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Determination of Isocyanates on Industrial Sampling Filters by MALDI-MS and Associated Studies

By

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For my mum, I could not have done it without her.

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Abstract

The primary aim of the work presented in this thesis was to develop methodologies for the characterisation of isocyanate compound fragments by LC/MS/MS and MALDI/MS analysis of isocyanates (NCO) on workplace sampling devices. The secondary aim was to perform related studies of veterinary medicine and pesticide compounds for possible quantitation from crop samples.

NCO are a group of highly reactive compounds widely used in the manufacture of polyurethane products such as flexible and rigid foams, fibres, elastomers and coatings such as paints and varnishes. NCO are known respiratory sensitisers and are the most common cause of occupationally induced asthma, bronchitis and emphysema. The determination of NCO functionality and any contributions to understanding more about NCO chemistry is of significant value to occupational hygiene bodies globally.

Here we demonstrate the application of MALDI-TOF-MS and tandem MS to the analysis of derivatised isocyanate monomers and prepolymers. NCO as 1-(2-methoxy) piperazine (MP) derivatives are analysed using MALDI-TOF-MS. The work was carried out to gauge the selectivity obtainable from the analysis of NCO mixtures without prior separation. Derivatised monomers and prepolymer mixtures are analysed and the potential of MALDI-TOF-MS for an NCO monitoring program is assessed. The results obtained show the possibility for mixture separation by this method, enabling the resolution of monomeric and prepolymeric species in spectra. Tandem MS is used for primary elucidation of fragment structures in the prepolymer samples and to designate proposed structures for unknown molecular parts.

Quantitation of MDI-MP was performed on spot samples, deuterated MDI-MP (d6 MDI-MP) and MDI derivatized with the novel derivatizing reagent 1-(2-ethoxyphenyl) piperazine (EP) to give MDI-EP were used as internal standards. Quantitation of MDI-MP on filters was also performed using a direct filter calibration with d6 MDI-MP as the internal standard.

Veterinary medicines are widely used to treat farm animals whether as a preventative measure (prophylactic use) or on a remedy basis. Treatment includes curing disease, improving feed conversion and promoting growth. A mixture of drug and its subsequent metabolites is excreted from the animal and the manure slurry is used as a fertiliser to treat crops destined for human consumption. The leaching of these substances into water/soil systems and consequently the human food chain is therefore of great concern.

The work presented shows the preliminary analysis of a range of compounds to attempt quantitation and determine the content from a number of crop samples. Ionic Liquid Matrices are employed to reduce background noise and improve the appearance of sample spectra.

Contents

Chapter 1

Introduction

1.1 Occupational Hygiene

Occupational hygiene is concerned with the identification, control and ultimate prevention of hazards that can generate damage to health or to the environment [1]. These problems arise from work activities and can take many forms [2], although the protection of workers and the environment, as well as promotion of good environmental health are paramount [3]. The penalties for accidents and occurrence of occupational illness in the workplace are not only financial, but can also damage company reputation leading to loss of custom [4]. The maintenance of a safe working environment by any employer is of utmost importance especially when there are known dangers apparent, such as chemical reagents. Most occupational illnesses caused by chemicals are progressive, with gradual degeneration of health, meaning the realisation of illness is usually when the damage has already been done.

Occupational hygiene encompasses a wide range of compounds and practices. Obvious offending materials are known solvents, poisons, chemicals and substances such as acids/alkalis in high concentrations. Biological hazards such as microorganisms and disease can be of significant risk in work environments and can lead to occupational illness. Airborne industrial toxic gases (such as carbon monoxide), fibres (asbestos for example), dust, aerosols, volatile solvents and vapours must also be considered. These substances may be present on plant as a raw material, could be a product of industry or even a by-product from the processes being carried out. Another factor that is governed by occupational hygiene law is environmental pollution. The responsibility of employers to maintain a safe working environment for their staff also extends to the outside world, the general public and any person that could be affected by poor conditions, such as delivery people or contract staff. Occupational deafness and blindness can be induced by unsatisfactory working conditions, and should also be considered when safeguarding the workplace. Risks from improper emergency procedures and practices (such as steps to be taken in the case of a fire) and furthermore all general areas of safety are of significant importance. The regulation and testing of electrical items (Electricity at Work Regulations, 1989) and machinery (Provision and use of Work Equipment Act, Revised 1998) in the work place aim to eliminate dangers that

could result in injury, illness and even fatality in extreme cases. The use of Personal Protective Equipment (PPE), when working with chemicals and biological tissues is a necessity, the responsibility lying with workers. According to Personal Protective Equipment at Work Regulations, 1992, employers have a duty to provide the relevant PPE of the correct type and quality for the job. Employees must wear the stated PPE and it is their duty to report any loss or defect in this equipment.

1.1.1 *Exposure*

Exposure routes for occupational hazards are just as varied as the substances themselves. The main routes for exposure to chemical or biological agents are dermal contact, respiratory inhalation and oral ingestion. The potential irritation arising from dermal contact will appear in a form depending on the substance and situation causing it. Solvents for example can degrease natural oils from the skin whereas acids/alkalis and chemical or biological compounds will denature dermal proteins [5]. Respiratory inhalation is a rapid and dangerously direct route for substances to enter the tract, lungs and consequently the circulatory system. The absorption rate of the toxic substance is swift due to the large surface area of pulmonary alveoli and the abundance of capillaries present, the very same efficient mechanism which enables life [6]. Oral ingestion is equally as dangerous, but is much less common due to accidental ingestion being difficult. These kinds of contact can lead to metabolic changes in the body from absorption, or production of localised toxic effects, such as induced eczema and dermatitis. Factors determining entry into the body include concentration of the substance, duration of exposure, solubility, reactivity in blood and human tissue and the dynamics of the substance upon entry into the body [7].

1.1.2 *Legislation*

Legislation and regulations have been apparent in industry since the mid twentieth century, especially in the mining and factory areas. However, the first legislation to encompass all industries was the Health and Safety at Work Act, 1974 (HASWA) [8].

HASWA is an enabling act, not giving instruction, just listing the duties of employer and employee in the maintenance of a safe working environment. This act allows for regulations to be made and updated as it becomes necessary, to avoid the occurrence of health and safety incidents. HASWA governs not only the safety of employees in a work task but also the safety of the premises and the environment. Control measures are placed on emissions, both chemical and noise, and the storage or disposal of waste products. The main act is sub-divided into regulations, which are more specific and give fine detail of processes or practice. One regulation which is part of HASWA is the Reporting of Industrial Diseases and Dangerous Occurrences Regulations, 1995 (RIDDOR) [9].

Every company in industry must possess an accident book of approved design for recording incidents. This book must be accessible to all employees, and must contain a record of all incidents, even if they seem minor or insignificant at the time. Incidents, however trivial, should be thoroughly investigated by the company to enable the prevention of similar occurrences in the future. Under the RIDDOR stipulations, certain major injuries and incidents must be reported to the relevant local authority or Health and Safety Executive (HSE) immediately. Some of these injuries and incidents are listed below;

- Fatalities
- Fractures
- Loss of sight of an employee
- The collapse of a wall on premises
- Certain workplace diseases
- Explosion of gas storage containers
- Any dangerous occurrence which renders a work area out of action for more than 24 hours
- Any accident/injury that hospitalises or stops a workforce member, or a member of the public working

Operations involving chemical substances are governed by health and safety regulations in the form of Control of Substances Hazardous to Health, 1988 (COSHH, revised 1999) [10]. The underlying principles of the COSHH regulations are the performance of risk assessments before a substance is

used, the correct safety precautions for a substance to be implemented, and ultimately to prevent incident when using potentially dangerous substances. A hazardous substance is defined by COSHH regulations as one which falls into any of the following categories;

- Substances classified as toxic, harmful, corrosive, irritant, flammable, explosive or radioactive
- Substances that have Maximum Exposure Limits (MEL) or Occupational Exposure Standards (OES)
- Substances that cause cancer (carcinogenic), mutations (mutagenic) or birth defects (teratogenic)
- Micro-organisms that can transmit disease or create any hazard to a worker
- Any dust in substantial concentration (>10 mg/m^3 8-hour TWA of total inhalable dust and >4 mg/m^3 similarly of respirable dust)
- Substances that present comparable hazards to any of the above

The formulation of a hazardous substance has to be taken into account prior to classification. For instance, a substance which is relatively non-hazardous as a lump may be extremely hazardous when finely powdered due to the greatly increased risk of inhalation.

The HSE have constructed theoretical limits for the safe use of hazardous substances, called Occupational Exposure Limits (OEL). OELs are standards that determine the amount of a substance allowable in the work place air [11]. This category is divided into two, depending on the substance in question, the extent of its use and the formulation employed. Occupational Exposure Standards (OES, legally binding standards) and Maximum Exposure Limits (MEL, set for substances which may cause the most harm) are enforced for all substances that could pose potential hazard. These standards are measured as a concentration of hazardous substance in air over a specified time period. Common measurements are ppm and $\mu g/m^3$ as time-weighted averages (TWA). The duration of sampling for the TWA value also depends on the substance in question, but 8-hours (average calculated shift length) and 15-minutes (Short-Term Exposure Level, STEL) are frequently used.

The methods used for monitoring procedures depend entirely on the applications of the substances involved. Industrial sampling is performed to enable the measurement of toxic substances when compared with a pre-developed standard scale. High performance liquid chromatography (HPLC) has been the favoured method for analysis of collected samples, but advances in technology and instrumentation mean that there is now a wide range of methods for use. The majority of monitoring analysis is still performed by HPLC, the detector being decided from the sample information required. Simple quantitation can be performed using an ultra-violet (UV) detector and by employing an internal standard. Structural or degradational information can be provided by standard or tandem mass spectrometry (MS).

1.2.1 *Swab Sampling*

This technique is used to determine amounts of substance in the workplace that are present due to either the result of spillage, or drift from formulations such as dry powders. The areas tested can be workstations, floors and other surfaces, both vertical and horizontal. The distance of swab site from the initial point of possible contamination is extremely important as it demonstrates the effectiveness of safety procedures for containing dangerous substances.

Generally, a sterile section of cloth or cotton, which can either be moistened with solvent or dry (depending on the substance of interest), is wiped in a snake-like pattern over the chosen surface. This is usually performed through a template of specified size, to enable the calculation of substance amount per unit area. This cloth is left to extract in a solvent system comparable with that of the chosen analysis technique, which is usually HPLC. The comparison with maximum safe working levels determined by governing bodies can indicate problems with exposure and identify risks that an employer may be taking.

1.2.2 *Personal Dust Sampling*

To establish the amount of potentially harmful substance a worker is exposed to, their personal workspace can be monitored using a 'personal dust sampler' containing an air-sampling filter. An expanded view of a sampler of this type is shown in Figure 1.1.

The filter cassette is attached to the inlet nozzle of a small pump and once sampling has commenced, the pump will be worn for a set amount of time (TWA value obtained). The personal sampler is usually worn on the outside of the worker's clothing at a set distance from the mouth, and hence the respiratory inlet. The glass fibre filter is normally moistened with solvent, depending on the reactivity of the substance being analysed, coated with a derivatising reagent. This provides a stable sample filter that can be transported to the laboratory for analysis. Generally, the filter content will be extracted before HPLC analysis is performed.

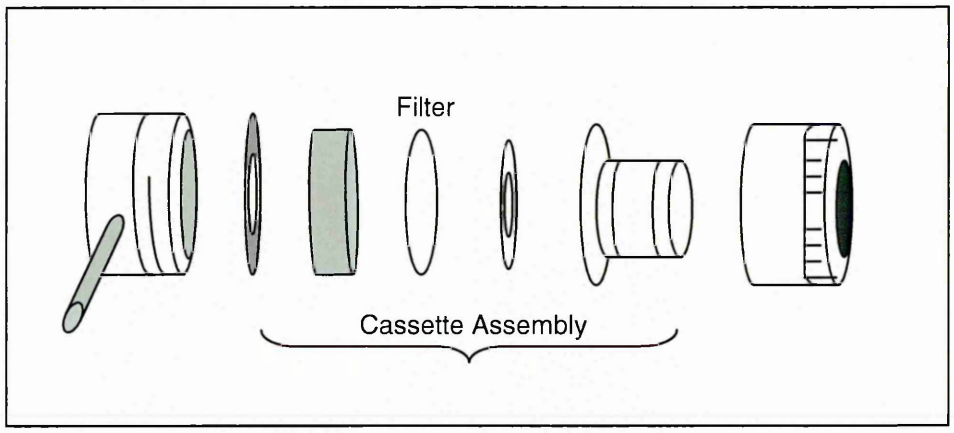


Figure 1.1 Expanded view of a workplace monitoring personal dust sampler [12]

1.2.3 *Impinger System Monitoring*

Sometimes referred to as bubbler systems, these employ the same principles as a filter sampler but do not contain a filter. Usually, a small volume of solvent/derivatising reagent solution is placed in the bottom of a sampling jar and this is attached to a pump, drawing air in from the surrounding environment [13]. As the air 'bubbles' through the solution for a set amount of time, the

substance of interest is derivatised to give a relatively stable sample liquor. This sample can be analysed in the laboratory without the need for an intermediate extraction step.

1.2.4 *Environmental Sample Monitoring*

When collecting samples in the open, it is impossible to use swabbing, impinger or filter systems. The way to enable external sample analysis is by collecting samples that could be contaminated with the substance of interest. The major examples of environmental samples are as follows;

- Vegetation – This could be in the form of crops, grasses, weeds or plants surrounding the area of interest. These can then be treated and prepared for analysis in the laboratory
- Soil samples – Which can be 'washed' to extract possible toxic substances
- Water samples – From aquatic systems such as rivers and streams. Pre-concentration stages are performed prior to analysis

1.3 Analysis Techniques used in this Study

1.3.1 *Mass Spectrometric Principles*

Mass spectrometry has been used in a wide range of applications for many years now, including genomics, proteomics, small molecule analysis, archaeology, environmental evaluation and engineering. MS is a powerful analytical tool that can provide quantitative as well as qualitative information on a varying range of chemical and biological compounds. Molecular weight, structural and compositional information can be elucidated for the characterisation of samples. Gaseous phase ions (either positive or negative, depending on the mode of operation) are separated according to their mass-to-charge ratios (m/z), thus enabling detection and assessment of decomposition and fragment patterns [14].

The basic components of a mass spectrometer are shown in Figure 1.2. Instrumentation comprises of a sample inlet, ionisation source, mass analyser and detector.

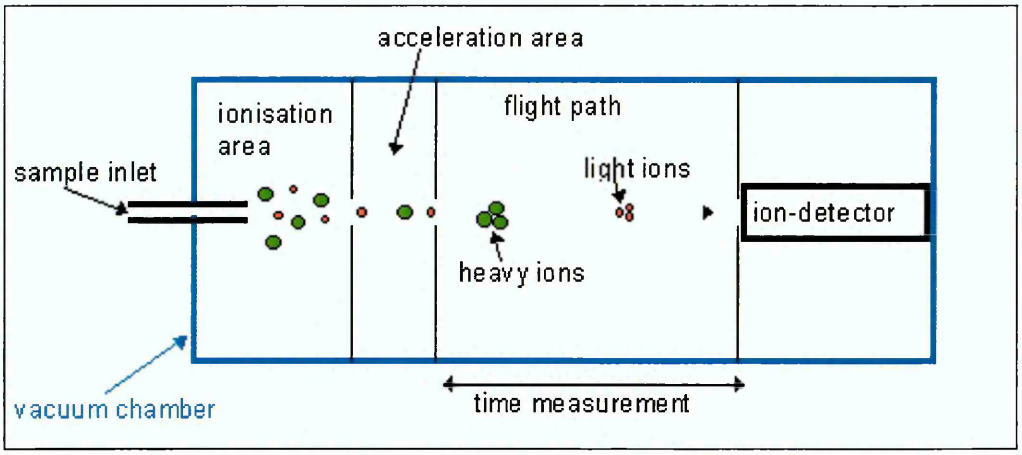


Figure 1.2 Schematic diagram of a simple time of flight mass spectrometer [15]

1.3.1.1 Sample Introduction

The introduction of samples into the mass spectrometer is by inlet interface. Vaporisation and ionisation ensue by thermal or high-energy desorption procedures.

The growing area of 'hyphenated techniques' such as LC/GC-MS and the improvement of instrumentation with new methodology have proved invaluable for sample analysis and have also provided a convenient method of sample introduction. Capillary infusion introduces small quantities of sample into the mass spectrometer without compromising vacuum strength. LC and GC can be used in this manner to perform sample separation into different components, prior to introduction. Negligible sample concentration and relatively high flow rates have previously caused problems with LC-MS interfacing, but the development of new ionisation techniques (electrospray for example) has meant analysis by this method is performed more frequently.

1.3.1.2 Ionisation Sources

Ionisation is the process of changing a neutral molecule into a charged particle. Ionisation techniques are classed as 'hard' or 'soft'. Hard techniques, such as electron ionisation (EI) involve high-energy, gas phase interaction and the formation of ions by bond dissociation. Often, a pre-separation step is necessary with this type of ionisation to produce useful spectral data. GC is usually the preferred method due to the fact that both separation and ionisation are performed in the gaseous phase. LC produces phase incompatibility but is more suitable for larger molecules, especially polymers. The high vacuum can be sustained by the use of atmospheric pressure techniques, facilitating LC coupling. Soft ionisation techniques, for instance MALDI, produce deprotonated or protonated molecules, depending on the mode of operation, with relatively no bond dissociation.

Atmospheric Pressure Ionisation (API)

API is a well established method of analysis that alleviates the need for high vacuum, whilst suitably linking liquid flow to the mass spectrometer [16]. There are two main API techniques, APCI and ESI.

Atmospheric Pressure Chemical Ionisation (APCI)

The APCI source contains a thermal vapourising probe which facilitates desolvation/vapourisation of liquid molecules and carries them through an ionic/molecular reaction region maintained at atmospheric pressure. Ions are produced from solvent vapour by means of a corona discharge. This technique can perform in both negative and positive ion modes and forms resultant deprotonated or protonated molecules, respectively. APCI is a relatively soft ionisation method, creating intact molecules with minimal fragmentation [17].

Electrospray Ionisation (ESI)

This method generates gaseous ions, at atmospheric pressure, directly from the condensed phase (usually aqueous or mixed organic system) rendering ESI-MS

ideal as a detection system for HPLC. An electric field is used to produce a fine spray of electrically charged droplets, present as the phenomenon known as a Taylor cone [18]. Ions are electrostatically directed into the mass analyser, before vapourisation occurs. Usually singly or doubly charged ions are produced which create a spectrum containing multiple peaks corresponding to the different charged-states of the same molecule.

Matrix Assisted Laser Desorption / Ionisation (MALDI)

MALDI-MS is a soft ionisation technique that uses a pulsed laser to generate gaseous ions. This method was introduced for the ionisation of large bio-molecules by Tanaka et al. using an inorganic matrix [19]. Previously, laser desorption/ionisation was used (LDI), which relied on the dissociation of thermally labile bonds. The method was further developed by Hillenkamp and Karas in 1988 with the use of organic acid matrices for involatile compounds [20, 21]. The use of a matrix extended the m/z range of mass spectral instrumentation by providing a source of gas-phase protons for ionisation in the positive ion mode. The matrix acts as a shield around the sample molecules, preventing direct exposure of the analyte to incident energy from the laser. The analyte can become protonated (+H), deprotonated (-H), or undergo catonisation with alkali metals to form salts. Adduct formation results from the addition of naturally abundant metals such as sodium and potassium to give the neutral molecule an overall positive charge. This is sometimes the preferred pathway for ionisation [22, 23]. The main factor in successful MALDI ionisation is the co-crystallisation properties of matrix and analyte. The chosen matrix should be suitable for use with both the system laser and the analyte in question. The matrix crystals absorb energy from laser irradiation (at the wavelength of the laser) and desorption causes the surface to disintegrate into the gaseous phase. During rapid evaporation of the matrix, electron and proton transfers impart energy to the analyte molecules which are also excited into the gaseous phase. The resultant mixture is composed of protonated/deprotonated molecules and adduct ions of matrix and analyte.

MALDI-MS on simple instruments can give poor mass spectral resolution, due to the following problems;

- There is an energy spread of the ions due to differences in kinetic energies (KE)
- Metastable ions / decomposition. Ions leave the source as a single ion, but can break up during flight without external influence to yield a fragment ion plus a neutral molecule. This is Post Source Decay (PSD)
- Matrix adduct ions occur

Common lasers for MALDI use are UV; nitrogen, λ = 337 nm and infra-red (IR); CO_2, λ = 10.6 μm and Nd:YAG (Neodymium:Yttrium Aluminum Garnet), λ = 1064 nm (although the Nd:YAG laser can operate at different wavelengths, eg. frequency doubling produces green light at 532 nm, half the wavelength of the primary light) [24]. For MALDI purposes, the frequency tripled Nd:YAG laser is employed. Nitrogen lasers are most frequently used due to their relatively low cost and good reliability.

Other Ionisation Sources

There are various other ionisation sources frequently used for mass spectrometric analysis [25];

- Electron Ionisation (EI)

  Involves high-energy, gas phase interaction and the formation of ions by bond dissociation, suitable for low molecular weight compounds. The gaseous phase sample passes into an electron ionisation region where it interacts with an electron beam. Electron ejection and fragmentation occur.
- Chemical Ionisation (CI)

  Similar to EI, this technique uses ion-molecule reactions in the gas phase to produce ions from the sample molecule. A reagent gas, such as methane, isobutane, or ammonia initiates the ionisation process. This reagent gas is primarily ionised by electron ionisation.
- Fast Atom Bombardment (FAB)

  The FAB ionisation technique is a soft ionisation method like MALDI, which employs a non-volatile liquid material as the matrix and a high

energy beam to sputter the sample and matrix from a probe surface [26]. The matrix is usually m-nitrobenzyl alcohol and the beam made up of xenon or caesium atoms. The matrix performs a similar function as in MALDI by absorbing particle beam energy, therefore minimizing sample damage and facilitating ionisation.

- Inductively Coupled Plasma (ICP)
  ICP-MS is predominantly used for elemental analysis, but has strong advantages over some atmospheric pressure techniques in other areas too. The plasma (gaseous ions) is produced by inductively coupling high-frequency energy to an argon gas flow. Analyte solution is injected into the centre of the plasma as an aerosol, where vapourisation and ionisation occur.

1.3.1.3 Mass Analysers

Ions are separated and sorted in the analyser according to m/z ratio. This can occur either by electric/magnetic field deflection or by the measurement of the time that an ion takes to travel a fixed distance [27].

Quadrupole Mass Filter

This analyser comprises of four precisely positioned parallel rods (elliptical, circular or hyperbolic cross section) with an applied direct current voltage (DC) and a superimposed, alternating radio frequency potential (RF). Mass ranges can be selected for analysis by varying the RF field. The applied potential is equal on opposite rods, whereas adjacent rods have contrary polarity.

Quadrupole Ion Trap

The same principles are employed as with a regular quadrupole mass filter. An ion trap enables isolation of one or more selected ions by ejecting unimportant species from the trap. The trap is actually an enclosed radio frequency quadrupole field. This analyser is most often used with an electrospray ionisation source which generates the ions that are consequently injected into

the trap. Collision-induced dissociation can follow ion trapping in the same analyser to generate fragment structures.

