in size, the electrical charge density at the surface of the droplets increases. After sufficient solvent has evaporated and the resulting droplets have disintegrated into smaller droplets by 'coulombic explosion' due to the natural repulsion between like charges, the stage is reached where the electric field at the droplet surface is so high that solute ions are evaporated from the liquid phase into the gas phase in the ion source (see Figure 1.3). The charged residue mechanism$$^{39}$$ assumes that "microdroplets" are initially formed due to field induced surface instability of the initial electrospray droplets. These microdroplets are essentially a solute ion in a shell of solvent molecules. Solvent evaporates from these microdroplets yielding molecular ions (see Figure 1.6).

Figure 1.6: Schematic diagram of the proposed mechanism for the formation of ions in the gas phase by ES+
In electrospray ionisation, the droplets are broken up by collisions with a "curtain" or "drying" gas (typically nitrogen), by heat, or by a combination of the two techniques. In both the VG and Perkin-Elmer Sciex sources, a stream of dry nitrogen gas continually passes over the sampling orifice to prevent solvent molecules from entering the vacuum chamber and to break hydrogen bonded ion/solvent clusters which might complicate the mass spectrum. In the Chait design source\(^\text{40}\) employed by Finnigan-MAT (see Figure 1.7), ionised analyte is transferred from atmospheric pressure to vacuum through a heated metal capillary. The capillary is heated at approximately 85°C and allows controlled evaporation of solvent from the droplets. The remaining solvent is desolvated from the analyte ions by applying an electric field between the capillary and the skimmer, resulting in collision-induced desolvation of the ions. Ions leaving the capillary are then focused by a tube lens before passing through a skimmer which is slightly off-centre to the tube lens exit. The skimmer acts as a momentum separator and heavier sample molecules tend to pass through whilst lighter solvent and drying gas molecules can be more readily pumped away in the intermediate vacuum stage. The 'pepperpot' in the VG source (see Figure 1.10) also aids desolvation. In both the Finnigan-MAT and VG API sources, a multipole lens is used to focus the ions and ensure optimum transmission into the mass analyser, thereby increasing sensitivity.

![Figure 1.7: Schematic diagram of the Chait electrospray ion source](image-url)
Electrospray ionisation produces protonated positive molecular ions or deprotonated negative molecular ions from polar, ionic compounds. Some compounds are susceptible to adduct-ion formation with cations which are present in the solution. Sodium, potassium and ammonium adducts are commonly observed.

The most interesting feature of electrospray ionisation is that multiply charged ions can be generated from compounds which have multiple charge sites (for example, peptides and oligonucleotides). A mass spectrometer analyses for mass to charge ratio (m/z) and, therefore, these ions appear in the mass spectrum at apparent masses of:

\[ \frac{M + nH}{n} = \frac{m}{z} \]  

(equation 1)

where \( M \) = actual mass  
\( n \) = number of charges  
\( H \) = mass of one proton

This is advantageous when looking at high molecular weight species because multiply charged ions may be produced with mass/charge ratios within the mass range of a quadrupole instrument. This allows analysis of compounds with molecular weights greater than 130,000 daltons. In peptides, the extent of multiple charging increases as the number of basic amino acids (ie arginine, histidine, lysine) present in the peptide sequence increases. For larger protein molecules, a bell-shaped distribution of multiply charged ions is frequently observed in the electrospray spectrum with the maxima generally observed between m/z 500 and 2000. Figure 1.8 shows the positive ion electrospray spectrum of horse heart myoglobin (molecular weight 16951.48). The charge states are indicated on the spectrum, but generally, for an unidentified species the charge states are generally unknown.
Any two adjacent ions in a series of multiply charged ions will differ by one charge, therefore, for ions with masses of $M_1$ and $M_2$:

$$n_1 = n_2 + 1 \quad \text{(equation 2)}$$

where $n_1$ and $n_2$ are the number of charges on $M_1$ and $M_2$ respectively.

Substituting $M_1$ and $M_2$ into equation 1:

$$\frac{M + n_1H}{n_1} = M_1$$

$\Rightarrow$

$$\frac{M + n_2H}{n_2} = M_2 \quad \text{(equation 3)}$$

Substituting equation 2 into equation 3:

$$\frac{M + (n_2 + 1)H}{n_2 + 1} = M_1$$

$\Rightarrow$

$$\frac{M + n_1H}{n_1} = M_1$$

$\text{(equation 5)}$
Rearrangement of the simultaneous equations 4 and 5:

\[ M = n_2M_2 - n_2H \]  (equation 6)

\[ M + (n_2 + 1)H = (n_2 + 1)M_1 \]  (equation 7)

Substituting \( M \) in equation 7:

\[ n_2M_2 - n_2H + (n_2 + 1)H = (n_2 + 1)M_1 \]

\[ n_2M_2 + H = n_2M_1 + M_1 \]

\[ n_2 = \frac{M_1 - H}{M_2 - M_1} \]

The values of \( M_1 \) and \( M_2 \) can be obtained directly from the spectrum and therefore \( n_2 \) can be calculated to the nearest whole number. Once \( n_2 \) is known, \( M \), the molecular weight of the analyte, can be determined using equation 1. Fortunately, it is not usually necessary to manually calculate the molecular weight because modern data systems can easily transform the electrospray spectrum to produce a molecular weight profile spectrum. Figure 1.9 shows the transformed horse heart myoglobin spectrum of the electrospray spectrum presented in Figure 1.8.

![Figure 1.9: Transformed horse heart myoglobin spectrum](image)
Low molecular mass polar organic molecules can also be analysed by electrospray to produce singly charged pseudo-molecular ions with little or no fragmentation. Structural information can be obtained from these ions by applying a voltage difference between two regions of the electrospray source to induce fragmentation of the molecular ion in the high pressure region. This is known as cone voltage fragmentation (CVF). In the systems which employ a multipole lens, this can be tuned to induce fragmentation before the ions enter the mass analyser, thereby providing structural information.

Electrospray is a low flow rate technique, typically using flow rates of less than 10 - 20μl/min. When the flow rate is increased for a particular sample concentration, the sample ion response does not increase but remains constant. The mass-flow sensitivity therefore decreases, which means that electrospray ionisation is concentration-sensitive rather than mass flow-sensitive. This is unlike the situation for other ionisation techniques. This phenomenon is due to a reduction in the ionisation efficiency at higher flow rates attributed to a decrease in the droplet-charging efficiency and an increase in the diameter of the droplets with increased flow rates. The lower ionisation efficiency is compensated for by the increase in the quantity of sample entering the source and, therefore, the sample ion response remains relatively constant. If, however, the flow rate is increased too much, the ion signal will eventually decrease and become unstable. A sheath gas, typically nitrogen, can be used to assist with the spray generation process. This is known as ionspray or pneumatically assisted electrospray and allows flow rates of up to 300 μl/min to be tolerated.

Electrospray is a sensitive technique often capable of producing a spectrum from less than 1 pmole of material or detecting 1 fmol as a specific detector.

The third API technique is atmospheric pressure chemical ionisation (APCI) and a typical interface is shown in Figure 1.10. Here, the LC eluent flows through a narrow bore stainless steel tube directly into the nebuliser, where it is converted into a fine aerosol by a high velocity jet of air or nitrogen. Droplets are swept by a mantle gas through a quartz tube heater which vaporises the droplets and analyte in the gas stream. The heated gas (typically at 120°C) and sample mixture flows from the quartz tube into the APCI source. A corona discharge is obtained by applying a voltage to a metal needle. This discharge
ionises the solvent vapour to create reagent ions (for example, $\text{H}_3\text{O}^+$ from water, $\text{CH}_3\text{OH}_2^+$ from methanol, and $\text{CH}_3\text{CNH}^+$ from acetonitrile) which ionise the gaseous analyte molecules by charge transfer as observed in the conventional CI process (see Section 1.2). In general, the vaporised LC eluent acts as the reagent gas. The APCI source can be operated in both the positive and negative ionisation modes with the ions analysed being dependant upon the polarity of the corona discharge needle which ionises the reagent gas. A curtain of nitrogen passing over the sampling orifice prevents solvent and buffer from entering the analyser region and breaks up solvent/ion clusters as described previously for ES. Both volatile and involatile buffers can be used because the nebulising gas sweeps non-evaporated particulate matter through the spray chamber and the source, thus preventing significant contamination of the analyser region. A major advantage of APCI is its compatibility with mobile phases containing up to 100% water at flow rates up to 2 ml/min.

Figure 1.10 : Schematic of an APCI source

The API techniques now make up the majority of the new LC-MS interface market\textsuperscript{43,44}. They are routinely used in many laboratories for both qualitative and quantitative analyses. Their design is relatively simple and therefore they are very robust and easy to maintain. The hardware required for these techniques is similar and most commercial systems can be readily interconverted between the configurations.
1.5.1.4 Continuous-Flow Fast Atom Bombardment (CF-FAB)

LC was first coupled to a fast atom bombardment (FAB) ion source indirectly using a moving-belt interface\(^45,46\). The LC eluent containing the FAB matrix (if desired) was sprayed directly onto the moving-belt where the mobile phase was removed by a combination of heat and vacuum (see Section 1.5.2.1). The FAB ion gun was targeted onto the belt to induce ionisation of the analyte. This method has been used to analyse peptides and other polar compounds to provide useful structural information.

The first direct coupling of LC and FAB used a frit-FAB interface\(^47\) (see Figure 1.11). The interface basically consisted of a porous stainless steel frit whose rim was glued to the end of a glass tube containing a fused silica capillary inside a stainless steel tube. The mobile phase vaporised from the surface of the frit leaving the solute and the matrix (10% glycerol) on the surface. This was bombarded by the argon beam. The pumping capabilities of the vacuum system allowed maximum flow rates of only 0.5 \(\mu\)l/min. Reproducible peak heights were observed for replicate injections of 20 ng of sodium taurocholate but peak tailing was noted.

![Schematic diagram of the frit-FAB interface](image)

The properties of the frit have been found to be important in optimising the sensitivity of the technique by minimising peak broadening\(^48\). Smaller pore sizes (3 \(\mu\)m) and thinner frits (0.30 mm) were found to produce less peak broadening and larger ion responses. Stainless steel and silver frits produced better results than glass, paper and PTFE frits, which could be due to their good "wettability" by glycerol, leading to stable and efficient ionisation of glycerol and the analytes. The temperature of the ionisation chamber was also found to
affect the stability and intensity of the ion beam but the optimum temperature was found to be dependant on the mobile phase composition. The frit-FAB interface has been successfully used for the analysis of prostaglandins and a tryptic digest of glucagon.

An alternative FAB probe was developed in 1986 which used an open copper tip instead of the stainless steel frit and allowed flow rates of up to 5 µl/min. This technique is known as dynamic or continuous-flow FAB. Linear responses were obtained following 0.7-135 ng injections of a peptide of molecular mass 1347.

A typical commercial CF-FAB probe target is shown in Figure 1.12. The LC eluent containing a low concentration of matrix (typically 95% water and 5% glycerol) is pumped through a silica capillary and flows onto the probe target. A filter pad or wick around the probe tip can improve the ion beam stability by absorbing excess LC eluent as it flows from the target, thereby ensuring a steady rate of evaporation. Unfortunately, non-volatile species accumulate on the wick and, therefore, this requires changing regularly. The probe tip is warmed to a temperature of 40 - 60°C to prevent freezing and to assist in evaporation of the LC eluent. On reaching the probe tip, the analyte and matrix are bombarded by atoms or ions as in the static mode and typical FAB spectra are obtained. This interface is therefore amenable to the analysis of involatile, thermally labile and high molecular mass species. CF-FAB has been used routinely for both quantitative and qualitative analyses.

Figure 1.12: Schematic of a CF-FAB probe tip
The advantage of CF-FAB, relative to conventional FAB, is not only that it is a direct LC/MS interface, but also that it produces a significant increase in sensitivity. The sensitivity of CF-FAB can be as much as 200 times greater than that provided by static FAB. This is partially due to increased ion production, but is primarily the result of a decrease in background chemical noise therefore producing a lower detection limit. The decrease in background ions from the degradation of the matrix is due to the continual replacement of the matrix at the probe tip in CF-FAB.

The major disadvantage of CF-FAB is the difficulty in obtaining a stable system. Optimum performance is achieved when the liquid sample is maintained as a thin film on the target surface. Accumulation of liquid around the probe tip region causes peak broadening from stagnancy of liquid flow, instability of the ion source pressure due to bubbling and dripping of the mobile phase, and film instability from the freezing of the mobile phase in the capillary. Careful temperature control of the probe tip is, therefore, required in order to ensure that the rate of liquid flow onto the surface is balanced by the rate of evaporation at the probe tip. Another disadvantage of CF-FAB arises from the pumping capability of the mass spectrometer which limits the eluent flow rate to typically 5 - 10 μl/min. Flow-splitting is, therefore, required for coupling to conventional or microbore LC systems which typically use flow rates of up to 1 ml/min. Capillary LC columns may be used to overcome this.

Matrix, typically glycerol, can be added pre- or post column. The addition of a viscous matrix into the mobile phase pre-column causes an increased pressure drop across the column. This can be offset by maintaining the column at a higher temperature to decrease the viscosity. This increased column temperature and the presence of a polar matrix will, however, change the conditions within the column and affect the chromatographic separation. The chromatographic resolution also decreases and peak broadening is observed with an increase in the viscosity of the mobile phase. If glycerol is to be added post-column, it is necessary to add it in a solvent mixture because it is difficult to mix pure glycerol with LC eluent. Post-column addition, therefore, results in a greater dilution of the eluent but this has the advantage that the chromatographic separation is not affected.
By either method, the continuous flow of eluent containing the involatile matrix, unfortunately results in contamination of the ion source and, therefore, reduces sensitivity.

**1.5.2 Solvent Removal Prior to Eluent Reaching the Ion Source**

**1.5.2.1 Moving Belt Interface (MBI)**

The first commercial moving belt interface was introduced in 1976\(^6\) and a typical system is shown schematically in Figure 1.13.

![Figure 1.13: Schematic of an MBI](image)

LC eluent is sprayed onto a moving, continuous polyimide belt which passes under an infra-red heater to vaporise a large proportion of the mobile phase. The belt then passes through three seals in a two stage vacuum lock, where the pressure is reduced to that of the source housing and any remaining solvent is removed. The sample remains on the belt and passes over a flash heater at the nose of the probe, which vaporises the sample in the ion source prior to ionisation under EI or CI conditions (see Section 1.2.

The method of depositing the LC eluent onto the belt has been found to be important\(^7\). In early designs the eluent was allowed to drip directly onto the belt but this resulted in the formation of droplets. This gave rise to uneven surface coverage which led, in turn, to
distorted peak shapes. A jet spray depositor was developed by Smith and Johnson to overcome this problem. The end of the stainless steel tubing from the LC was crimped and an inert gas was used to nebulise the solvent. The inert gas was preheated to aid solvent evaporation. This method allowed higher flow rates of less volatile solvents (e.g., water) to be readily handled and led to improvement in peak shapes.

The MBI is compatible with both normal and reverse phase solvent systems. Involatile buffers cannot be utilised because these accumulate on the belt and reduce the sensitivity of the system. Flow rates up to 1.5 ml/min are possible with organic solvents but the efficiency of the pumping system limits this to less than 1 ml/min when the aqueous content is significant. Optimisation of the solvent evaporator temperature and belt speed is necessary to ensure that almost all the solvent is evaporated in the interface housing prior to reaching the first vacuum lock. A major reduction in transfer efficiency is observed if too much solvent enters the pumping region due to the solvent (and analyte) being blown off the belt as it passes through the high pressure drop. However, it has also been observed that a lower sample transfer efficiency is obtained if all the solvent is evaporated at this stage due to evaporation of the analyte as the belt temperature increases. Further loss of the sample, particularly highly volatile species, prior to its reaching the ion source can also be reduced by water cooling of the probe. The transfer efficiency of the sample is, therefore, dependent on mobile phase composition, flow rate and sample volatility.

The major advantage of the MBI is that it produces library searchable EI data which is particularly useful in compound identification. The only other interface capable of producing such data is the particle beam interface (see Section 1.5.2.2). Quantitative analyses have been demonstrated in the EI mode using polynuclear aromatic hydrocarbons. A detection limit of less than 200 pg was observed with a linear dynamic range of four orders of magnitude.

A disadvantage of the MBI is the narrow volatility range of compounds amenable to analysis using this interface. Very volatile species are lost to the pumping system before reaching the source whereas very involatile species are not detected under EI or CI conditions, because they are not vaporised from the belt. Attempts have been made to overcome the latter problem by using FAB ionisation (see Section 1.5.1.4). This method
has been employed for the analysis of thermally labile and involatile species. Detection limits of less than 1 ng were observed for several amino acids. In MBI FAB-MS, the analytes on the belt are directly bombarded by a stream of atoms. Unlike probe FAB, no matrix is required because the continual movement of the belt ensures that the analytes are replenished on the belt prior to FAB ionisation. Excess sample is removed from the belt with a methanol washbath located in the atmospheric pressure region of the interface. MBI FAB produces greater fragmentation than probe FAB because the matrix in the latter technique not only provides a continually replenished sample surface for FAB ionisation, but also absorbs excess internal energy created in the process. In MBI FAB, no matrix is available, therefore, the excess internal energy results in increased fragmentation. Another disadvantage of the technique is that it is a mechanical device and therefore needs careful handling to obtain meaningful results. Memory effects may also be observed even when the belt is cleaned by a heating and scrubbing system. Even so, the MBI has been used by many groups to analyse a wide variety of compound classes but it has now been superceded by other, more efficient interface methods.

1.5.2.2 Particle Beam Interface (PBI)

This interface is based on the Monodisperse Aerosol Generation Interface (MAGIC) first described by Willoughby and Browner in 1984 and later improved by Winkler and co-workers in 1988. A typical design is shown schematically in Figure 1.14.

Figure 1.14 : Schematic of a PBI
The LC eluent is pumped through a narrow fused-silica capillary (typically 25 μm internal diameter) which forms a liquid jet. This liquid flow interacts with the surrounding gas at near atmospheric pressure resulting in the spontaneous break up of the liquid into droplets with a narrow size distribution. A stream of helium gas, perpendicular to the liquid jet, disperses the droplets and prevents coagulation of the drops. This aerosol then passes through a warmed desolvation chamber where the solvent evaporates rapidly leaving a mixture of solvent vapour, helium gas, and less volatile analyte particles. This mixture is directed through a nozzle into a low pressure region (typically 1-10 Torr) where it undergoes a rapid expansion to form a high velocity gas jet containing suspended analyte particles. The higher mass of the analyte particles compared to the helium and solvent molecules results in the latter having relatively less momentum and therefore being dispersed further from the core of the expansion jet. The lighter solvent vapour can be skimmed off and pumped away. The remaining gas jet is therefore enriched with analyte particles and then passes through a sampling orifice into a second momentum separator where the process is repeated. The pressure is reduced here to approximately 0.1-1 Torr. The analyte particles enter the mass spectrometer ion source via a transfer line where they undergo flash vaporisation prior to ionisation under EI or CI conditions (see Section 1.2). Using this system, the source pressure can be kept low enough to produce true EI spectra. The sensitivity of the PBI has been shown to be affected by solvent composition and flow rate, nebulisation gas pressure, ion source and desolvation chamber temperatures, and position of the capillary within the nebuliser. An increase in sensitivity of nearly an order of magnitude can be gained if these parameters are optimised. Sensitivity is reduced as solvent polarity and boiling point are increased, possibly due to the decreased efficiency of particle formation and desolvation resulting in larger droplets. The optimum flow rates for the operation of the interface are 0.4 - 0.6 ml/min, although this is reduced when mobile phases with high water content are employed. The temperature of the desolvation chamber is critical and dependant on the mobile phase composition and nature of the analyte. If it is operated at too low a temperature, then solvent will condense on the chamber, resulting in poor transfer efficiency of the analyte. The desolvation temperature, therefore, has to be increased as the percentage of water is increased in the mobile phase in
order to maintain optimum sensitivity. The source temperature is dependant on the chemical properties of the analyte. The nebulisation gas pressure has a significant effect on the transmission of analyte molecules through the interface and, therefore on the response. The optimum position of the fused-silica capillary within the nebuliser is flush to, or slightly protruding from, the nebuliser body.

The PB interface is mechanically simpler and easier to install than the moving-belt interface which is the only other interface capable of producing classical EI data (see Section 1.5.2.1). The PB interface also has a wider sample volatility and thermal lability range although it has been shown that the sensitivity of this interface decreases as the volatility of the analyte increases. This is due to an increase in the loss of the more volatile species in the momentum separator\textsuperscript{66}. The level of background noise for the PBI is also significantly less than that of the moving-belt interface. The MBI has a high level of background contamination due to the continual thermal desorption of phthalate plasticizers from the polyimide belt\textsuperscript{63}.

A major drawback of the PBI is that the transmission of analyte through the separator becomes non-linear at low concentrations. A mathematical model, based on the hypothesis that the PBI has a particle size cut off level, was proposed to describe this phenomenon\textsuperscript{67}. Below this level, small particles are pumped away in the momentum separator and, above it, larger particles are transferred more efficiently into the ion source of the mass spectrometer. The actual cut off level is unknown. The model assumes that the size of the desolvated particle is dependant on the initial droplet size and on the concentration of the sample. As the sample concentration is reduced, the particle size decreases until the average is below the hypothetical cut off limit. The response factor of the analyte is, therefore, reduced at lower concentrations and eventually reaches zero. This results in a linear calibration plot at the higher concentrations with a pronounced deviation from this linearity in the lower concentration region.

The addition of semi-volatile mobile phase additives (for example, ammonium acetate and ammonium oxalate) has been shown, in some cases, to improve both the linearity and sensitivity of the PBI\textsuperscript{67}. This is because the overall concentration of species in each droplet is increased, therefore leading to an increase in the size of the desolvated particle and
hence an increase in the transfer efficiency through the interface. The effects of ten mobile phase additives on twenty-four analytes were investigated and it was found that no single additive enhanced the response for all analytes. Some additives worked well for particular analytes whilst other additive-analyte combinations actually had a negative effect. In general, acid and neutral additives enhanced acidic analytes whilst basic additives enhanced basic analytes. It would, therefore, appear that the effectiveness of mobile phase additives depends on the chemical characteristics of both the additive and the analyte. This suggests that the effect involves chemical interactions and is not just a simple physical process.

PB-LC/MS has been used by many groups to analyse a variety of compounds both qualitatively and quantitatively, although the overall sensitivity of the technique is generally lower than that of the API interfaces.