Fourier Transform (FT)

FT-MS is a high resolution, tandem MS-capable analyser, first introduced in 1974 by Comisarow and Marshall [28]. This technique, which is also commonly referred to as FTICR (ion cyclotron resonance), uses the principle of a charged particle orbiting in the presence of a magnetic field. RF is utilised for excitation purposes producing a detectable ion current, on a time dependent scale. Fourier transformation translates this data into a more useful frequency read-out for the multiply analysed ions, related to their m/z ratios.

Magnetic Sectors

This mass analyser employs a magnetic field for the separation of ions according to their mass-to-charge ratio. Acceleration occurs by use of an electric field, prior to the charged particle entering the magnetic sector. The particle will travel in a spiral pattern through the sector with a radius dependent on the m/z and speed of the ion. Detection points are theoretically constructed along the sector according to curvature radius, so only ions with the correct m/z will be detected. A disadvantage of this technique is relatively low resolution compared with other mass analysers. In an effort to combat this complexity, double-sector instruments have been developed to replace single-sector magnetic analysers. The magnetic sector is complemented with an electrostatic analyser, which acts as a kinetic energy (KE) filter for ions with uniform energy. Linkage of magnetic sector devices with FAB and EI sources is frequent.

Time of Flight (TOF)

This method was first introduced as a mass analysis technique for spectrometry purposes in 1955 [29]. Basically, ions produced at the source are separated by m/z ratio. This process takes place in a flight tube, where the flight times of the various ions are measured and a mass-to-charge scale is derived. Spectra are

collected and summed per laser shot, therefore TOF requires a very fast data acquisition system.

Linear TOF instruments are the simplest form of time of flight analysers. An applied voltage (electric field) accelerates ions before they are directed toward the flight tube to give all ions the same kinetic energy. The ions are separated based upon their mass-to-charge ratios (m/z), consequently giving the ions different flight times. The larger the ion, the slower it will travel along the flight path and will therefore be detected after smaller, faster ions. Ions of diverse masses will exhibit different yet constant velocities. The mass range of these linear TOF instruments is theoretically unlimited, but kinetic energy distribution caused by metastable ions can give poor resolution at high masses.

The following equations show how the ions are separated based on linear flight times and the relationship to the m/z ratio. Equation 1.1 explains the relationship between KE of an ion in a constant electrical field and its velocity.

$$KE = \frac{1}{2}mv^2 = zeV \qquad \text{..Equation 1.1}$$

Where m = mass of the ion, v = velocity of the ion, z = Number of charges, e = unit of electric charge (1.6×10^{-19} C) and V = applied acceleration voltage. Combination of the two equations produces Equation 1.2.

$$\frac{mv^2}{2} = zeV \qquad \text{..Equation 1.2}$$

The time (t) it takes the ion to travel down the flight tube can be calculated by Equation 1.3.

$$t = \frac{l}{v} \qquad \text{..Equation 1.3}$$

Where l = length of the flight tube and v = velocity of the ion

Combination of equations 1.2 and 1.3 explains the relationship between time of flight and the mass of an ion, Equation 1.4.

$$t^2 = \frac{m}{z}\left(\frac{l^2}{2Ve}\right) \qquad \text{..Equation 1.4}$$

Linear TOF analysers can make use of delayed extraction to improve mass resolution. In this technique, the high-voltage field is switched on several nano/micro seconds after the laser hits the target instead of simultaneously. This reduces the impact of the initial analyte velocity distribution on detected peak width. If the voltage is turned on simultaneously with sample bombardment, then the fastest initial ions will be nearer to the grid and will therefore be accelerated further, making velocity separation greater. During delay time, ions move to new positions as a function of their initial velocities. If the voltage is turned on after a short period of time, the fastest initial ions will have already passed the grid and the slower ions will be closer, thus being accelerated to draw alongside the faster ions in the flight tube [30].

The Reflectron TOF analyser is more expensive than the linear instrument and can give more complex spectra with extra peaks. The reflectron analyser is basically the same as the linear instrument, but it uses an ion mirror at the end of the flight path that reflects ions back into the tube. This enables focusing of different mass and velocity ions. The reflectron TOF analyser can separate metastable ions, which accounts for the complicated spectra obtained.
See Figure 1.3.

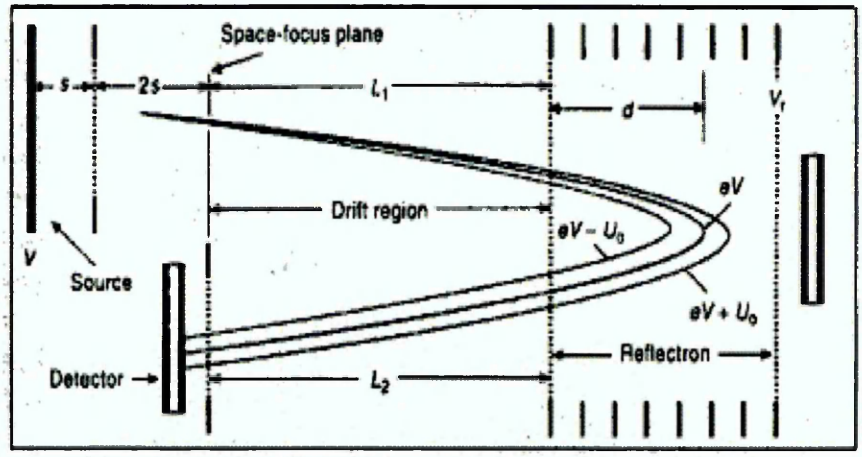


Figure 1.3 Diagrammatic Representation of a reflectron TOF [31]

Differences in energy of similarly charged ions produce different times of flight and therefore broader peaks with lower mass accuracy. The reflectron compensates for this effect by allowing ions with slightly faster velocities to penetrate deeper into the ion mirror, thus delaying them fractionally. When they exit the mirror, the ions of larger mass that were traveling slower, have reached the delayed, faster ones. This gives better resolution and mass accuracy.

Tandem Mass Spectrometry

Due to the lack of degradation with most ionisation techniques in frequent use (not including EI), this method induces fragmentation and is used to obtain more structural information about target molecules and samples. Fragments from a selected ion are generated by collision, and mass analysis is performed on the resulting fragments. Ionic/molecular induced collision is referred to as collision induced dissociation (CID). CID is primarily achieved by selecting an ion of interest with a mass analyser and then launching that ion into a gas-containing collision cell (most frequently helium or argon). The subsequent products can be analysed to obtain a product ion spectrum. A single analysis run will give the constituent products of the test analyte. Further tandem analysis will break down each product into smaller identifiable components, and so on. These spectra can be 'reconstructed' to provide a characteristic molecular fingerprint of the starting compound. Tandem mass spectrometry is sometimes referred to as MS^2. MS analysis of initial ions is followed by MS^2, analysis of first

generation products. Ensuing generations of product ions can be explained by MS^3, MS^4 etc.

There are four main types of tandem mass spectral analysis;

- Product Ion Scans (Daughter Ion): This experiment can be used to determine the product ions which arise from a selected precursor ion
- Precursor Ion Scans (Parent Ion): This type of scan can be used to establish the precursor ions which give rise to a selected product ion
- Constant Neutral Loss Scans: Analysis by this method can show which ions undergo the loss of a given neutral product
- Multiple Reaction Monitoring: Specific product ions of pre-selected precursor ions are recorded i.e. the precursor ions must undergo a specific reaction or transformation to allow detection.

Figure 1.4 shows the different scan modes available for analysis by a triple quadrupole instrument. The second quadrupole has no direct current component and is operated in radio frequency mode only. This mode of operation allows maximum transmission of ions through the quadrupole into the second stage of analysis.

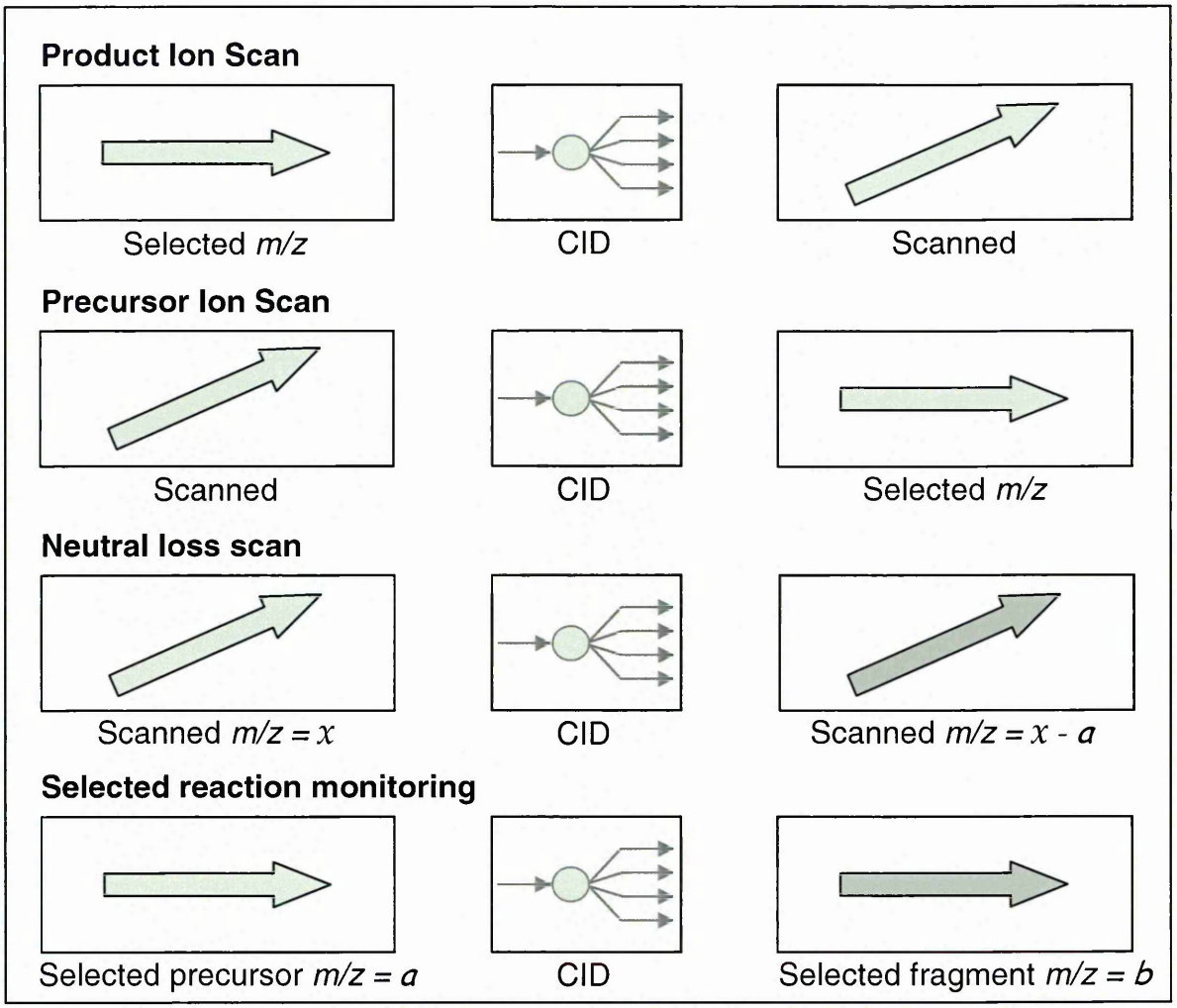


Figure 1.4 A schematic representation of the tandem MS modes of operation of a triple quadrupole mass spectrometer, adapted from the original [32]

1.3.1.4 Ion Detection

Detection allows collection and processing of amplified, secondary electron signals from the primary ion beam, or inducing a current generated by a moving charge. This allows transformation of information into a mass spectrum. Some common detectors are;

- Faraday Cup
- Multistage Electron Multiplier
- Micro-channel Plate
- Photomultiplier

A Faraday cup converts an ion striking a dynode surface (constructed of a secondary emitting material) into a flow of electrons, consequently a current, slightly amplifying the signal.

An electron multiplier is made from a series of dynodes maintained at increasing potentials, achieving much higher sensitivity than the single dynode of a Faraday cup. Ions strike the dynode surface producing a secondary emission. These electrons are drawn into the next dynode, and so on, creating a cascade effect. If one ion enters the electron multiplier, typically a one-million fold current will be produced.

The micro-channel plate detector is constructed from a large number of micro continuous dynode multipliers arranged in an array. Each plate is coated in a semi-conductive material and acts as a dynode. This detector allows simultaneous detection for ions of different mass and is therefore suitable for employment with time of flight mass analysers.

The photomultiplier conversion dynode detector is similar to an electron multiplier where the ions initially strike a dynode, resulting in the emission of secondary electrons. The photomultiplier contains a phosphorescent screen as opposed to a metal plate. The secondary signal is made up of positive and negative ions, electrons and neutral molecules. A cascade of electrons will finally result in a sufficiently amplified, detectable current.

1.3.2 *MALDI Principles*

Possibly the most important area of MALDI analysis is the successful co-crystallisation of matrix and analyte molecules. The co-crystallisation of sample and matrix when pipetted onto a spot target ("dried-droplet" method) can suffer from poor homogeneity of matrix/sample mix with differing particle sizes [33]. For analysis by MALDI-TOF-MS (imaging or thin layer chromatography (TLC) processes), the matrix material must be applied by a suitable method in order to obtain a matrix crystal layer with surface homogeneity sufficient to yield chromatographic data [34-36]. Uneven matrix coverage gives higher chances of

ionisation in heavily loaded areas. It is possible also that matrix surface heterogeneity can lead to differing times of flights for TOF mass analysis.

Air-spray and electrospray deposition techniques have been applied to MALDI slide preparation, to try and alleviate these problems. Fine matrix layer deposition is facilitated by using an air-gun or by means of a charged needle, ensuring a homogeneous layer forms on the target surface, with uniform crystal size. Both methods ensure that the matrix solution is dispersed and deposited as very fine droplets [37, 38].

The matrix is selected to ensure the peaks obtained are away from the sample mass range so as not to interfere. The matrix must be soluble in similar solvents to the sample and give reproducible signal results. The matrix also needs to have good vacuum stability, promote intense ionisation and have high molar absorptivity at the laser wavelength employed. Other important qualities that the matrix should possess are lack of reactivity toward the analyte, high lattice structure and high heat of sublimation. The basis of MALDI-MS is the study of ionised molecules and their fragments after co-crystallisation with a solid organic matrix. The analyte sample needs to be incorporated into the matrix crystal when micro-litre amounts of both are added onto a MALDI target [39]. The use of a matrix enables the analysis of samples with up to 500 kDa molecular weight at femtomole sensitivities, without sample decomposition. A simplified schematic diagram of the desorption process is shown in Figure 1.5.

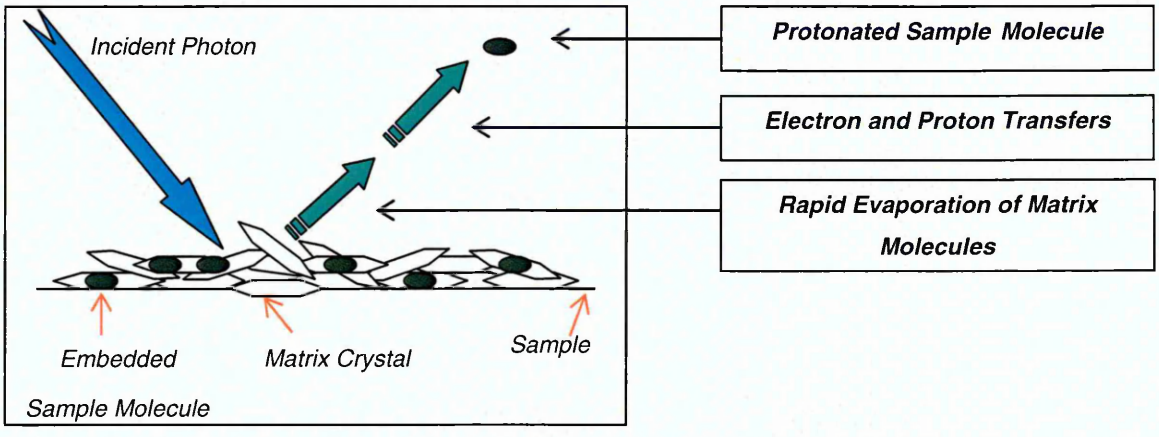


Figure 1.5 Schematic diagram of photon energy absorption [40]

1.3.2.1 Organic Acid Matrices

Some common MALDI matrices are shown in Figure 1.6.

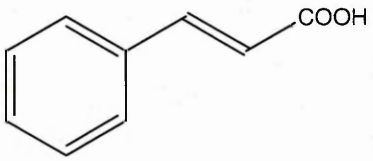
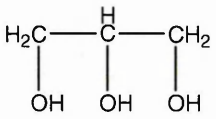
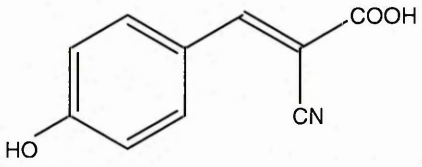
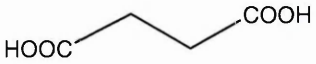
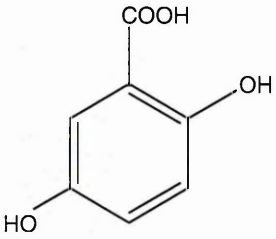
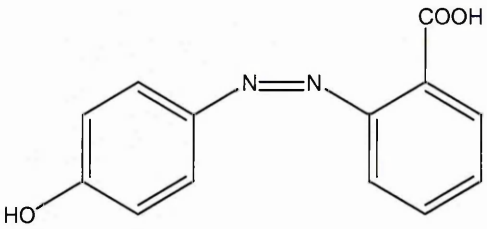
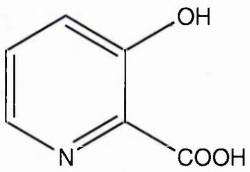
UV Matrices	IR Matrices
 Trans-Cinnamic Acid (CA)	 Glycerol
 α-Cyano-4-Hydroxycinnamic Acid (α-CHCA)	 Succinic Acid
 2,5-Dihydroxybenzoic Acid (DHB)	
 2-(4-Hydroxyphenylazo)benzoic Acid (HABA)	
 3-Hydroxypicolinic Acid (3-HPA)	

Figure 1.6 Common MALDI Matrices

1.3.2.2 Ionic Liquid Matrices

The recent development in MALDI sample preparation has brought about ionic liquid matrices (ILM) [41]. ILMs have excellent solubilising properties, possess a broad liquid temperature range and have high vacuum stability in comparison with standard liquid and solid matrices. These matrices provide mass spectra with greatly reduced background ions, yielding a single peak for matrix influence. There are many experimental uses for ILMs, but they have proved particularly successful in the analysis of low molecular weight compounds [42]. ILMs are often called room temperature ionic liquids (RTILs). For a fuller description of ILMs, see Chapter 4 of this thesis.

1.3.2.3 Sample Preparation

There are a number of sample preparation techniques used to homogeneously incorporate the analyte into the matrix for crystal layer formation [43].

Dried-Droplet

This is the original sample preparation technique, used by Hillenkamp and co-workers [44]. Aqueous matrix compound is mixed with the analyte solution and deposited onto the sample target. Sample surfaces obtained using this method tend to be heterogeneous in nature, with an uneven crystal layer, both in size and number. Analysis data proves this can lead to poor mass accuracy and spectral quality.

Fast Matrix Evaporation

This technique was introduced in order to combat problems associated with the dry-droplet method [45]. It involves application of the matrix in a highly volatile solvent, such as acetone, which when evaporated forms a thin layer of matrix on the target. The crystal layer is homogeneous with more uniform crystals, with analyte applied on top of the matrix layer.

Electrospray Deposition

This application method was introduced in the late 1990's as a suitable way to apply MALDI matrices onto analysis surfaces [46, 47]. A small amount of matrix solution is sprayed through a charged stainless steel or capillary needle onto a grounded sample plate (matrix/analyte mixture could also be sprayed). The applied voltage used is usually between 3 and 5 Kilo Volts. This method produces even coverage and with inclusion of an internal standard has been shown to be quantitative.

Air-spray Deposition

This method (also known as pneumatic spraying) has been introduced and has demonstrated reproducible results. Adapted from the electrospray method, this is simply the spraying of matrix, without an applied voltage. Air-spray has been used for a number of applications, but has proved especially successful in the coverage of membranes for imaging MALDI [48].

Other Sample Preparation Techniques

There are many other ways of introducing matrix onto the sample target, but these tend to be fairly crude methods, with little homogeneity in the resultant crystal layer. Pressing, brushing and crushed-crystal are the most basic methods.

Crushed-crystal is where matrix is initially applied to the sample probe and crushed, prior to addition of matrix/analyte mixture. This technique was developed to enhance crystal formation when working with high concentrations of involatile solvents.

Vacuum drying is based on the dried-droplet technique, although the crystal size is reduced and homogeneity improved by rapid drying of the matrix solution in a sample chamber.

The overlayer method combines crushed crystal and fast evaporation. Solvent evaporation forms the initial layer of small crystals, which is then followed by deposition of a mixture or matrix/analyte solution on top of the layer (as in the matrix/analyte deposition step of the crushed-crystal method).

The sandwich process is derived from the fast-evaporation method and the overlayer method. The analyte is not pre-mixed with matrix, but is applied on top of a fast-evaporated matrix-only bed. This is followed by the deposition of a second layer of matrix in a traditional (non-volatile) solvent, sandwiching the sample between two matrix layers.

Spin-coating was introduced in 1995 to evenly coat samples of large biomolecules [49]. It is performed by placing 3-10 μl of pre-mixed sample solution onto a 1" diameter target plate. The spin coater operated at ~ 300 rpm producing evenly spread crystal deposits in air. This method proved reproducible but is not suitable for all types of MALDI analysis, or MALDI sample targets.

The 'quick and dirty' matrix application technique separates matrix and analyte. In this method a drop of matrix solution is added on top of a drop of analyte solution. Both solutions are mixed thoroughly with the pipette tip before the mixture is dried under an air or nitrogen stream. This method has the same homogeneity and crystal size problems as the early methods, such as fast evaporation.

1.3.3 *Liquid Chromatography (LC)*

The use of LC for environmental sample analysis provides a separation technique that gives further information regarding the compounds of interest by resolving the individual species prior to detection.

1.3.3.1 High Performance Liquid Chromatography (HPLC)

HPLC is a well established separation technique that has been successfully linked to many detection systems for an extensive range of applications.

Analyte molecules are separated into individual components based on their interactions with two phases, a mobile liquid phase and a solid stationary phase. In liquid chromatography the stationary phase is packed into a column and the mobile phase is the eluent used. The relative affinities of the components present in the analyte mixture for either phase gives rise to different rates of migration through the column. Various different detectors can be used with LC applications, two of the most commonly used being the UV/Visible wavelength spectrophotometer and the mass spectrometer.