1.6 REFERENCES


3. “Supercritical Fluid Chromatography”, edited by R.M. Smith, Royal Society of Chemistry


19. C.H. Kenyon, Biomed. MS, 10, 535, (1083)


43. S. Preece, MicroMass, Altrincham, Personal Communication
44. M.E. Harrison, Finnigan-MAT, Hemel Hempstead, Personal Communication

48
     Spectrom., 15, 179, (1988)
58. M.J. Hayes, E.P. Lankmayer, P. Vouros, B.L. Karger, J.M. McGuire, Anal.
     (1988)
CHAPTER 2 : IDENTIFICATION OF THE METABOLITES OF HOE 127490, A SULFONYL UREA HERBICIDE, IN RAT URINE BY TANDEM MASS SPECTROMETRY WITH ELECTROSPRAY IONISATION
2.1 INTRODUCTION

Sulfonylurea herbicides were discovered by George Levitt of DuPont in 1975 and since then, hundreds of sulfonylurea herbicide patents have been issued. There is extensive interest in this class of compounds due to their low application rates (typically 2-100 g/hectare), broad spectrum of weed control, good crop selectivity and low mammalian toxicity which are all desirable factors in the development of a new herbicide.

Sulfonyl urea herbicides are thermally labile and cannot be analysed directly by GC/MS unless they are initially derivatised to form stable species. LC/MS has been employed in their analysis, using a variety of interfaces including direct liquid introduction (DLI), moving-belt (MBI), thermospray (TSP), continuous-flow fast atom bombardment (CF-FAB) and electrospray (ES). DLI, MBI with chemical ionisation and TSP all produced weak protonated molecular ions of the sulfonyl urea herbicides. CF-FAB produced the protonated molecular ion as the base peak with structurally useful fragment ions, whilst ES produced predominantly pseudo molecular ions. Tandem mass spectrometry was employed to provide structural information on the sulfonyl ureas when ES ionisation was used. Only CF-FAB has been reported for the analysis of metabolites of sulfonyl urea herbicides.

Metabolic reactions in rats reported include alkyl and aryl oxidation followed by glucuronide and sulfate conjugation, dealkylation and hydrolysis of the sulfonyl bridge. Methyl-2-(4,6-dimethoxy(pyrimidin-2-ylcarbamoylsulfamoyl)-4-(N-methyl-isobutyramido) benzoate (Hoe 127490, see Figure 2.1) shows potential as a herbicide for the control of broad leaf weeds in cereals.

\[
\text{C}_{20}\text{H}_{25}\text{N}_{5}\text{O}_{8}\text{S} (495)
\]

Figure 2.1 : Hoe 127490

As is the case for all agrochemicals at the development stage, it is essential to investigate
the metabolic pathway to help understand the toxicological profile of Hoe 127490 in animals, particularly food producing species and man. In order to generate preliminary information on the metabolic fate of Hoe 127490, an initial study was undertaken on rodents. In this study, a male rat was dosed at a level of 10 mg/kg (bodyweight) and the urine was collected over a 24 hour period. The urine was then injected directly onto an HPLC column without any sample clean-up prior to LC/MS and LC/MS/MS analysis with electrospray ionisation.

This chapter describes the identification of twenty metabolites of Hoe 127490 using these techniques.

2.2 TANDEM MASS SPECTROMETRY (MS/MS)

Many compounds which are ionised using a soft ionisation technique (for example APCI, ES and FAB) produce only pseudo molecular ions with no, or only weak, fragment ions being observed. This is useful if only molecular weight information is required, but often, further information about the structure is desirable.

Tandem mass spectrometry using, for example, a triple quadrupole mass spectrometer can be used to fragment pseudo-molecular ions, thereby yielding information about the chemical structure. A triple quadrupole mass spectrometer consists, as the name implies, of three quadrupole mass analysers in tandem (see Figure 2.2). Quadrupoles 1 and 3 are operated with the normal combination of radio-frequency (RF) and direct current (DC) voltages. Quadrupole 2 has only a RF voltage applied and acts as a collision cell. Ions produced in the ion source are passed into the first quadrupole analyser in the usual manner. This analyser is set to allow only the ions of interest, in this case the pseudo molecular ions, to be transmitted into the second quadrupole. This second quadrupole contains an inert gas, typically argon at low pressure, which collides with the ions of interest to induce fragmentation. This process is known as collision-activated dissociation (CAD). The fragment ions thus produced are focused into the third quadrupole where they are separated according to their mass to charge ratio. The resulting spectrum therefore depicts the fragment ions produced from, in this case, the pseudo molecular ions. This process is known as a daughter or product ion experiment.
Other modes of MS/MS operation are the parent (or precursor) ion scan and the constant neutral loss scan. The former is used for the identification and confirmation of known groups of compounds which produce a common daughter ion on fragmentation. In this case, quadrupole 3 is used to select an ion formed in the collision cell and quadrupole 1 is scanned in the usual way to produce a spectrum showing its parent ions. The constant neutral loss experiment is again used for the identification and confirmation of known groups of compounds. In this case, the compounds do not produce the same daughter ions but lose a common neutral fragment instead. In these experiments, quadrupoles 1 and 3 are scanned simultaneously with a mass offset equal to the mass of the neutral fragment which is lost. The resulting mass spectrum shows the parent ions.

LC/MS with electrospray ionisation was initially employed for the work described here. Pseudo molecular ions were observed for the metabolites with only weak fragment ions being observed. A variety of daughter ion experiments was therefore performed on the protonated molecular ions in order to fully characterise each metabolite. Parent ion experiments were also utilised to ensure that all metabolites were detected.

2.3 EXPERIMENTAL

2.3.1 Reagents and Materials

Hoe 127490 was synthesised at AgrEvo GmbH, Frankfurt and its purity was established by melting point, proton nuclear magnetic resonance spectroscopy, mass spectrometry and HPLC. A standard solution containing Hoe 127490 (5 mg) in 50 ml of 50% acetonitrile / 50% water (0.1% trifluoroacetic acid [TFA]) was prepared. Acetonitrile and water (both HPLC grade) were obtained from Fisons Scientific Equipment, Loughborough. TFA was obtained from Fluorochem Limited, Old Glossop.

The urine sample was collected over a 24 hour period from a rat which had been dosed with Hoe 127490 at a level of 10 mg/kg (body weight). Administration of Hoe 127490 to
the rat and collection of the urine were carried out at AgrEvo UK Limited, Chesterford Park and were completely separate from the analytical aspects of this study, therefore, details are not reported here. No pre-treatment of the urine sample was performed.

2.3.2 Instrumentation

LC/MS and LC/MS/MS investigations were performed using a Hewlett Packard 1090 liquid chromatograph with autosampler coupled to a VG Biotech Quattro II triple quadrupole mass spectrometer fitted with a standard electrospray interface. LC/MS operations were carried out with optimised source temperature and cone voltage of 170°C and 30 V respectively, with alternating positive and negative ionisation scans over the mass range 50-800 Da at 1 scan/second.

LC/MS/MS analyses were performed in the positive ionisation mode only. Appropriate mass ranges were selected for each experiment and one scan was obtained every second. Argon was employed as the collision gas at a pressure of 1.0 mbar. A collision energy of 22 eV was employed for the parent ion experiments because this maximised the intensity of the base peak in the daughter ion spectrum of Hoe 127490. This collision energy was reduced slightly to 20 eV for the daughter ion experiments in order to enhance the weaker fragment ions.

LC separation was achieved using a 250 × 4.6 mm Hypersil 5 ODS column. A flow rate of 1 ml/min was employed with a mobile phase of 100% A for 3 minutes increasing with a linear gradient to 50% A / 50% B after 20 minutes and finally to 100% B after 27 minutes (solvent A = 0.1% aqueous TFA, solvent B = acetonitrile [0.1% TFA]). The total chromatographic run time was 45 minutes. 30% of the flow was split to the mass spectrometer and the remaining 70% flowed to waste. A UV wavelength of 238 nm was monitored and 25 µl injections were made.
2.3.3 Analysis

2.3.3.1 Parent compound (Hoe 127490)

Hoe 127490 was examined by LC/MS using the conditions described above. Daughter ion LC/MS/MS experiments were also performed on its protonated molecular ion (m/z 496) over a mass range of 50-500 Da.

2.3.3.2 Urine sample

The urine sample was initially investigated by LC/MS using the conditions described above. On the basis of results obtained from this investigation (see Section 2.4.2.1), 7 subsequent LC/MS/MS experiments were performed to provide additional information on suspected metabolites detected in the sample. These experiments were as follows:

i. LC/MS/MS daughter ion experiments on the potential MH⁺ ion, m/z 482

Daughter ion spectra were generated for m/z 482 for the complete chromatographic run over a mass range of 50-500 Da.

ii. LC/MS/MS daughter ion experiments on potential MH⁺ ions, m/z 315, 398, 412, 468 and 496

Daughter ion spectra were obtained in a single chromatographic run on m/z 398 (16-18 minutes), m/z 315 (18-19 minutes), m/z 468 (19-19.5 minutes and 21-23.5 minutes), m/z 412 (19.5-21 minutes) and m/z 496 (23.5-27 minutes). The mass range was scanned over the region 50-500 Da.

iii. LC/MS/MS daughter ion experiments on the potential MH⁺ ion, m/z 512

Daughter ion spectra were generated for m/z 512 for the complete chromatographic run over a mass range of 50-520 Da.

iv. LC/MS/MS daughter ion experiments on the potential MH⁺ ion, m/z 498

Daughter ion spectra were generated for m/z 498 for the complete chromatographic run over a mass range of 50-510 Da.
In order to ensure that all metabolites containing the pyrimidine ring have been detected, LC/MS/MS parent ion experiments were performed on the base peak fragment ions, m/z 168, 182 and 198. Parent ion spectra were obtained for the ions m/z 168, 182 and 198 in a single chromatographic run using alternating parent ion scans. The mass range was scanned over the region 170-1000 Da for 45 minutes.

After examination of the data obtained in the parent ion experiments, the following daughter ion experiments were performed:

v. LC/MS/MS daughter ion experiments on m/z 674 and m/z 688

Daughter ion spectra were obtained for m/z 674 and 688 in a single chromatographic run using alternating daughter ion scans. The mass range was scanned over the region 50-690 Da.

vi. LC/MS/MS daughter ion experiments on m/z 301, 454, 578 and m/z 592

Daughter ion spectra were obtained in a single chromatographic run on m/z 301 (10-16.5 minutes, mass range 50-310 Da), m/z 578 and m/z 592 (alternating scans 16.5-20 minutes, mass range 50-600 Da) and m/z 454 (20-24.5 minutes, mass range 50-460 Da).

2.4 RESULTS

2.4.1 Parent Compound (Hoe 127490)

Under the conditions employed, Hoe 127490 had a retention time of 24.8 minutes (see Figure 2.3).
Full scan LC/MS analysis in the positive ionisation mode produced a base peak at m/z 496 corresponding to the protonated molecular ion (see Figure 2.4). Significant ions were also observed at m/z 518 and m/z 534 due to [M + Na]$^+$ and [M + K]$^+$ respectively. A fragment ion was observed at m/z 182 corresponding to the pyrimidinyl isocyanate ion (see Figure 2.6). Only weak data was obtained in the negative ionisation mode (see Figure 2.4) showing a base peak at m/z 494 due to [M-H].
LC/MS/MS daughter ion experiments on the MH\(^+\) ion (m/z 496) produced significant ions at m/z 298 and 182 (see Figure 2.5). Weaker ions were also detected at m/z 464, 315, 298, 156 and 84. Proposed structures for these fragment ions are shown in the fragmentation pathway in Figure 2.6.

Figure 2.5 : Daughter ion spectrum of Hoe 127490 (m/z 496)
2.4.2 Urine Sample

Examination of the urine sample by LC/MS with electrospray ionisation in both the positive and negative modes yielded a number of prominent MH$^+$ and [M-H]$^-$ ions respectively which could correspond to potential metabolites of Hoe 127490. Important structural information on these species was also available by consideration of the weaker fragment ions which were observed. Further structural information was obtained from LC/MS/MS investigations. Key fragment ions observed in the LC/MS and/or LC/MS/MS experiments described below were assigned as shown in Figure 2.7.
R = H, R1 = H (m/z 168)  
R = Me, R1 = H (m/z 182)  
R = Me, R1 = OH (m/z 198)

R2 = H, R3 = H (m/z 270)  
R2 = Me, R3 = H (m/z 284)  
R2 = H, R3 = Me (m/z 284)  
R2 = Me, R3 = Me (m/z 298)

MH^+ - MeOH when species contains a methyl ester

Figure 2.7 : General fragment ions produced from Hoe 127490-related species

2.4.2.1 LC/MS

The UV chromatogram for the urine sample is shown in Figure 2.8.
The following potential metabolites were detected:

*i. Hoe 127490* (see Figure 2.9)

Parent compound appeared to be present in the urine. Spectra observed at a retention time of 24.6 minutes were comparable to those obtained for the standard sample (see Figure 2.4).
ii. Des-methyl-Hoe 127490 (see Figures 2.10-2.12)

Three species were detected at retention times of 17.6, 21.3 and 24.7 minutes (Metabolites IV, XI and XX respectively) which all appeared to have a molecular weight of 481 (m/z 482 and 480 observed in ES+ and ES- respectively). These may be due to des-methyl-Hoe 127490. The earliest eluting species produced a significant ion at m/z 168 (ES+) which suggests that the methyl group may have been lost from the pyrimidine ring. A significant ion was observed at m/z 182 in the ES+ spectra of the other two species which suggests that the pyrimidine ring remains intact and that demethylation has occurred at the opposite end of the parent molecule. Ions were detected at m/z 464 and m/z 450 in the ES+ spectra of Metabolites XI and XX respectively. The former ion may be due to [MH+ - H2O] of the ester demethylated species. The latter ion may correspond to [MH+ - MeOH] of the N-des-methyl species. LC/MS/MS experiments were performed to confirm the structures of these three metabolites (see Section 2.4.2.2 i).

Figure 2.10 : ES+ and ES- spectra of species eluting at 17.6 minutes (Metabolite IV)
iii. Des-dimethyl-Hoe 127490 (see Figures 2.13 and 2.14)

Two species were detected at retention times of 19.4 and 21.7 minutes (Metabolites IX and XIII respectively) which appeared to have a molecular weight of 467 (m/z 468 and 466 observed in ES+ and ES- respectively). These may be due to des-dimethyl-Hoe 127490. Metabolite XIII produced a significant ion at m/z 182 (ES+) which suggests that
the pyrimidine ring remains intact and that demethylation has occurred at the opposite end of the parent molecule. A significant fragment ion corresponding to the pyrimidine ring was not observed in the ES+ spectra of Metabolite IX but a weak ion was observed at m/z 436 ([MH<sup>-</sup> - MeOH] possibly) which suggests that one of the pyrimidine methyl groups has been lost together with the N-methyl group. LC/MS/MS experiments were performed to confirm the structures of these two species (see Section 2.4.2.2 ii).

![Relative Intensity](image1)

**Figure 2.13:** ES+ and ES- spectra of species eluting at 19.4 minutes (Metabolite IX)

![Relative Intensity](image2)

**Figure 2.14:** ES+ and ES- spectra of species eluting at 21.7 minutes (Metabolite XIII)
iv. Des-methyl-des-isobutyryl-Hoe 127490 (see Figure 2.15)

A species was detected at a retention time of 19.6 minutes (Metabolite X) which appeared to have a molecular weight of 411 (m/z 412 and 410 observed in ES+ and ES- respectively). This may be due to des-methyl-des-isobutyryl-Hoe 127490. An ion was observed at m/z 168 (ES+) which suggests that the methyl group may have been lost from the pyrimidine ring. LC/MS/MS experiments were performed to confirm the structure of this species (see Section 2.4.2.2 ii).

Figure 2.15: ES+ and ES- spectra of species eluting at 19.6 minutes (Metabolite X)

v. Des-dimethyl-des-isobutyryl-Hoe 127490 (see Figure 2.16)

A species (Metabolite II) was detected at a retention time of 16.9 minutes which appeared to have a molecular weight of 397 (m/z 398 and 396 observed in ES+ and ES- respectively). This may be due to des-dimethyl-des-isobutyryl-Hoe 127490. An ion was observed at m/z 168 (ES+) which suggests that the methyl group may have been lost from the pyrimidine ring. LC/MS/MS experiments were performed to fully identify the structure of this species (see Section 2.4.2.2 ii).
Relative Intensity

100-

100-

m/z

ES+

ES-

Figure 2.16: ES+ and ES- spectra of Metabolite II eluting at 16.9 minutes

vi. Sulfonamide (see Figure 2.17 for proposed structure and Figure 2.18 for ES+ spectrum)

C_{13}H_{18}N_{2}O_{3}S (314)

Figure 2.17: Sulfonamide (Metabolite VII)

A species (Metabolite VII) was detected at a retention time of 18.5 minutes which appeared to have a molecular weight of 314 (m/z 315 and 337 observed in ES+ which may correspond to MH+ and MNa+ respectively of the sulfonamide). An ion was observed at m/z 298 which may be due to the loss of ammonia from the protonated molecular ion of this species. No significant ES- data was obtained for this species. LC/MS/MS experiments were performed to confirm the structure of this species (see Section 2.4.2.2 ii).
Figure 2.18: ES+ spectrum of species eluting at 18.5 minutes (Metabolite VII)

**vii. Des-methyl-sulfonamide** (see Figure 2.19 for proposed structure and Figure 2.20 for the ES+/- spectra)

![Des-methyl sulfonamide structure](image)

C<sub>12</sub>H<sub>16</sub>N<sub>2</sub>O<sub>3</sub>S (300)

Figure 2.19: Des-methyl sulfonamide (Metabolite I)

A species was detected at a retention times of 15.3 minutes (Metabolite I) which appeared to have a molecular weight of 300 (m/z 301 and 323 observed in ES+ which may correspond to MH<sup>+</sup> and MNa<sup>+</sup> respectively of the des-methyl-sulfonamide and m/z 299 due to [M-H]<sup>-</sup> was observed in ES-). An ion was observed at m/z 284 in ES+ which may be due to the loss of ammonia from the protonated molecular ion of this species and an ion at m/z 255 in ES- may be due to the loss of CO<sub>2</sub> suggesting that the methyl ester may not be present. LC/MS/MS experiments were performed to confirm the structure of this species (see Section 2.4.2.5).
viii. Hydroxy-Hoe 127490 or Hoe 127490-N-oxide (see Figures 2.21-2.23)

Three species were detected at retention times of 21.3, 22.4 and 23.0 minutes (Metabolites XII, XVI and XVIII respectively) which all appeared to have a molecular weight of 511 (m/z 512 and 534 observed in ES+ which may be due to MH⁺ and MNa⁺ respectively). These may be due to hydroxy-Hoe 127490 or Hoe 127490-N-oxide. No significant ES- data were obtained for these species. There are no literature references to N-oxides being rat metabolites of sulfonylurea herbicides but hydroxylate species have been reported77. It might be assumed therefore, that these three metabolites are hydroxylated species.

Metabolite XII produced a significant ion at m/z 198 (ES+) which suggests that the pyrimidine ring is hydroxylated. A significant ion was observed at m/z 182 in the ES+ spectra of the other two species which suggests that the pyrimidine ring remains intact and that hydroxylation has occurred on the opposite end of the molecule. LC/MS/MS experiments were performed to fully identify the structures of these three metabolites (see Section 2.4.2.2 iii).
Figure 2.21: ES+ spectrum of Metabolite XII eluting at 21.3 minutes

Figure 2.22: ES+ spectrum of Metabolite XVI eluting at 22.4 minutes
ix. Hydroxy-des-methyl-Hoe 127490 or des-methyl-Hoe 127490-N-oxide (see Figures 2.24-2.26)

Three species were detected at retention times of 21.8, 22.3 and 23.3 minutes (Metabolites XIV, XV and XIX respectively) which all appeared to have a molecular weight of 497 (m/z 498 and 520 observed in ES+ which may be due to MH⁺ and MNa⁺ respectively and m/z 496, [M-H]⁻ under ES- conditions). These may be due to hydroxy-des-methyl-Hoe 127490 or des-methyl-Hoe 127490-N-oxide. As discussed above, there are no literature references to N-oxides being rat metabolites of sulfonylurea herbicides whereas hydroxylated species are reported, therefore it might be assumed that these three metabolites are all hydroxylated species.

The earliest eluting species (Metabolite XIV) produced a significant ion at m/z 198 (ES+) which suggests that the pyrimidine ring is hydroxylated and that demethylation has occurred on the opposite end of the molecule. A significant ion was observed at m/z 182 in the ES+ spectrum of the latest eluting species (Metabolite XIX) which suggests that the pyrimidine ring remains intact and that hydroxylation and demethylation has occurred on the opposite end of the molecule. No conclusions could be made on the other species (Metabolite XV). LC/MS/MS experiments were performed to fully identify the structures of these three metabolites (see Section 2.4.2.2.iv).
Figure 2.24: ES+ and ES- spectra of Metabolite XIV eluting at 21.8 minutes

Figure 2.25: ES+ and ES- spectra of Metabolite XV eluting at 22.3 minutes
2.4.2.2 LC/MS/MS daughter ion experiments on potential $MH^+$ ions

i. LC/MS/MS daughter ion experiments on the potential $MH^+$ ion, $m/z$ 482

Figures 2.27-2.29 show the MS/MS data obtained for the three species referred to in Section 2.4.2.1.ii above, which confirms the presence of three des-methyl-Hoe 127490 isomers. A proposed structure for each metabolite is included with each spectrum.
Figure 2.28: Daughter ion spectrum of Metabolite XI eluting at 21.2 minutes

Figure 2.29: Daughter ion spectrum of Metabolite XX eluting at 24.7 minutes
ii. LC/MS/MS daughter ion experiments on potential MH⁺ ions (m/z 315, 398, 412, 468 and 496)

Figures 2.30-2.35 show the MS/MS data obtained for the species referred to in Sections 2.4.2.1.i and iii-vi above. A proposed structure for each metabolite is included with each spectrum. Figure 2.35 confirms the presence of Hoe 127490 in the urine.

Figure 2.30: Daughter ion spectrum of Metabolite VII eluting at 18.5 minutes

Figure 2.31: Daughter ion spectrum of Metabolite II eluting at 16.9 minutes
Figure 2.32: Daughter ion spectrum of Metabolite X eluting at 19.6 minutes

Figure 2.33: Daughter ion spectrum of Metabolite IX eluting at 19.4 minutes
iii. LC/MS/MS daughter ion experiments on the potential MH⁺ ion, m/z 512

Figures 2.36-2.38 show the MS/MS data obtained for the three species referred to in Section 2.4.2.1.viii above.
Figure 2.36: Daughter ion spectrum of Metabolite XII eluting at 21.3 minutes

Figure 2.37: Daughter ion spectrum of Metabolite XVI eluting at 22.4 minutes
Very weak data was obtained for the species eluting at 21.3 minutes (see Figure 2.36) but a base peak was observed at m/z 198 confirming that hydroxylation has occurred on the pyrimidine ring as shown in Figure 2.39.