Modes of Liquid Chromatography

The mode of separation selected for chromatography relies upon optimal conditions for analyte separation. Separation through the chromatographic system is partitioned into two areas, isocratic and gradient elution.

Isocratic elution employs constant conditions for the separation of analyte components.

Gradient elution comprises of variations in mobile phase composition, temperature or flow rate. Varying the composition of the mobile phase involves altering the elution strength from low to high.

Partition Chromatography

The term partition refers to the retention mechanism employed for separation, normal or reverse phase. Depending on the properties of the analyte in question, the polarity of the mobile phase is varied.

Normal phase chromatography involves a polar stationary phase and a non-polar mobile phase, the opposite being applied for reverse phase partition. Normal phase depends on the polar groups of sample interacting with the polarity of the stationary phase. When operated in reverse mode, reliance lies with the hydrophobicity of the stationary phase and solute molecules.

Coupled or hyphenated techniques have revolutionised chemical analysis. Separation of the analyte through the LC column provides peak information for the components present. Mass spectrometry, combined with LC separation, provides spectral information about the molecular weights of singly/multiply charged molecules and structural information about the compound of interest. The signals produced are interpreted by a data station and presented in the form of a mass spectrum [50].

Nanospray Analysis is a method of transferring ions from liquid to gaseous phase at atmospheric pressure, rendering this technique ideal for coupling an LC system to a mass spectrometer. The analyte solution is passed through a nano-capillary needle, charged with a large electric field. Droplet dispersion occurs as the solution reaches the needle tip and the liquid flow is nebulised. Dole et al. (1969) first recorded nanospray application as a means of ion formation, for use with mass spectrometry [51, 52]. Major problems with the nanospray interface have been encountered with the fragility of the needle and the need for a uniform conical tip. Issues are also encountered with the maintenance of a continuous flow controlled by a steady electric field.

1.4 Compound Classes Studied

In statistics published by the Health and Safety Executive (HSE), isocyanates (NCO) are cited as the primary cause of occupationally induced asthma, bronchitis and emphysema in the UK. The Trades Union Congress (TUC), the organisation responsible for expressing the views and concerns of Britain's Working Unions, has predicted that occupational asthma could cost the UK economy over £2 billion over the next ten years [53]. The ever increasing need to perform analysis rapidly and more cost-effectively has meant that MALDI-MS has been considered as a screening technique.

Veterinary medicines are widely used to treat and protect the health of livestock and also as growth promoters. If animal slurry is used for crop fertilization then it

is deemed possible that veterinary compounds are introduced into soil, water systems and possibly the human food chain [54]. Pesticides are of extreme benefit to society and are used in a wide range of crop cultivation and storage processes, particularly to increase crop preservation times for export and shelf life. However, if content of these substances appeared in the human food chain then it would pose an extreme threat to health [55, 56].

1.4.1 *Isocyanate Induced Occupational Asthma*

Prolonged exposure to airborne monomeric NCO has been demonstrated as a cause of a range of respiratory disorders in both laboratory animals and humans [57, 58]. The relationship between NCO and respiratory sensitisation effects is very well established and NCO are cited as the most common cause of occupationally induced asthma, bronchitis and emphysema [59-63]. Repeated exposure to low concentrations of NCO leads to an immunological response characterised by progressive impairment of pulmonary function with symptoms such as wheezing, coughing, chest tightness, general breathing difficulties and increased stress on the heart. Additional symptoms include runny eyes and nose, or in severe cases life threatening anaphylactic shock or delayed pulmonary oedema, which can cause death [64, 65].

It has been observed that inhalation of aerosol formations of non-volatile NCO species can result in the same respiratory effects as inhalation of monomeric NCO vapours [66]. It has been shown that the effects of NCO sensitisation are not monomer-specific. An individual who is sensitised by exposure to one type of NCO will exhibit the symptoms of sensitisation in an atmosphere of a different NCO [67]. NCO prepolymers and monomers are commonly encountered in the same environments. For these reasons, the measurement of NCO compound levels in the working environment is a matter of major concern.

In a sensitised individual, exposure to NCO, even for a short period of time, will trigger an asthmatic response [68, 69]. Once sensitisation of the respiratory tract has occurred then the sufferer's asthma can be triggered by the far smaller concentrations of the chemical agent, rendering them unable to continue working with these compounds. Sensitisation may be the result of a single,

acute case of high exposure (eg. leak or spill) or prolonged exposure to low levels (eg. poor quality atmosphere in the workplace, i.e. a chronic toxic effect) [70-72]. The degree of injury as a result of NCO exposure is proportional to the type of compound (singular or diisocyanate compounds for example) and exposure level endured. Occupational asthma is a "disease characterised by variable airflow limitation and/or airway hyper-responsiveness due to causes and conditions attributable to a particular occupational environment and not to stimuli encountered outside the workplace" [73]. The asthmatic symptoms triggered by occupational influences do not necessarily appear immediately upon exposure to compounds, which can lead to problematic diagnosis.

Isocyanates are frequently used as industrial intermediates for products as they contain the highly reactive functional group, ~N=C=O. However, this readily conjugates with proteins to act as a respiratory sensitiser. The isocyanate is not antigenic by itself, but when it complexes with human proteins, the product formed is considered foreign to the body and an allergic reaction is initiated. There are two categories of occupational asthma in the workplace, which are;

- Work-Aggravated Asthma
- True Occupational Asthma

Work-Aggravated asthma is the worsening of pre-existing asthma caused by contact with an irritant. True occupational asthma is specifically caused by sensitisation to a particular chemical agent.

1.4.2 *Exposure Limits*

The HSE created a common maximum exposure limit system including all organic isocyanate species, based upon the number of reactive isocyanate ~N=C=0 groups per cubic unit metre of air. This was to overcome the problems associated with many different measurement units being used [74]. The hazard associated with the use of NCO depends not only on the toxicity of the NCO, but also on volatility and quantity used in a particular operation. 2,4- and 2,6-toluene diisocyanate (2,4- and 2,6-TDI) are much more volatile than 4,4-methylenebiphenyl diisocyanate (MDI) at room temperature and therefore

poses significantly increased hazards when compared with similar quantities of MDI.

The MEL has been set to;

- 20 μg/m^3 (NCO), 8-hour TWA
- 70 μg/m^3 (NCO), 15-minute TWA [75]

The 8-hour MEL for monomeric and polymeric forms is equivalent to approximately 0.036 mg/m^3 depending on the NCO in question.

1.4.3 *Isocyanate Properties*

NCO compounds are widely used in industry. Mono-functional NCO are used as intermediates in the production of herbicides, crop-protection agents and anti-diabetic drugs [76]. However, the most important class of NCO for industrial purposes are the di-functional group. Diisocyanates, for example, are widely used in the manufacture of polyurethane products such as flexible and rigid foams, fibres, coatings such as paints and varnishes, and elastomers. Hexamethylene diisocyanate (HDI), MDI, 2,4- and 2,6-TDI account for more than 90% of the commercial use of diisocyanates in the United Kingdom [77]. Isocyanates can be liquids or solids at room temperature and are soluble in most organic solvents. Synthesis of isocyanates is most often achieved by reacting phosgene with a primary amine. This gives the isocyanate product plus an acid chloride, as in Figure 1.7.

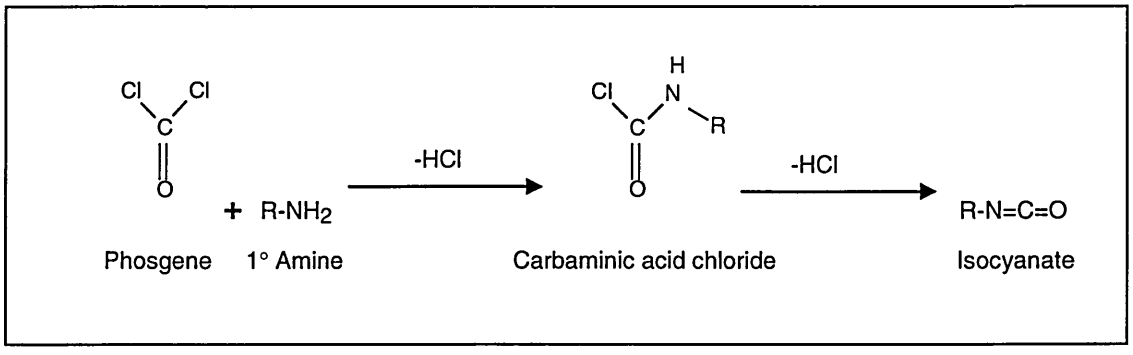


Figure 1.7 Reaction scheme for the formation of isocyanate compounds

Isocyanates undergo a variety of addition reactions with compounds containing a doubly-bonded carbon or metal-halogen complex. Isocyanates react with water to form carbaminic acid (carboxylic acid system) and elimination of carbon dioxide yields a primary amine product.

Analysis was concentrated on HDI (aliphatic isocyanate) and MDI (aromatic isocyanate). The structures of these diisocyanates are very similar to each other and are shown in Figure 1.8. Also depicted are the analogous structures of 2,4- and 2,6-TDI. NCO prepolymers are combinations of NCO monomers that have similar chemistry to the parent molecule but they are less volatile as a result of their greater molecular mass. Shown in Figure 1.8 is an example of such an NCO prepolymer, the commonly produced biuret form of HDI [78].

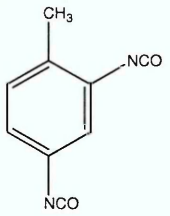
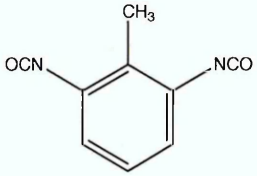
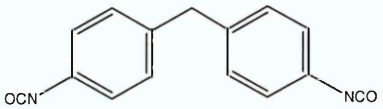
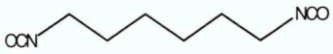
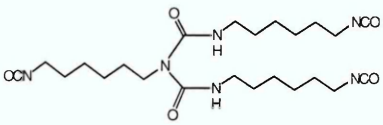
Compound Name	Abbreviation	Mwt.	Structural Formula
2,4-Toluene diisocyanate	2,4-TDI	174.2	
2,6-Toluene diisocyanate	2,6-TDI	174.2	
4,4-Methylenebiphenyl diisocyanate	MDI	250.3	
Hexamethylene diisocyanate	HDI	168.2	
Hexamethylene diisocyanate biuret adduct	-	478.6	

Figure 1.8 Compound name, common abbreviation, molecular weight (Mwt.) and the structural formulae of the four NCO with the highest occurrence in UK occupational hygiene analysis. An example of a biuret HDI NCO prepolymer is also shown

The rapid rate reaction with amines enables the collection of the isocyanates by using 1-(2-methoxy) piperazine, (MP) as set out by the Methods for Determination of Hazardous Substances [79]. The filter is impregnated with a solvent solution of MP and reaction with the airborne isocyanate occurs on contact to give stable urea derivatives. The structure of the MP group is shown in Figure 1.9.

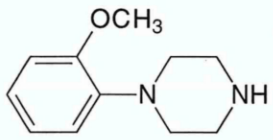
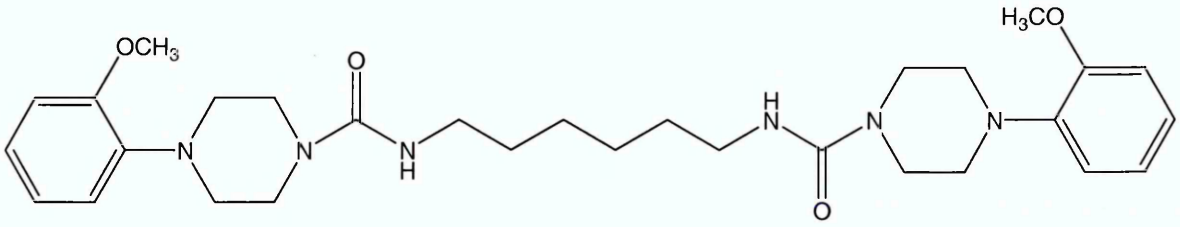
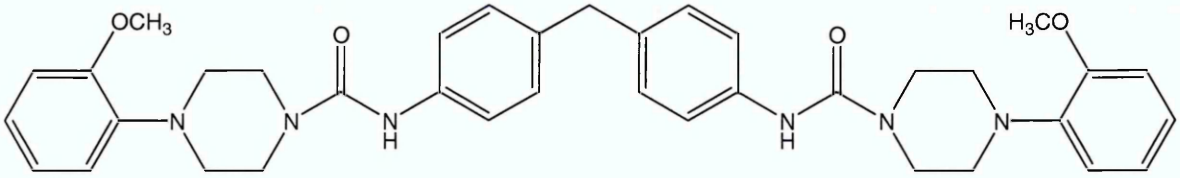


Figure 1.9 The structure of MP derivatising reagent

The derivatised isocyanate products of HDI-MP and MDI-MP are therefore collected and analysed. The structures of the derivatised products with the appropriate relative molecular masses (RMM) are shown below in Figure 1.10.



HDI-2MP : RMM 552.34



MDI-2MP : RMM 634.33

Figure 1.10 Stable isocyanate derivative structures of HDI and MDI

1.4.4 *Previous Determination of Isocyanates*

When MALDI is compared with high performance liquid chromatography (HPLC) [80] and other methods that require sample extraction, it was hoped that using MALDI-TOF-MS could develop a more time-effective method for the direct analysis of sampling filters. HPLC methods with a non-selective ultraviolet detector (UV) are qualitatively effective only for simple sample mixtures in the absence of oligomers (consisting of a finite number of monomer units). Although

many methods have been reported for the analysis of NCO, HPLC is still favoured, with on-line detection by one of several detectors.

The method recommended by the HSE is MDHS 25/3 [81]. This method uses derivatisation of NCO with MP and detection by electrochemical (EC) and UV detectors in series. This analysis technique is commonly referred to as the response ratio method. NCO functionality of an eluting peak is determined by the ratio of the peak areas from the EC and UV detectors. If the ratio of the EC to UV peaks lies within predetermined limits then the peak is ascribed to NCO functionality and outside the limits is dismissed as interference. Calibration can be carried out with appropriate standards for quantitation and complex mixtures can be analysed with multiple UV detectors in series, by comparing the peak ratios from the different instruments [82].

The National Institute for Occupational Safety and Health (NIOSH) improved upon this method with the use of a UV-photodiode array detector (UV-PDA) [83]. This method was able to identify oligomer peaks in polymeric, aromatic isocyanate samples but was not quantitative. Rudzinski et al. [84] investigated capillary-zone-electrophoresis (CZE) for analysis of MP-isocyanate derivatives. They found that the MP compound actually decomposed the capillary during analysis giving non-reproducible results. HPLC-electrospray-MS (LC-ES-MS) was discovered to be an effective method for obtaining qualitative information about complex mixtures by taking information directly from the mass-spectrum peaks [85]. This method is concentration sensitive and can be used to obtain limited quantitative results.

However, none of these methods is capable of direct analysis of the filter.

Carr et al. have reported the analysis of NCO with MALDI-MS [86]. In their study, the MDI polymers and prepolymers were treated with methanol to produce stable urethanes. Polymers are large organic molecules formed by combining an infinite number of monomer units, whereas prepolymers are substances formed by pre-reacting an isocyanate with a polyol. The urethanes were analysed using MALDI-TOF-MS with dithranol as the matrix. Compounds were identified in the mass spectrum as sodium adducts.

1.4.5 *Veterinary Medicines and Pesticide Residues*

1.4.5.1 Veterinary Medicines

In order to treat and protect the health of animals a wide range of veterinary medicines and other therapeutic agents are used across the UK. Due to the possible damaging effect that may be caused by the indirect release of these substances into the environment, there is now increasing concern over the use of veterinary medicines.

Veterinary medicines are used for a variety of purposes in the treatment of livestock. The main applications are prophylactic use (preventative measures), treating diseased conditions, growth promotion and to improve feed conversion (less food, maximum weight gain). After animal administration of these substances, the drug is metabolised. A mixture of drug and its metabolites is excreted in urine and faeces from the animal and this excrement is then used to produce manure slurry. The manure is conserved in tanks before being dispersed onto fields to cultivate agricultural crops. The main concern of the HSE is that a number of these veterinary medicines, (if not fully degraded) could have harmful effects upon non-target organisms and that these substances could be found in agricultural products, which have been grown for human consumption.

Other possible routes for veterinary drugs entering the environment are shown in Figure 1.11.

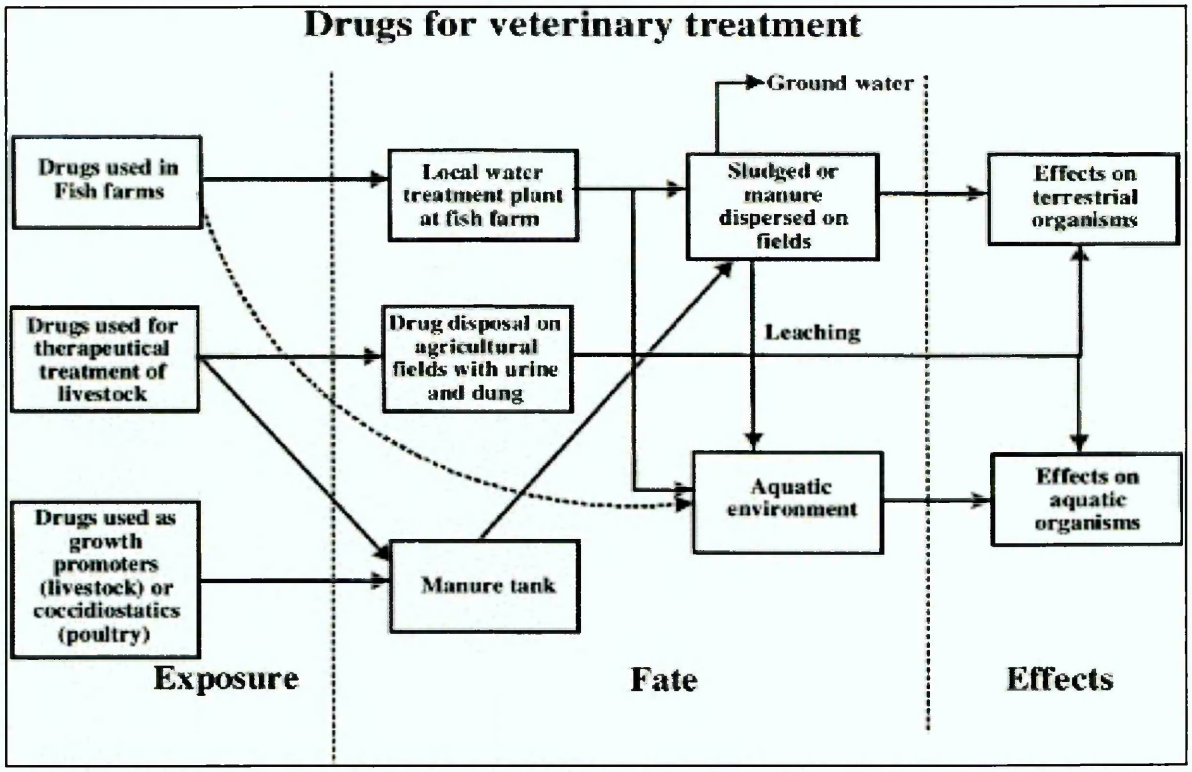


Figure 1.11 Expected exposure routes of veterinary drugs into the environment, adapted from the original [87]

1.4.5.2 Pesticides

A pest can be one of any number of living organisms that cause undesirable damage to food crops. Pests can be animal, including insects, mice and larger animals or biological such as weeds, fungi and other microorganisms. Pesticides or agrochemicals are substances which are intended to deter, destroy or completely remove specific pests on a given crop. The term pesticide incorporates herbicides, fungicides and various other pest controlling substances [88].

Although pesticides can pose risk or harm to the environment and population, they are of significant benefit to society. The use of pesticides during stages of agricultural cultivation and storage of harvested crops is important, particularly for increased quality of preservation, for transportation purposes and shelf-life.

With the extensive use of various pesticide substances in food production, the monitoring of levels that could have implications in human food sources is imperative.

1.4.6 *Veterinary Medicine and Pesticide Properties*

The analysis of a selected range of common veterinary medicines and pesticides was undertaken. The compounds chosen for study were selected due to their expected greatest potential to reach the environment, to show highest toxicity and persistence.

1.4.6.1 Veterinary Medicines

Trimethoprim [89] - $C_{14}H_{18}N_4O_5O_3$

Trimethoprim is a broad spectrum antibiotic with excellent activity against most gram-negative organisms and against Staphylococci bacteria in the skin. This renders trimethoprim as an ideal choice for the treatment of skin infections or as a general antibiotic.

Trimethoprim has the ability to penetrate into exudates and infected tissues that other antibiotics are unable to infiltrate. This means trimethoprim can enter not only abscessed tissue but also the prostate gland, blood brain barrier and eye, therefore treating localised infections.

Trimethoprim interferes with folate synthesis in susceptible bacteria. It binds tightly to bacterial dihydrofolate reductase, thousands of times more readily than to the same human enzyme. This action interferes with the uptake of *p*-aminobenzoic acid (PABA) into folic acid, an essential component of bacterial development. Trimethoprim is used in conjuction with sulfadiazine, which inhibits bacterial dihydrofolate synthetase, the enzyme immediately preceding dihydrofolate reductase. Therefore, the combination of trimethoprim with a sulfonamide (such as sulfadiazine) can produce a synergistic effect.

Trimethoprim is a crystalline solid with a mass of 290.32. The structure of trimethorpim is given in Figure 1.12.

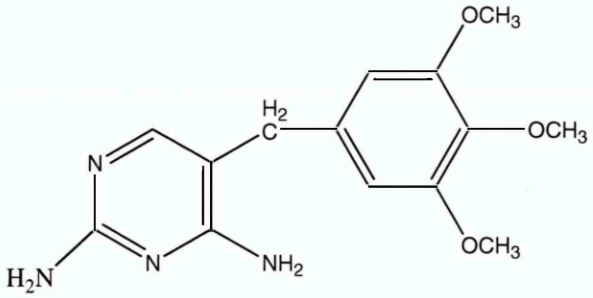


Figure 1.12 Trimethoprim structure

Sulfadiazine [90] - $C_{10}H_{10}N_4O_2S$

Sulfadiazine is a sulfonamide antibiotic, which exhibits in vitro inhibitory activity against a broad spectrum of gram-positive and gram-negative bacteria.

Sulfadiazine combined with trimethoprim is an antibiotic used to treat respiratory, urinary tract, skin, and gastrointestinal infections including infections with coccidia.

Sulphadiazine is a folic acid antagonist and is considered most effective against pathogenic protozoa when administered in combination with trimethoprim. Sulfadiazine is a structural analogue of p-aminobenzoic acid (PABA), which is a precursor, required for bacterial synthesis of folic acid. Sulfadiazine inhibits bacterial dihydropteroate synthase by competing for binding sites with p-aminobenzoic acid (PABA), retarding bacterial growth. Sulfonamides do not affect mammalian cells or bacterial cells that use folic acid precursors or preformed (dietary) folic acid.

Sulfadiazine is a crystalline solid with a mass of 250.28. The structure is shown in Figure 1.13.

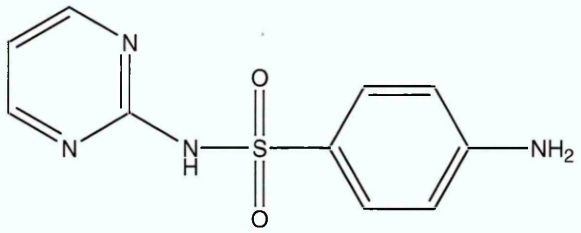


Figure 1.13 Sulfadiazine structure

Oxytetracycline Hydrochloride [91] - $C_{22}H_{24}N_2O_9.HCl$

Oxytetracycline hydrochloride is an inhibitor of bacterial growth (bacteriostatic), a broad spectrum antibiotic belonging to the tetracyclines class. These drugs are amphoteric which means they will form salts with both acids and bases, therefore able to exist as adducts of sodium or chloride.

Oxytetracycline interferes with the protein synthesis of bacteria needed for multiplication and division, therefore stopping the spread of infection. This process occurs as the drug irreversibly binds to the 30s (small subunit) of bacterial ribosomes and therefore interferes with the binding of aminoacyl tRNA to the acceptor site on the mRNA-ribosome complex. Only multiplying organisms are affected.

Oxytetracycline is a yellowish crystalline solid with a mass of 496.90. The structure of oxytetracycline hydrochloride is depicted in Figure 1.14.

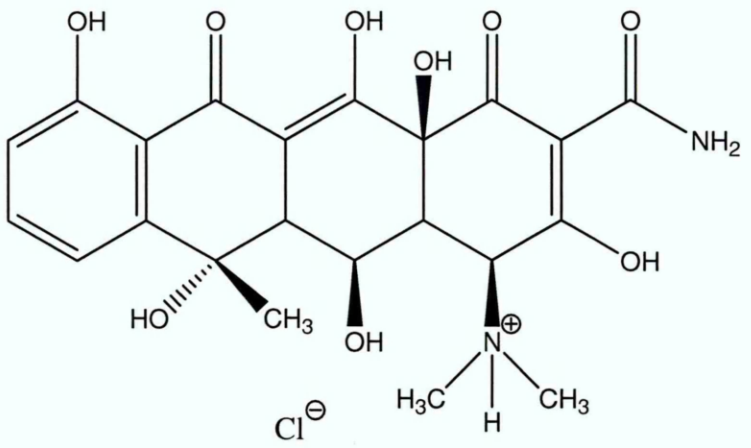


Figure 1.14 Oxytetracycline hydrochloride structure

Florfenicol [92] - $C_{12}H_4Cl_2FNO_4S$

Florfenicol is a bacteriostatic broad spectrum antibiotic, which exhibits activity against bacterial respiratory pathogens. Florfenicol is a fluorinated analogue of thiamphenicol and both are derivatives of chloramphenicol. It is particularly useful for treating pneumonia, especially where resistance to more common antibiotics has developed.

Florfenicol inhibits protein synthesis in bacteria by binding to the 50s subunit on the ribosome. This leads to the inhibition of peptidyl transferase, preventing the transfer of amino acids to growing peptide chains and subsequent protein formation. Florfenicol also inhibits mitochondrial protein synthesis in bacterial cells by binding to the 70s ribosome at these sites.

Florfenicol is a white crystalline solid with a mass of 358.21. The structure is shown in Figure 1.15.

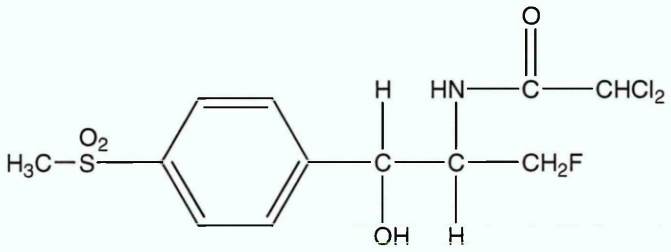


Figure 1.15 Florfenicol structure

Levamisole Hydrochloride [93] - $C_{11}H_{12}N_2S.HCl$

Levamisole is a broad spectrum anthelmintic agent (destroys or causes the expulsion of parasitic intestinal worms) of the imidazothiazole group. It is used in veterinary medicine for the control of gastrointestinal parasites in cattle, sheep and pigs. Levamisole can also be used as an antineoplastic agent (cancer medication), by interfering with the growth of cancer cells, thus slowing their spread.

Levamisole is a cholinergic agonist like nicotine and causes nicotinic stimulation of ganglia and the CNS in the parasite. Parasites are usually paralyzed and expelled alive. At higher concentrations, levamisole interferes with nematodal carbohydrate metabolism where it blocks fumarate reductase and succinate oxidase.

Levamisole is a white crystalline solid, the structure of which is given in Figure 1.16, with a mass of 240.80.

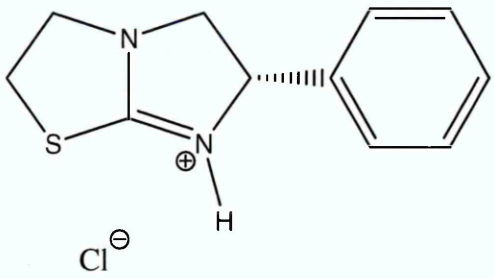


Figure 1.16 Levamisole hydrochloride structure

Enrofloxacin [94] - $C_{10}H_{10}N_4O_2S$

Enrofloxacin is a bactericidal, broad spectrum fluoroquinolone antibiotic. Its activity is concentration dependent.

Enrofloxacin deactivates bacterial enzymes necessary for the transcription of DNA. Segments to be used must be uncoiled by an enzyme called DNA gyrase, which is deactivated by enrofloxacin, making the reading of DNA impossible.

Irreversible damage is made to the DNA, which leads to cell death, although mammalian DNA gyrase is a different shape and therefore remains unharmed.

Enrofloxacin is a crystalline solid with a mass of 359.40. The structure of enrofloxacin is given in Figure 1.17.

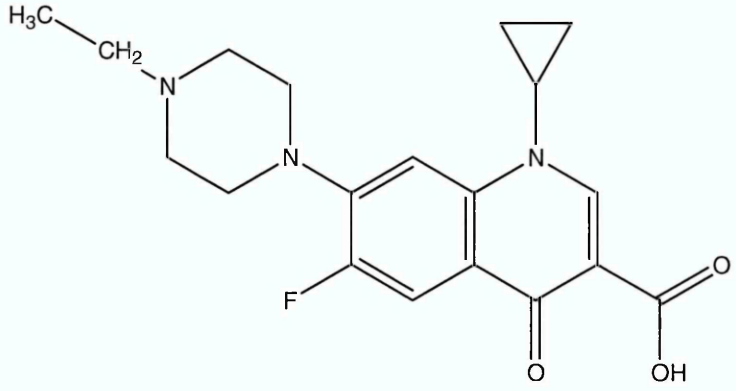


Figure 1.17 Enrofloxacin structure

Tylosin Hemitartrate Salt Dehydrate [95] - $C_{48}H_{80}NO_{20}.2H_2O$

Tylosin is a bacteriostatic antibiotic that belongs to the macrolide class. It is made naturally by bacteria and acts to inhibit protein synthesis in prokaryotic cells by inhibiting the 50s ribosome. This cellular structure is only present in certain bacteria as a method of making internal proteins.

Tylosin is used as an antibiotic in the treatment of livestock infection and for anti-inflammatory properties in the intestine of small animals.

Tylosin is an off-white crystalline powder, the structure of which is shown in Figure 1.18, with a mass of 1027.17.

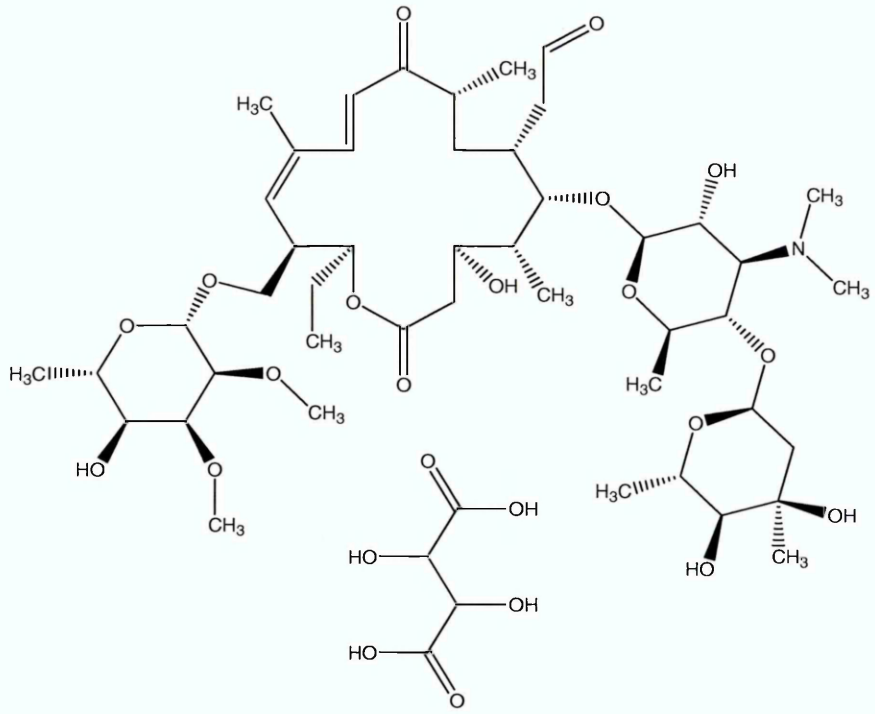


Figure 1.18 Tylosin hemitartrate salt dehydrate structure

1.4.6.2 Pesticide Residues

Cypermethrin [96] – $C_{22}H_{19}Cl_2NO_3$

Cypermethrin is a synthetic pyrethroid insecticide and a permethrin analogue. It works by killing insects that eat or come into contact with the compound.

Cypermethrin is a fast acting neurotoxin that rapidly degenerates the central nervous system, primarily acting on the basal ganglia of the CNS. This causes repetitive nerve action through prolongation of sodium permeability which occurs during the recovery phase of neuron action potential.

Cypermethrin is not a plant systemic as it is readily degraded on soil or plants but has good residual activity on inert surfaces.

Cypermethrin is a crystalline solid with a mass of 416.30. As is evident from the structure shown in Figure 1.19, the molecule contains two chlorine atoms which will give rise to characteristic isotopic patterns in the mass spectra.

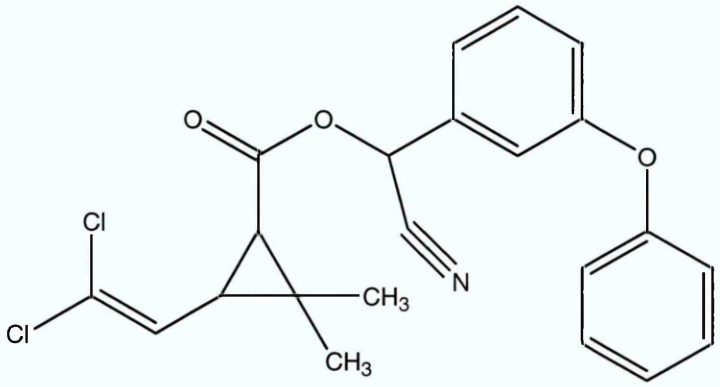


Figure 1.19 Cypermethrin structure

1.4.7 *Previous Determination of Veterinary Medicines and Pesticide Residues*

Extensive work has been undertaken when analyzing veterinary medicines and pesticide residues, although only recently has their danger been fully recognized as a source of possible environmental pollution.

The HSL has performed varying analyses on wide range of veterinary medicine and pesticide substances, from water and soil samples in addition to vegetation matrices. Solid phase (SPE) and solvent extraction methods have been performed in association with sample concentration and clean-up steps. A multi-residue method was developed, employing multiple reaction monitoring (MRM) by LC-MS-MS [97]. A gradient elution method was utilsed in positive electrospray mode. The results obtained were reproducible and concurred with results from previous veterinary medicine analysis [98].

Quantitative analysis has been performed using gas chromatography (GC) linked with flame photometric detection (FPD) on similar matrices, without the necessity for pre-concentration and cleaning phases [99].

Other work has combined parts of previous techniques to achieve an SPE-GCMS rapid, small scale method [100].

Exposure of the workforce or human population to substances that have a detrimental effect can be completely avoided with the correct precautions in place. Potentially harmful isocyanate and veterinary medicine compounds are now starting to be strictly monitored by organisations such as the HSE to avoid unnecessary illness or ailment. Occupational exposure limits (OEL) have been set out for isocyanate compounds, whereas, maximum residue limits (MRL) govern veterinary medicine and pesticide restrictions. Part of the responsibility still lies with individuals, who must follow procedures to avoid personal contamination.

There is a definite necessity for new analytical techniques within the occupational hygiene field, for both the introduction and enforcement of laws and regulations. The continual desire for faster and more accurate instrumentation and methodologies ensures rapid progression of analysis schemes. By combining established analytical procedures with new inventions and ideas, advances in technologies are immense.

Mass spectrometry has already proved invaluable in many areas of research and compound examination. The development of matrix application methods, improved matrix formats and higher quality instrumentation, all enable the use of MALDI as a rapid screening technique with the possibility of analyte quantitation. It was hoped that adaptation of the MALDI analysis technique would lead to a direct filter analysis method by utilising a matrix on the sampling filters.

The fusion of separation methods, such as LC, with mass spectrometric detection has provided elemental, compositional and structural information about analyte substances.

It was hoped that the work described in this thesis could lead to a rapid screening method using MALDI-TOF-MS, with the potential for investigation of compound distribution in thin slices of vegetation.

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Chapter 2

Isocyanate Characterisation

Isocyanates are a very important class of compounds in the occupational hygiene forum due mainly to their behaviour as respiratory sensitisers. The determination of NCO functionality and any contributions to understanding more about NCO chemistry are of significant value to occupational hygiene bodies globally.

This chapter demonstrates the application of MALDI-TOF-MS and tandem MS to the analysis of derivatised isocyanate monomers and prepolymers. Our NCO as MP derivatives are analysed using MALDI-TOF-MS. The aim of the work has been to gauge the selectivity obtainable from the analysis of NCO mixtures without prior separation. Derivatised monomers and prepolymer mixtures as MP derivatives are analysed and the potential of MALDI-TOF-MS for an NCO monitoring program is assessed. The results obtained show the possibility for mixture separation by this method, enabling the resolution of monomeric and prepolymeric species in analysis spectra.

Analysis was concentrated on HDI and MDI isocyanate compounds. NCO prepolymers are combinations of NCO monomers that have similar chemistry to the parent molecule but they are less volatile as a result of their greater molecular mass. Often, the true structures and molecular masses of these polymeric compounds are unknown; all that is known is the way in which they polymerise. Dimeric, trimeric and tetrameric forms are all believed to have been analysed, along with higher polymeric configurations of HDI and MDI.

Tandem mass spectrometry induces fragmentation and is used to obtain more structural information about target molecules and samples. Fragments from a selected ion are generated by collision, and mass analysis is performed on the resulting portions, producing fragment ion spectra. A single analysis run will give the constituent fragments of the test analyte. These spectra can be 'reconstructed' to provide a characteristic molecular fingerprint of the start compound. Product ion scanning (Daughter Ion) was employed for these experiments, determining the fragment ions which arise from a selected precursor ion.

The developed methods were then applied to the analysis of derivatised swabs from a HSE monitoring scheme, producing viable identification of unknown structures.

LC-MS affords separation of analyte moieties before detection to enable the collection of more useful sample data. The separated products are introduced into the mass spectrometer in solution either on or off-line. On-line LC-MS is where the chromatograph is connected straight to the mass spectrometer and the mass spectra are acquired as the subsequent compounds of the mixture are eluted.

This chapter demonstrates the application of liquid chromatography separation linked with mass spectrometry for the analysis of derivatised isocyanate monomers and prepolymer mixtures. The results obtained show the possibility for mixture separation by this method, enabling the resolution of monomeric and prepolymeric species in analysis spectra.

2.2 Experimental

2.2.1 *Chemicals and reagents*

NCO monomers (HDI, MDI, 2,4- and 2,6-TDI) and commercial formulations were provided as 1-(2-methoxyphenyl) piperazine derivatives by the Health and Safety Laboratory (HSL, Buxton, UK). HPLC grade methanol and trifluoroacetic acid (TFA) were obtained from Fisher Chemicals (Loughborough, Leicester, UK). α-Cyano-4-hydroxy cinnamic acid (α-CHCA) was purchased from Sigma Aldrich (Poole, Dorset, UK). Mirrorcryl MS paint hardener® was obtained from Mirror Products (Sheffield, UK).

2.2.1.1 Preparation of Derivatised Standards and Formulations

Stock solutions of HDI, MDI, 2,4-TDI and 2,6-TDI as MP derivatives were prepared, 100 µg/ml in methanol (0.1% TFA). Commercial formulations were prepared by dissolving the MP-derivatised material in methanol/TFA to achieve

a comparable concentration of approximately 100 µg/ml. Solubility issues with MP- derivatised NCO prepolymers (> 1000 Da) were noted and it was necessary to sonicate several samples for periods of up to 5 minutes. Care was taken to maintain the temperature of the sonic bath at 37°C.

2.2.1.2 Preparation of Swab Sample Solutions

Swabs were taken from the monitoring process using cotton buds dipped in toluene/MP solution (as prepared in MDHS 25/3). The swabs were left to extract in 1 ml methanol (0.1% TFA) for an hour.

2.2.2 *Instrumentation*

All analyses were performed using an Applied Biosystems (Foster City, USA)/MDS Sciex (Ontario, Canada) 'Q-Star' Pulsar i hybrid quadrupole time of flight instrument, fitted with an Applied Biosystems/MDS Sciex oMALDI 2 ion source. This instrument utilises a nitrogen laser with a 337nm wavelength. Data was collected and manipulated using the 'Analyst–QS' software supplied with the instrument.

The HPLC system used was a Waters 600MS quaternary pump with a Perkin-Elmer IS200 autosampler. The detector employed was a Waters 480 set to a wavelength of 279 nano meters.

2.2.3 *Experimental*

2.2.3.1 MALDI Methodology

The matrix used for the MALDI analysis was α-CHCA (25 mg/ml) in methanol (0.1% TFA). The sample and the matrix solutions were mixed (4:1) and 1µl of the resulting solution was placed on the specialised stainless steel target for analysis. All spectra were the result of the cumulative acquisition of laser shots from a 2-minute run time with a laser repetition rate of 20 Hz. The instrument resolution was 10,000 fwhm and the mass calibration was adjusted as required. The normal calibration was performed by product ion scan of renin substrate (peptide species).

2.2.3.2 Tandem Mass Spectrometry Methodology

The sample preparation method employed was the same as in the previous section (2.2.3.1), mixing analyte with matrix in a 4:1 ratio. Product ion scans were performed by entering the mass of interest and inducing fragmentation by applying collision energy (CE) in the form of a cone voltage. The cone voltage level was commenced at 20 Volts and gradually increased over the run up to the maximum value of 120 Volts. The higher the cone voltage utilised, the greater the fragmentation, resulting in the appearance of more ions at lower m/z values.

2.2.3.3 LC-MS Methodology

Reverse phase partition was employed for this study with a non-polar stationary phase and polar medium as the mobile phase. The reverse phase medium was composed of hydrophobic, non-polar C_{18} hydrocarbon chain, packed into a 2.1 μm diameter column, 100 mm in length. Gradient elution was performed by varying the composition of the mobile phase over time, keeping the flow rate, pH and temperature constant.

The solvents used were acetonitrile (A) and 0.1 molar ammonium acetate, pH adjusted to 4.7 (B). Gradient elution was employed; the composition of the mobile phase is explained in Table 2.1.

Time (Minutes)	Flow rate (μL/minute)	Percentage Composition A	Percentage Composition B
0	200	60	40
10.0	200	60	40
10.5	200	70	30
15.0	200	100	0
25.0	200	100	0
30.0	200	60	40

Table 2.1 Mobile phase composition for gradient elution method

The samples were prepared as 100 μg/ml solutions in acetonitrile, to be compatible with the mobile phase. Overall run-time was 45 minutes and a mass range for analyses was set at m/z 170 – 3000. The ion gas source value (IGS) and the curtain gas were set to values stipulated in the software (70 and 40, respectively). The ionspray voltage employed (IS) was 4500 Volts and the sample injection volume was maintained at 10 μl.

2.3 Results and Discussion

2.3.1 *Application of MALDI-MS to the Analysis of Derivatised NCO Standards*

MALDI mass spectra of MP-derivatised NCO monomers exhibited intense peaks representative of protonated molecules $[M+H]^+$ as well as metal ion adducts $[M+Na]^+$ and $[M+K]^+$ (Figures 2.1, 2.2 and 2.3). The peaks appeared at 553, 575 and 591 Th for HDI (Figure 2.1), 635, 657 and 673 for MDI (Figure 2.2) and 559, 581 and 597 for TDI (Figure 2.3).

An abundant peak was observed in all spectra corresponding to $[MP+H]^+$ at 193 Th. This protonated molecule originates from the MP reagent (192 Da) that is present in large excess in the samples. The presence of excess derivatising reagent has been previously confirmed by LC-MS [1]. MP alkali metal adduct ions were present in the NCO monomers analysed but only in low abundance, which varied frequently and with no apparent correlation to the monomer type. Also present in the mass spectra acquired were peaks corresponding to $[M+H-192]^+$ which corresponds to the loss of one derivatising reagent group from the protonated derivatised NCO monomer (361 Th HDI, 443 MDI and 367 for TDI). Ions were observed as a result of similar losses of the derivatising reagent from the sodium and potassium adduct ions of the NCO monomers i.e. $[M+Na-192]^+$ and $[M+K-192]^+$, which appear at 383 and 399 Th HDI, 465 and 481 MDI and 389 and 405 TDI.

Any low mass isocyanate ions produced from the samples are suppressed by the abundant matrix ions present in the mass spectra, 190, 212 and 228 Th for protonated, sodium and potassium adducts, respectively.