![Proposed structure for Metabolite XII eluting at 21.3 minutes]

\[ C_{20}H_{25}N_3O_9S \ (511) \]

The other two species (see Figures 2.37 and 2.38), eluting at 22.4 and 23.0 minutes, show base peaks at m/z 182, indicating that no changes have been made to the pyrimidine ring and therefore, hydroxylation has occurred on the opposite side of the molecule. The ions observed at m/z 314 support this. The species eluting after 22.4 minutes (Metabolite XVI) showed a weak ion at m/z 100 which may correspond to the species shown in Figure 2.40.
If so, then Metabolite XVI can be assumed to have the structure shown in Figure 2.41. By elimination of options for the position of hydroxylolation, Metabolite XVIII, eluting after 23.0 minutes is thought to be hydroxylated on the phenyl ring as shown in Figure 2.41. Unfortunately, the exact point of substitution of the hydroxyl group on the phenyl ring could not be established by MS alone, and proton nuclear magnetic resonance (NMR) spectroscopy would be required in order to determine the exact identity of this metabolite.

\[ \text{Metabolite XVI} \]
\[ \text{C}_{20} \text{H}_{25} \text{N}_{5} \text{O}_{9} \text{S (511)} \]

Figure 2.41 : Proposed structures for Metabolites XVI and XVIII, respectively eluting at 22.4 and 23.0 minutes

iv. *LC/MS/MS daughter ion experiments on the potential } M+ \text{ ion, } m/z \text{ 498*}

Figures 2.42 and 2.43 show the MS/MS data obtained for the species referred to in Section 2.4.2.1.ix above, eluting at 22.3 and 23.3 minutes.
Both species produced base peaks at m/z 182, indicating that no changes have been made to the pyrimidine ring. Hydroxylation and demethylation have therefore occurred on the opposite side of the molecule. The ions observed at m/z 300 support this. The earlier eluting species (Metabolite XV) showed a significant ion at m/z 466 which suggests that...
the ester methyl is intact and therefore it can be assumed that N-demethylation has occurred. The species eluting after 23.3 minutes (Metabolite XIX) did not produce an ion at m/z 466 but it was observed on closer examination of the LC/MS ES+ data. It has therefore been concluded that N-demethylation has also occurred with this metabolite. Unfortunately, no ions were detected which could determine the exact position of the hydroxyl group on either metabolite. On the assumption that they will probably be the N-demethylated versions of the species described in Section 2.4.2.2.iii, structures have been proposed for these two metabolites as shown in Figure 2.44. A comparison of the relative retention times with those of the corresponding N-methylated species (Metabolites XVI and XVIII), has been used to distinguish between the two isomers. It has been assumed, therefore, that the phenolic metabolite will elute after the hydroxymethyl metabolite as in the case of the N-methyl metabolites in Section 2.4.4.2.iii.

C_{19} H_{23} N_{5} O_{9} S (498)

Figure 2.44: Proposed structures for Metabolites XIV, XV and XIX eluting at 21.8, 22.3 and 23.3 minutes respectively
No data was obtained for the species eluting at 21.8 minutes (Metabolite XIV) therefore the LC/MS ES+ data had to be examined more closely in order to identify this metabolite (see Figure 2.23). As discussed previously, an ion was detected at m/z 198 suggesting that the pyrimidine ring is hydroxylated and that demethylation has occurred at the opposite end of the molecule. A weak ion was also observed at m/z 466 which may correspond to [MH⁺ - MeOH]. This indicates that the ester methyl is still intact and therefore, it can be concluded that the metabolite is N-demethylated. The structure shown in Figure 2.44 has therefore been proposed for Metabolite XIV.

2.4.2.3 LC/MS/MS parent ion experiments on the pyrimidine fragment ions
The base peak observed in all daughter ion spectra corresponded to the pyrimidine fragment ion (m/z 168, 182 or 198). In order to ensure that all metabolites containing these fragments had been identified, parent ion experiments were performed on these three ions.

i. LC/MS/MS parent ion experiments on m/z 168
Four species were observed which appeared to contain a demethylated pyrimidine ring (see Figure 2.45 for the mass chromatograms of the parent ions detected). The parent ions were observed at m/z 398 (retention time 17.0 minutes), m/z 482 (retention time 18.0 minutes), m/z 412 (retention time 19.6 minutes) and m/z 468 (retention time 19.7 minutes). All four metabolites have been previously identified (Metabolites II, IV, X and IX respectively, see Section 2.4.2.2) and no other related species were detected.
ii. LC/MS/MS parent ion experiments on m/z 182

Nine species were observed which appeared to contain the pyrimidine ring intact (see Figure 2.46 for the mass chromatograms of the parent ions detected). The parent ions were observed at m/z 454 (retention time 22.9 minutes), m/z 468 (retention time 21.7 minutes), m/z 482 (two isomers, retention times 21.3 and 24.8 minutes), m/z 496 (retention time 24.6 minutes), m/z 498 (two isomers, retention times 21.8 and 23.3 minutes) and m/z 512 (two isomers, retention times 22.3 and 23.0 minutes). Eight of the species have been previously identified (Metabolites XIII, XI, XX, XV, XIX, XVI, XVIII and parent compound, see Section 2.4.2.2). The species eluting at 22.9 minutes may be due to a metabolite of molecular weight 453. On further examination of the LC/MS ES+ data, this appears to be the case (see Figure 2.47). Significant ions were detected at m/z 454 and 476 which may correspond to MH+ and MNa+ respectively. No pseudo molecular ion was observed under ES- conditions. Further fragmentation data is required in order to identify this species (see Section 2.4.2.5).
Figure 2.46: Mass chromatograms for parents of m/z 182
iii. LC/MS/MS parent ion experiments on m/z 198

Five species were observed which appeared to contain the hydroxylated pyrimidine ring intact (see Figure 2.48). The parent ions were observed at m/z 498 (retention time 17.8 minutes), m/z 512 (retention time 21.3 minutes), m/z 592 (retention time 17.3 minutes), m/z 674 (retention time 18.7 minutes) and m/z 688 (retention time 18.1 minutes).

The m/z 512 species (Metabolite XII) eluting at 21.3 minutes has been previously identified (see Section 2.4.2.2.iii). The species eluting at 17.8 minutes was not observed in the daughter ion experiment on m/z 498 (see Section 2.4.2.2.iv). Examination of the LC/MS ES+ and ES- data (see Figure 2.49), indicates that it may be a fragment ion of a species of molecular weight 577 (m/z 578 and 600 observed under ES+ conditions, and m/z 576 and 598 observed under ES- conditions). Figure 2.50 shows a proposed structure for this metabolite. An ion was not detected at m/z 578 in the parent ion experiment corresponding to this species (see Figure 2.48) but instead, the parent ion was observed at m/z 498 which is probably due to the loss of SO$_3$ from the protonated molecular ion. LC/MS/MS daughter ion experiments on m/z 578 are required to confirm this structure (see Section 2.4.2.5).
Figure 2.48: Mass chromatograms for parent ions of m/z 198
The species eluting after 17.3 minutes with a base peak at m/z 592 may be due to a metabolite of molecular weight 591. On closer examination of the LC/MS ES+ and ES- data, this appears to be the case (see Figure 2.51). Significant ions were detected at m/z 592 and 614 under ES+ conditions, and m/z 590 and 612 under ES- conditions due to the protonated and deprotonated molecular ions respectively and their corresponding sodium adducts. A proposed structure for this metabolite is shown in Figure 2.52. LC/MS/MS daughter ion experiments on m/z 592 were performed to confirm this structure (see Section 2.4.2.5).
Figure 2.51: ES+ and ES- spectra of Metabolite III eluting at 17.3 minutes

Figure 2.52: Proposed structure for Metabolite III eluting at 17.3 minutes

C_{20}H_{25}N_5O_{12}S_2 (591)

The species eluting after 18.7 minutes with a base peak at m/z 674 may be due to a metabolite of molecular weight 673. On closer examination of the LC/MS ES+ and ES- data, this appears to be the case (see Figure 2.53). Significant ions were detected at m/z 674 and 696 under ES+ conditions, and m/z 672 and 694 under ES- conditions due to the protonated and deprotonated molecular ions respectively and their corresponding sodium adducts. A proposed structure for this metabolite is shown in Figure 2.54. LC/MS/MS daughter ion experiments on m/z 674 were performed to confirm this structure (see Section 2.4.2.4).
The species eluting after 18.1 minutes with a base peak at m/z 688 may be due to a metabolite of molecular weight 687. On closer examination of the LC/MS ES+ and ES- data, this appears to be the case (see Figure 2.55). Significant ions were detected at m/z 688 and 710 under ES+ conditions, and m/z 686 and 708 under ES- conditions due to the protonated and deprotonated molecular ions respectively and their corresponding sodium adducts. A proposed structure for this metabolite is shown in Figure 2.56. LC/MS/MS daughter ion experiments on m/z 688 were performed to confirm this structure (see Section 2.4.2.4).
2.4.2.4 LC/MS/MS daughter ion experiments on m/z 674 and m/z 688
Figures 2.57 and 2.58 show the MS/MS data obtained for these two species. The relevant proposed structures from Section 2.4.2.3.iii are included.
Both spectra show a significant ion at m/z 198 indicating the presence of a hydroxylated pyrimidine ring. The ion at m/z 374 in both spectra is due to the glucuronide of the hydroxylated pyrimidine species. The methyl ester appears to be intact in both metabolites because ions are detected at m/z 480 and m/z 466 which are due to [m/z 512 - MeOH] of the methylated species and [m/z 498 - MeOH] of the des-methyl species respectively. The MS/MS data are therefore consistent with the proposed structures.
Figure 2.57: Daughter ion spectrum of Metabolite VI eluting at 18.1 minutes

Figure 2.58: Daughter ion spectrum of Metabolite VIII eluting at 18.7 minutes

2.4.2.5 LC/MS/MS daughter ion experiments on m/z 301, 454, 578 and m/z 592
Figures 2.59-2.62 show the daughter ion spectra (with the corresponding structures, where relevant, proposed in Sections 2.4.2.1.vii and 2.4.2.3.iii) for these four species.
Figure 2.59: Daughter ion spectrum of Metabolite I eluting at 15.3 minutes

Figure 2.60: Daughter ion spectrum of Metabolite V eluting at 17.8 minutes
The spectrum shown in Figure 2.59 confirms the presence of the des-methyl-sulfonamide, whose structure is shown on the spectrum, because the ion at m/z 84 indicates that the species is N-methylated.
The spectra shown in Figures 2.60 and 2.61 show a base peak at m/z 198 indicating the presence of a hydroxylated pyrimidine ring. The ion at m/z 278 in both spectra is due to the sulfated pyrimidine ion. Metabolite V is presumed to be N-demethylated in preference to ester demethylated because it has a longer retention time than that of Metabolite III and it has been noted that all the other N-demethylated metabolites elute after their corresponding methylated species. The LC/MS/MS data are therefore consistent with the proposed structures for Metabolites III and V.

A base peak was observed at m/z 182 in the daughter ion spectrum of Metabolite XVII (see Figure 2.62) indicating that the pyrimidine ring is unchanged. A significant ion at m/z 256 indicates that the changes to the parent compound have probably occurred on the opposite side of the molecule. Unfortunately, no other ions were observed to aid identification of this metabolite and the structure shown in Figure 2.63 has therefore been proposed.

![Figure 2.63: Tentative structure for Metabolite XVII](image)

This metabolite is believed to have been produced by the decarboxylation of de-esterified Metabolite XIX. Decarboxylation in sulfonyl urea herbicides has not been previously reported but decarboxylation of an aromatic carboxylic acid is a known metabolic step. Isolation of the metabolite for proton NMR investigations would be required in order to confirm the identity of this species.

### 2.5 DISCUSSION

Twenty metabolites of Hoe 127490 in the rat have been identified by LC/MS and LC/MS/MS analysis of the urine sample without the need for sample pretreatment and
clean-up. Of these twenty metabolites, only three could not be confidently identified (Metabolites XVII, XVIII and XIX) using mass spectrometric techniques alone. Isolation of these metabolites is required prior to NMR investigations. Detection of Metabolites III, V, VI and VIII using parent ion scans shows the importance of using a variety of MS/MS experiments to ensure that all metabolites have been detected. If daughter ion scans alone had been used, these four metabolites would probably not have been detected and identified.

The aim of this work was to identify the metabolites present in the urine, and quantification of the levels present was not of primary importance. Even so, semi-quantitative results were available from the UV chromatogram which had been obtained on the urine (see Figure 2.64). It can be assumed that all of the metabolites will possess a similar chromophore and therefore the relative peak areas on the UV trace will correspond to the levels present. The elution time of each of the metabolites is indicated on the chromatogram.

Figure 2.64 : UV (238 nm) of metabolites identified in rat urine sample

This chromatogram indicates that a significant proportion of the parent compound has not been metabolised and is excreted in the urine. From the UV response, the major metabolites appear to be Metabolites IV (4-hydroxy-6-methoxypyrimidinyl-Hoe 127490), VI (5-hydroxypyrimidinyl-Hoe 127490-glucuronide conjugate), XII (5-hydroxypyrimidinyl Hoe 127490) and XX (des-N-methyl-Hoe 127490).
A proposed metabolic pathway is shown in Figure 2.65. Nine routes of metabolism are proposed in this pathway, featuring one or more of the following:

i. Hydroxylation of the pyrimidine ring which may subsequently be conjugated to form the sulfate or the glucuronide

ii. Hydroxylation of the phenyl ring

iii. Hydroxylation of the isopropyl side chain

iv. N-demethylation

v. Ester demethylation

vi. Methoxypyrimidinyl-demethylation

vii. Decarboxylation

viii. Hydrolysis of the amide bridge

ix. Hydrolysis of the sulfonyl urea bridge

All of these routes have been previously reported for sulfonyl urea herbicides excluding the decarboxylation step although, as discussed previously, this is a known metabolic pathway in other aromatic methyl esters and therefore might be expected.
Figure 2.65 Proposed metabolic pathway for Hoe 127490 in the rat
2.6 CONCLUSIONS
This study has demonstrated the power of LC/MS and LC/MS/MS as a technique for the rapid detection and identification of metabolites in a complex biological matrix such as urine. Untreated urine collected from a rat which had been dosed with Hoe 127490 was analysed directly by electrospray LC/MS and LC/MS/MS and twenty metabolites were identified. The compound is extensively metabolised in the rat by nine different routes and a metabolic pathway has been proposed.

2.7 REFERENCES
CHAPTER 3: DEVELOPMENT OF A SEMI-AUTOMATED METHOD FOR THE IDENTIFICATION OF DIPEPTIDES IN PROTEIN HYDROLYSATES
3.1 INTRODUCTION

Protein (or at least a source of certain amino acids) is an essential nutritional requirement for man. Ingested proteins are broken down in the digestive system and are then used in the synthesis of proteins essential to all aspects of development and growth.

The nutritional value of a protein is determined by its amino acid content. Man is able to biosynthesise about half of the amino acids in quantities required for growth and for maintenance of normal nitrogen balance. These are known as the non-essential amino acids. The other amino acids must be provided in the diet to meet man's metabolic needs and are called the essential amino acids. These are histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine.

The amino acids ingested by man are generally in the form of proteins. Amino acids can be absorbed in the gastrointestinal tract either as free amino acids or as di- and tripeptides so dietary protein must be hydrolysed by proteolytic enzymes in the stomach, pancreas and small intestine before absorption can occur. Free amino acids or protein hydrolysates may be administered to patients undergoing treatment who are too ill to be able to readily digest whole proteins. The smaller peptides in the hydrolysed protein and the free amino acids do not require hydrolysis in the body and are therefore absorbed more easily. It has been shown that di- and tripeptides are absorbed more rapidly from the small intestine than are free amino acids and therefore preparations of partially hydrolysed proteins containing a predominance of small peptides are of greater nutritional value than an equivalent mixture of free amino acids. The nutritional value of a protein hydrolysate is dependant on the initial protein (i.e. the amino acid content) and on the degree of hydrolysis (i.e. the length of the resulting peptides).

Another advantage of short chain peptides over free amino acid mixtures is the poor solubility or stability of some of the free amino acids. Free tyrosine and cysteine are relatively insoluble (0.4 g/l and 0.1 g/l respectively), whilst free glutamine, cysteine and tryptophan are relatively unstable during sterilization procedures and on storage. These 'problem' free amino acids can be replaced with soluble and stable short-chain peptides containing glutamine, cysteine, tyrosine and tryptophan. The short-chain peptides can either be synthesised individually or produced by hydrolysis of a protein. The latter method is considered to be the cheapest option but the protein hydrolysate needs to be
characterised prior to clinical use. A method for identifying the peptides present in protein hydrolysates would therefore be valuable in characterising these preparations.

Protein hydrolysates used for nutritional purposes are extremely complex mixtures of peptides and free amino acids. For example, in a mixed polypeptide with random sequence, there are 400 and 8000 possible di- and tripeptides, respectively. In reality, for a hydrolysate of a single protein, there are fewer possibilities, however, analysis of these mixtures poses a considerable challenge to the chemist. Identification of the peptides present requires their separation and sequencing and, to date, gas chromatography-mass spectrometry (GC/MS) or size exclusion chromatography followed by reversed-phase liquid chromatography (LC) have been applied to protein hydrolysates. In the former method, the protein hydrolysates were initially desalted by cation exchange chromatography and the resulting peptides were separated from free amino acids by copper-chelex chromatography. Copper (II) was removed from the purified peptides with a borate buffer. After removal of the borate by treatment with acidified methanol, the purified peptides were dried and derivatised with N,O-bis(trimethylsilyl)trifluoroacetamide prior to GC/MS analysis. As discussed in Section 1.2, GC requires the analytes to be vaporised prior to chromatographic separation and, unfortunately, only the smaller derivatised dipeptides had the required volatility to facilitate analysis. Derivatised tripeptide standards produced only degradation products by GC/MS. Even so, a total of fifteen dipeptides were identified in ovalbumin and casein hydrolysates using this GC/MS method.

The latter LC method initially involved the fractionation of the peptides present in two casein hydrolysates by size exclusion chromatography. The fractionated peptides were then separated by reversed phase LC on a C\textsubscript{18} column using an aqueous trifluoroacetic acid and acetonitrile gradient. 213 and 187 peptides from dephosphorylated and phosphorylated hydrolysates respectively were isolated and subsequently automatically identified on a Waters Pico-Tag amino acid analysis system. This method therefore appears to be extremely effective but, unfortunately, it has the major drawback of being very time-consuming due to the extensive separation and isolation steps involved.

Routine analysis of peptides and proteins by LC/MS using tandem mass spectrometry has been reported, particularly since the introduction of the atmospheric pressure ionisation
techniques but, to date, this technique has not been applied to the analysis of protein hydrolysates.

This chapter describes the development of an efficient, semi-automated liquid chromatography-tandem mass spectrometry (LC/MS/MS) method for this purpose using atmospheric pressure chemical ionisation (APCI). The method allows the rapid identification of dipeptides in complex mixtures and has been applied to a casein hydrolysate. The separation power of MS/MS is utilised to simplify the analytical process by eliminating the requirement for optimum chromatographic separation of the dipeptides prior to their identification. In the work described, no chromatography was necessary in the initial experiments on peptide mixtures. MS/MS was used to separate and examine each peptide in turn with fragmentation information, via daughter ion experiments, being obtained for each species present. A computer programme has been developed in-house which automatically examines this data and subsequently produces a list of dipeptides which appear to be present in the sample. The presence of these peptides can then be confirmed rapidly by on-line LC/MS using a crude chromatographic separation.

3.2 SEQUENCING PEPTIDES BY TANDEM MASS SPECTROMETRY

As discussed previously (see Section 2.2), many compounds which are ionised using a soft ionisation technique produce only pseudo molecular ions with no, or only weak, fragment ions being observed. This is useful if only molecular weight information is required but, further information about the structure is often desirable.

Tandem mass spectrometry using, for example, a triple quadrupole mass spectrometer can be used to fragment pseudo-molecular ions, thereby yielding information about the chemical structure. A triple quadrupole mass spectrometer produces ions with low kinetic energies of between 20 and 100 eV and fragmentation of peptides occurs mainly through simple cleavages of the peptide backbone. Cleavage of the bonds in the peptide backbone can occur in three places and the charge can be retained on either side of the fragmentation, depending on the basicities of the amino acids in the peptide chain. Theoretically therefore, six different series of sequence ions, known as the a-, b-, c-, x-, y- and z-ion series, may be produced as shown in Figure 3.1. The resulting set of fragment ions for a peptide is therefore indicative of its amino acid sequence. For low
energy collisions, the predominant fragment ions are b- and y-type ions and ions resulting
from neutral losses of water or ammonia. The low mass immonium ions are indicative of
the amino acids present but are not related to their positions in the peptide chain.

\[
\text{C-terminus ions}
\begin{array}{c}
\text{x}_3 \text{ y}_3 \text{ z}_3 \\
\text{x}_2 \text{ y}_2 \text{ z}_2 \\
\text{x}_1 \text{ y}_1 \text{ z}_1
\end{array}
\]

N-terminus ions

\[
\frac{[\text{H}_2\text{N-CH-CO-NH-CH-COOH}]}{\text{CHR}^1
\frac{\text{R}}{\text{CHR}^1}}
\]

\[
\frac{[\text{HN=CH-CO-NH-CH-COOH}]}{\text{CHR}^1
\frac{\text{R}}{\text{CHR}^1}}
\]

side-chain ions

\[
\frac{[\text{CH-CO-NH-CH-COOH}]}{\text{CHR}^1
\frac{\text{R}}{\text{CHR}^1}}
\]

w ions

\[
\frac{[\text{H}_2\text{N}=\text{CH}]}{\text{CHR}^1
\frac{\text{R}}{\text{CHR}^1}}
\]

d ions

\[
\frac{[\text{HN}=\text{CH-CO-NH-CH-COOH}]}{\text{CHR}^1
\frac{\text{R}}{\text{CHR}^1}}
\]

v ions

immonium ions

\[
\frac{[\text{H}_2\text{N}=\text{CH}]}{\text{CHR}^1
\frac{\text{R}}{\text{CHR}^1}}
\]

Figure 3.1: Peptide fragment ions

The isobaric amino acids, leucine and isoleucine, cannot be differentiated using a triple
quadrupole mass spectrometer because the ions formed reflect the mass of each amino acid
side chain and not the structure. In order to discriminate between these isobaric amino
acids, high energy collisions are required to produce the d-, v- and w-ion series which are
dependant on the structure of the amino acid at the point of cleavage from the backbone
chain (see Figure 3.1). A magnetic sector instrument is required where the precursor ions
have kinetic energies of 5000 - 10000 eV. The other pair of isobaric amino acids, lysine
and glutamine, can be differentiated using high energy collisions or by analysis at low
collision energies after acetylation of the peptides with acetic anhydride. Acetylation
occurs on the free amino-terminus of the peptide and on the amino-group of any lysine
side chains.

Tandem mass spectrometry provides a rapid method for amino acid sequencing of
peptides. However, the sequence data produced can be complicated and full spectral
interpretation can be time consuming. Computer programs are now available from most
mass spectrometer manufacturers to simplify the process. These can predict sequence ions from a given peptide structure or, conversely, propose structures for unknown peptides from CAD and in-source fragmentation spectra.