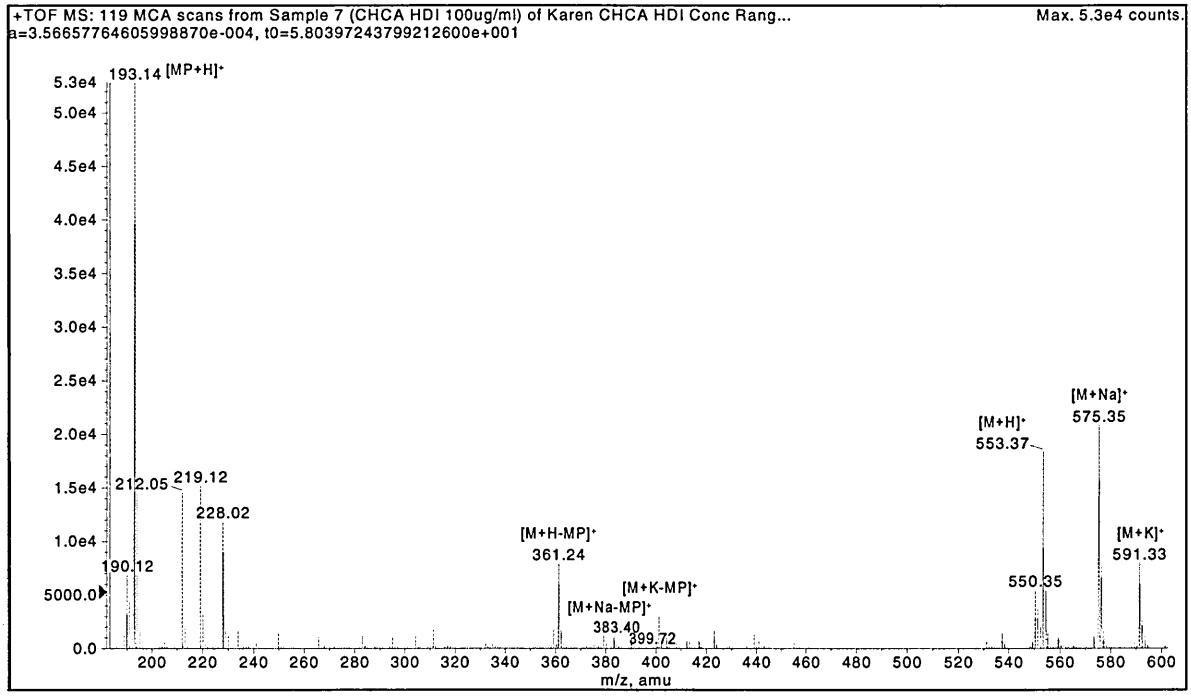


Figure 2.1 A MALDI mass spectrum acquired from the analysis of 100 µg/mL of MP-derivatised HDI monomer with α-CHCA matrix (25 mg/ml) in methanol (0.1 % TFA). The sample and the matrix were mixed (4:1) and 1µl of the resulting solution was placed on the stainless steel target of the instrument for analysis

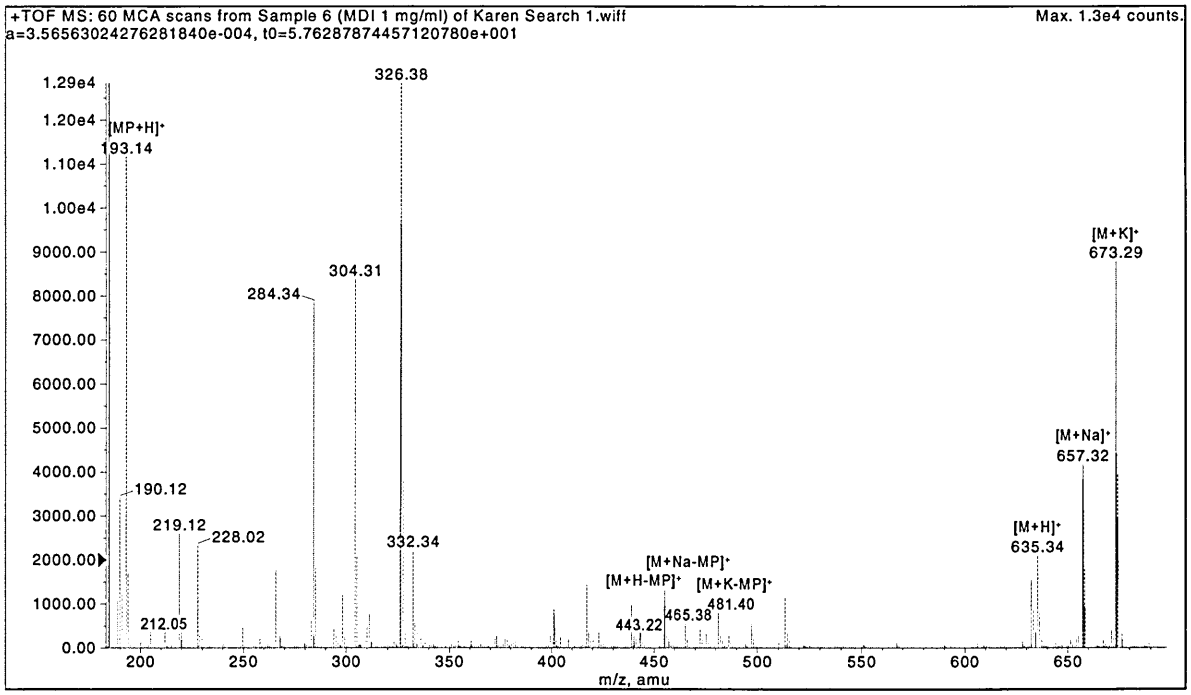


Figure 2.2 A MALDI mass spectrum acquired from the analysis of 100 µg/ml of derivatised MDI monomer with α-CHCA matrix (25 mg/ml) in methanol (0.1 % TFA). The sample and the matrix were mixed (4:1) and 1µl of the resulting solution was placed on the stainless steel target of the instrument for analysis

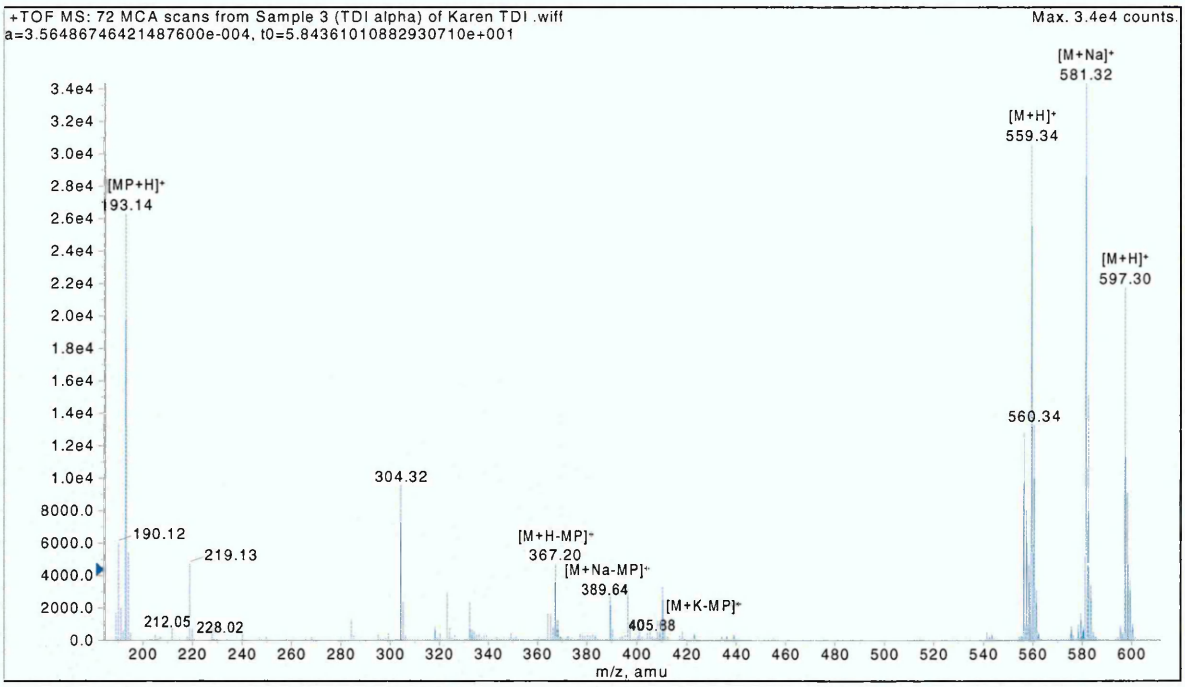


Figure 2.3 A MALDI mass spectrum acquired from the analysis of 100 µg/ml of derivatised 2,4-TDI monomer with α-CHCA matrix (25 mg/ml) in methanol (0.1 % TFA). The sample and the matrix were mixed (4:1) and 1µl of the resulting solution was placed on the stainless steel target of the instrument for analysis

2.3.2 *Application of MALDI-MS to the Analysis of Derivatised NCO Commercial Formulations*

2.3.2.1 HDI Formulation

Derivatised NCO commercial formulations were analysed using MALDI-MS. The individual formulations analysed were known to contain only monomers or prepolymers derived from one type of NCO. It was therefore not necessary to consider ions from several NCO species during the interpretation of the mass spectra generated. The identity of the base monomer was known for all samples but the identities of individual NCO components of the formulations were not (regulatory authorities often question the NCO composition of commercial formulations). A limitation of MALDI-MS is the difficulty of on-line separation, as would be afforded by LC-MS for example. Omitting sample separation prior to ionisation can result in a complex mass spectrum from mixture analyses. This complexity is alleviated somewhat by using a 'soft' ionisation technique, such as MALDI. Soft ionisation techniques produce fewer dissociation products of the parent molecule and a greater number of molecular

species. We have shown that MALDI-MS will yield not only a protonated molecule for NCO monomers but will also generate other monomer-related ions. The presence of ions such as $[M+H+MP]^+$ (where M is the NCO monomer and MP the derivatising reagent) and related group 1 metal ion adducts can act to complicate the assignment of ambiguous peaks in acquired mass spectra.

The mass spectrum shown next (Figure 2.4) is an example of the data acquired from MALDI-MS analysis of an HDI-based prepolymer commercial formulation.

The mass spectrum shows the monomeric protonated molecule along with the respective sodium and potassium adducts (553, 575 and 591 Th). The middle mass range of the spectrum is dominated by ions at 1081, 1103 and 1119 Th from protonated, sodium adduct and potassium adduct species of the polymeric form, respectively (Figure 2.4 inset). From literature sources [2] it is possible to assign this ion to a protonated HDI cyclic trimer containing 3 derivatising groups. Related sodium and potassium ion adducts are observed for this species at 1103 and 1119 Th, respectively. An ion corresponding to a derivatised HDI cyclic trimer minus one derivatising group is present at 889 Th. Ions of this type, $[M+H-192]^+$ have been identified previously for NCO monomers.

Ions with m/z values higher than that of the potassium ion adduct of the derivatised HDI trimer are observed. There are groups of ions in the ranges 1200-1300, 1400-1500 and 1600-1700 Th of the mass spectrum. The author is at present unaware of a HDI prepolymer that could produce derivatised ions that correspond to the group at range 1600-1700 Th. Examination of the spectrum yields a relationship between the ions at 1230, 1422 and 1614 Th (present as part of the groups, but are presented unlabelled). This relationship is the result of multiple losses of 192 Da (derivatising reagent) from the presumed precursor ion, at 1614 Th, indicating that this compound possesses 5 HDI and 4 MP groups.

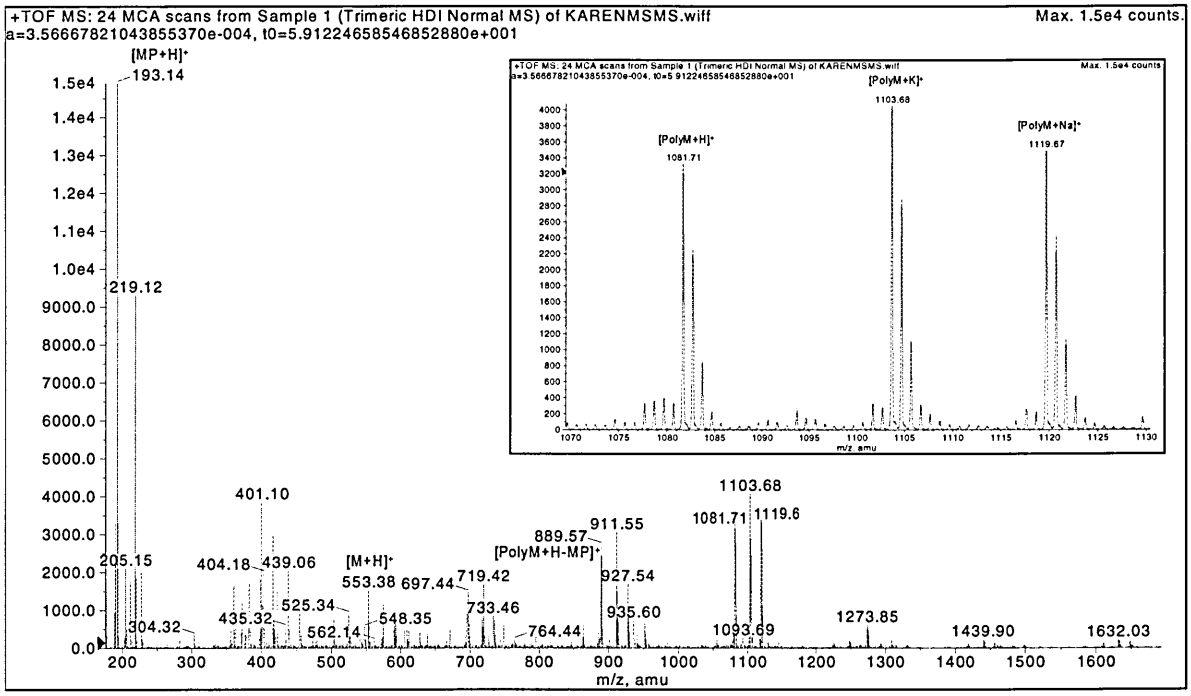


Figure 2.4 A MALDI mass spectrum of a derivatised HDI prepolymer sample. The spectrum was acquired from the analysis of a 100 µg/ml sample with α-CHCA matrix (25 mg/mL) in methanol (0.1 % TFA). The sample and the matrix were mixed (4:1) and 1µL of the resulting solution was placed on the stainless steel target of the instrument for analysis. The inset spectrum displays an expanded peak pattern that arises from the protonated molecule, sodium and potassium adducts at 1081, 1103 and 1119 Th

2.3.2.2 MDI Formulation

The mass spectrum obtained from the analysis of an MP-derivatised MDI-based commercial formulation (Figure 2.5) is now discussed.

This shows an ion corresponding to the protonated derivatised MDI molecule (635 Th); this is useful diagnostic information in the instance of the analysis of a sample of unknown composition. Sodium and potassium ion adducts of the MDI monomer are also present in the mass spectrum (657 and 673 Th, respectively). Several abundant ions at m/z values higher than that of the protonated derivatised NCO monomer were observed which are suspected derivatised NCO prepolymer ions. Possible sodium and potassium ion adducts are identifiable as satellite peaks for several of the suspected protonated prepolymer ions. For example, the ions at 1269, 1291 and 1307 Th are likely to

be related by an $[M+H]^+$, $[M+Na]^+$, $[M+K]^+$ type relationship, where M is an unidentified derivatised prepolymer molecule or related dissociation product (Figure 2.5 inset). A repeating common difference of 192 Da is observed between several ions present in Figure 2.5. The rounded mass values for these peaks were calculated at 1413, 1221, 1029 and 837, suggesting successive losses of MP derivatising reagent. In cases where the base monomer information is unavailable, the presence of a diagnostic ion such as the protonated molecule of the derivatised NCO monomer can be of significant value. A repeating common difference of 323 Da is also observed in the spectrum. The mass values for these peaks are 958, 1281 and 1604 (635 + 323n Th).

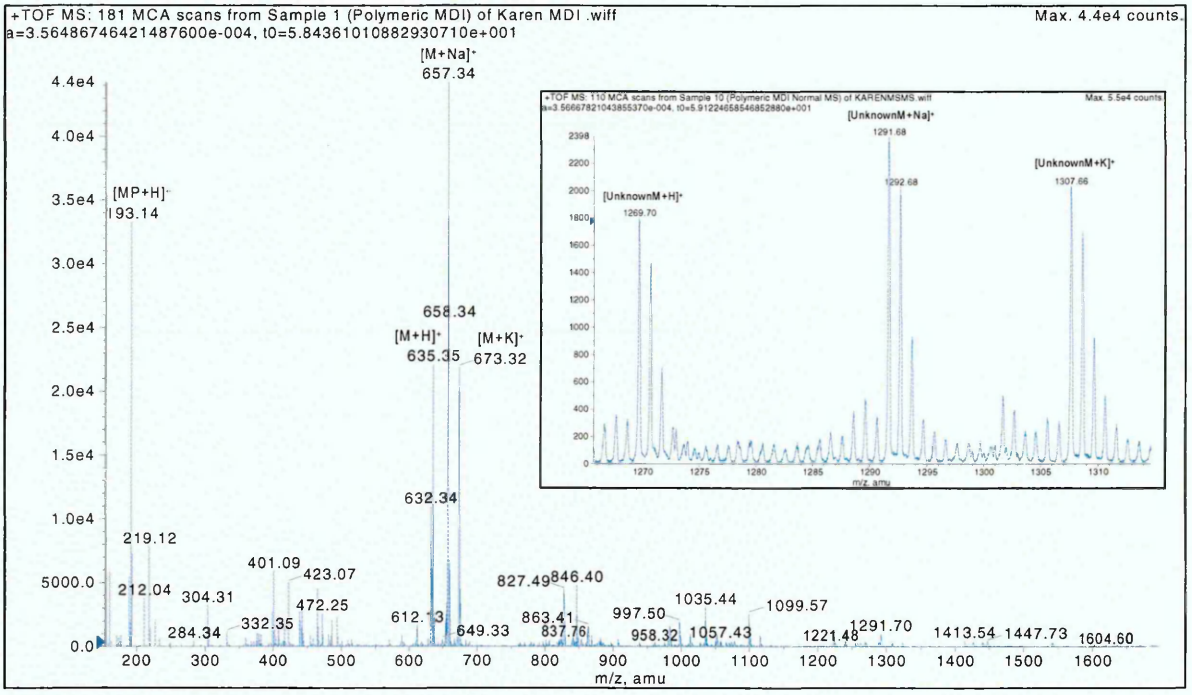


Figure 2.5 A MALDI mass spectrum of a derivatised MDI prepolymer. The spectrum was acquired from the analysis of a 100 µg/ml sample with α-CHCA matrix (25 mg/ml) in methanol (0.1 % TFA). The sample and the matrix were mixed (4:1) and 1µl of the resulting solution was placed on the stainless steel target of the instrument for analysis. The inset spectrum displays an expanded peak pattern that arises from the protonated molecule, sodium and potassium adducts at 1269, 1291 and 1307 Th

2.3.3 *Tandem Mass Spectrometric Analysis for Structural Elucidation*

2.3.3.1 HDI Formulation

By employing product ion scanning, peaks can be forced to dissociate to show possible constituent groups. Patterns of mass differences were evident in the spectra, i.e. that of derivatising reagent loss, 192 Da (Figure 2.6). The elucidated structure of mass 1080 Da (Figure 2.7) possesses 3 outer isocyanate functional groups with 3 MP derivatising reagent clusters.

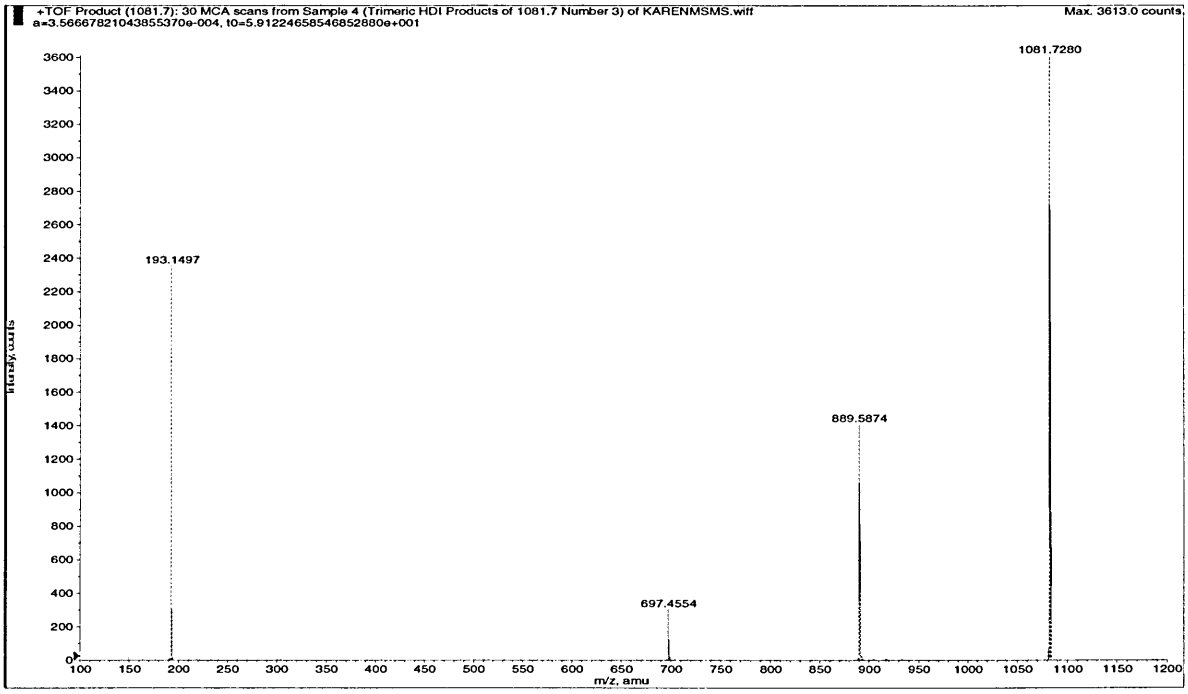


Figure 2.6 A tandem mass spectrum from analysis of the derivatised polymeric HDI sample, obtained by product ion scanning of the protonated parent molecule 1081 Th

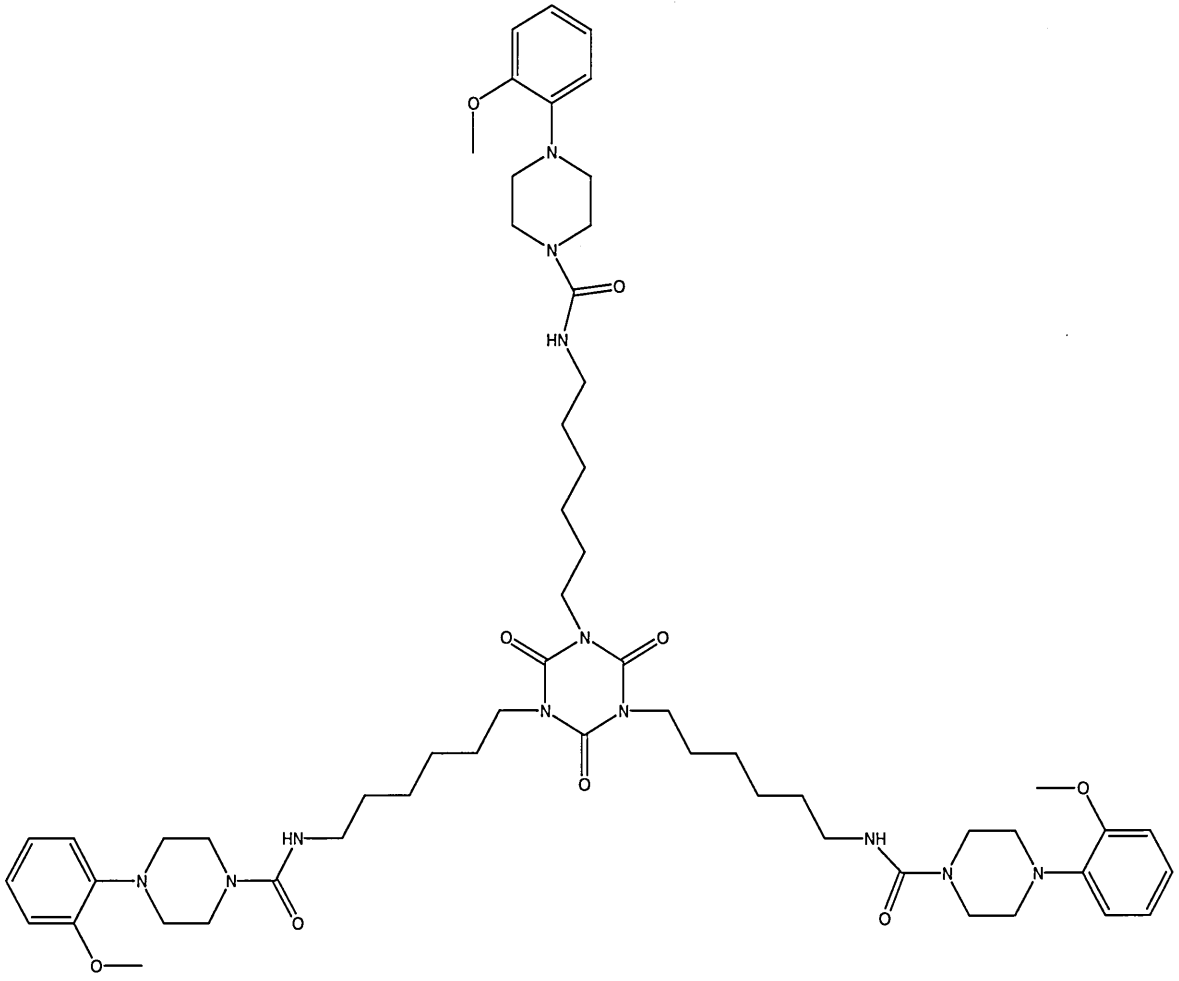


Figure 2.7 Depicts a proposed structure obtained by tandem MS analysis of a fragment from the polymeric HDI sample, 1080 Da

2.3.3.2 MDI Formulation

Data that enabled the proposal of possible structures for constituents of the MDI formulation were obtained by carrying out tandem MS. The proposed structure for mass 1412 Da contains 5 isocyanate functional groups and 4 derivatising reagent molecules (Figure 2.8). By progressively removing derivatising groups, the resultant masses of 1220, 1028 and 836 Da were determined. By employing product ion scanning, patterns of mass differences were evident in the spectra, i.e. that of derivatising reagent loss, 192 Da (Figure 2.9).