As discussed previously, protein hydrolysates which are used for nutritional purposes are complex mixtures of peptides and free amino acids. On-column LC/MS analysis with and without source fragmentation could be employed to identify the peptides present with the aid of the manufacturers peptide identification software, but the data obtained would be extremely complex and would rely upon the development of an extremely efficient LC separation. The effective identification of all peptides present would therefore be very tedious and time-consuming therefore a simpler process is required.

The work described here uses the separation power of MS/MS to provide structural information on individual peptides in mixtures, thereby reducing the need for optimum chromatographic separations.

### 3.3 EXPERIMENTAL

A method needed to be established whereby the peptides could be easily selected. Initial investigations were based on work reported by D.S. Millington et al.\textsuperscript{93} for the rapid analysis of amino acids in blood and urine. Here, the amino acids were derivatised with n-butanol prior to FAB MS/MS analysis. A neutral loss scan of 102 Da in the MS/MS mode due to the loss of butyl formate from the protonated molecular ion, was used to rapidly identify the butylated amino acids (see Figure 3.2). The applicability of this method was investigated for the rapid identification of peptides in complex mixtures in attempts to select the peptides by constant neutral loss scans.

![Figure 3.2: Schematic representation of the fragmentation of butyl ester derivatives of amino acids](image)

Figure 3.2: Schematic representation of the fragmentation of butyl ester derivatives of amino acids
3.3.1 Reagents and materials

Peptide standards were obtained from Bachem Feinchemikalien AG (Bubendorf, Switzerland), Serva Feinbiochemica (Heidelberg, Germany) and Sigma Chemical Company (Poole, England). A standard mixture containing a broad spectrum of approximately equal amounts of 77 dipeptides was prepared. The peptides used are listed in Table 3.1. Acetonitrile (far UV grade), water (HPLC grade), acetic acid, methanol, ethanol, i-propanol, n-butanol, n-pentanol and n-octanol were obtained from Fisons Scientific Equipment (Loughborough, England). n-Propanol was obtained from May and Baker Limited (Dagenham, England). Dithiothreitol and iodoacetamide were obtained from Sigma Chemical Company (Poole, England), trifluoroacetic acid (TFA) from Fluorochem Limited (Old Glossop, England) and acetylchloride from Aldrich Chemical Company (Gillingham, England). The alcohols were dried over molecular sieve (type 4A) prior to use. The hydrolysed casein sample was obtained from Laboratoire Roger Bellon (France).

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<td>Thr-Val</td>
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Table 3.1: Dipeptides in standard mixture
3.3.2 Instrumentation

A Finnigan-MAT TSQ 7000 triple quadrupole mass spectrometer was used in combination with Finnigan-MAT APCI or ES interfaces. Operation was carried out in the positive ionisation mode. Optimum vaporiser (APCI mode only) and capillary temperatures of 500°C and 220°C respectively, were employed. A corona current of 5 µA was used in the APCI mode. Source fragmentation was minimised by operating at an octapole voltage of 0 eV whereas an octapole voltage of 25 eV was employed when source fragmentation was required. Argon was used as the collision gas at a pressure of 2.5 mT unless stated otherwise.

Sample solutions were infused using a Harvard Model 22 syringe pump. The make-up solvent was delivered for these infusions using a Waters 600MS LC pump. The latter pump was also employed to deliver the LC eluent for on-column and loop injection analyses. 10 µl sample injections were performed using a Waters 717 autosampler.

On-column LC/MS analyses were carried out on a 30 cm x 3.9 mm Phenomenex Bondclone 10 C18 column.

3.3.3 Initial investigations

A mixture of six peptides (approximately 1mg of each of the following; Leu-Leu, Val-Ala, Leu-Ala-Pro, Leu-Leu-Val-Phe, Gly-Ala-Leu and Pro-Phe-Gly-Lys) was treated with n-butanol (200 µl) and acetyl chloride (1 drop) at 65°C for 30 minutes in a capped 2 ml glass vial. The solvent was evaporated under helium at 40°C and the dried sample was redissolved in methanol (1 ml).

The resulting solution was infused at 5 µl/min through the ES probe. The ion source conditions were optimised to achieve maximum intensities of the protonated molecular ions. The detectors were then set to monitor neutral losses of 102 Da and the argon collision gas pressure and collision energy were adjusted to maximise the signal reaching the second detector. Unfortunately, no conditions could be established whereby a significant response could be observed. It would therefore appear that butyl formate is not lost from butylated peptides to form a stable ion. This was investigated further by examining the daughter ion spectra for each of the individual protonated molecular ions.
The butylated peptide mixture was again infused at 5 μl/min through the ES probe. Each MH$^+$ ion was selected separately and the collision energy was optimised for each to produce maximum sensitivities for the [MH - 102]$^+$ daughter ions.

Daughter ion spectra were subsequently acquired for the MH$^+$ ion of each peptide and the resulting spectra are shown in Figures 3.3-3.8. As can be seen, the [MH - 102]$^+$ ions are almost insignificant except in the case of Leu-Leu. This would explain the reason for no significant data being obtained in the original investigations involving neutral loss of 102 Da. Other competing fragmentation pathways, particularly those resulting in cleavage of the peptide bonds, are probably responsible for the reduced [MH - 102]$^+$ signals observed here, compared to those obtained for butylated amino acids reported by D.S. Millington et al$^{03}$.

![Figure 3.3: Daughter ion spectrum of MH$^+$ of butylated Val-Ala (m/z 245)](image)

Figure 3.3: Daughter ion spectrum of MH$^+$ of butylated Val-Ala (m/z 245)
Figure 3.4: Daughter ion spectrum of $\text{MH}^+$ of butylated Leu-Leu ($m/z$ 301)

Figure 3.5: Daughter ion spectrum of $\text{MH}^+$ of butylated Gly-Ala-Leu ($m/z$ 316)
Figure 3.6: Daughter ion spectrum of MH⁺ of butylated Leu-Ala-Pro (m/z 356)

Figure 3.7: Daughter ion spectrum of MH⁺ of butylated Pro-Phe-Gly-Lys (m/z 504)
Closer examination of the daughter ion spectra show, as expected, that major fragment ions arise from backbone fragmentation producing the $y$-type ions (see Figure 3.9). Significant responses are also observed for the immonium ions (see Figure 3.9), particularly in the analysis of the butylated dipeptides. These ions have been labelled appropriately on each spectrum (the immonium ions are labelled in blue and the $y$-type ions in red).

It was felt that the presence of these significant fragment ions could be used to develop an automated method for the identification of dipeptides.
3.3.4 Investigations into the automated identification of dipeptides

The aim of this work was to develop a computer programme in-house which could automatically identify and list the dipeptides present in peptide mixtures. The aim was to automatically examine the CAD or in-source fragmentation spectra of the dipeptides for the presence of a common fragment ion. This ion could subsequently be used to identify one of the amino acids present in the dipeptide, and then, by mass difference and a knowledge of the molecular weight of the dipeptide, the dipeptide sequence could ultimately be determined.

Both the immonium and the y-type ions were considered for this process. The immonium ion would not be suitable for the full sequence identification of a dipeptide because, although they are indicative of the amino acids present, they are not related to the position of the amino acid in the peptide chain. Conversely, the y-type ions are related to the C-terminal amino acid, therefore, if the molecular mass of the dipeptide and the identity of the C-terminal amino acid are known, the full identification of the dipeptide is possible (see Figure 3.10).

As discussed in Section 1.5.1.3, adduct ions are commonly observed with ES ionisation and are generally less significant when APCI is employed. Therefore, in order to obtain the simplest data possible, it was decided to investigate the use of APCI for the automated
identification of the dipeptides. The separation power of MS/MS was investigated in preference to on-column LC/MS with in-source fragmentation in order to simplify the analytical process.

3.3.5 Optimisation of collision energy

Separate aliquots (approximately 2 x 1 mg) of each of the following dipeptides were taken: Val-Ala, Lys-Arg, Met-Asn, Glu-Asp, Ala-Cys, Ala-Glu, Val-Gln, Ala-Gly, Gly-His, Ser-Leu, Val-Ile, Gly-Lys, Ser-Met, Asp-Phe, Gly-Pro, Phe-Ser, Ala-Thr, Ile-Trp, Ile-Tyr and Thr-Val. A sample of each dipeptide was individually dissolved in 50% acetonitrile/50% water (1 ml) and the remaining aliquots were treated separately with dried n-butanol (200 μl) and acetyl chloride (1 drop) at 65°C for 30 minutes. On cooling, these n-butanol solutions were diluted with 50% acetonitrile/50% water (800 μl).

A mobile phase of 50% methanol/50% water (500 μl/min) was employed with vaporiser and capillary temperatures of 500°C and 220°C respectively. A corona current of 5 μA was used. Argon was employed as the collision gas at a pressure of 2.5 mT. The effect of the collision energy on the intensities of the y-type ions for both the non-derivatised and butylated dipeptides was examined during the tuning process. A collision energy of approximately 15 to 20 eV appeared to produce maximum intensities for the y-type ions. This was investigated further by analysing the non-derivatised and butylated dipeptides separately at collision energies of 15, 18 and 21 eV.

Loop injections (10 μl) were made for each solution at these three collision energies. Daughter ion experiments were performed on the MH⁺ ion for each non-derivatised and butylated dipeptide. Typical data is shown in Figures 3.11-3.14.
Figure 3.11: Daughter ion spectra of MH⁻ for non-derivatised Val-Ile (m/z 231) and MH⁻ for butylated Val-Ile (m/z 287) [collision energy 18 eV]
Figure 3.12: Daughter ion spectra of MH⁺ for non-derivatised Ile-Trp (m/z 318) and MH⁺ for butylated Ile-Trp (m/z 374) [collision energy 18 eV]
Figure 3.13: Daughter ion spectra of MH⁺ for non-derivatised Phe-Ser (m/z 253) and MH⁺ for butylated Phe-Ser (m/z 309) [collision energy 18 eV]
Figure 3.14: Comparison of daughter ion spectra of MH⁺ for non-derivatised Lys-Arg (m/z 303) at collision energies of 15, 18 and 21 eV
The resulting intensities of the y-type ion for each species were compared at the three voltages from their mass chromatograms. Maximum peak intensities for the y-type ions occurred at collision energies of either 15 or 18 eV for all non-derivatised and butylated dipeptides except in the case of Lys-Arg whose y-type ion maximised at 21 eV (see Figure 3.14 for typical data). A collision energy of 18 eV was therefore selected as being the most general and optimum collision energy to be used for all further analyses.

3.3.6 Investigation into the effect of the alkylating alcohol on the intensity of the y-type ion

In the previous experiment, it was observed that the relative intensities of the y-type ion in the daughter ion spectrum of the butylated dipeptides were more significant than those of the corresponding non-derivatised dipeptides and therefore, the effect of the alkylating alcohol was investigated further. The significance of the alkyl chain length on the carboxy end of the C-terminus amino acid on the relative intensities of the y-type ions was
investigated more extensively for the following alcohols: methanol, ethanol, n-propanol, i-propanol, n-butanol, n-pentanol and n-octanol. Each alcohol was dried over 4A molecular sieve prior to its use.

A homogeneous mixture of ten dipeptides (approximately 1 mg of each of the following: Met-Val, Ala-Ala, Pro-Ser, Ser-Gly, Pro-Val, Phe-Ser, Cys-Gly, Trp-Tyr, Asp-Gly and Ile-Ser) was split into eight equal portions. Seven fractions were alkylated with a different alcohol (200 µl) in the presence of acetyl chloride (1 drop) at 65°C for 30 minutes. N-octanol appeared to be less reactive than the other alcohols due to reduced solubility, therefore this solution was heated for 1 hour. On cooling, each reaction solution and the remaining dipeptide portion were diluted separately with 50% acetonitrile/50% water (200 µl or 400 µl in the case of the non-derivatised fraction). The resulting solutions were infused separately at 2 µl/min through the APCI probe with a make-up solvent comprising 49.75% acetonitrile/49.75% water/0.5% acetic acid (200 µl/min). It was found that the n-octanol solution was immiscible with acetonitrile/water and therefore the spray from the APCI probe was less efficient than for the other alcohols resulting in weaker data being obtained. Even so, it was possible to perform the necessary MS/MS experiments in order to complete this alcohol comparison exercise.

Daughter ion experiments were performed on the MH⁺ ion for each alkylated dipeptide. A collision energy of 18.0 eV and an argon gas pressure of 2.5 mT were employed. Typical spectra are shown in Figure 3.15.
Daughter ion spectrum of non-derivatised Ser-Gly (m/z 163)

Daughter ion spectrum of methylated Ser-Gly (m/z 177)

Figure 3.15: Daughter ion spectra of MH⁺ for alkylated Ser-Gly
Daughter ion spectrum of ethylated Ser-Gly (m/z 191)

Daughter ion spectrum of n-propylated Ser-Gly (m/z 205)

Figure 3.15 continued
Figure 3.15 continued

 Daughter ion spectrum of i-propylated Ser-Gly (m/z 205)

 Daughter ion spectrum of n-butylated Ser-Gly (m/z 219)

Figure 3.15 continued
Daughter ion spectrum of n-pentylated Ser-Gly (m/z 233)

Daughter ion spectrum of n-octylated Ser-Gly (m/z 275)

Figure 3.15 continued
The relative peak heights of the y-type ions for each experiment were measured and plotted as a bar graph in order to observe the effect of the alkylating agent on the intensity of these ions (see Figure 3.16).

![Bar graph showing relative intensity of y-type ions for different derivatising alcohols](image)

In general, as the carbon chain length of the alkylating group increased, so did the relative intensity of the y-type ion and, for the non-derivatised dipeptides, these ions were significantly weaker. In the case of the i-propanol, the relative intensities of the ions of interest were generally lower than those for the corresponding n-propanol. The relative peak height of the corresponding n-pentylated Cys-Gly y-type ion was significantly lower than might be expected. This was found to be due to a non-related base peak ion in the spectrum at m/z 104 which reduced the relative peak height of the y-type ion of the n-pentylated Cys-Gly (m/z 146) from 100% to 36% (see Figure 3.17). The m/z 104 ion corresponds to the methionine immonium ion from unreacted Met-Val (MW 248) which was also shown to be present in the infusion mixture.
Figure 3.17: Daughter ion spectrum of MH⁺ for pentylated Cys-Gly (m/z 249)

Figure 3.18: Daughter ion spectrum of MH⁺ for non-derivatised Met-Val (m/z 249)
Figure 3.18 shows the daughter ion spectrum of non-derivatised Met-Val and the ions detected can also be clearly observed in the daughter ion spectrum shown in Figure 3.17 corresponding to n-pentylated Cys-Gly (MW 248) where m/z 104 is the base peak. Maximum peak heights for the y-type ions of the C-terminal amino acids are desirable to aid detection, therefore the longer chained alcohols need to be employed. The viscosity of the n-octanol eliminates its efficient use, therefore n-butanol and n-pentanol were selected as the derivatising alcohols to be used in further investigations.

3.3.7 Development of a method for the reduction of cystine bridges

The automated determination of cysteine containing peptides would be complicated if there are internal disulfide bonds present. Methods were therefore investigated to establish a suitable reduction step whose product could subsequently be used in the alkylation reaction with minimal sample pretreatment.

The mass spectrometer was operated under the previously described APCI+ conditions using 50% acetonitrile/ 50% water at a flow rate of 500 µl/min.

3.3.7.1 Adaption of a FAB method using ammonium hydroxide solution

Ala-Cys-S-S-Cys-Ala (1 mg) was treated with n-butanol (200 µl) and 35% ammonium hydroxide solution (2 drops). The resulting mixture was shaken prior to dilution with 50% acetonitrile/ 50% water (1 ml). The mixture was acidified with TFA (1 drop) and a 10 µl loop injection was made.

The resulting spectrum (see Figure 3.19) showed a base peak at m/z 383 corresponding to MH⁺ of the oxidised peptide. The reduced peptide showed a peak at m/z 193 (MH⁺) with an intensity of only 6.2%. This method appears therefore not to be sufficiently effective for the reduction of cysteine bridges due to the reduced solubility of the dipeptide in n-butanol compared to water.
3.3.7.2 Reduction using aqueous dithiothreitol (DTT)⁹⁶

Ala-Cys-S-S-Cys-Ala (1 mg) was treated with an aqueous solution of 50 mM DTT (200 µl) at 50°C for 15 minutes. On cooling, this reaction solution was diluted with 50% acetonitrile/50% water (800 µl) and a 10 µl loop injection was made. The resulting spectrum (see Figure 3.20) showed a base peak at m/z 193 corresponding to MH⁺ of the reduced peptide. A significant ion (90% relative intensity) was also observed at m/z 234 due to an acetonitrile adduct of this species. No peak was observed at m/z 383 for the oxidised peptide. This method therefore appears to be suitable for the effective reduction of cystine bridges.
3.3.7.3 *Reduction using dithiothreitol (DTT) in n-butanol*

The DTT method (see Section 3.3.7.2) was adapted to investigate the use of an alcohol instead of water in attempts to make the method more compatible with the subsequent alkylation step.

A 50 mM DTT solution was prepared in a 5% water/ 95% n-butanol. Ala-Cys-S-S-Cys-Ala (1 mg) was treated with this solution (200 μl) at 50°C for 15 minutes. On cooling, a 10 μl loop injection was made.

The resulting spectrum (see Figure 3.21) showed a base peak at m/z 383 corresponding to MH⁺ of the oxidised peptide. Peak intensities of only approximately 15% and 16% were observed respectively for the ions of interest at m/z 193 and m/z 234 due to MH⁺ and [MH⁺ + CH₃CN] respectively of the reduced peptide. It would therefore appear that the reduced solubility of the peptide in n-butanol significantly affects the rate of reaction.
3.3.7.4 Conclusions of cystine bridge reduction step

It would appear that the reduction of the cystine bridges proceeds best in an aqueous solvent. This is probably due to the poor solubility of the peptide in n-butanol. Future reductions will therefore be carried out in water as described in Section 3.3.7.2. Unfortunately, as water is incompatible with the butylation and pentylation steps, the reaction mixture requires freeze-drying prior to alkylation of the dipeptides.

3.3.8 S-Carboxyamidation of cysteine containing dipeptides with iodoacetamide

Peptides containing reduced cystine bridges are prone to oxidation, therefore the HS-group requires protection with a stable group. Iodoacetamide (ICH$_2$CONH$_2$) is an alkylation agent commonly used for this purpose.

Ala-Cys-S-S-Cys-Ala (1 mg) was reduced using DTT (200 µl) as described in Section 3.3.7.2. On cooling, the solution was treated in two separate experiments with 45 mM iodoacetamide in water at room temperature. In the first experiment, 50 µl of the
iodoacetamide solution was added for 15 minutes and in the second experiment, 100 µl of the iodoacetamide solution was used for 20 minutes. The resulting solutions were freeze-dried prior to being redissolved in 50% acetonitrile/50% water (1 ml) and analysed as described above.

Both experiments produced spectra (see Figures 3.22 and 3.23) showing a base peak at m/z 250 corresponding to MH⁺ of the acetamide-derivatised peptide. Significant ions (15-20% relative intensity) were also observed at m/z 291 due to the acetonitrile adduct of this species. Ions were observed at m/z 383 (approximately 10% relative intensity) corresponding to MH⁺ of the oxidised peptide. Ions at m/z 193 and m/z 234 corresponding to MH⁺ and [MH+CH₃CN]⁺ of the reduced, non-S-carboxyamidated form are significantly weaker in the spectrum from the second experiment than from the first.

Figure 3.22: Full scan spectrum of Ala-Cys-S-S-Cys-Ala reduced with aqueous DTT and treated with 50 µl iodoacetamide for 15 minutes
It would therefore appear that the reaction proceeds to almost completion when the reduced peptide is treated with a greater proportion of iodoacetamide for a longer period. This method was therefore selected for all future S-carboxyamidations.

### 3.3.9 Identification of dipeptides in a standard mixture

#### 3.3.9.1 Sample preparation

A homogeneous mixture (20 mg) containing the broad spectrum of 77 standard dipeptides was treated with dithiothreitol (200 μl, 45 mM) at 50°C for 20 minutes to reduce the cystine bridges. The resulting cysteine groups were protected by the addition of iodoacetamide (200 μl, 100 mM) and left to stand at room temperature for 20 minutes. After freeze drying, the mixture was split into two equal portions and each portion was alkylated at 65°C for 30 minutes with either n-butanol or n-pentanol (300 μl) in the presence of acetylchloride (3 drops). On cooling, each reaction solution was diluted with 49.75% acetonitrile/49.75% water/0.5% acetic acid (300 μl) prior to their individual infusion and subsequent analysis by APCI MS/MS.
3.3.9.2 Calibration of the mass spectrometer

In order to obtain optimum responses for the daughter ion experiments using integer masses for the parent ions, it was necessary to recalibrate the mass spectrometer to ensure that the mass of the ions were within 0.1 Da of their nominal masses. This was achieved by infusing a small portion of the mixture of butylated standards and setting the masses for the protonated molecular ions in both Q1 and Q3 to the nominal mass ± 0.1 Da.

3.3.9.3 Analysis

The remaining butylated solution was infused at 2 µl/min through the APCI probe with a make-up solvent comprising 49.75% acetonitrile/ 49.75% water/ 0.5% acetic acid (200 µl/min). The conditions used in the mass spectrometer are as described previously (see Section 3.3.2) with a collision energy of 18.0 eV. Daughter ion experiments were performed automatically on all ions starting at m/z 1 using a macro file. Three scans were acquired for each daughter ion experiment and these were subsequently averaged automatically. Only one averaged scan was therefore obtained for each daughter ion experiment and hence the scan number corresponds to m/z of the parent ion. The peak labelling in the total ion chromatogram, therefore, not only corresponds to the scan number, as is normally the case, but also to the mass of the parent ion. The chromatograms were plotted in a bar chart format in preference to the typical distribution curve layout to allow the species present to be more easily selected. The whole process was repeated using the pentylated sample.