Considering possible condensation products from the synthesis of MDI, a series of assigned ions present in Figure 2.5 can be diagrammatically depicted (Figure

2.10). Only the first product of condensation for MDI is shown with a combined molecular weight of 958 Da.

This figure shows the singular increase in MDI prepolymer size, characterised by the addition of one tolyl isocyanate group. The molecular mass of an MP-derivatised tolyl isocyanate group is equivalent to the repeating mass difference of 323 Da observed in the spectra. The 1281 and 1604 Th are formed by progressively adding benzyl groups (analogous structures to the 958 Da molecule).

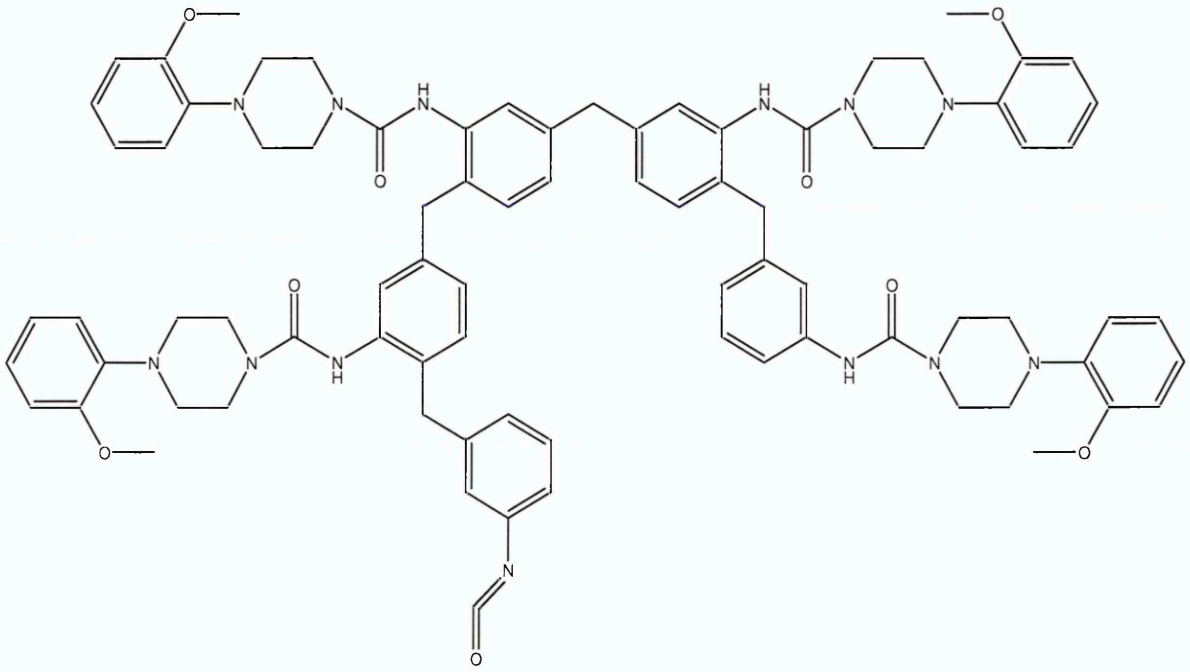


Figure 2.8 Depicts a proposed structure obtained by tandem MS analysis of a fragment from the polymeric MDI sample, 1412 Da

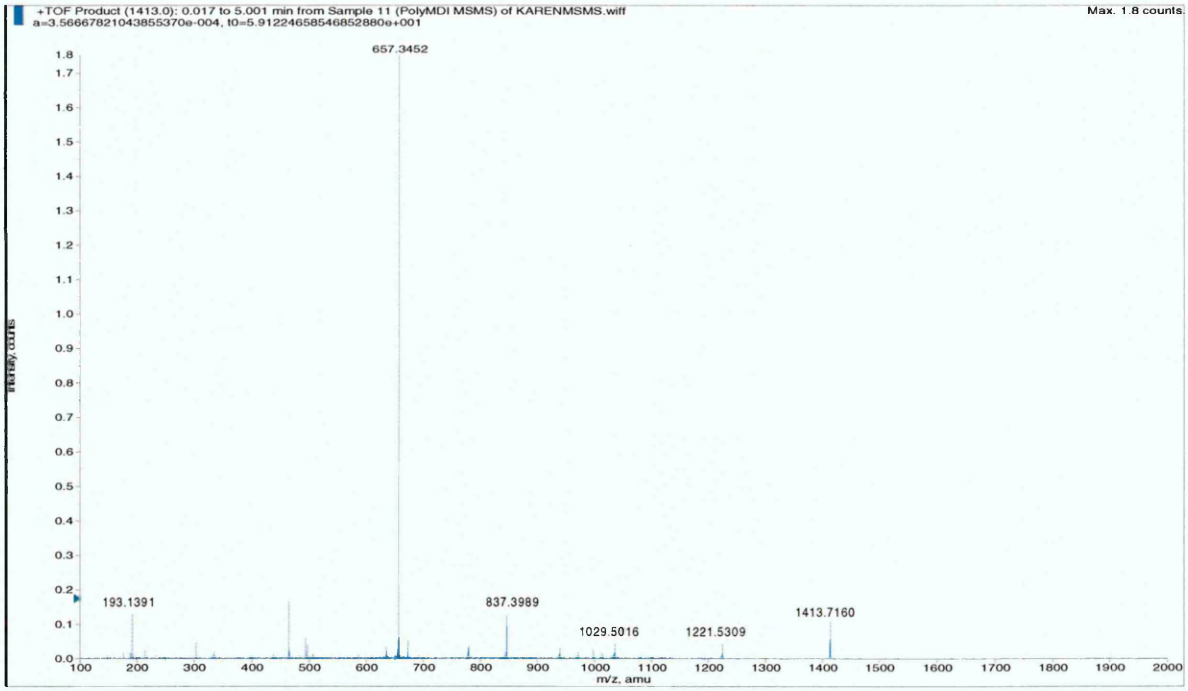


Figure 2.9 A tandem mass spectrum from analysis of the polymeric MDI sample, obtained by product ion scanning of the protonated parent molecule 1413 Th

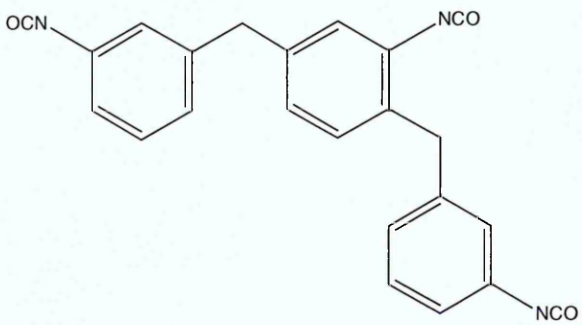
Mwt. of MP derivative	Mwt.	Pre-polymer Structural Formula
958.2	381.4	

Figure 2.10 The Structural formula of an MDI prepolymer condensation product with a molecular weight of 958 Da. The molecular weight (Mwt.) of the pre-polymer and the MP derivative are presented

2.3.4 *Application of LC-MS to the analysis of derivatised NCO commercial formulations*

2.3.4.1 HDI Formulation

It was concluded from the chromatograms obtained that the derivatising agent (MP, RMM 192) was detected between 1.85 and 2.75 minutes. Other isocyanate related peaks appeared from roughly 3 to 29 minutes. The peaks showed good separation with baseline resolution.

Analysis of monomeric HDI-MP gave 2 major peaks at masses of 553 (protonated molecule) and 193 (protonated derivatising reagent). Figure 2.11 shows the base peak chromatogram from the analysis of monomeric HDI with a peak relating to 553 (protonated molecule) being the most prominent. An extracted ion chromatogram is shown in Figure 2.12 depicting the MP derivatising reagent at elution time of approximately 2.5 minutes. The mass spectrum from the analysis of monomeric HDI-MP is presented in Figure 2.13. LC-MS analysis has proved that the derivatising reagent is in excess, which complies with the preparation of the samples by the HSE.

Analysis of trimeric HDI produced a chromatogram, Figure 2.14 in which appeared both the derivatising reagent and multiple trimeric HDI peaks. Figure 2.15 is a mass spectrum showing excess derivatising reagent and peaks related to polymeric HDI. The previously elucidated structure of 1081 Th is present with other peaks analogous to the trimeric structure.

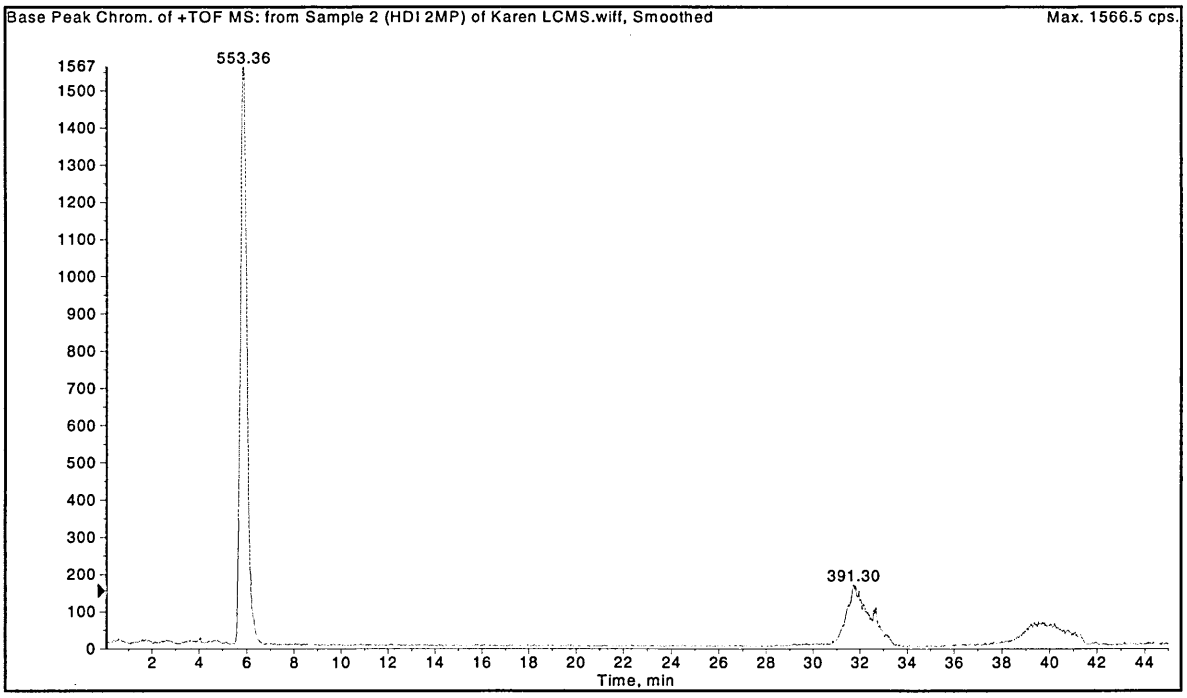


Figure 2.11 LC-MS base peak chromatogram of HDI-MP showing a HDI related peak at an elution time of approximately 6 minutes. The samples were prepared as 100 μg/ml solutions in acetonitrile, to be compatible with the mobile phase. The overall run-time was 45 minutes with a sample injection volume of 10 μl

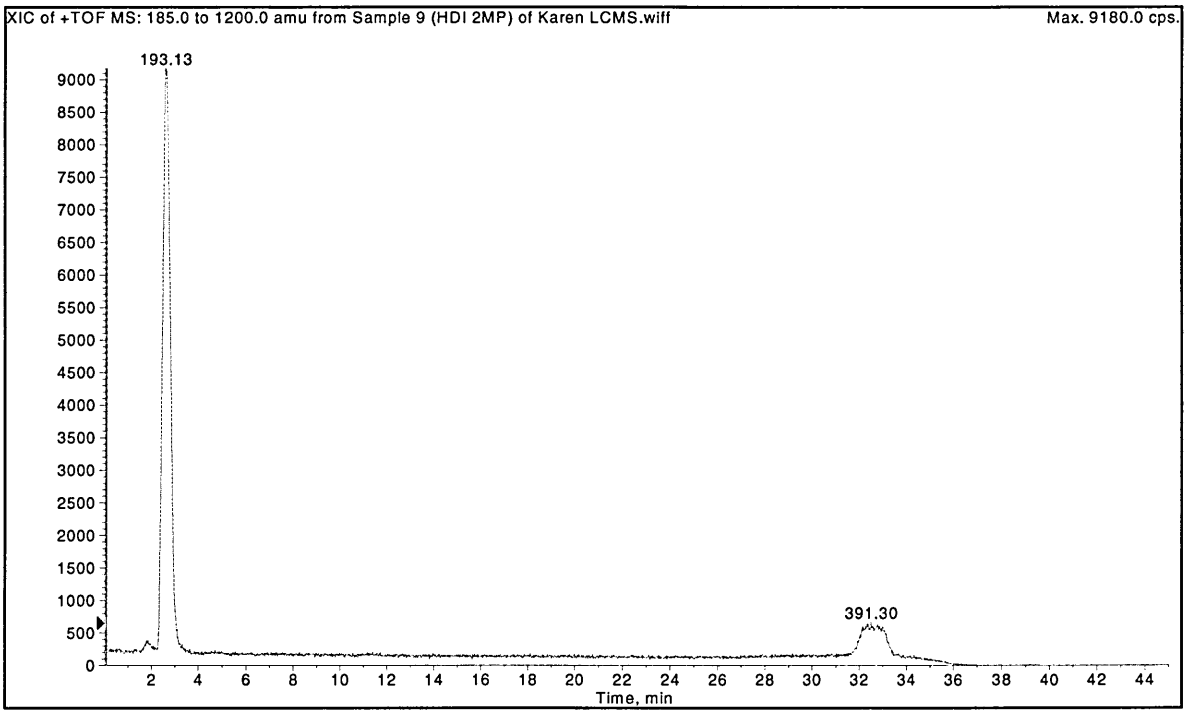


Figure 2.12 LC-MS extracted ion chromatogram of HDI-MP showing MP derivatising reagent at elution time 2.5 minutes

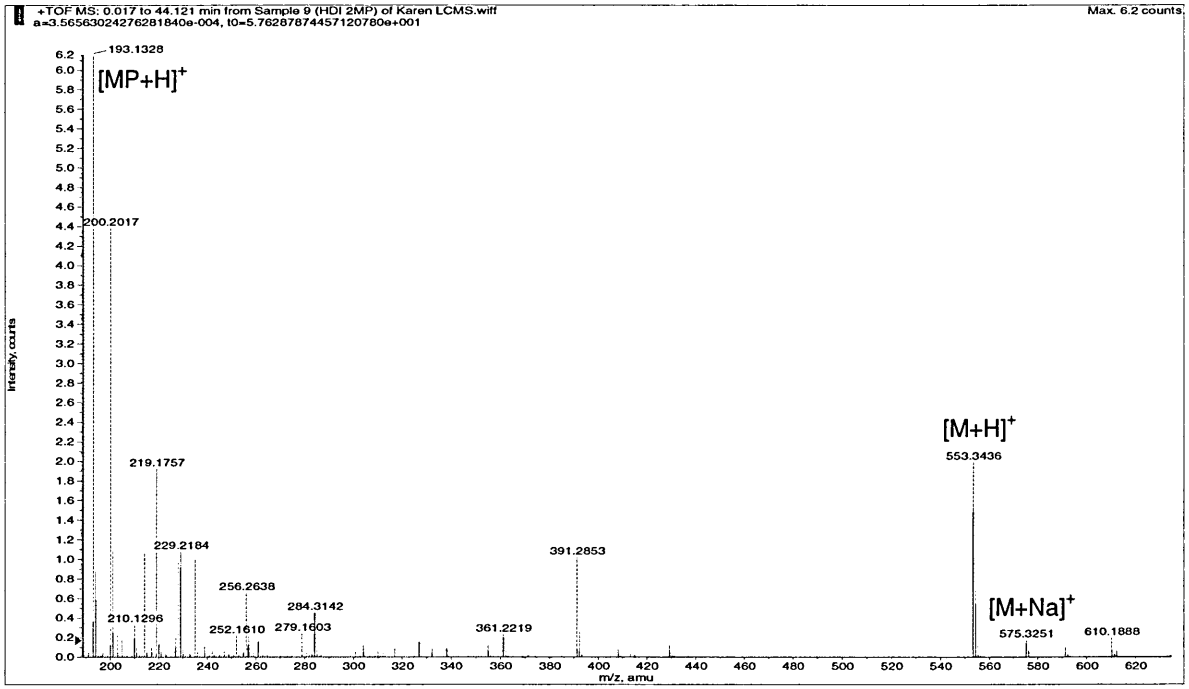


Figure 2.13 Mass Spectrum from the analysis of monomeric HDI-MP showing HDI at mass 553 and MP at 193

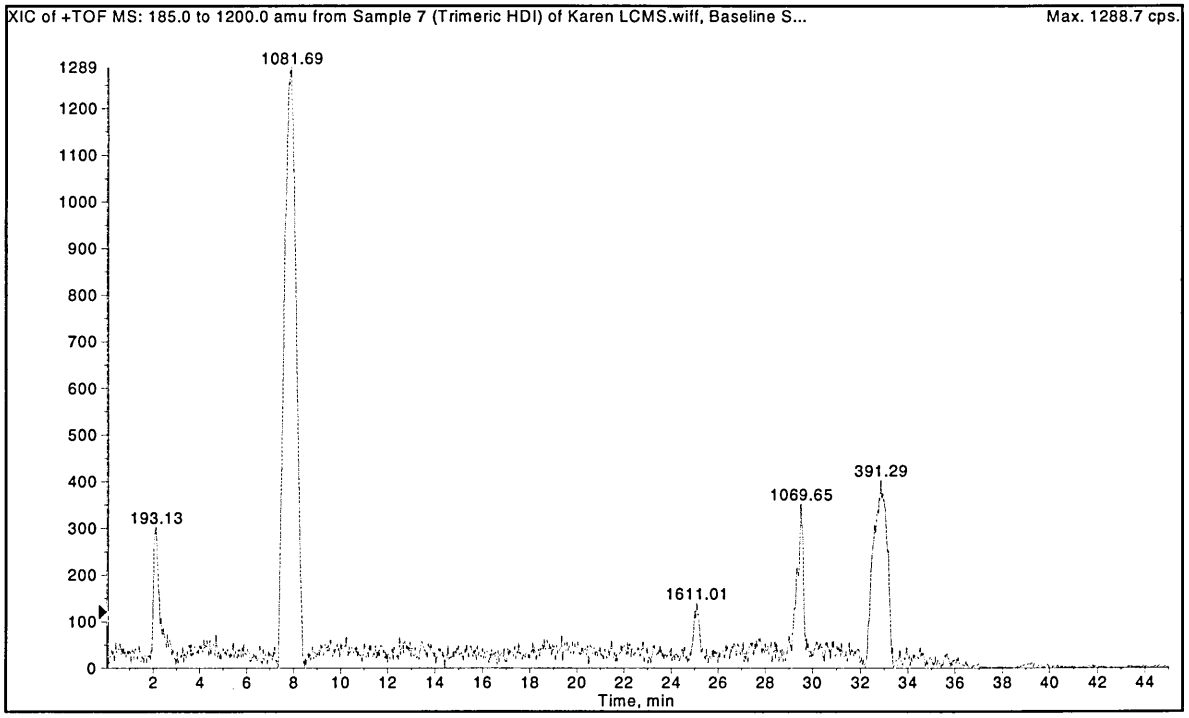


Figure 2.14 LC-MS Extracted Ion Chromatogram of Trimeric HDI. Elution times for derivatising reagent and HDI related polymeric peak 1081 Th are 2.5 and 8 minutes, respectively

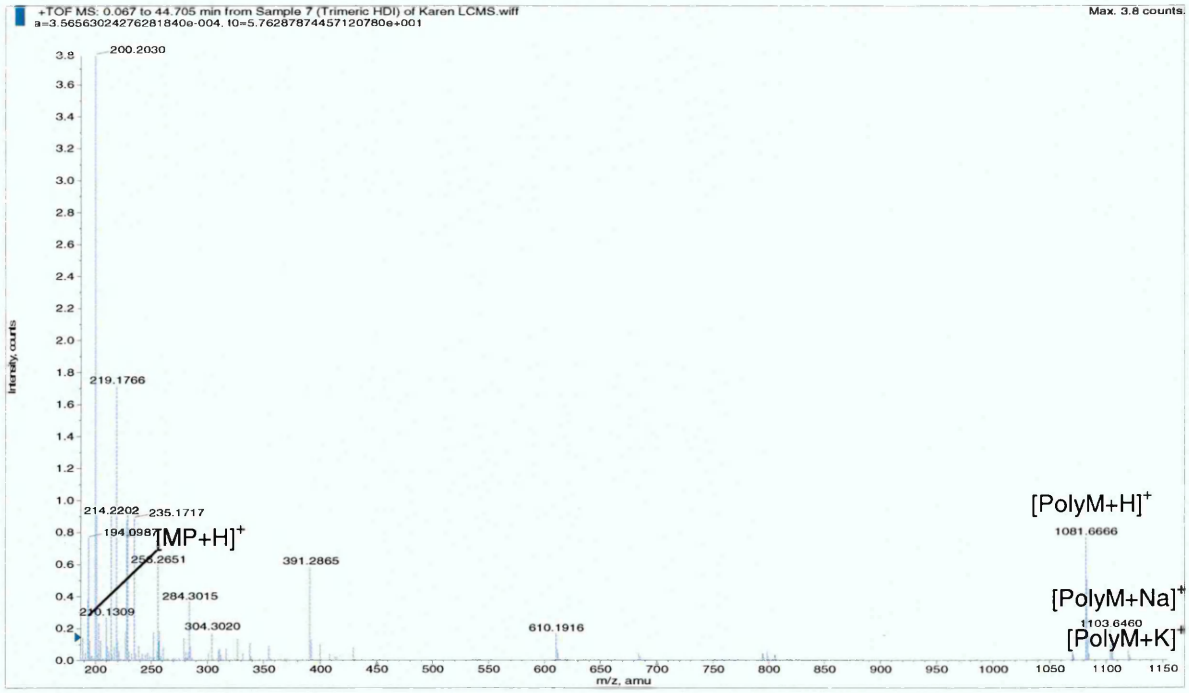


Figure 2.15 Mass Spectrum of trimeric HDI showing a peak corresponding to the protonated elucidated structure of HDI at mass 1081 Th

2.3.4.2 MDI Formulation

Results from MDI analyses seemed to show the MP being detected slightly later than in the HDI samples. Analysis of monomeric MDI proved difficult.

Figure 2.16 presents a base peak chromatogram from the analysis of monomeric MDI. Figure 2.17 shows a representative mass spectrum. MP is present at 193 Th, but no MDI related peaks are visible.

Analysis of polymeric MDI showed a monomer related MDI peak (635 Th) at 4.3 minutes, Figure 2.18. The resultant mass spectrum demonstrates the presence of peaks relating to MP at 193 Th, and the protonated, sodium and potassium adducts of the monomeric MDI molecule (635, 657 and 673 Th, respectively, Figure 2.19).

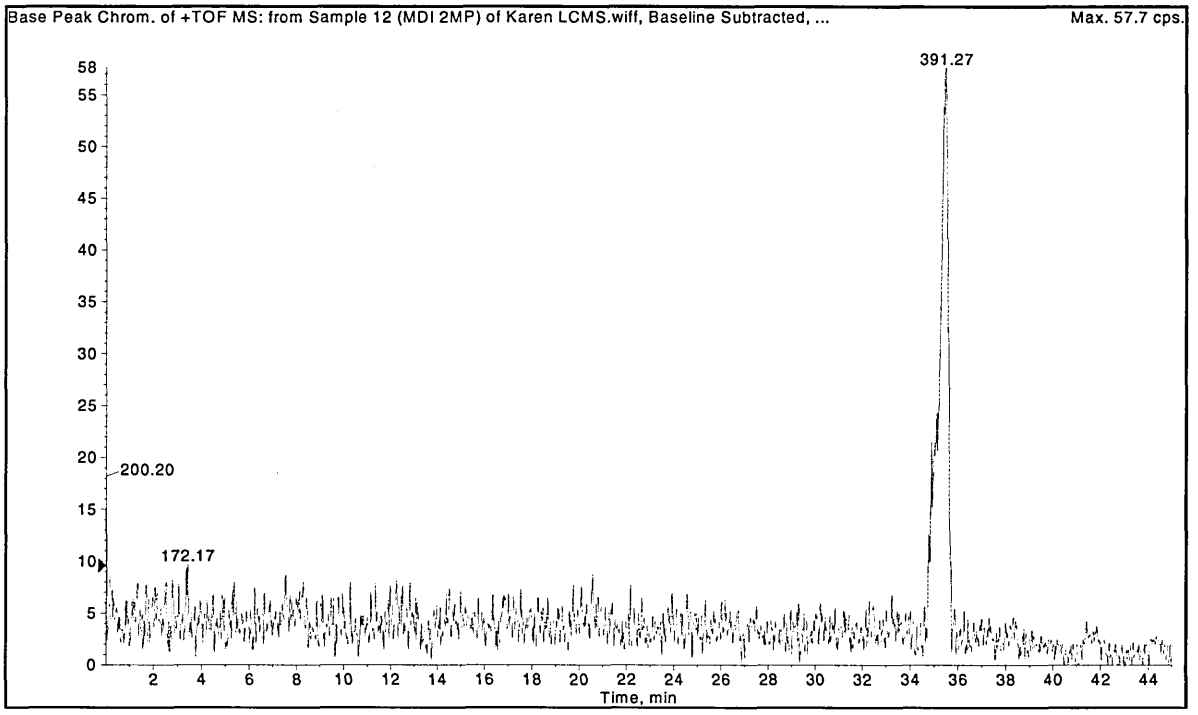


Figure 2.16 LC-MS base peak chromatogram showing no MDI or derivatising reagent related peaks. The samples were prepared as 100 µg/ml solutions in acetonitrile, to be compatible with the mobile phase. The overall run-time was 45 minutes with a sample injection volume of 10 µl

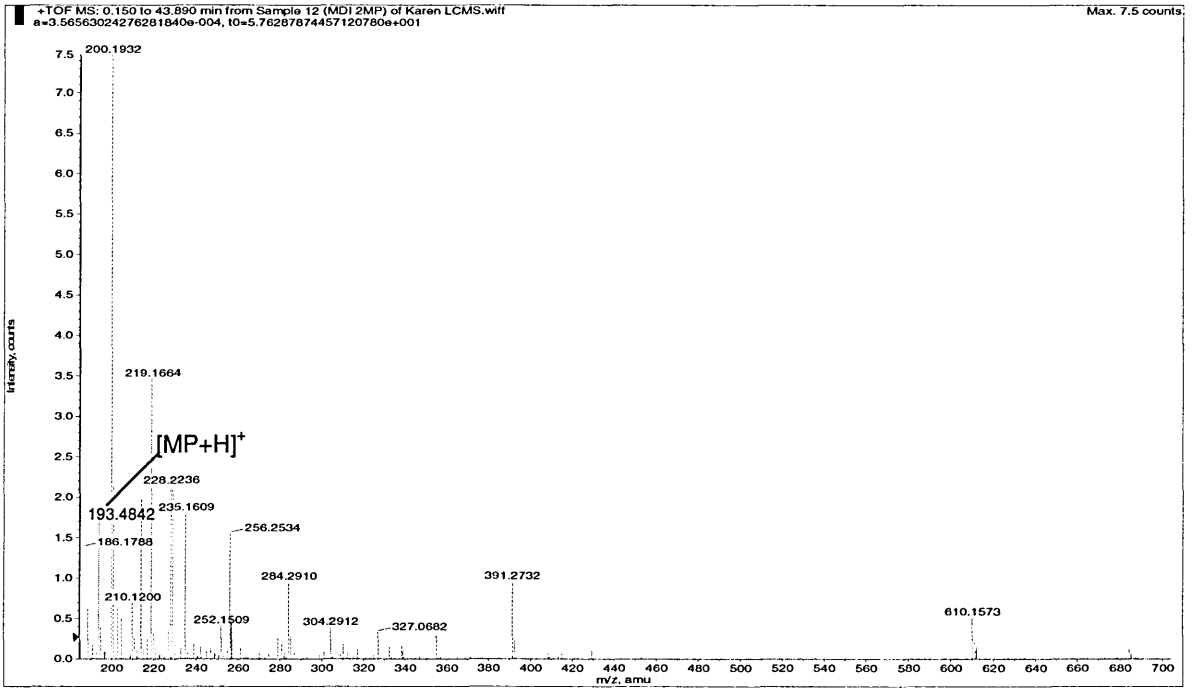


Figure 2.17 Mass Spectrum of monomeric MDI showing only a peak relating to MP at 193 Th

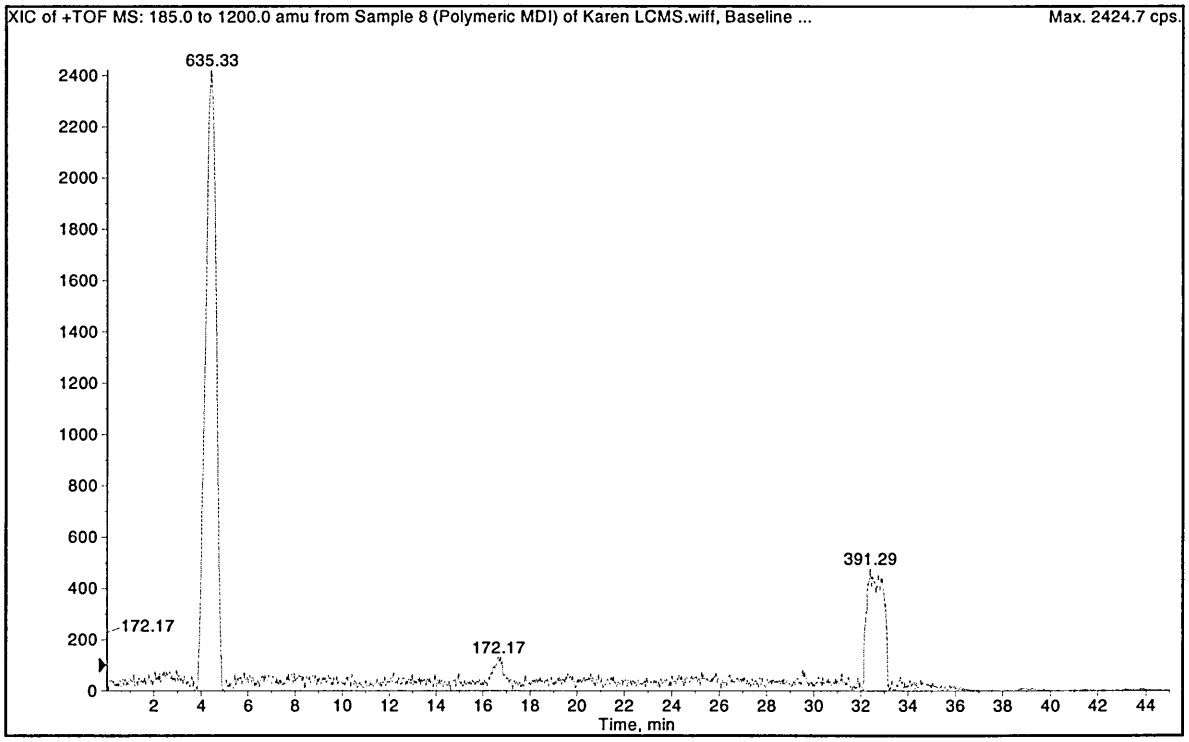


Figure 2.18 LC-MS extracted ion chromatogram of polymeric MDI showing a peak related to monomeric MDI at 4.2 minutes

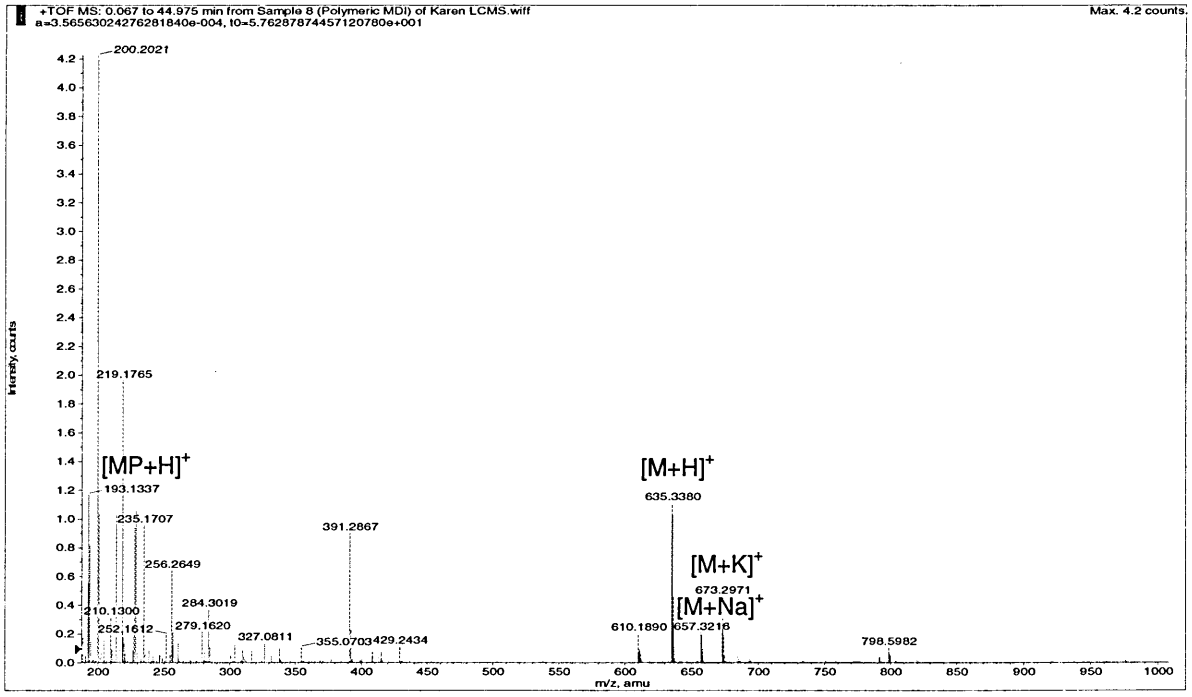


Figure 2.19 Mass Spectrum of polymeric MDI showing a peak relating to MP at 193 Th and monomeric MDI peaks

2.3.5 *Comparison with Previous Work*

The analysis of isocyanate compounds has previously been performed by a diverse range of analytical techniques, to obtain both qualitative and quantitative data. Determination of HDI, MDI and TDI compounds as MP derivatives was first performed using reverse-phase HPLC by Hardy and Walker [3]. Developments in isocyanate analysis saw isocratic methods evolve for the analysis of other monomeric compounds [4]. More recently, gradient HPLC techniques have been used to achieve separation of complex mixtures. These procedures made use of carbon-type columns and acetate buffers, the main portion of the organic eluent consisting of either methanol or acetonitrile [5].

Various derivatising reagents have been utilised for isocyanate analysis, some of the earliest important examples being MP and 9-(N-methylaminomethyl) anthracene, (MAMA) [6, 7]. In studies comparing both limits of detection for isocyanate compounds and the two derivatising reagents by HPLC methods, MP yielded better resolution at the same detection sensitivity than MAMA [8].

Carr and Jackson [9] attempted the direct analysis of pre-polymeric and polymeric MDI by MALDI-MS, without success. Instead of using a derivatising reagent to stabilise the isocyanate compound, this group denatured the compound with methanol forming the respective converted methyl urethane groups. The resultant compounds were named 'methanol-killed' isocyanates. MALDI-MS analysis of the 'methanol-killed' polymeric MDI compounds produced acceptable spectra with the expected peak series of sodiated methyl urethane ions. The polymeric isocyanate pattern allowed Carr and co-workers to follow the compound peak pattern up to a maximum of ten aromatic rings. However, the presence of multiple peaks relating to non methanol affected species (up to nine aromatic rings) and other reaction related products rendered the spectra difficult to interpret. The analysis of 'methanol-killed' pre-polymeric MDI compounds yielded more simple spectra with the most intense peak relating to the sodiated adduct of the intact molecule. The results obtained were used to illustrate the usefulness of MALDI-MS for isocyanate characterisation.

Karlsson et al. combined novel derivatisation, liquid chromatography and mass spectrometric methods for the analysis of complex mixtures of airborne isocyanates [10]. The primary derivatising reagent used in the study was dibutylamine (DBA), as Karlsson and his group discovered that the reaction with DBA was at least twice as fast as the reactions of isocyanate compounds with MP. This LC-MS method aimed to provide separation and detection for complex aromatic isocyanates and mixed isocyanate samples, by drawing upon experience and knowledge and working with Tinnerberg [11]. For quantitation purposes a tetradeuterium-labeled and derivatised isocyanate analogue was incorporated into analysis mixtures as an internal standard.

LC-MS analysis showed an elution order related to increasing molecular mass and the number of isocyanate-derivatised urea groups in the compounds. The tetradeuterium-labeled isocyanate species eluted just prior to the standard derivatised isocyanate. Mass spectrometry produced spectra depicting the expected peaks relating to $[M+H]^+$, $[M+Na]^+$, dimer $[2M+H]^+$ and $[M+H-DBA]^+$ as also seen in the MP analysis of isocyanates discussed in this thesis.

Although quantitative studies of isocyanate compounds have been performed numerous times by differing methods, the underestimation of total isocyanate concentration is of concern. The amount of chemical isocyanate variants and fragment related species in resultant spectra make it almost impossible to ultimately conclude definite isocyanate content. Karlsson's work involved a comparison of DBA derivatising reagent with the more widely used MP. The work uncovered problems with MP as there was an influence from and interfering peaks caused by the derivatising compound. Although the group had success with the tetradeuterium-labeled isocyanate when implemented as an internal standard, they demonstrated the importance of internal standard choice for quantitation purposes.

In this thesis, investigations centred round the more recently developed methods.

A gradient elution technique was employed utilising an acetonitrile mobile phase incorporating an ammonium acetate buffer. The instrument column was a

Hypersil 100 mm, 2.1 μm C18 column, which had been determined as suitable for use with the acetonitrile/buffer solvent system.

MP derivatising reagent was used throughout all the experiments as the resolution and appearance of the derivatising reagent related peaks were ideal for these types of analysis. The MP reagent was added in excess to the samples, but even at low sensitivities this peak was always apparent in resultant chromatograms and spectra. The derivatisation by MP rendered the samples stable, which enabled analysis to be performed. No instability problems were encountered with the derivatised isocyanate compounds and therefore Carr's method of 'methanol-killing' to denature the samples was not investigated. Karlsson and co-workers preferred DBA derivatising reagent for the analysis of isocyanate mixtures, but only due to the speed of the combination reaction. MP derivatisation was adequate for the purposes of this thesis as the samples were obtained as previously derivatised standards. Had the combination of derivatising reagent and isocyanate compound been a part of this study then obviously DBA would have been tested and considered. The use of MP during analyses did not produce any interfering peaks as suggested by Karlsson et al.. All compounds considered as matrices or internal standards were carefully investigated and subsequently eliminated if interference had occurred.

For LC-MS purposes, quantitation was not attempted in this study. However, the diversity found when studying deuterated isocyanate compounds for LC-MS enabled the direct transfer to a MALDI-MS quantitation method, yielding acceptable results.

Quantitation of isocyanate content proved extremely difficult as highlighted by Karlsson and his group. Even with relatively simple mixtures of isocyanates, the number of isocyanate related peaks was immense. The most import peaks relating the protonated molecules were often swamped in spectra by the presence of adduct related peaks and those from isocyanate fragment compounds.

The developed MALDI methods were then applied to the analysis of derivatised swabs from a HSE monitoring scheme, producing viable identification of unknown structures

The hardener used in a spray-painting monitoring process scheme had an unknown content and quantity of HDI compounds as the manufacturers were unwilling to disclose this information. Analysis was performed to identify isocyanates present in the sample. The sample showed a combination of derivatised monomeric and polymeric HDI (Figure 2.20). The mass spectrum shows the monomeric protonated HDI molecule along with the respective sodium and potassium adducts (553, 575 and 591 Th, Figure 2.20 inset). Higher mass polymeric structures are also present up to ~ 1500 Th.

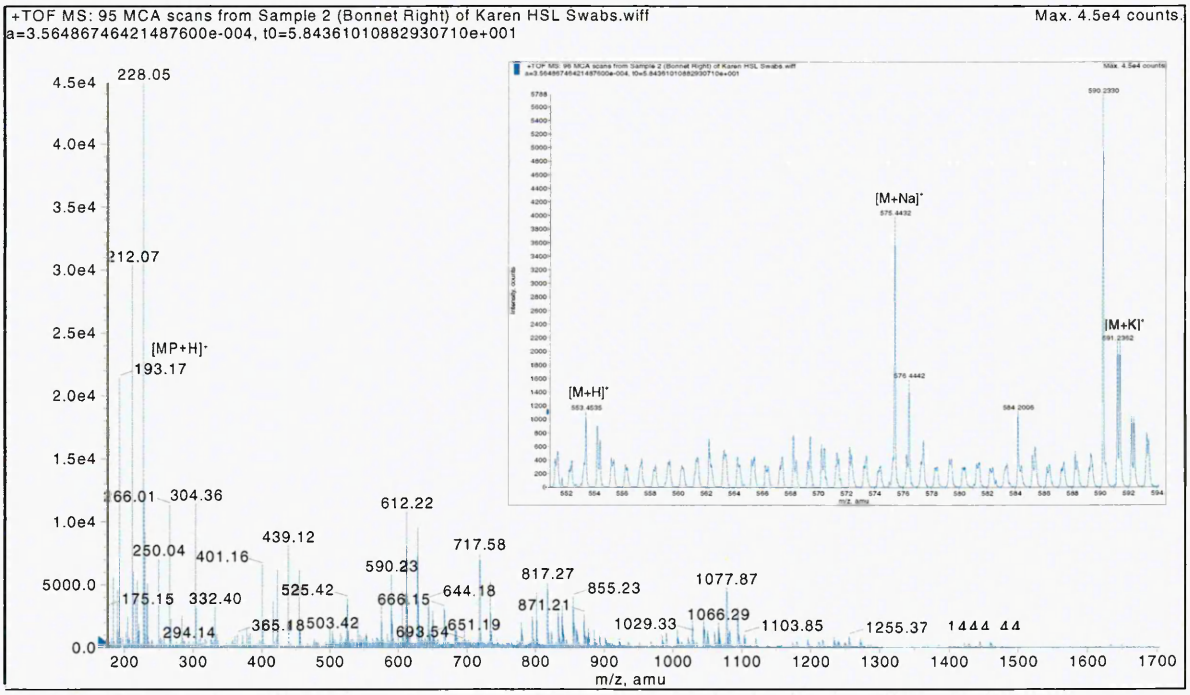


Figure 2.20 A mass spectrum from the analysis of monitoring program swab samples displaying peaks that correspond to the protonated molecule, sodium and potassium adducts of monomeric HDI (553, 575 and 591 Th). Also present are polymeric peak patterns in the higher mass range

2.5 Conclusions

MALDI-MS has successfully been applied to the analysis of NCO monomer and prepolymer samples. NCO monomers have been shown to readily form protonated molecules when subjected to MALDI-MS. The application of MALDI-MS to the analysis of derivatised NCO prepolymers in commercial formulations has been demonstrated. The interpretation of the results from the analysis of commercial formulations has been reported for both unknown HDI and MDI prepolymer samples. The results presented here are only a small fraction of the data produced from the analyses of derivatised commercial formulations, based on the four most commonly encountered NCO monomers (HDI, MDI, 2,4- and 2,6-TDI). Interpretation of mass spectra from MP derivatised NCO is hindered by the readiness of the derivatised NCO to form adducts with sodium/potassium ions and also with excess derivatising reagent present in the sample. These effects are clearly depicted and labeled on the sample spectra.