The expected masses of the characteristic y-type ions for each C-terminal amino acid, [H₃NCHRCOOX]⁺, produced from butylated and pentylated dipeptides were determined (see Table 3.2). It should be noted that both glutamic acid and aspartic acid containing dipeptides would be derivatised with two equivalents of alcohol due to the extra carboxylic acid group present in these two amino acids.
<table>
<thead>
<tr>
<th>Amino acid</th>
<th>R</th>
<th>Butylated species</th>
<th>Pentylated species</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ala</td>
<td>-CH₃</td>
<td>146</td>
<td>160</td>
</tr>
<tr>
<td>Arg</td>
<td>-(CH₂)₄NHC(NH₂)=NH</td>
<td>231</td>
<td>245</td>
</tr>
<tr>
<td>Asn</td>
<td>-CH₂CONH₂</td>
<td>189</td>
<td>203</td>
</tr>
<tr>
<td>Asp</td>
<td>-CH₂COOH</td>
<td>246</td>
<td>274</td>
</tr>
<tr>
<td>Cys (acetamide deriv.)</td>
<td>-CH₂SCH₂CONH₂</td>
<td>235</td>
<td>249</td>
</tr>
<tr>
<td>Glu</td>
<td>-CH₂CH₂COOH</td>
<td>260</td>
<td>288</td>
</tr>
<tr>
<td>Gln</td>
<td>-CH₂CH₂CONH₂</td>
<td>203</td>
<td>217</td>
</tr>
<tr>
<td>Gly</td>
<td>-H</td>
<td>132</td>
<td>146</td>
</tr>
<tr>
<td>His</td>
<td></td>
<td>212</td>
<td>226</td>
</tr>
<tr>
<td>Ile</td>
<td>-CH(CH₃)CH₂CH₃</td>
<td>188</td>
<td>202</td>
</tr>
<tr>
<td>Leu</td>
<td>-CH₂CH(CH₃)CH₃</td>
<td>188</td>
<td>202</td>
</tr>
<tr>
<td>Lys</td>
<td>-(CH₂)₄NH₂</td>
<td>203</td>
<td>217</td>
</tr>
<tr>
<td>Met</td>
<td>-(CH₂)₂SCH₃</td>
<td>206</td>
<td>220</td>
</tr>
<tr>
<td>Phe</td>
<td>-CH₂Ph</td>
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<td>236</td>
</tr>
<tr>
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<td>162</td>
<td>176</td>
</tr>
<tr>
<td>Thr</td>
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<td>190</td>
</tr>
<tr>
<td>Trp</td>
<td></td>
<td>261</td>
<td>275</td>
</tr>
<tr>
<td>Tyr</td>
<td>-CH₂-Ph-(4-OH)</td>
<td>238</td>
<td>252</td>
</tr>
<tr>
<td>Val</td>
<td>-CH(CH₃)₂</td>
<td>174</td>
<td>188</td>
</tr>
</tbody>
</table>

Table 3.2: Structures and expected masses of the y-type ions for each C-terminal amino acid
The MS/MS data from the two infusion experiments were analysed, using mass chromatograms, for the presence of each of the respective y-type ions for the twenty commonly occurring amino acids (see Figure 3.24 for typical mass chromatograms for C-terminal methionine residues). The masses of the parent ions from which each y-type ion was produced could be obtained directly from the mass chromatograms for each y-type ion because, as stated earlier, scan number on the chromatogram also corresponds to m/z of the parent ion.

The mass chromatograms were compared for the butylated and pentylated y-type ion for each amino acid. Peaks were assumed to be derived from an alkylated species when an ion was observed 14 Da higher (28 Da for a species containing one aspartic or glutamic acid residue, or 42 Da for a dipeptide containing two of these acidic residues) in the pentylated chromatogram compared to the corresponding butylated chromatogram. These alkylated species were then considered to be possible dipeptides and have been labelled in red in the example shown in Figure 3.24. This process was repeated for each of the amino acids.
Figure 3.24: Mass chromatograms (in bar chart format) of parent ions of m/z 206 and m/z 220 corresponding to butylated and pentylated respectively C-terminal methionine residues.
The C-terminal arginine containing dipeptides were not observed in the m/z 231 (butylated sample) and m/z 245 (pentylated sample) mass chromatograms due to the protonated molecular ions, as had been predicted and therefore the relevant daughter ion spectra were examined more closely. Typical data for Lys-Arg are shown in Figure 3.25.

Figure 3.25: Daughter ion spectra of m/z 359 (butylated sample) and m/z 373 (pentylated sample) [MH⁺ for Lys-Arg]
It can be seen that the y-type ions of interest (m/z 231 and 245) are not present in the spectra, therefore accounting for the absence of the MH$^+$ ions in the respective mass chromatograms. Ions at m/z 189 in the butylated spectrum and m/z 203 in the pentylated spectrum are observed due to the loss of NCNH$_2$ from the predicted y-type ions. Mass chromatograms were therefore produced for these fragment ions, leading to the detection of the parent ions of the two C-terminal arginine containing dipeptides (Ala-Arg and Lys-Arg) (see Figures 3.26a and 3.26b). The ions of interest are labelled in red on the chromatograms.

![Mass chromatograms](image)

**Figure 3.26a**: Mass chromatograms (in bar chart format) of parent ions of m/z 189 corresponding to butylated C-terminal arginine residues
Having established a method for detecting the C-terminal arginine containing dipeptides, the next step in the identification process was to identify the dipeptides present. By comparing the mass of the butylated parent ion with the known mass of the y-type ion (due to the C-terminal amino acid), the mass of the N-terminal amino acid could then be calculated. From a knowledge of the mass of each of the 20 commonly occurring amino acids, the N-terminal amino acid could be easily identified from this calculated mass. Subsequently, the structure of the whole dipeptide could be determined (see Figure 3.27 for a typical calculation).
Butylated species of molecular weight 306 containing C- terminal methionine residue

\[ R \quad \text{CH}_2\text{CH}_2\text{SCH}_3 \]

\[ \Rightarrow \text{Partial Structure: } H_2\text{NCHCNHCHCOBu} \]
\[ \quad \parallel \quad \parallel \quad O \quad O \]

\[ R = 306 - 261 \]
\[ \Rightarrow R = 45 \]
\[ \Rightarrow R = -\text{CH} - \text{OH} \]
\[ \quad \text{CH}_3 \]
\[ \Rightarrow \text{N- terminal threonine residue} \]
\[ \Rightarrow \text{DIPEPTIDE: ThrMet} \]

Figure 3.27: Identification of the species labelled with an asterisk (*) in Figure 3.24

Calculations were performed on all of the species which had been selected by comparison of the corresponding butylated and pentylated mass chromatograms, and a total of 93 dipeptides were identified (see Table 3.3). As discussed in Section 3.2, this method does not allow the isobaric amino acid (glutamine and lysine, nor leucine and isoleucine) containing dipeptides to be differentiated. Lxx therefore corresponds to leucine or isoleucine, and Gln is used to represent glutamine and lysine.

Twenty-six of the dipeptides identified were not actually present in the standard mixture used (see Table 3.1) and these are indicated in Table 3.3 with an asterisk (*). These false identifications may be due to artifacts of the experiment arising from fragmentation of the larger dipeptides. A method therefore needed to be established whereby these false identifications could be omitted. The use of on-column LC/MS with and without source fragmentation was investigated (see Section 3.3.10).
Table 3.3 : Dipeptides identified using butylated and pentylated mass chromatograms

Of the 77 dipeptides known to be present in the standard mixture, 10 were not detected by this infusion MS/MS method. These were Arg-Ala, Trp-Ala, Cys-Gly, Ala-His, Gly-His, Gln-Gln, His-Leu, His-Phe, Arg-Phe and Trp-Tyr. Closer examination of the butylated and pentylated mass chromatograms showed that in all cases, the parent ion was not present in the appropriate pentylated mass chromatogram. Further examination of the relevant pentylated daughter ion spectrum showed that the pentylated y-type ion was not detected for these ten dipeptides. In fact, the daughter ion data was extremely weak/non-existent for these pentylated species which might suggest that they were not actually present in the
pentylated mixture. The derivatisation step therefore required further investigation to establish its efficiency in order to ensure optimum derivatisation (see Section 3.3.14).

3.3.10 On-column LC/MS

3.3.10.1 Instrumentation

On-column LC/MS analysis with APCI ionisation in the positive ionisation mode was performed with and without source fragmentation using alternating octopole voltages. Source fragmentation was minimised by operating at an octapole voltage of 0 eV and an octapole voltage of 25 eV was employed to induce source fragmentation. Optimum vaporiser and capillary temperatures of 500°C and 220°C respectively, were employed. A corona current of 5 µA was used. On-column LC/MS analysis was carried out on a 30 cm × 3.9 mm Phenomenex Bondclone 10 C₁₈ column. A flow rate of 1 ml/min was employed with a gradient of 100% A to 100% B in 100 minutes (Solvent A = 99.9% water/0.1% TFA, Solvent B = 90% acetonitrile/9.01% water/0.09% TFA). An injection volume of 10 µl was used.

3.3.10.2 Analysis

The butylated solution (100 µl) was diluted with 49.75% acetonitrile/49.75% water/0.5% acetic acid (400 µl) and analysed in a single chromatographic run by on-column APCI LC/MS with and without source fragmentation. The molecular weights for the butyl esters of the dipeptides identified using the MS/MS infusion method and listed in Table 3.3 were calculated. Mass chromatograms were produced for the MH⁺ ions of these species on the data acquired with an octopole offset of 0 eV and used to obtain spectra for these species from both the 0 eV and 25 eV data. Manual examination of the spectra was used to confirm/deny the presence of the dipeptides listed in Table 3.3. Figure 3.28 shows the total ion chromatogram (TIC) obtained.
All the species which were known to be actually present in the mixture were detected and the corresponding retention times are listed in Table 3.4. As predicted, the species labelled with an asterisk in Table 3.3 were not detected by LC/MS.
<table>
<thead>
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<th>Dipeptide Pair</th>
<th>Retention Time</th>
<th>Dipeptide Pair</th>
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<tr>
<td>Ala-Ala</td>
<td>19.31</td>
<td>Glu-Gly</td>
<td>36.54</td>
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<td>24.43</td>
<td>Gly-Lxx</td>
<td>33.43</td>
</tr>
<tr>
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<td>47.54</td>
<td>Lxx-Lxx</td>
<td>41.06</td>
</tr>
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<td>Met-Asn</td>
<td>24.00</td>
<td>Phe-Lxx</td>
<td>44.08</td>
</tr>
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<td>Pro-Asn</td>
<td>18.08</td>
<td>Pro-Lxx</td>
<td>35.54</td>
</tr>
<tr>
<td>Glu-Asp</td>
<td>51.55</td>
<td>Ser-Lxx</td>
<td>32.46</td>
</tr>
<tr>
<td>Gly-Asp</td>
<td>18.02</td>
<td>Thr-Lxx</td>
<td>34.03</td>
</tr>
<tr>
<td>Ala-Cys</td>
<td>19.19</td>
<td>Val-Lxx</td>
<td>37.30</td>
</tr>
<tr>
<td>Gly-Cys</td>
<td>17.24</td>
<td>Gly-Met</td>
<td>28.57</td>
</tr>
<tr>
<td>Ala-Glu</td>
<td>40.07</td>
<td>Ser-Met</td>
<td>28.30</td>
</tr>
<tr>
<td>Glu-Glu</td>
<td>53.15</td>
<td>Thr-Met</td>
<td>29.54</td>
</tr>
<tr>
<td>Val-Glu</td>
<td>43.54</td>
<td>Ala-Phe</td>
<td>35.54</td>
</tr>
<tr>
<td>Asp-Gln</td>
<td>32.14</td>
<td>Asp-Phe</td>
<td>49.12</td>
</tr>
<tr>
<td>Gly-Gln</td>
<td>13.16</td>
<td>Gly-Phe</td>
<td>35.37</td>
</tr>
<tr>
<td>Val-Gln</td>
<td>23.36</td>
<td>Lxx-Phe</td>
<td>43.31</td>
</tr>
<tr>
<td>Ala-Gly</td>
<td>15.08</td>
<td>Gly-Pro</td>
<td>22.37</td>
</tr>
<tr>
<td>Asp-Gly</td>
<td>34.07</td>
<td>Phe-Pro</td>
<td>37.37</td>
</tr>
</tbody>
</table>

Table 3.4: Retention times (in minutes) of identified butylated dipeptides (Lxx = Leu or Ile, Gln = Gln or Lys)

3.3.11 Development of computer programme

Manual identification of the dipeptides is extremely time consuming, and therefore, a computer programme using the C programming language under the Ultrix operating system, has been developed for use with the Finnigan DEC data system, which
automatically carries out the calculations. A flow chart of the process is shown in Figure 3.29 and the full programme is shown in the Appendix.

**Figure 3.29 : Flow chart of steps involved in automatic identification of dipeptides**
The programme initially creates, for both the butylated and pentylated samples, the mass chromatograms for each y-type ion of the C-terminal amino acids. The corresponding butylated and pentylated mass chromatograms are then compared and the masses of possible dipeptides are listed. If a species is listed in both experiments, calculations are automatically performed as described previously on these ions to identify the N-terminal amino acids. The resulting identified dipeptides which appear to be present in the sample are listed and this list is printed automatically.

3.3.12 Summary of method developed to identify dipeptides in complex mixtures

3.3.12.1 Sample preparation

The sample (20 mg) is treated with dithiothreitol (200 μl, 45 mM) at 50°C for 20 minutes to reduce the cystine bridges. The resulting cysteine groups are protected by the addition of iodoacetamide (200 μl, 100 mM) and left to stand at room temperature for 20 minutes. After freeze drying, the sample is split into two equal portions and each portion is alkylated at 65°C for 30 minutes with either n-butanol or n-pentanol (300 μl) in the presence of acetylchloride (3 drops). On cooling, each reaction solution is diluted with 49.75% acetonitrile/49.75% water/0.5% acetic acid (300 μl) prior to its individual infusion and subsequent analysis by APCI MS/MS. The butylated solution (100 μl) is also diluted further with 49.75% acetonitrile/49.75% water/0.5% acetic acid (400 μl) and analysed in a single chromatographic run by on-column APCI LC/MS with and without source fragmentation.

3.3.12.2 Analysis

The resulting butylated and pentylated solutions are infused separately at 2 μl/min through the APCI probe with a make-up solvent comprising 49.75% acetonitrile/49.75% water/0.5% acetic acid (200 μl/min). The conditions used in the mass spectrometer are as described previously (see Section 3.3.9.3). Daughter ion experiments are performed automatically on all ions starting at m/z 1 using the previously described macro file (see Section 3.3.9.3) and the computer programme described in Section 3.3.11 is employed to automatically analyse the resulting data for dipeptides.
The presence of each dipeptide identified by the programme can be confirmed by rapid examination of the on-line LC/MS data, with and without source fragmentation, obtained from the butylated sample.

Full sample preparation and analysis can be easily completed within one working day for each sample.

3.3.13 Analysis of a casein hydrolysate

A casein hydrolysate prepared by enzymic hydrolysis of bovine casein was analysed using the method described above. In conjunction with a knowledge of the amino acid sequences of the bovine casein proteins to differentiate, where possible, between the isobaric amino acids, leucine and isoleucine, and glutamine and lysine, fifty-two dipeptides were identified (see Table 3.5).

<table>
<thead>
<tr>
<th>Ala-Arg</th>
<th>Ala-Gln</th>
<th>Ala-Ile</th>
<th>Ala-Met</th>
<th>Ala-Phe</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ala-Tyr</td>
<td>Ala-Val</td>
<td>Arg-Ser</td>
<td>Gln-Leu</td>
<td>Gxx-Trp</td>
</tr>
<tr>
<td>Glu-Gly</td>
<td>Gly-Arg</td>
<td>Gly-Tyr</td>
<td>His-Pro</td>
<td>Ile-His</td>
</tr>
<tr>
<td>Ile-Gln</td>
<td>Ile-Lys</td>
<td>Ile-Thr</td>
<td>Ile-Val</td>
<td>Leu-Gln</td>
</tr>
<tr>
<td>Leu-His</td>
<td>Leu-Lys</td>
<td>Leu-Thr</td>
<td>Leu-Tyr</td>
<td>Lxx-Asn</td>
</tr>
<tr>
<td>Lxx-Trp</td>
<td>Lxx-Glu</td>
<td>Lxx-Lxx</td>
<td>Lys-Glu</td>
<td>Phe-Leu</td>
</tr>
<tr>
<td>Phe-Phe</td>
<td>Phe-Tyr</td>
<td>Pro-Glu</td>
<td>Ser-Lxx</td>
<td>Ser-Met</td>
</tr>
<tr>
<td>Ser-Thr</td>
<td>Ser-Trp</td>
<td>Thr-Gln</td>
<td>Thr-Glu</td>
<td>Thr-Lxx</td>
</tr>
<tr>
<td>Thr-Tyr</td>
<td>Tyr-Leu</td>
<td>Tyr-Tyr</td>
<td>Val-Ala</td>
<td>Val-Asn</td>
</tr>
<tr>
<td>Val-Asp</td>
<td>Val-Gxx</td>
<td>Val-Lxx</td>
<td>Val-Phe</td>
<td>Val-Ser</td>
</tr>
<tr>
<td>Val-Thr</td>
<td>Val-Tyr</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(Lxx = Leu or Ile, Gxx = Gln or Lys)

Table 3.5: Dipeptides identified in a casein hydrolysate

3.3.14 Investigation into the method of derivatisation for dipeptides

As discussed in Section 3.3.9.3, the derivatisation step requires further investigation to ensure that the dipeptides are efficiently alkylated and therefore producing significant MS/MS responses to allow the automated identification of each dipeptide. The method
used to date involved alkylation with n-butanol and n-pentanol at 65°C in the presence of a few drops of acetyl chloride. In this method, hydrogen chloride (HCl) was produced in situ from the reaction of acetyl chloride with the alcohol. The exact concentration of HCl produced was therefore unknown and it was felt that improvements in the method might be achieved if a known and specific concentration of HCl were used instead. The method reported by D. Millington et al. was therefore investigated.

3.3.14.1 Instrumentation

A Hewlett Packard 1090 liquid chromatograph and autosampler coupled to a VG Biotech Quattro II triple quadrupole mass spectrometer with a standard electrospray interface was employed. Operation was carried out in the positive ionisation mode with a source temperature and cone voltage of 150°C and 30 V respectively. A Vydac 250 x 2.1 mm 201HS C18 column and equivalent guard column were used. A flow rate of 0.3 ml/min was employed with a mobile phase of 100% A for 5 minutes increasing to 100% B after 95 minutes (solvent A = 1% acetonitrile/99% water [0.2% TFA], solvent B = 90% acetonitrile/10% water [0.2% TFA]). An injection volume of 10 μl was made and a UV wavelength of 210 nm was used.

3.3.14.2 Sample preparation

The following dipeptides were investigated under various derivatisation conditions: Gly-Ala, Gly-Asn, Gly-Asp, Gly-Cys-S-S-Cys-Gly, Gly-Glu, Gly-Gln, Gly-Gly, Gly-His, Gly-Leu, Gly-Lys, Gly-Met, Gly-Phe, Gly-Pro, Gly-Ser, Gly-Thr, Gly-Trp, Gly-Tyr, Gly-Val and Ala-Arg. HCl gas was bubbled through separate aliquots of methanol, ethanol, n-propanol and n-butanol (dried over 4A molecular sieve) to produce 3M HCl solutions. Each dipeptide (approximately 1 mg) was treated with the stated acidified alcohol (100 μl) under the following conditions:

A : Butanol at 65°C for 20 minutes
B : Propanol at 65°C for 20 minutes
C : Ethanol at 65°C for 20 minutes
D : Methanol at 65°C for 20 minutes
E : Ethanol at ambient temperature for 45 minutes
F : Methanol at ambient temperature for 45 minutes

On completion, each reaction solution (50 μl) was diluted with mobile phase (1% acetonitrile/99% water [0.2% TFA], 1 ml) prior to LC/MS analysis.

3.3.14.3 Analysis

The resulting solutions were analysed separately under the conditions described in Section 3.3.14.1. Peak areas for the alkylated products, starting materials, and any bi-products were measured for each sample on the resulting UV chromatogram (see Figure 3.30 for typical data) and the percentages of desired products were calculated. Graphs were plotted showing this percentage for each derivatised dipeptide in order to compare the various conditions employed.

![UV (210 nm) of Gly-Tyr derivatised with EtOH/HCl at ambient temperature for 45 minutes](image)

Figure 3.30 : UV (210 nm) of Gly-Tyr derivatised with EtOH/HCl at ambient temperature for 45 minutes

Figure 3.31 shows a comparison of the four alcohols at 65°C for 20 minutes (methods A-D). The general trend (excluding Gly-Asn and Gly-Gln) indicates that methanol produces optimum results and that, as the alkyl chain length of the alcohol increases, then so the efficiency of the derivatisation decreases. In fact, when butanol was employed, less than 20% of the dipeptides, Ala-Arg, Gly-Cys, Gly-His and Gly-Lys, were butylated. This may be attributed to a reduction in the solubility and reactivity of these dipeptides in the larger, less polar alcohols due to steric hindrance as the alkyl chain length is increased.
In the case of the asparagine and glutamine containing dipeptides, it appeared that methanol was the least efficient alcohol. Examination of the respective chromatograms (see Figure 3.32 for typical data) showed that the corresponding dimethylated aspartic and glutamic acid containing dipeptides had been produced (see Figure 3.33 for proposed reaction sequence). As the chain length of the alcohol increased, so the level of the corresponding diester decreased due presumably, to the decreasing reactivity of the alcohol.
Figure 3.32: UV traces (210 nm) for Gly-Asn ethylated at ambient temperature and 65°C

Figure 3.33: Alkylation of Gly-Asn and Gly-Gln to produce the corresponding ‘diester’

Figure 3.34 shows a comparison of the derivatisation method using both methanol and ethanol at both 65°C and ambient temperature (methods C-F). At ambient temperature, the reaction mixtures were shaken in an ultrasonic bath for 45 minutes to ensure complete reaction. It can be seen that the higher temperature generally leads to a greater percentage of the desired product but unfortunately, as described above, the corresponding diesters were formed when Gly-Asn and Gly-Gln were alkylated. These diesters were insignificant when the milder conditions were employed in the ambient temperature experiments therefore these milder conditions might be considered to be more suitable. The percentages
of the desired esters are generally greater than 90% and in the worst cases, no less than 80% at ambient temperature. This was felt to be adequate for this application.

- 65°C MeOH - Ambient MeOH
- 65°C EtOH - Ambient EtOH

Figure 3.34: Comparison of efficiency of alkylation using methanol and ethanol at ambient temperature and 65°C

It was concluded that under the conditions used, n-butanol and therefore n-propanol were unsuitable derivatising agents. Methanol and ethanol at ambient temperature were considered to be significantly better alternatives.

3.4 RESULTS AND DISCUSSION

A semi-automated method has been developed to allow the rapid identification of dipeptides in complex mixtures without the necessity of achieving optimum chromatographic separations. A standard mixture containing a broad spectrum of 77 dipeptides was examined using this method and 67 (i.e. 87%) of the dipeptides present were identified.

The dipeptides which were not identified were found to give data which were too weak to be discerned by the computer programme. This was probably due to the derivatisation step
not being fully efficient. More extensive investigations into the method of alkylation have shown that n-butanol is a poor alkylating agent and for some dipeptides, derivatises less than 20% of the dipeptide to the butylated product. To ensure maximum conversion of the dipeptides into an alkylated species, derivatisation using methanol and ethanol in the presence of hydrochloric acid at room temperature has been found to give significantly improved results probably due to greater solubility and reactivity of the peptides in these two alcohols. The computer programme which has been devised can be easily adapted to analyse methylated and ethylated dipeptides. It is anticipated that the response for the relevant methylated and ethylated y-type ions of the C-terminal amino acids, although weaker than those for the corresponding butylated and pentylated species, should be large enough to allow the identification of the dipeptides using this method. Unfortunately, access to a Finnigan system was withdrawn and therefore the applicability of this potentially improved method could not be fully evaluated. Further work is therefore required in this area. It would also be useful to investigate the sensitivity of the method in order to establish its usefulness in other areas.

As discussed previously, the low energy collisions which occur in a triple quadrupole mass spectrometer cannot differentiate between glutamine and lysine, nor isoleucine and leucine. It is therefore impossible for the computer programme to distinguish between dipeptides containing residues of these isobaric pairs. In protein hydrolysate samples, a knowledge of the originating protein sequence would allow the peptides containing these isobaric amino acids to be identified more confidently.

The work described in this chapter has concentrated on the identification of dipeptides only. Further method development would be necessary before the higher peptides could be identified in order to fully characterize the protein hydrolysates. Electrospray, being a softer ionisation technique, would probably be required for the efficient ionisation of the larger peptides. A method needs to be established whereby the peptides could be easily located if LC/MS is to be used. Constant neutral loss experiments on the derivatised peptides may identify the molecular masses of these peptides. It has been shown that the loss of 102 Da from the protonated molecular ion of the butylated peptide does not produce significant data, therefore alternative derivatisations could be considered (for example, acetylation on the N-terminal amino acid which might result in the loss of 100
Da and 42 Da respectively, or methylation of the C-terminal amino acid which might then lose 32 Da). Once the masses of the parent ions of the individual peptides have been determined, daughter ion experiments could then be performed on these ions in order to identify the amino acid sequence of each peptide. The commercial software available (for example, BioLynx from MicroMass, Altrincham) could be used to assist in the sequencing step.

An alternative approach might be to improve the LC chromatographic separation of the peptides and then obtain sequence information using in-source fragmentation. Capillary electrophoresis (CE) may offer advantages in the separation of the peptides and therefore CE-MS could be investigated.

3.5 CONCLUSION

A semi-automated method has been developed which allows the rapid identification of dipeptides in complex mixtures, in particular in protein hydrolysates. Improvements to the assay have also been identified but need to be evaluated fully. To date, 52 dipeptides have been identified in a casein hydrolysate. This is a significant improvement on the GC/MS method described in Section 3.1 but, although quicker and easier than the LC isolation method reported previously, it has the major drawback of only being useful for the identification of dipeptides. With further method development though, LC/MS (or CE/MS) is considered to offer significant advantages in the identification of the higher peptides.

Methods have been suggested for the identification of all peptides present in protein hydrolysates which could lead to the full characterisation of such mixtures and hence, allowing them to be used in clinical applications. The significance of the nutritional value of an hydrolysate might also be established, particularly if it were possible to characterise the peptides excreted in a patients urine. Unfortunately, no work has been performed to date on determining the detection limit for the method described in this chapter. Sensitivity is not an issue when analysing the protein hydrolysates as sufficient sample would usually be available. Problems may be encountered though when looking at urine samples, therefore it may be necessary to combine urine fractions. The infusion MS/MS method
may not need to be performed on the urine. LC/MS could be used to look specifically for the peptides that have been identified previously in the hydrolysate fed to the patient.

3.6 REFERENCES

98. J.C. Mercier, F. Grosclaude, B. Ribadeau Dumas, Milchwissenschaft, 27, 402, (1972)
CHAPTER 4: DEVELOPMENT OF A RAPID METHOD FOR THE QUANTIFICATION OF SN 609369 IN SHEEP PLASMA
4.1 INTRODUCTION

2-[5-chloro-1-(2,6-dichloro-4-trifluoromethylphenyl)-3-methyl-1H-pyrazol-4-yl]-1H-imidazole-4,5-dicarbonitrile (SN 609369, see Figure 4.1) shows potential as an insecticide for use against blood-sucking ectoparasites and some endoparasites in both sheep and cattle. It is believed to be a gamma-aminobutyric acid (GABA) antagonist which leads to insect mortality through nerve synapses 'twitching' continually.

\[
\begin{align*}
&\text{N} & & \text{N} \\
&M & & \text{Me} \\
&\text{N} & & \text{NH} \\
&\text{N} & & \text{Cl} \\
&\text{Cl} & & \text{Cl} \\
&\text{CF}_3 & & \\
\end{align*}
\]

\[C_{16}H_6Cl_3F_3N_6 = 444\]

Figure 4.1: SN 609369

In this study, a sheep was dosed intravenously with an aqueous solution of SN 609369 sodium salt at a rate of 5 mg/kg bodyweight. The level of SN 609369 remaining in the animals blood was monitored by removing 10 ml of blood from the treated sheep at the following intervals: 3 hours, 6 hours, daily for 7 days, 9, 11, 14, 17 and 20 days.

An existing method to determine the level of SN 609369 remaining in the blood involved a lengthy extraction process followed by HPLC analysis with UV detection. The extraction involved shaking the plasma with 6M hydrochloric acid and diethyl ether for one hour, centrifuging the resulting mixture for fifteen minutes and then separating the organic layer. This process was repeated, the two organic layers were combined and then evaporated prior to HPLC analysis using nitrobenzene as the internal standard. The whole process
took about three hours. This was deemed to be too long for a routine assay and therefore a faster analytical procedure was sought. HPLC coupled to TSP MS was investigated as the alternative analytical technique. The detection limits of SN 609369 using a variety of ionisation techniques were also compared.

4.2 GAMMA-AMINOBUTYRIC ACID (GABA)
GABA serves as an inhibitory transmitter in the central nervous system\textsuperscript{105}. It interacts with the post synaptic receptors of an inhibitory synapse and leads to an increase in the chloride ion permeability of the membrane. This results in an outward flow of current across the post synaptic membrane and a corresponding inward flow across the surrounding cell membrane. The increase in chloride ion conductance leads to membrane hyperpolarisation which helps maintain the membrane at or near to its resting value so that its excitability is reduced.

SN 609369 is believed to interact with the post synaptic receptors of the inhibitory synapse and therefore prevent the interaction of GABA. The excitability of the receiving cells is not reduced and the nerve synapses 'twitch' continually leading to insect mortality.

4.3 QUANTITATIVE ASPECTS OF MASS SPECTROMETRY
Quantification by mass spectrometry is not a straightforward technique because the response to a sample at the detector depends on several parameters which are difficult to reproduce. The condition, temperature and pressure of the ion source, for example, all have an effect and equimolar amounts of different compounds do not give an equal response because the ionisation efficiency of a species depends on molecular structure and vapour pressure. Despite these complications, quantification by mass spectrometry is possible and it relies on the fact that the ion current obtained from a certain analyte is proportional to the amount of the analyte present. The responses for different components in a mixture are rarely the same and so a standard sample of each component is generally required to determine the relative response factor so that accurate quantification can be performed.
Selected ion recording (SIR) is the usual technique for quantitative mass spectrometric measurements. It originates from the work of Sweeley et al.\textsuperscript{106}, and was developed by Hammer et al.\textsuperscript{107} for the analysis of drugs. Unlike a conventional mass spectrum in which the mass range is continuously scanned, only a few ions which are characteristic of the compounds under study are monitored. The mass spectrometer is switched from one selected mass to another ignoring all other values. This leads to high sensitivity because the ion currents of interest can be monitored for much longer than in the case of a full scan spectrum.

Quantitative mass spectrometry usually uses chromatographic separation prior to selected ion monitoring, although the conventional direct insertion probe\textsuperscript{108} or fast atom bombardment\textsuperscript{109} can be used. Data are obtained by integration of the area under the evaporation profile. The combination of chromatography with mass spectrometry can give quantitative data with a high degree of accuracy and precision.

A standard is required when performing quantitative analysis with which the response from the analyte can be compared. An external standard method can be used but this technique is prone to error since changes in the condition of the instrument, for example, slight variations in the ion source pressure, affect the magnitude of the signals obtained. The use of an internal standard produces greater accuracy. In the latter method, the internal standard should be added at the earliest stage possible to compensate for errors in the sample preparation and work-up. As long as the internal standard and analyte behave identically during the work-up, unavoidable or accidental losses of the analyte, due to, for example, incomplete extraction or spillage, do not affect the ratio of the two compounds and so the result is valid. Similarly, instrumental changes from one run to another are made irrelevant because they affect the absolute values and not the ratios. The internal standard provides its own characteristic ions which are continuously monitored together with those characteristic of the analyte. Three types of internal standard are applicable to mass spectrometry:

a. Stable isotopically-labelled standard
b. Homologue standard
c. Analogue standard
Isotopically labelled standards provide the greatest accuracy because they are chemically the same compound as the analyte and therefore have the same extraction, chromatographic and ionisation properties. Internal standards labelled with $^{13}$C, $^{15}$N and $^{18}$O are more similar chemically to the corresponding unlabelled compound than those labelled with deuterium, but they are not as popular because they are expensive and generally difficult to synthesize. The internal standard should contain a sufficient number of labelled atoms to shift the molecular weight by at least three mass units for compounds containing C, H, N and O only. The ions monitored will therefore not be common to both compounds and hence cross contamination is avoided. Compounds containing elements such as chlorine or bromine, whose heavier isotopes contribute significantly to the ion current, may require a greater mass shift if precise quantification is required. As the number of isotopic labels incorporated into a structure increases, the greater is the likelihood that the labelled and unlabelled analogues will behave differently. The optimum number of deuterium atoms is therefore usually three to five. The disadvantages of using a stable isotopically-labelled internal standard are that they are often difficult and expensive to synthesize, and they require a second channel in SIR which leads to a reduction in sensitivity.

Homologue standards are very similar in chemical structure to the analyte and therefore have similar extraction efficiencies. If a suitable homologue standard can be found that contains an ion in its mass spectrum which is common to that of the analyte, then only one ion needs to be monitored in the analysis to detect both compounds. This leads to higher sensitivities than if several ions are monitored. If this type of internal standard is employed, the analyte and standard need to be separated chromatographically prior to mass spectrometric detection otherwise the responses from each compound would be superimposed.

The use of an analogue internal standard is usually the least accurate method since extraction ratios and derivatisation can differ considerably. More than one ion usually has to be monitored which reduces sensitivity. Analogues however, have the advantage of being convenient and cheap.
In the development of this assay, analogue internal standards were investigated because they were readily available.

4.4 EXPERIMENTAL

4.4.1 Reagents and Materials

SN 609369, SN 606078, SN 608154 and SN 609369 sodium salt (hepta-hydrate) were synthesised at AgrEvo UK Limited, Chesterford Park and their purities were ascertained by melting point, proton nuclear magnetic resonance spectroscopy, mass spectrometry and elemental analysis. All solvents were of analytical grade and were obtained from Rathburn Chemicals Limited, Walkerburn or Fisons Scientific Equipment, Loughborough. Ammonium acetate was obtained from BDH Limited, Poole or Lancaster Synthesis Limited, Morecombe and glacial acetic acid was supplied by BDH Limited, Poole. The plasma samples were obtained from a trial carried out at AgrEvo UK Limited, Chesterford Park. The administration of SN 609369 and the blood sample collection were completely separate from the analytical aspects of this study and are not reported here.

4.4.2 Instrumentation

Chromatographic separations were developed using a Waters Model 6000A HPLC pump connected to a 7125 Rheodyne injection valve with a 20 μl sample loop. UV absorption at 265 nm was monitored using an Applied Biosystems Model 757 Absorbance Detector. Thermospray experiments were performed on a VG MassLab 12-250 quadrupole mass spectrometer linked to a VG 11250 data system. A standard VG thermospray interface with no filament or discharge facilities was used. The LC eluent was pumped with a Gilson 302 LC pump and the samples were injected via a 7125 Rheodyne injection valve with a 20 μl sample loop.

4.4.3 Development of Chromatographic Conditions

SN 609369 had previously been analysed on a 250 x 4.6 mm Hypersil 5-ODS column with a mobile phase containing 68% acetonitrile : 32% 0.01 M phosphate buffer (pH 3). This was used as a starting point for these chromatographic investigations.
A 250 x 4.6 mm Hypersil 5-ODS column with a Whatman pellicular ODS guard column was employed. Five mobile phases were investigated at a flow rate of 1.0 ml/min.

A. 45% acetonitrile : 55% 0.1M aq. ammonium acetate pH 4.3  
B. 50% acetonitrile : 50% 0.1M aq. ammonium acetate pH 4.3  
C. 55% acetonitrile : 45% 0.1M aq. ammonium acetate pH 4.3  
D. 65% methanol : 35% 0.1M aq. ammonium acetate pH 4.3  
E. 70% methanol : 30% 0.1M aq. ammonium acetate pH 4.3

The ammonium acetate solution was prepared by adjusting 0.1 M aqueous ammonium acetate buffer to pH 4.3 with glacial acetic acid.

2-[2-(2,6-dichloro-4-trifluoromethylphenyl)-5-methyl-2H-1,2,3-triazol-4-yl]-1H-imidazole-4,5-dicarbonitrile (SN 608154) and 2-[2-(2,6-dichloro-4-trifluoromethylphenyl)-2H-1,2,3-triazol-4-yl]-1H-imidazole-4,5-dicarbonitrile (SN 606078) were both investigated as potential internal standards for the assay (see Figure 4.2 for structures of these species).

\[
\text{C}_{14}\text{H}_4\text{Cl}_2\text{F}_3\text{N}_7 = 397}  
\text{SN 606078} 
\]

\[
\text{C}_{15}\text{H}_6\text{Cl}_2\text{F}_3\text{N}_7 = 411}  
\text{SN 608154} 
\]

Figure 4.2 : SN 606078 and SN 608154
Table 4.1 shows the retention times which were observed for SN 609369, SN 606078 and SN 608154 (where applicable) for each of the solvent systems. Under the conditions used, SN 608154 and SN 609369 could not be fully resolved whereas SN 606078 and SN 609369 were separated. SN 606078 therefore appeared to be a more suitable internal standard for the analysis than SN 608154.

<table>
<thead>
<tr>
<th>Solvent System</th>
<th>Retention Time (minutes)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SN 609369</td>
</tr>
<tr>
<td>A</td>
<td>11.6</td>
</tr>
<tr>
<td>B</td>
<td>7.4</td>
</tr>
<tr>
<td>C</td>
<td>7.0</td>
</tr>
<tr>
<td>D</td>
<td>&gt;30</td>
</tr>
<tr>
<td>E</td>
<td>7.4</td>
</tr>
</tbody>
</table>

Table 4.1: Observed Chromatographic Retention Times

Of the mobile phases investigated, solvent system A gave the best separation of SN 609369 (retention time 11.6 minutes) and SN 606078 (retention time 8.0 minutes) with a relatively short run time. It was therefore concluded that SN 606078 would be used as the internal standard and that the solvent system employed would consist of 45% acetonitrile : 55% 0.1 M aqueous ammonium acetate at pH 4.3.

4.4.4 Development of Thermospray Conditions

4.4.4.1 Comparison of Positive and Negative Thermospray Ionisation Modes

The combination of LC and negative ionisation MS has been shown to be a valuable analytical tool for the study of halogenated compounds in crude biological matrices. Ionisation of electronegative compounds followed by detection of negative ions rather than positive ions can lead to significant increases in sensitivity for these species whilst most biological constituents are "transparent" to detection. Bearing in mind the fact that plasma samples are to be analysed and that SN 609369 is a highly halogenated species, SN
609369 was initially examined in both the positive and negative thermospray ionisation modes to determine the most sensitive and specific mode of detection.

A solution of SN 609369 in the mobile phase was prepared. This was injected directly into the thermospray interface operating in the negative ionisation mode. The temperatures and repeller voltages were optimised such that the spectrum for SN 609369 showed predominantly the [M-H]⁻ species with little fragmentation. This process was repeated in the positive ionisation mode such that the spectrum showed predominantly the [M+H]⁺ species with little [M+ NH₄]⁺ or fragmentation. The resulting spectra are shown in Figures 4.3 and 4.4.

Figure 4.3: SN 609369 negative ion thermospray (TSP-) spectrum
In the negative ionisation mode, the spectrum shows predominantly ions at m/z 443, 445, 447, 449 (tri-chloro substitution pattern) corresponding to [M-H]⁻ of SN 609369. In the positive ionisation mode, the spectrum shows ions at m/z 445, 447, 449, 451 (tri-chloro substitution pattern), m/z 462, 464, 466, 468 (tri-chloro substitution pattern) and m/z 463, 465, 467, 469 (tri-chloro substitution pattern) which correspond to [M+H]⁺, [M+NH₄]⁺ and possibly [M+CH₃COONH₄.CH₃COOH]⁺ respectively.

In order to check the relative sensitivities in the positive and negative ionisation modes, a standard solution was prepared containing 50 mg/l of SN 609369. Three 20 µl injections were made in each mode and the resulting chromatograms are shown in Figures 4.5 and
4.6. As predicted, the signal to noise ratio and sensitivity were considerably better for the detection of SN 609369 in the negative ionisation mode. All future analyses were therefore carried out in this mode.

Figure 4.5: SN 609369 TSP+ (m/z 445 ion chromatogram)

Figure 4.6: SN 609369 TSP- (m/z 443 ion chromatogram)
4.4.4.2 Optimisation of Thermospray Conditions

The optimum conditions chosen for subsequent analyses were:

- **Mobile phase**: 45% acetonitrile : 55% water (0.1M ammonium acetate at pH 4.3)
- **Flow rate**: 1.0 ml/minute
- **Temperatures**: evaporation chamber 250°C, source 200°C, vaporiser 245°C
- **Repeller voltage**: 250 - 300 V

The intensity of the pseudo-molecular ion continued to increase as the vaporiser temperature increased but problems arose with blockages of the capillary in the thermospray probe when the temperature rose above 250°C. All experiments were therefore performed with a vaporiser temperature of 245°C to overcome this problem.

The optimum repeller voltage varied on a daily basis depending on the degree of contamination of the thermospray source. It appeared that the cleaner the source, the lower the repeller voltage had to be for optimisation of the pseudo molecular ion. The repeller voltage was therefore optimized daily.

SN 606078 was examined under the optimum conditions to ensure that it gave a thermospray spectrum. The spectrum obtained is shown in Figure 4.7. Prominent ions are detected at m/z 396, 398, 400 (di-chloro substitution pattern) corresponding to [M-H]⁻ of SN 606078.
4.4.5 Calibration of the Mass Spectrometer
Mass calibration was performed in the negative ionisation mode with a polyethylene glycol (PEG) mixture (250 ng/ml each of PEG 200, PEG 300 and PEG 400, 1:1:1) dissolved in 70% methanol in 0.1 M ammonium acetate buffer. At a vaporiser temperature of 230°C, an evaporation temperature of 250°C and a repeller voltage of 200 V, this mixture produced \([M+CH_3COO]^+\) ions of PEG oligomers.

4.4.6 Linearity Test of LC-MS System using SN 606078 as Internal Standard
All experiments were performed under the optimum thermospray conditions outlined in the previous section. The mass spectrometer was operated in the SIR mode in preference to
full scan detection to increase the sensitivity and improve the precision. Four ions were monitored: m/z 396, 398 (corresponding to [M-H]$^-$ of SN 606078) and m/z 443, 445 (corresponding to [M-H]$^-$ of SN 609369).

Nine standard solutions were made up containing SN 609369 covering the range 0 - 50 mg/l and internal standard (SN 606078) at 10 mg/l in acetonitrile. Three 20 µl injections were made of each solution via the 250 x 4.6 mm Hypersil 5-ODS column with a Whatman pellicular ODS packed guard column. Under the conditions used, SN 606078 and SN 609369 eluted at retention times of approximately 8.5 and 11.75 minutes respectively. The resulting peak areas for SN 606078 and SN 609369 on the m/z 396 and 443 ion traces respectively were measured for each of the standard solutions and a calibration curve was produced (see Figure 4.8) by plotting the ratio of the two peak areas against the SN 609369 concentration.

![Figure 4.8: SN 609369 Calibration Curve (25cm column, SN 606078 internal standard, acetonitrile solutions).](image_url)
The resulting plot showed considerable scatter and the correlation coefficient of 0.9663 indicates poor precision for the calibration curve. Table 4.2 shows the precision of the method at each of the nine standard concentrations. The variation of the response factor (CV) ranged from 0.9 - 22.6%.

<table>
<thead>
<tr>
<th>Amount Injected (mg/l)</th>
<th>Area Ratio</th>
<th>CV</th>
<th>n</th>
</tr>
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<td></td>
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<td>SD</td>
<td>%</td>
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<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>0.2</td>
<td>0.002</td>
<td>0.9</td>
</tr>
<tr>
<td>2</td>
<td>0.53</td>
<td>0.054</td>
<td>10.2</td>
</tr>
<tr>
<td>5</td>
<td>0.9</td>
<td>0.142</td>
<td>15.6</td>
</tr>
<tr>
<td>10</td>
<td>2.35</td>
<td>0.532</td>
<td>22.6</td>
</tr>
<tr>
<td>20</td>
<td>7.38</td>
<td>1.029</td>
<td>13.9</td>
</tr>
<tr>
<td>30</td>
<td>12.30</td>
<td>2.350</td>
<td>19.1</td>
</tr>
<tr>
<td>40</td>
<td>22.24</td>
<td>1.345</td>
<td>6.0</td>
</tr>
<tr>
<td>50</td>
<td>18.48</td>
<td>2.272</td>
<td>12.3</td>
</tr>
</tbody>
</table>

Table 4.2: Analytical precision for SN 609369 in acetonitrile

A possible explanation for the poor correlation coefficient and the generally high variation in response factors could be that the thermospray conditions varied slightly during the run. Slight changes in the temperatures or pressures in the ion source would lead to fluctuations in the thermospray response.

This was investigated further by analysis of the same standard solutions without the 250 mm column such that SN 609369 and SN 606078 co-eluted. This would eliminate the problem of slight variations in the stability of the thermospray beam during each run.

Three 20 μl injections of each of the nine standard solutions were made via the Whatman pellicular ODS packed guard column under the optimum thermospray conditions described in Section 4.4.4.2. The resulting peak areas for m/z 396 and m/z 443 were measured and the ratios of the two peak areas were plotted against the SN 609369 concentrations. The calibration curve produced (Figure 4.9) was linear with a correlation coefficient of 0.9965. There appears to be considerably less scatter for replicate injections than that observed in the calibration curve produced from the column analysis (Figure 4.8). This suggests that the proposed explanation about thermospray conditions varying with time is plausible.
An advantage of performing this analysis with only a guard column was the faster speed of analysis - three replicate injections could be made in three minutes compared to a total time of 45 minutes via the 250 mm column. Unfortunately, this advantage was considerably outweighed by the poor peak shape produced when no column was present (see Figure 4.10).

Figure 4.9: SN 609369 Calibration Curve (loop injections, SN 606078 internal standard, acetonitrile solutions)

Figure 4.10: SN 609369 TSP- (loop injections, m/z 443 ion chromatogram)
Some separation was also required when the actual plasma samples were analysed so that any impurities could be separated from the species of interest. For these reasons, it was felt that an alternative internal standard should be investigated.

Watson et al.\textsuperscript{112} have encountered problems with intrinsic instability of the thermospray ion beam during quantitation of Vitamin D. They employed an isotopically labelled internal standard in the development of an assay for 1,25-dihydroxyvitamin D\textsubscript{3}. The use of an isotopically labelled internal standard was considered for this SN 609369 assay but it was felt to be impractical in this instance because a suitable isotopic analogue would be difficult and expensive to synthesise. The mass increase would have to be at least 8 amu to avoid interferences with the chlorine and carbon isotope peaks from SN 609369. An alternative internal standard therefore had to be found and a species which co-eluted with SN 609369 was felt to be necessary. SN 608154 was therefore investigated (see Table 4.1).

### 4.4.7 Linearity Test of LC-MS System using SN 608154 as Internal Standard

Initially it was necessary to determine whether SN 608154 would give a thermospray spectrum in the negative ionisation mode. A solution of SN 608154 in the mobile phase was injected directly into the thermospray interface via the Rheodyne injection valve. The resulting spectrum is shown in Figure 4.11. It consists predominantly of ions at m/z 410, 412, 414 (di-chloro substitution pattern) corresponding to [M-H]\textsuperscript{-} of SN 608154.

Nine standard acetonitrile solutions were prepared containing various levels of SN 609369 covering the range 0 - 50 mg/l and the internal standard SN 608154 at 10 mg/l. Three 20 μl injections were made of each solution via the 250 x 4.6 mm Hypersil 5-ODS column with a Whatman pellicular ODS packed guard column. The analyses were performed under the optimum thermospray conditions outlined in Section 4.4.4.2 and the mass spectrometer was operated in the SIR mode. Ions at m/z 443, 445 (corresponding to [M-H]\textsuperscript{-} of SN 609369) and m/z 410, 412 (corresponding to [M-H]\textsuperscript{-} of SN 608154) were monitored.
Figure 4.11: SN 608154 TSP- spectrum

Under the conditions used SN 609369 and SN 608154 co-eluted at a retention time of approximately 11.75 minutes. The resulting peak areas for SN 609369 and SN 608154 on the m/z 443 and m/z 410 ion traces respectively, were measured for each of the standard solutions and the ratios of the two peak areas were plotted against the SN 609369 concentrations. The resulting calibration curve is shown in Figure 4.12. The calibration curve was linear with a correlation coefficient of 0.9985 down to a level of 1 mg/l. Table 4.3 shows the precision of the method at each of the nine standard concentrations. The CV ranged from 0.2 - 11.4%.
Figure 4.12: SN 609369 Calibration Curve (25cm column, SN 608154 internal standard, acetonitrile solutions)

<table>
<thead>
<tr>
<th>Amount injected (mg/l)</th>
<th>Peak Area Ratio</th>
<th>CV</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SD ×10⁻³</td>
<td>%</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>0.112</td>
<td>8.8</td>
<td>7.8</td>
</tr>
<tr>
<td>2</td>
<td>0.319</td>
<td>36.4</td>
<td>11.4</td>
</tr>
<tr>
<td>5</td>
<td>0.650</td>
<td>21.9</td>
<td>3.4</td>
</tr>
<tr>
<td>10</td>
<td>1.454</td>
<td>24.8</td>
<td>1.7</td>
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<td>20</td>
<td>3.520</td>
<td>51.4</td>
<td>1.5</td>
</tr>
<tr>
<td>30</td>
<td>5.342</td>
<td>8.1</td>
<td>0.2</td>
</tr>
<tr>
<td>40</td>
<td>7.593</td>
<td>52.5</td>
<td>0.7</td>
</tr>
<tr>
<td>50</td>
<td>9.307</td>
<td>283.3</td>
<td>3.0</td>
</tr>
</tbody>
</table>

Table 4.3: Analytical precision for SN 609369 in acetonitrile
SN 608154 would therefore appear to be more suitable as an internal standard for the assay than SN 606078.

4.4.8 Reduction of Assay Time

The aim of the project was to develop a fast and selective assay. It was therefore felt that a shorter LC column should be investigated in order to reduce the run times as the need to separate SN 609369 from the internal standard had been eliminated. A 33 x 4.6 mm Supelcosil LC-8-DB (3 micron) column was used for this purpose.

Nine standard solutions containing SN 609369 covering the range 0 - 50 mg/l and the internal standard SN 608154 at 10 mg/l in acetonitrile were prepared. Three 20 µl injections of each solution were made via the 33 mm column with the Whatman pellicular ODS packed guard column. The analyses were performed using the previously developed optimum conditions and the mass spectrometer was operated in the SIR mode monitoring ions at m/z 445, 443, 412 and 410. Under these conditions SN 609369 and SN 608154 co-eluted at a retention time of 1.75 minutes. The resulting peak areas were measured and the calibration curve obtained is shown in Figure 4.13.

The calibration curve was linear with a correlation coefficient of 0.9984. Table 4.4 shows the precision of the method at each of the nine standard concentrations. The CV ranged from 1.9 - 8.8%.
Table 4.4: Analytical precision for SN 609369 in acetonitrile
4.4.9 Analysis of Plasma Samples

4.4.9.1 Sample Preparation

A sheep plasma sample containing 32.3 mg/l of SN 609369 (determined by the original lengthy extraction procedure - see Section 4.1) was used as the stock standard solution. The sample had been taken from a sheep two days after oral dosage with SN 609369. Plasma samples containing 0, 2.0, 4.0, 8.1, 16.2 and 32.3 mg/l of SN 609369 were prepared by diluting the stock plasma sample appropriately with sheep plasma taken from an untreated sheep. 0.5 ml of SN 608154 in acetonitrile (20 mg/l) was added to 0.5 ml of each of the standard plasma samples. The tubes were vortexed for 10 seconds and then centrifuged for 2 minutes. The supernatant was separated and analysed.

4.4.9.2 Linearity and Precision

The analyses were performed under the optimum thermospray conditions (see Section 4.4.4.2) and the mass spectrometer was operated in the SIR mode, monitoring ions at m/z 445, 443, 412 and 410. Three 20 μl injections of each solution were made at 4.5 minute intervals via the 33 mm analytical column and the guard column. Figure 4.14 shows the four ion traces for the 2.0 mg/l standard solution. The resulting peak areas for each standard solution were measured and the resultant calibration curve is shown in Figure 4.15.

A linear plot was obtained with a correlation coefficient of 0.9989. Table 4.5 shows the precision of the method at each of the six standard concentrations. The CV ranged from 18% at the lower end of the standard curve to 2% at the high end.
Figure 4.14 : 2 mg/l plasma standard ion chromatograms
Figure 4.15: SN 609369 Calibration Curve (3.3cm column, SN 608154 internal standard, plasma solutions)

<table>
<thead>
<tr>
<th>Amount injected (mg/l)</th>
<th>Peak Area Ratio</th>
<th>CV</th>
<th>n</th>
</tr>
</thead>
<tbody>
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<td>0.046</td>
<td>8.5</td>
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<tr>
<td>2.0</td>
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<td>11.0</td>
<td>11.0</td>
</tr>
<tr>
<td>4.0</td>
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<td>15.0</td>
<td>6.8</td>
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<td>8.1</td>
<td>0.495</td>
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<td>5.1</td>
</tr>
<tr>
<td>16.2</td>
<td>1.032</td>
<td>18.8</td>
<td>1.8</td>
</tr>
</tbody>
</table>

Table 4.5: Analytical precision for SN 609369 in plasma
4.4.9.3 Reproducibility and Stability of SN 609369 in Plasma

The reproducibility of the assay and the stability of the analyte and internal standard in solution were investigated by repeating the previous experiment on the same standard solutions which had been stored at 5°C for 48 hours. The average peak area ratios for both experiments are shown in Figure 4.16.

![Figure 4.16](image)

Figure 4.16: SN 609369 Calibration Curve on Days 1 and 3 (3.3 cm column, SN 608154 internal standard, plasma solutions)

The results show that the two plots are almost identical, the gradients being 0.0647 and 0.0652 with correlation coefficients of 0.9989 and 0.9968 respectively. This indicates that, under the same thermospray LC/MS conditions, the method is reproducible and that SN 609369 and SN 608154 are relatively stable in plasma / acetonitrile solutions for a minimum of 48 hours, if stored at 5°C.
4.4.10 Preparation and Analysis of Plasma Standards using SN 609369 Sodium Salt

The standard plasma solutions used so far originated from a plasma sample obtained from a sheep which had been dosed with SN 609369. The amount of SN 609369 present in this sample was determined by the original, lengthy extraction procedure followed by HPLC with UV detection. The accuracy of this extraction method therefore determined the accuracy of the standards used in the TSP LC-MS assay. In order to obtain optimum results, standards should be prepared from accurately weighed amounts of SN 609369 in plasma. SN 609369 is not soluble in plasma or water and therefore the sodium salt was used to prepare the standards.

A stock solution was prepared containing 100 mg/l of SN 609369 sodium salt (hepta-hydrate) dissolved in plasma taken from an untreated sheep. This was diluted appropriately with more blank sheep plasma to yield standard SN 609369 plasma solutions covering the range 0 - 100 mg/l. 1.0 ml of SN 608154 in acetonitrile (20 mg/l) was added to 1.0 ml of each of the standard solutions. The tubes were vortexed for 10 seconds and then centrifuged for 2 minutes. The supernatant was separated and analysed.

The analyses were performed under the previously developed optimum thermospray conditions and the mass spectrometer was operated in the SIR mode monitoring ions at m/z 445, 443, 412 and 410. Three 20 μl injections were made of each solution at 4.5 minute intervals via the 33 mm column and guard column. The resulting peak areas for each standard solution were measured and the calibration curve obtained is shown in Figure 4.17. The calibration plot was linear with a correlation coefficient of 0.9985. Table 4.6 shows the precision of the method at each of the nine standard concentrations. The CV ranged from 0.3 - 8.2%.
Figure 4.17: SN 609369 Calibration Curve using SN 609369 sodium salt in plasma

<table>
<thead>
<tr>
<th>Amount injected (mg/l)</th>
<th>Peak Area Ratio</th>
<th>CV</th>
<th>n</th>
</tr>
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</tr>
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<td>4.1</td>
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<td>0.9</td>
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<td>40.9</td>
<td>2.235</td>
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Table 4.6: Analytical precision for SN 609369 in plasma prepared from SN 609369 sodium salt
4.4.11 Recovery Check

A stock solution was prepared containing 100 mg/l of SN 609369 sodium salt (heptahydrate) dissolved in plasma taken from an untreated sheep. This was diluted appropriately with more blank sheep plasma to yield standard SN 609369 plasma solutions covering the range 0 - 100 mg/l. 0.5 ml of SN 608154 in acetonitrile (20 mg/l) was added to 0.5 ml of each of the standard solutions. The tubes were vortexed for 10 seconds and then centrifuged for 2 minutes. The supernatant was separated and analysed.

This process was repeated using a stock solution of SN 609369 sodium salt (heptahydrate) in distilled water.

Each standard solution was analysed as described in the previous section and the resulting peak areas for each standard solution were measured. The calibration curve obtained for the water standards is shown in Figure 4.18. The calibration plot was linear with a correlation coefficient of 0.9945. This plot was used to calculate the actual concentrations of SN 609369 injected in the plasma standards and from these results, the percentage of SN 609369 recovered in each plasma standard was calculated (Table 4.7).

![Figure 4.18: SN 609369 Calibration Curve using SN 609369 sodium salt in water](image)
<table>
<thead>
<tr>
<th>Theoretical Amount injected (mg/l)</th>
<th>Mean Area Ratio</th>
<th>Actual concn (mg/l)</th>
<th>Recovery %</th>
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<td>111</td>
</tr>
<tr>
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<td>86</td>
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<td>0.157</td>
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</tr>
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<td>1.034</td>
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</tr>
<tr>
<td>54.16</td>
<td>1.650</td>
<td>36.61</td>
<td>68</td>
</tr>
</tbody>
</table>

Table 4.7: Recovery check of SN 609369 from plasma

The level of SN 609369 recovered ranged from 48% to 111% but was consistently about 70% at the higher concentrations. The higher recovery values for the lower SN 609369 concentrations probably arise due to errors in measuring the small volumes of spiked plasma stock solution required for these standard solutions. The low recovery (48%) for the 11 mg/l standard compared to the other standards could not be explained as easily, although it is suspected that pipetting errors again may be the source of the problem.

4.4.12 Limit of Detection of SN 609369

The requirement of the assay was to determine the concentration of SN 609369 in plasma down to a detection limit of approximately 1 mg/l. This appeared to be close to the limit of detection under the conditions used during this thermospray assay (see Figure 4.19).
4.4.13 Final Assay developed for Analysis of Sheep Trial Samples

The final assay that had been developed is described below:

4.4.13.1 Reagents

a. SN 609369 sodium salt (hepta-hydrate)
b. SN 608154
c. Plasma from an untreated sheep
d. Ammonium acetate
e. Acetonitrile (analytical grade)
f. Glacial acetic acid
g. Distilled water
4.4.13.2 Procedure

i. Preparation of mobile phase

Ammonium acetate (approximately 7.7 g) was dissolved in distilled water (550 ml). The solution was acidified with glacial acetic acid to pH 4.3 and diluted with acetonitrile (450 ml).

ii. Preparation of standard and sample solutions

SN 608154 (approximately 2 mg accurately weighed) was dissolved in acetonitrile (100 ml) to give a 20 mg/l internal standard solution.

SN 609369 sodium salt, hepta-hydrate (approximately 1.2 mg accurately weighed) was dissolved in plasma from an untreated sheep (10 ml). 0, 0.01, 0.02, 0.05, 0.1, 0.2, 0.3, 0.4 and 0.5 ml of this stock solution was diluted appropriately with further sheep plasma to a total volume of 0.5 ml yielding standard solutions of SN 609369 (free base) covering the range 0 - 100 mg/l.

Internal standard solution (0.5 ml) was added to each standard solution and sample (0.5 ml) in a tapered centrifuge tube. Each tube was vortexed for 10 seconds and then centrifuged for 2 minutes. The supernatant was separated.

iii. Thermospray LC-MS

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Specification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mobile phase</td>
<td>45% acetonitrile : 55% water (0.1M ammonium acetate at pH 4.3)</td>
</tr>
<tr>
<td>Flow rate</td>
<td>1.0 ml/minute</td>
</tr>
<tr>
<td>Column</td>
<td>33 x 4.6 mm Supelcosil LC-8-DB (3 micron) with Whatman pellicular ODS packed guard column</td>
</tr>
<tr>
<td>Temperatures</td>
<td>evaporation chamber 250°C, source 200°C, vaporiser 245°C</td>
</tr>
<tr>
<td>Repeller voltage</td>
<td>250 - 300 V</td>
</tr>
</tbody>
</table>

The mass spectrometer was operated in the negative ionisation mode monitoring ions at m/z 445, 443, 412 and 410.
iv. Analysis of samples and standards

Three 20 μl injections of each standard and sample solution were made at 4.5 minute intervals.

The areas of the peaks of m/z 445, 443, 412 and 410 corresponding to SN 609369 and the internal standard were determined. The area ratios of m/z 445 to m/z 443, and m/z 412 to 410 were calculated and the presence of SN 609369 in the sample extracts were confirmed if each of the following criteria were satisfied:

a. the component had the same LC retention time as that of the standard solutions
b. the ions m/z 445 and 443 exhibited simultaneous maxima for the LC peak
c. the chlorine isotope ratio for this pair of ions agreed to that obtained for the standards
d. the signal of all peaks was greater than three times the noise.

If SN 609369 was confirmed to be present, the area ratios of m/z 443 (SN 609369) over m/z 410 (internal standard) were calculated. Those for the standard solutions are used to produce a calibration curve of area ratio of SN 609369 over internal standard against concentration of SN 609369.

The concentration of SN 609369 in each sample was twice the value which was read directly from the calibration curve in mg/l.

4.4.14 Analysis of Sheep Trial Samples

A sheep was dosed intravenously with an aqueous solution of SN 609369 sodium salt at a rate of 5 mg/kg bodyweight. The level of SN 609369 remaining in the animals blood was monitored by removing 10 ml of blood from the treated sheep at the following intervals: 3 hours, 6 hours, and 2, 3, 4, 5, 6, 7, 9, 11, 14, and 17 days. The blood samples were centrifuged and the plasma was isolated. Techniques used in the administration of SN 609369 and the isolation of the plasma were totally independent of the analytical aspects of this study. The plasma samples were analysed by the final assay described in the previous section and the results were calculated using three independent calibration curves:

a. Standards in acetonitrile
b. Standards in plasma from trial sample diluted appropriately
c. Standards in plasma using plasma spiked with sodium salt.

The results are tabulated in Table 4.8 and are compared with the results obtained using the original, time consuming extraction method. A graphical representation of the results is shown in Figure 4.20.

<table>
<thead>
<tr>
<th>Time</th>
<th>SN 609369 Concentration (mg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Long extraction</td>
</tr>
<tr>
<td>3 hours</td>
<td>32.3</td>
</tr>
<tr>
<td>6 hours</td>
<td>27.0</td>
</tr>
<tr>
<td>2 days</td>
<td>24.0</td>
</tr>
<tr>
<td>3 days</td>
<td>23.5</td>
</tr>
<tr>
<td>4 days</td>
<td>19.8</td>
</tr>
<tr>
<td>5 days</td>
<td>13.9</td>
</tr>
<tr>
<td>6 days</td>
<td>10.2</td>
</tr>
<tr>
<td>7 days</td>
<td>10.4</td>
</tr>
<tr>
<td>9 days</td>
<td>6.7</td>
</tr>
<tr>
<td>11 days</td>
<td>5.7</td>
</tr>
<tr>
<td>14 days</td>
<td>4.4</td>
</tr>
<tr>
<td>17 days</td>
<td>3.3</td>
</tr>
</tbody>
</table>

Table 4.8: Level of SN 609369 in plasma samples from sheep trial

In general, the results determined in this study were lower than those which had been determined by the long extraction procedure. The curve produced from the latter method deviated further from an exponential curve than those produced in this study (see Table 4.9) therefore suggesting that the results calculated by the method described in Section 4.4.10 were closer to the true values than those previously determined because an exponential curve would be predicted rather than more random values.
Figure 4.20: SN 609369 residue in sheep plasma (intravenous dosage at 5 mg/kg bodyweight)

Table 4.9: Regression values of deviation from exponential curve

<table>
<thead>
<tr>
<th>Calibration curve used</th>
<th>Regression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Long extraction assay</td>
<td>0.981</td>
</tr>
<tr>
<td>Acetonitrile standards</td>
<td>0.974</td>
</tr>
<tr>
<td>Diluted sheep plasma standards</td>
<td>0.987</td>
</tr>
<tr>
<td>Spiked plasma standards</td>
<td>0.992</td>
</tr>
</tbody>
</table>

Generally, the levels of SN 609369 in the trial samples appeared to be higher when the calibration curve was produced using plasma standards in preference to acetonitrile standards. This was not surprising because it had been shown in Section 4.4.11 that only about 70% of SN 609369 was extracted from plasma and this had not been taken into
consideration when the concentration of SN 609369 was determined in the trial samples using the acetonitrile calibration curve.

The accuracies of the results were uncertain when the plasma standards produced by diluting the 3 hour sheep trial sample were used because the accuracies depended on the accuracy of the concentration of SN 609369 which had been measured in the three hour sheep trial sample by the long extraction procedure.

4.5 ALTERNATIVE IONISATION TECHNIQUES

LC/MS utilising short LC columns is routinely used in many laboratories when the rapid quantitative analysis of numerous samples is required, for example, in clinical investigations or environmental sample analyses\textsuperscript{113-115}. The atmospheric pressure ionisation techniques are generally employed due to their ability to achieve low detection limits. The sensitivities of electrospray (ES) and atmospheric pressure chemical ionisation (APCI) were therefore investigated for the analysis of SN 609369.

4.5.1 Instrumentation

LC/MS investigations were performed using a Hewlett Packard 1090 liquid chromatograph and autosampler coupled to a VG Biotech Quattro II triple quadrupole mass spectrometer fitted with standard ES or APCI interfaces. ES operations were carried out with optimised source temperature and cone voltage of 170°C and 30 V respectively, whilst APCI operations were performed using a source temperature 160°C, a probe temperature of 650°C and a cone voltage of 50 V. 20 µl injections were made. The mass spectrometer was operated in both the positive and negative ionisation modes under full scan (m/z 100-800) and SIR conditions.

LC conditions were identical to those described previously in Section 4.4.13.2iii. An LC eluent split of 0.3 ml/min to the mass spectrometer and 0.7 ml/min to waste was employed for the LC/MS ES experiments.
4.5.2 Analysis

A stock solution containing 10 mg/l of SN 609369 in 50% acetonitrile/50% water (0.1 M ammonium acetate at pH 4.3) was prepared. This solution was diluted accordingly to produce 100, 10, 1, 0.5 and 0.25 µg/l standard solutions. Full scan data were acquired on the 100 µg/l standard solution in both the positive and negative ionisation modes using both ES and APCI ionisations. No significant data were obtained using either interface in the positive mode.

Figure 4.21 shows the ES- spectrum obtained under the optimised source conditions described in Section 4.5.1. The only significant ions detected corresponded to the deprotonated molecular ions at m/z 443 (tri-chloro substitution pattern).

![Relative Intensity](image)

Figure 4.21: ES- spectrum of SN 609369

Figure 4.22 shows the APCI- spectrum obtained under the optimised source conditions described in Section 4.5.1. Significant ions were detected at m/z 443 (tri-chloro substitution pattern), m/z 409 (di-chloro pattern), m/z 375 (mono-chloro pattern), m/z 341 and m/z 367 which may be due to [M-H]-, [M-Cl]-, [M-2Cl+H]-, [M-3Cl+2H]- and [M-NC=CH-C=CN]- respectively.
The detection limits for the two interfaces were determined in the SIR mode. Three replicate injections of the standard solutions were made and ions at m/z 445 and 443 were monitored during ES- investigations, whilst ions at m/z 411 and 409 were monitored in the APCI- mode. Figures 4.23 and 4.24 show the ion traces for the weakest standards which could be clearly detected in these experiments.
Figure 4.24: APCI- ion chromatograms for SN 609369 (0.25 µg/l)

The detection limits were 1 µg/l and 0.25 µg/l for ES- and APCI- respectively. These values are significantly better than that obtained using the thermospray interface (1 mg/l, see Section 4.4.12), therefore if lower detection limits are required, either interface could be employed in preference to thermospray.

4.6 CONCLUSIONS

The major objective of this project was to reduce the assay time for determining the level of SN 609369 in sheep plasma. This has been achieved. The procedure had involved a lengthy extraction followed by LC with UV detection which took approximately three hours to analyse each sample. The total analysis time for each sample using the method developed here was approximately twenty minutes.

The lengthy extraction was eliminated by analysing the samples by thermospray LC-MS in the negative ionisation and SIR modes. Minimal chromatographic separation was shown to be necessary and therefore a short column was employed to reduce the assay time further. Two readily available internal standards were investigated - SN 608154, which co-eluted with SN 609369, and SN 606078, which had an earlier retention time. The former appeared to be the most suitable because it overcame the problem of slight variations in the stability of the thermospray beam during each run and led to linear calibration curves.
The detection limit for the analysis of approximately 1 mg/l was adequate for the requirements of the assay. The results obtained for the trial samples using the final assay described in Section 4.4.13 were comparable with those previously obtained by the original lengthy procedure. In general, the results tended to be lower than the original values but the results are felt to be more accurate because they produced a smoother exponential curve.

A faster and more accurate analytical method for the quantification of SN 609369 in sheep plasma samples has therefore been developed using LC/MS with TSP- ionisation. Both APCI- and ES- have been shown to offer significantly greater sensitivities over TSP- if lower SN 609369 detection limits are required.

If improvements in the detection limit become necessary and TSP- ionisation is the only available technique, then extraneous materials in the plasma would have to be removed prior to the analysis. A more efficient clean-up procedure would therefore be required in order to separate the analyte from co-eluting species which might suppress the signal of the analyte. A longer LC column may also be required to enhance this further but this would contradict the aims of this project. An alternative solvent system using methanol and/or a different buffer instead of acetonitrile and ammonium acetate may also lead to enhanced sensitivities in all ionisation modes. The separation power of MS/MS should also be considered. Even though the ion transmission is reduced when MS/MS is employed, increased detection limits can be obtained for samples whose detection limits are controlled by the background noise from the sample matrix. Background ions are removed in the first quadrupole thereby improving the signal-to-noise ratio for a particular species and hence overall detection limits are improved.

4.7 REFERENCES
105. R.J. Dowson, Pestic. Sci., 8, 651, (1977)


CHAPTER 5: CONCLUSIONS
5.1 CONCLUSIONS

The coupling of liquid chromatography with mass spectrometry provides a very powerful analytical tool which allows the rapid detection, identification and quantification of both unknown and known species in complex biological matrices with minimal or no sample pre-treatment and clean-up. This thesis has described a variety of LC/MS and LC/MS/MS techniques to solve three very different problems.

Chapter 2 described the structure elucidation of twenty metabolites using electrospray LC/MS and LC/MS/MS analysis of a sample of rat urine taken from an animal treated with Hoe 127490, a sulfonyl urea herbicide. No sample pretreatment was required. A metabolic route involving nine metabolic steps was proposed. The work showed the importance of using both parent and daughter ion experiments in order to detect all metabolites present. Quantitative MS data was not required for this work although UV data were available in order to provide semi-quantitative information.

Chapter 3 described the development of a semi-automated method using LC/MS and infusion MS/MS with atmospheric pressure chemical ionisation for the identification of dipeptides in complex mixtures. Both LC and tandem mass spectrometry were used independently to separate the individual peptides. The method was applied to a casein hydrolysate sample and fifty-two dipeptides were identified. The method is significantly quicker than a method previously employed involving the isolation of the individual peptides prior to amino acid analysis, but unfortunately, as yet, this new method is not as effective and is unable to be used for the identification of higher peptides. Areas have been identified in order to improve the assay and suggestions have been made to enable the method to be extended for the examination of the higher peptides. Again, no quantitative data were required for this work.

Chapter 4 described the development of a rapid method for the quantification of the insecticide SN 609369 in plasma taken from treated sheep. A lengthy extraction of the plasma was avoided by analysing the samples by LC/MS with negative ion thermospray ionisation in the selected ion recording mode after a rapid extraction with acetonitrile. The use of a short LC column allowed very short run times with minimal regard to chromatographic separation, and therefore rapid analysis times. Detection limits of 1 mg/l
were obtained. Both electrospray and atmospheric pressure chemical ionisation were shown to provide more than a thousand-fold improvement in sensitivity.

This thesis has shown the power of LC/MS and LC/MS/MS for complex mixture analysis in a variety of areas and has shown the ability of the techniques to provide both quantitative and qualitative information, depending on the nature of the particular problem to be solved.
APPENDIX
#!/bin/csh
#
# Procedure to predict di-peptides sequences from MS/MS data
# In a mixture by infusion experiments
#
# Usage: pep_predict data_file1 data_file2
#
set ECHO = /bin/echo
set DIR = /usr/users/finnigan/bin
set CURRENT = 'acquisition_path'
set LIST = $1

if (-e $DIR$LIST)_PEPS.nl then
    
else
    $ECHO* --------------------------------------------------------- > $LIST)_PEPS.nl
    $ECHO* Dipeptides Identified * >> $LIST)_PEPS.nl
    $ECHO* For Data Files $1 and $2* >> $LIST)_PEPS.nl
    $ECHO* ** ** >> $LIST)_PEPS.nl

    $ECHO* --------------------------------------------------------- > $LIST)_UNPEPS.nl
    $ECHO* Possible Dipeptides * >> $LIST)_UNPEPS.nl
    $ECHO* For Data Files $1 and $2* >> $LIST)_UNPEPS.nl
    $ECHO* ** ** >> $LIST)_UNPEPS.nl
    $ECHO* ** ** >> $LIST)_UNPEPS.nl

endif

#--------------------------------- TAKE INTO ACCOUNT N-TERMINAL ACIDS 
#---------------------------------

foreach MASS ('cat $DIR/PEP_MASSES.lst')

    if ($MASS) == 148) then
        @ BUTYL_MASS = $(MASS) + 112
    else
        if ($MASS) == 134) then
            @ BUTYL_MASS = $(MASS) + 112
        else
            if ($MASS) == 175) then
                @ BUTYL_MASS = $(MASS) + 14
            else
                @ BUTYL_MASS = $(MASS) + 56
            endif
        endif
    endif

    if ($MASS) == 148) then
        @ PENTYL_MASS = $(MASS) + 140
    else
        if ($MASS) == 134) then
            @ PENTYL_MASS = $(MASS) + 140
        else
            # the rest of the code
if (${MASS} == 175) then
  @ PENTYL_MASS = ${MASS} + 28
else
  @ PENTYL_MASS = ${MASS} + 70
endif

${ECHO} : ************************************************************
${ECHO} : SET UP C_TERMINAL AMINO ACID
${ECHO} : ************************************************************

if (${MASS} == 90) then
  set CTERM = Ala
else
  if (${MASS} == 175) then
    set CTERM = Arg
  else
  if (${MASS} == 133) then
    set CTERM = Asn
  else
  if (${MASS} == 134) then
    set CTERM = Asp
  else
  if (${MASS} == 179) then
    set CTERM = Cys
  else
  if (${MASS} == 148) then
    set CTERM = Glu
  else
  if (${MASS} == 147) then
    set CTERM = Gln
  else
  if (${MASS} == 76) then
    set CTERM = Gly
  else
  if (${MASS} == 156) then
    set CTERM = His
  else
  if (${MASS} == 132) then
    set CTERM = Lxx
  else
  if (${MASS} == 150) then
    set CTERM = Met
  else
  if (${MASS} == 166) then
    set CTERM = Phe
  else

200
if (${MASS} == 116) then
set C_TERM = Pro
else
if (${MASS} == 106) then
set C_TERM = Ser
else
if (${MASS} == 120) then
set C_TERM = Thr
else
if (${MASS} == 205) then
set C_TERM = Trp
else
if (${MASS} == 182) then
set C_TERM = Tyr
else
if (${MASS} == 118) then
set C_TERM = Val
endif
endif

# ---------------------------------------------------------------
# PREPARE PENTYLATED SCAN LIST
# ---------------------------------------------------------------
${ECHO} " "Preparing Pentylated Scan List"
${ECHO} " "For ${1} ${C_TERM}"
${ECHO} " "---------------------------------------------------------------
send_to_chro i
send_to_chro-an ${1}
send_to_chro ${PENTYL_MASS}
wait_for_chro
send_to_chro -2
send_to_chro "fbr;fju 1,1;1l,1;d"
wait_for_chro
send_to_chro swa ${DIR}/PENT.sl
wait_for_chro
#send_to_chro "hopt go landscape;h"
#wait_for_chro
#sleep 10

# ---------------------------------------------------------------
# PREPARE BUTYLATED SCAN LIST
# ---------------------------------------------------------------
${ECHO} " "Preparing Butylated Scan List"
${ECHO} " "For ${1} ${C_TERM}"
${ECHO} " "---------------------------------------------------------------

201
send_to_chro i
send_to_chro an $(2)
send_to_chro $(BUTYL_MASS)
wait_for_chro
send_to_chro -2
send_to_chro "fbt;fi;u 1,1;11,1;d"
wait_for_chro
send_to_chro swa $(DIR)/BUT.sl
wait_for_chro
#send_to_chro "hopt go landscape;h"
#wait_for_chro
#sleep 10

get_numbers_in_correct_format_to_compare
for_all_except_n_terminal_acids

cd $(DIR)

$ECHO: ********************
$ECHO: * Preparing BUT_56 Scan List *
$ECHO: ********************
$ECHO: 
$ECHO: foreach NUM('cat BUT.sl')
$ECHO:     if $(MASS) == 148 then
$ECHO:         @ FRED = $(NUM) - 112
$ECHO:     else
$ECHO:         if $(MASS) == 134 then
$ECHO:             @ FRED = $(NUM) - 112
$ECHO:         else
$ECHO:             if $(MASS) == 175 then
$ECHO:                 @ FRED = $(NUM) - 14
$ECHO:             else
$ECHO:                 @ FRED = $(NUM) - 56
$ECHO:         endif
$ECHO:     endif
$ECHO:     if $(FRED) > 100 then
$ECHO:         $ECHO: "$(FRED)" >> BUT_56.sl
$ECHO:     else
$ECHO:         endif
$ECHO: end

$ECHO: ********************
$ECHO: * Preparing PENT_70 Scan List *
$ECHO: ********************
$ECHO: 
$ECHO: foreach NUMP('cat PENT.sl')
$ECHO:     if $(MASS) == 148 then
$ECHO:         @ FREDP = $(NUMP) - 140
$ECHO:     else
$ECHO:         if $(MASS) == 134 then
$ECHO:             @ FREDP = $(NUMP) - 140
$ECHO:         else
$ECHO:             if $(MASS) == 175 then
$ECHO:                 @ FREDP = $(NUMP) - 28
$ECHO:             else
202
@ FREDP = ${NUMP) - 70
endif

if (${FREDP} > 100)then
  ${ECHO} "${FREDP}" » PENT_70.sl
else
end if
end

#-------------------------------------------
# GET NUMBERS IN CORRECT FORMAT TO COMPARE
# FOR NTERMINAL ACIDS
#-------------------------------------------------------------------------------------

${ECHO} "  * Preparing BUT_112 Scan List
${ECHO} "  * For ${1} ${C_TERM} 

foreach ANUM ('cat BUT.si')
  if (${MASS} == 148)then
    $@ AFRED = ${ANUM} - 168
  else
    if (${MASS} == 134)then
      @ AFRED = ${ANUM} - 168
    else
      if (${MASS} == 175)then
        @ AFRED = ${ANUM} - 70
      else
        @ AFRED = ${ANUM} - 112
      endif
    endif
  endif
  if (${AFRED} > 100)then
    ${ECHO} "${AFRED}" » BUT_112.sl
  else
    endif
end

${ECHO} "  * Preparing PENT_140 Scan List
${ECHO} "  * For ${1} ${C_TERM} 

foreach ANUMP ('cat PENT.si')
  if (${MASS} == 148)then
    @ AFREDP = ${ANUMP} - 210
  else
    if (${MASS} == 134)then
      @ AFREDP = ${ANUMP} - 210
    else
      if (${MASS} == 175)then
        @ AFREDP = ${ANUMP} - 98
      else
        @ AFREDP = ${ANUMP} - 140
      endif
    endif
  endif
  if (${AFREDP} > 100)then
    ${ECHO} "${AFREDP}" » PENT_140.sl
  else
    endif
end
# COMPARE SCAN LISTS AND WRITE TO SAME.sl and ASAME.sl

```bash
comm -12 BUT_56.sl PENT_70.sl > SAME.sl
comm -12 BUT_112.sl PENT_140.sl > ASAME.sl
```

```bash
foreach ITEM ('cat SAME.si')
  @ N_TERM = ($ITEM - $MASS)
  @ MOL_WT = ($ITEM - 1)
end if
```

```bash
# CREATE N-TERMINUS LIST
#
#   --------------------------------------------
#
if ($MASS) == 148)
  @ BUTYL_SPEC = ($MOL_WT) + 113
else
  if ($MASS) == 134)
    @ BUTYL_SPEC = ($MOL_WT) + 113
  else
    if ($MASS) == 175)
      @ BUTYL_SPEC = ($MOL_WT) + 15
    else
      @ BUTYL_SPEC = ($MOL_WT) + 57
    endif
  endif
  if ($MASS) == 148)
    @ PENTYL_SPEC = ($MOL_WT) + 141
  else
    if ($MASS) == 134)
      @ PENTYL_SPEC = ($MOL_WT) + 141
    else
      if ($MASS) == 175)
        @ PENTYL_SPEC = ($MOL_WT) + 29
      else
        @ PENTYL_SPEC = ($MOL_WT) + 71
      endif
      if ($N_TERM) == 71)
        @ Echo *Ala[(C_TERM)] ($MOL_WT)* >> ($LIST)_PEPS.n1
        @ Echo $BUTYL_SPEC$PENTYL_SPEC >> ($LIST)_PRINT.lst
      else
        if ($N_TERM) == 156)
          @ Echo *Arg[(C_TERM)] ($MOL_WT)* >> ($LIST)_PEPS.n1
          @ Echo $BUTYL_SPEC$PENTYL_SPEC >> ($LIST)_PRINT.lst
        else
```
if (${N_TERM} == 114) then
$\{\text{ECHO}\} *\text{Asn}\{\text{C_TERM}\} \{\text{MOL_WT}\} * \{\text{LIST}\}_\text{PEPS.nl}
$\{\text{ECHO}\} \{\text{BUTYL_SPEC}\}\{\text{PENTYL_SPEC}\} >> \{\text{LIST}\}_\text{PRINT.lst}
else

if (${N_TERM} == 160) then
$\{\text{ECHO}\} *\text{Cys}\{\text{C_TERM}\} \{\text{MOL_WT}\} * \{\text{LIST}\}_\text{PEPS.nl}
$\{\text{ECHO}\} \{\text{BUTYL_SPEC}\}\{\text{PENTYL_SPEC}\} >> \{\text{LIST}\}_\text{PRINT.lst}
else

if (${N_TERM} == 128) then
$\{\text{ECHO}\} *\text{Gln}\{\text{C_TERM}\} \{\text{MOL_WT}\} * \{\text{LIST}\}_\text{PEPS.nl}
$\{\text{ECHO}\} \{\text{BUTYL_SPEC}\}\{\text{PENTYL_SPEC}\} >> \{\text{LIST}\}_\text{PRINT.lst}
else

if (${N_TERM} == 57) then
$\{\text{ECHO}\} *\text{Gly}\{\text{C_TERM}\} \{\text{MOL_WT}\} * \{\text{LIST}\}_\text{PEPS.nl}
$\{\text{ECHO}\} \{\text{BUTYL_SPEC}\}\{\text{PENTYL_SPEC}\} >> \{\text{LIST}\}_\text{PRINT.lst}
else

if (${N_TERM} == 137) then
$\{\text{ECHO}\} *\text{His}\{\text{C_TERM}\} \{\text{MOL_WT}\} * \{\text{LIST}\}_\text{PEPS.nl}
$\{\text{ECHO}\} \{\text{BUTYL_SPEC}\}\{\text{PENTYL_SPEC}\} >> \{\text{LIST}\}_\text{PRINT.lst}
else

if (${N_TERM} == 113) then
$\{\text{ECHO}\} *\text{Ile}\{\text{C_TERM}\} \{\text{MOL_WT}\} * \{\text{LIST}\}_\text{PEPS.nl}
$\{\text{ECHO}\} \{\text{BUTYL_SPEC}\}\{\text{PENTYL_SPEC}\} >> \{\text{LIST}\}_\text{PRINT.lst}
else

if (${N_TERM} == 147) then
$\{\text{ECHO}\} *\text{Phe}\{\text{C_TERM}\} \{\text{MOL_WT}\} * \{\text{LIST}\}_\text{PEPS.nl}
$\{\text{ECHO}\} \{\text{BUTYL_SPEC}\}\{\text{PENTYL_SPEC}\} >> \{\text{LIST}\}_\text{PRINT.lst}
else

if (${N_TERM} == 97) then
$\{\text{ECHO}\} *\text{Pro}\{\text{C_TERM}\} \{\text{MOL_WT}\} * \{\text{LIST}\}_\text{PEPS.nl}
$\{\text{ECHO}\} \{\text{BUTYL_SPEC}\}\{\text{PENTYL_SPEC}\} >> \{\text{LIST}\}_\text{PRINT.lst}
else

if (${N_TERM} == 87) then
$\{\text{ECHO}\} *\text{Ser}\{\text{C_TERM}\} \{\text{MOL_WT}\} * \{\text{LIST}\}_\text{PEPS.nl}
$\{\text{ECHO}\} \{\text{BUTYL_SPEC}\}\{\text{PENTYL_SPEC}\} >> \{\text{LIST}\}_\text{PRINT.lst}
else

if (${N_TERM} == 101) then
$\{\text{ECHO}\} *\text{Thr}\{\text{C_TERM}\} \{\text{MOL_WT}\} * \{\text{LIST}\}_\text{PEPS.nl}
$\{\text{ECHO}\} \{\text{BUTYL_SPEC}\}\{\text{PENTYL_SPEC}\} >> \{\text{LIST}\}_\text{PRINT.lst}
else

if (${N_TERM} == 186) then
$\{\text{ECHO}\} *\text{Trp}\{\text{C_TERM}\} \{\text{MOL_WT}\} * \{\text{LIST}\}_\text{PEPS.nl}
$\{\text{ECHO}\} \{\text{BUTYL_SPEC}\}\{\text{PENTYL_SPEC}\} >> \{\text{LIST}\}_\text{PRINT.lst}
else

if (${N_TERM} == 163) then
$\{\text{ECHO}\} *\text{Ty}\{\text{C_TERM}\} \{\text{MOL_WT}\} * \{\text{LIST}\}_\text{PEPS.nl}
$\{\text{ECHO}\} \{\text{BUTYL_SPEC}\}\{\text{PENTYL_SPEC}\} >> \{\text{LIST}\}_\text{PRINT.lst}
else

if (${N_TERM} == 99) then

205
${ECHO} "Val\{C_TERM\} \{MOL_WT\}" \n${ECHO} \$\{BUTYL\}_SPEC$\{PENTYL\}_SPEC >> \$\{LIST\}_PRINT.lst
\nendif

${ECHO} "???\{C_TERM\} \{MOL_WT\}" \nend

#--------------------------------------------------------
# SET UP LIST OF AMINO ACIDS
# FOR N_TERMINAL ACIDS
#--------------------------------------------------------

foreach ATEM (\'cat ASAME.sl\')

  \$ N_TERM = (\$ATEM - \$MASS)
  \$ MOL_WT = (\$ATEM - 1)
endif

#--------------------------------------------------------
# SET UP LIST OF AMINO ACIDS
#--------------------------------------------------------

if (\$MASS == 90) then
  set C_TERM = Ala
else
  if (\$MASS == 175) then
    set C_TERM = Arg
else
  if (\$MASS == 133) then
    set C_TERM = Asn
else
  if (\$MASS == 134) then
    set C_TERM = Asp
else
  if (\$MASS == 179) then
    set C_TERM = Cys
else
  if (\$MASS == 148) then
    set C_TERM = Glu
else
  if (\$MASS == 147) then
    set C_TERM = Gln
else
  if (\$MASS == 76) then
    set C_TERM = Gly
else

206
if ($\text{MASS} == 156)$ then
set $\text{C TERM} = \text{His}$
else
if ($\text{MASS} == 132)$ then
set $\text{C TERM} = \text{Lxx}$
else
if ($\text{MASS} == 150)$ then
set $\text{C TERM} = \text{Met}$
else
if ($\text{MASS} == 166)$ then
set $\text{C TERM} = \text{Phe}$
else
if ($\text{MASS} == 116)$ then
set $\text{C TERM} = \text{Pro}$
else
if ($\text{MASS} == 106)$ then
set $\text{C TERM} = \text{Ser}$
else
if ($\text{MASS} == 120)$ then
set $\text{C TERM} = \text{Thr}$
else
if ($\text{MASS} == 205)$ then
set $\text{C TERM} = \text{Trp}$
else
if ($\text{MASS} == 182)$ then
set $\text{C TERM} = \text{Tyr}$
else
if ($\text{MASS} == 118)$ then
set $\text{C TERM} = \text{Val}$
endif
endif
endif
endif
endif
endif
if ($\text{MASS} == 148)$ then
@ $\text{BUTYL SPEC} = (\text{MOL WT}) + 169$
else
if ($\text{MASS} == 134)$ then
@ $\text{BUTYL SPEC} = (\text{MOL WT}) + 169$
else
if ($\text{MASS} == 175)$ then
@ $\text{BUTYL SPEC} = (\text{MOL WT}) + 71$
else
@ $\text{BUTYL SPEC} = (\text{MOL WT}) + 113$
endif
else
if ($\text{MASS} == 148)$ then
@ $\text{PENTYL SPEC} = (\text{MOL WT}) + 211$
else
if ($\text{MASS} == 134)$ then

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@ PENTYL_SPEC = ${MOL_WT} + 211
else
  if (${MASS} == 175) then
    @ PENTYL_SPEC = ${MOL_WT} + 99
  else
    @ PENTYL_SPEC = ${MOL_WT} + 141
  end if
endif

if (${N_TERM} == 115) then
  ${ECHO} "Asp$(C_TERM) ${MOL_WT}" >> ${LIST}_PEPS.nl
  ${ECHO} $BUTYL_SPEC$PENTYL_SPEC >> ${LIST}_PRINT.lst
else
  if (${N_TERM} == 129) then
    ${ECHO} "Glu$(C_TERM) ${MOL_WT}" >> ${LIST}_PEPS.nl
    ${ECHO} $BUTYL_SPEC$PENTYL_SPEC >> ${LIST}_PRINT.lst
  else
    ${ECHO} "???$(C_TERM) ${MOL_WT}" >> ${LIST}_UNPEPS.nl
  endif
endif

rm -f BUT.sl
rm -f BUT_56.sl
rm -f BUT_112.sl
rm -f PENT.sl
rm -f PENT_70.sl
rm -f PENT_140.sl
rm -f SAME.sl
rm -f ASAME.sl
end

# print_spec.dsp $1 $2
exit(0)