Analysis of NCO prepolymers using MALDI-TOF-MS-MS has effectively confirmed previously determined structures and also aided the elucidation of other fragment structures from the unknown prepolymer samples. The application of tandem MS by-passed the need for a separate on-line separation technique, proving simple and fairly rapid to execute. Tandem MS enabled fragments from possible structures to be identified, consequently giving rise to proposed prepolymer constituents.

MALDI-MS facilitated the analysis of paint hardener from swab samples to obtain qualitative information about the isocyanate content. Derivatised monomeric and polymeric HDI compounds are both present, but further analysis is needed to determine higher mass polymeric structures.

2.6 References

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Chapter 3

Direct Filter Quantitation

3.1 Introduction

One important factor influencing the correct diagnosis of occupational asthma is the identification of NCO in the workplace atmosphere. Major problems associated with the sampling and analyses of workplace NCO are the high reactivity and relative involatility of these compounds, especially the polymeric NCO widely used in industry. Polymeric NCO (also called oligomeric, oligo-isocyanates, poly-isocyanates and NCO pre-polymers) have greater stability and possess a low vapour pressure. However, when the processes in which polymeric NCO are utilised, for instance spray-painting in workshops, are considered then concerns regarding atmospheric concentrations and workplace exposure are still relevant.

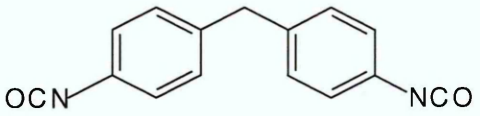
Workplace air sampling of vapour phase NCO is carried out using personal dust samplers (IOM head or similar), attached to the inlet nozzle of a small pump. Workplace air is drawn by the pump, through an MP coated glass fibre filter (GF/A or similar), so that the airborne NCO react with the MP and are trapped. The stable NCO-MP urea can then be transported to the laboratory and analysed. Sampling of NCO in workplace atmospheres is complex. Different chemical forms of NCO require different analytical and sampling conditions i.e. polymeric or monomeric, aliphatic or aromatic NCO. Different physical forms of NCO also require different sampling techniques, for example MDHS 25/3 recommends both vapour sampling using an MP doped filter and mixed phase aerosol sampling using an impinger filter combination.

Karlsson et al have reported the derivatisation of NCO with dibutylamine (DBA) [1] and were the first group to apply liquid chromatography/mass spectrometry (LC-MS) to the determination of NCO.

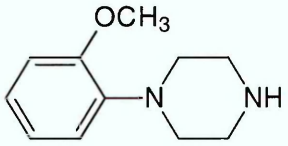
The aim of the work reported in this chapter was to develop a method for the direct quantitation of NCO on air sampling filters, negating the need for lengthy extraction procedures and a chromatographic separation step. The structures of the underivatised NCO studied (MDI), derivatised MDI (MDI-MP), the derivatising agent MP, novel derivatising reagent 1-(2-ethoxyphenyl) piperazine (EP) [2] and the internal standard used (D_6 MDI-MP) are given, Figure 3.1.

Quantitation of MDI-MP was performed on spot samples, using as internal standards, deuterated MDI-MP (D_6 MDI-MP) and MDI derivatised with the novel derivatising reagent 1-(2-ethoxyphenyl) piperazine (EP) to give MDI-EP. Good linearity (R^2 = 0.9664 {D_6 MDI-MP} and 0.9119 {MDI-EP}) and precision (average standard deviation = 0.0907 {D_6 MDI-MP} and 0.0662 {MDI-EP}, n=20) were obtained for MDI-MP on target using these two internal standards.

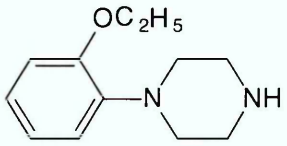
Quantitation of MDI-MP on filters was also performed using a direct filter calibration with D_6 MDI-MP as the internal standard. The degree of linearity (R^2 = 0.8474) and precision of the calibration (ASD = 0.1036, n=5) were acceptable for further quantitation work.



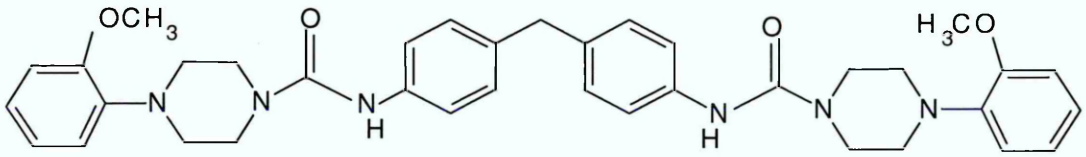
MDI



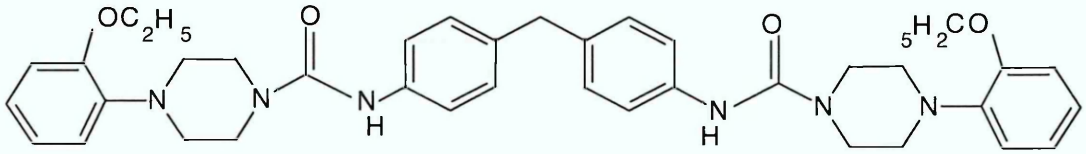
MP



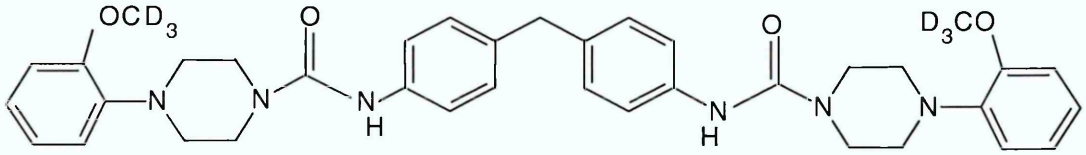
EP



MDI-MP: RMM 634.33



MDI-EP: RMM 662.83



Deuterated MDI-MP: RMM 640.36

Figure 3.1 Structures of the derivatised NCO compounds used

3.2 Experimental

3.2.1 *Chemicals and Reagents*

MP coated sampling filters (Whatman GF/A, prepared as described in MDHS 25/3), MDI-MP and MDI-EP derivatives were prepared by the Health and Safety Laboratory (HSL, Buxton, UK). Deuterated MDI-MP was purchased from Sythelec Ltd (Lund, Sweden). HPLC grade methanol was purchased from Fischer Chemicals (Loughborough, Leicester, UK). Trifluoroacetic acid (TFA), α-cyano hydroxy cinnamic acid (α-CHCA, organic acid MALDI matrix), underivatised MDI, MP and EP (hydrochloride salt) were obtained from Sigma Aldrich (Poole, Dorset, UK).

3.2.1.1 Preparation of Derivatised Standards and Matrix

Stock solutions of MDI-MP, D_6-deuterated MDI-MP and MDI-EP were prepared at 1mg/ml in methanol (0.1% TFA). α-CHCA matrix was dissolved in methanol (0.1% TFA), 25 mg/ml. Problems arose when preparing these solutions, especially with the NCO compounds. It was therefore necessary to sonicate several samples for periods of up to 5 minutes to ensure the samples dissolved. Care was taken to maintain the sonic bath temperature at 37°C to minimise evaporation and the possibility of degradation.

3.2.2 *Instrumentation*

All analyses were performed using an Applied Biosystems (Foster City, USA)/MDS Sciex (Ontario, Canada) "Q-Star" hybrid quadrupole time of flight instrument, fitted with an orthogonal MALDI ion source and ion imaging software. This instrument utilises a nitrogen laser with a 337nm wavelength. The instrument resolution is 10,000 fwhm and the mass calibration is adjusted as required. The normal calibration is performed by product ion scan of renin substrate (peptide species).

Data was collected and manipulated using the 'Analyst–QS' software supplied with the instrument.

The air gun employed was a Badger 100-G-F Gravity Feed, with a medium head and 200 μl solvent reservoir cup (The Airbrush Company, Lancing, West Sussex, UK).

3.2.3 *Analytical Procedure*

3.2.3.1 Matrix Coverage and Filter Derivatisation

It is essential that the matrix coverage is as homogeneous and complete as possible to enable reproducible ionisation of the analyte, by laser, over the whole filter area. It was also of interest to investigate the efficiency and uniformity of the MP coating process described in MDHS 25/3.

The MP coated filters were air-sprayed with 5 coats of α-CHCA (250 μl per layer = 1250 μl) and left under a heat lamp to dry between coats.
The filter was then mounted onto a recessed stainless steel MALDI target with double-sided sticky tape and imaged by moving the sample stage at 0.5 mm increments to build up a pixel representation. The laser power was set to 25%/14.3 μJ, with a repetition rate of 1000 Hz.

Before NCO analysis could continue, it had to be determined if the filter doping method carried out by the HSL successfully produces even coverage. It was thought that the distribution of MP might be a 'doughnut' shape, sparse in the centre and around the edge but with an intense ring pattern. The filter was imaged in the same way as described previously.

3.2.3.2 Preliminary MDI-MP Analysis

5 μl of 1 mg/ml MDI-MP was dropped into the centre of an MP impregnated, matrix coated filter with a micro-pipette tip and an imaging run was carried out. It was again thought that a 'doughnut' shape would occur.

3.2.3.3 Concentration Range Studies

The MP impregnated filter was coated with 25 mg/ml α-CHCA matrix by air-spray deposition (5 layers, left to dry under a heat lamp between coats). 1 μl

aliquots of MDI-MP standard solutions (concentrations of 1, 0.8, 0.6, 0.4 and 0.2 mg/ml) were spotted onto the filter, giving NCO amounts of ~132, ~106, ~80, ~53 and ~26 ng "on filter" (RMM MDI-MP 634, "RMM" NCO group = 42, MDI is a di-isocyanate, so 1 g MDI-MP is equivalent to 132.3 mg of NCO). The filter was then imaged by moving the sample stage, increment size of 0.5 mm and a laser power of 25%, 14.3μJ.

For quantitation by MALDI-MS it is usually necessary to use an internal standard to compensate for differences in sample/matrix surface homogeneity. Calibration was attempted using the 190 Th α-CHCA matrix ion as an internal standard for the MDI-MP compound. MDI-MP stock solutions (200 μl) were mixed with 80 μl α-CHCA matrix and 1 μl of this resultant mixture was spiked onto the stainless steel target. The MDI-MP range of amounts "on the target" was ~0.71, ~0.57, ~0.43, ~0.29 and ~0.14 μg, which corresponds to ~95, ~76, ~57, ~38 and ~19 ng of NCO. Twenty spots for each concentration level were analysed on a spot target using an automated search pattern with a 1 minute acquisition time. The analyte:matrix peak area ratio for each spot was plotted against the concentration of NCO used, to provide a concentration curve for use in further quantitation studies.

NCO-MP derivatives (HDI-MP and naphthyl diisocyanate-MP) were tested for use as an internal standard for the MALDI-MS analysis of MDI-MP but were found to be unsuccessful. The use of a deuterated analogue of the NCO compound under study as an internal standard has been reported previously [3, 4] and this approach was investigated by employing a deuterated analogue of MDI-MP (D_6 MDI-MP) for a separate quantitation calibration. These experiments were carried out using the method described previously in this section. MDI-MP stock solutions (200 μl of 1 to 0.2 mg/ml) were mixed with α-CHCA matrix (100 μl) and D_6-MDI-MP (200 μl) and 1 μl of the mixture spiked onto the target, giving amounts of NCO "on filter" of ~53 to ~11 ng. For each MDI-MP level, 20 spots were analysed, utilising an automated search pattern. The average analyte:internal standard peak area ratio was plotted to give a calibration curve, utilising the sodium adduct peak areas due to their superior spectral intensities for all analyses.

Again employing the method of 20 spot repetitions and an automated search pattern, MDI-EP was added to the sample mixture to be investigated as an internal standard. The concentrations used were as described earlier in this section. The average analyte:internal standard peak area ratios were charted against the initial concentration of NCO (sodium adduct peak areas).

3.2.3.4 Filter Calibration using a Deuterated Analogue Internal Standard

A filter calibration was performed to enable quantitation via direct method comparison. The spot sample calibration could not be applied to filter analysis due to large differences in ionisation efficiency. The peak intensities obtained from spot samples were much greater than for filter analysis work. Filters were spiked with 200 µl of varying MDI-MP concentrations (0.2 - 0.04 mg/ml, 5 filters for each) giving rise to amounts of NCO "on filter" of ~5 to ~1 µg. These filters were coated with a mixture of matrix and deuterated internal standard (1050 µl matrix with 200 µl internal standard), mounted and imaged as described in section 3.2.3.1 with an increment size of 2mm. The mass spectra were summed and the analyte:internal standard peak area ratios calculated (using the most abundant sodium adduct peaks). The average peak ratios were plotted against the original NCO concentration applied.

3.2.3.5 Acquisition Time Investigation

An investigation was performed to deduce whether the imaging increment size and consequently the time taken for analysis would have a significant bearing on the calculated results. Four identical filters (in triplicate) were prepared by adding 100 µl of MDI-MP solution (1 mg/ml, 13.2492 µg NCO) and coating the filters with a mixture of matrix and deuterated internal standard (using the method from section 3.2.3.4). The filters were imaged as described in section 3.2.3.1 with varying increment sizes. The increments used were 0.5, 1, 2 and 4 mm. The mass spectra obtained were summed and the analyte:internal standard peak area ratios were calculated (using the sodium adduct ion peaks).

3.2.3.6 WASP Filter Quantitation

By implementing previously developed methods and calibrations it was possible to quantify the MDI-MP concentrations from actual sample filters in the WASP analysis scheme (Workplace Analysis Scheme for Proficiency). The MP impregnated WASP sample filters were coated with 5 x 250 µl aliquots of an α-CHCA matrix (25 mg/ml, 1050 µl) / internal standard (0.5 mg/ml, 200 µl) mixture, again left to dry under a heat lamp between coats.

3.3 Results and Discussion

3.3.1 *Matrix Coverage and Filter Derivatisation*

Both the α-CHCA matrix application and the MP coating methods were shown to be successful. The images of the selected ions (α-CHCA = 190 Th and MP = 193 Th) show a homogeneous and complete coverage of the filter surface. The expected 'doughnut' shape of MP was not observed (Figures 3.2 and 3.3).

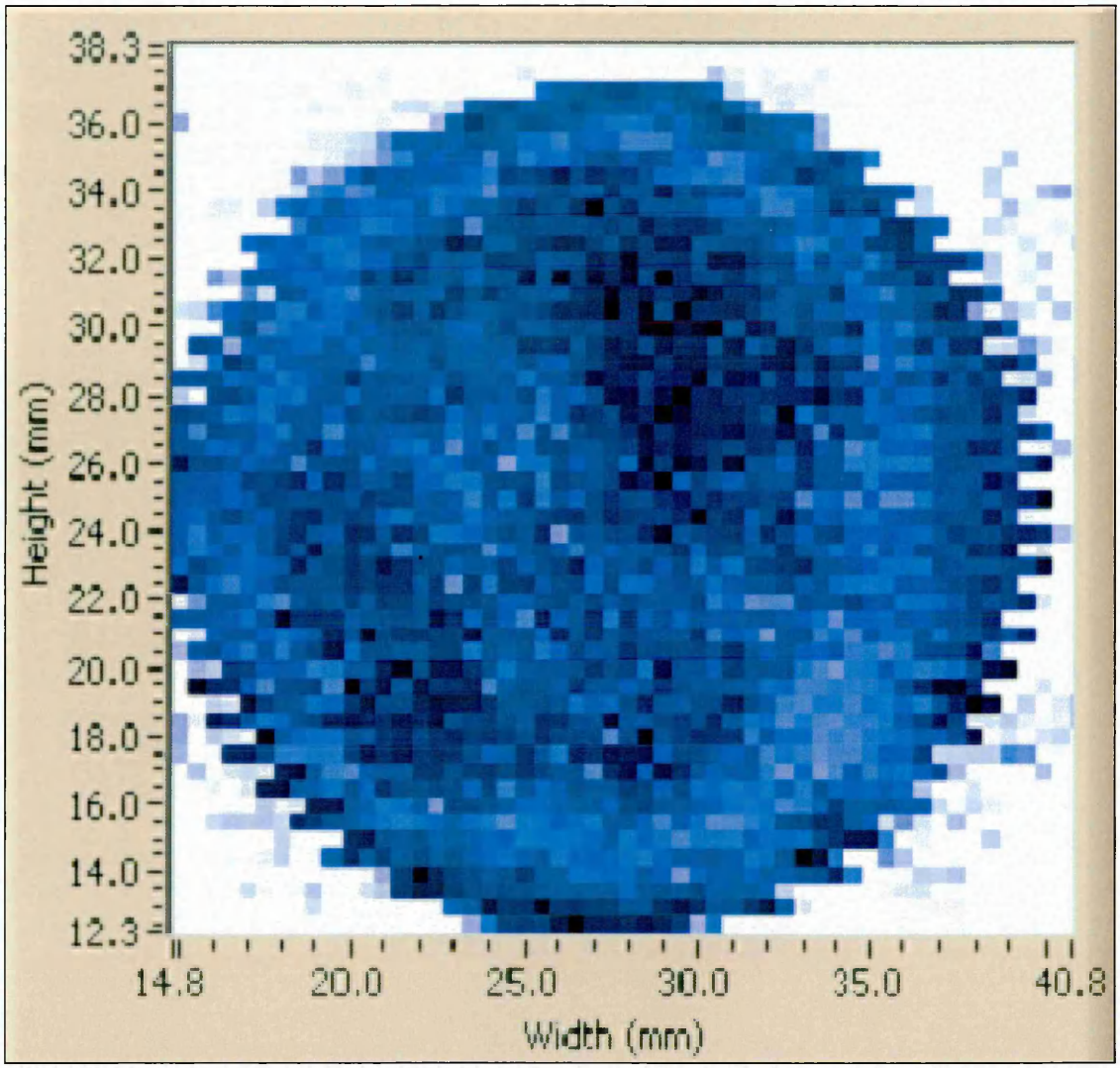


Figure 3.2 Image of the protonated ion of α-CHCA matrix from deposition onto an MP coated filter (190 Th)

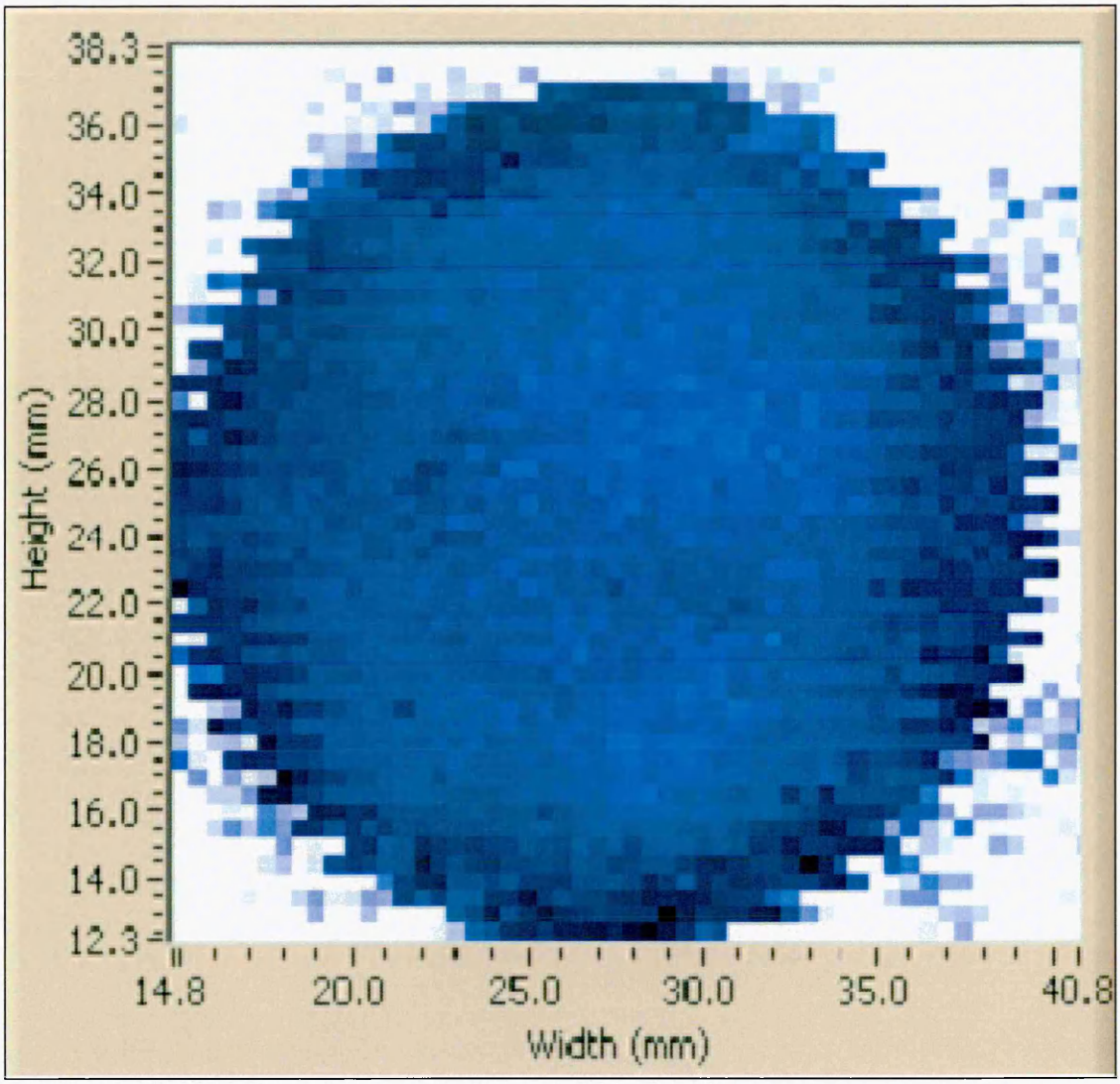


Figure 3.3 Image of the MP derivatising reagent protonated ion from an MP coated sample filter (193 Th)

3.3.2 *Preliminary MDI-MP Analysis*

In this case, the predicted "doughnut" pattern for the MDI-MP derivative was apparent in the image (Figure 3.4). A representative spectrum shows the presence of protonated MP (193 Th), protonated α-CHCA (190 Th) and protonated MDI-MP (635 Th) (Figure 3.5). However, these experiments clearly showed that MALDI-MS is able to image MDI-MP from a matrix coated filter.

The difference in pattern is probably due to the different volumes used to dope the filters with MP (200 µl) [5] and spike on the MDI-MP derivative (5 µl) (see section 3.2.3.2). The larger volume used to coat the filters with MP is sufficient to saturate the filter leading to homogeneous coverage. This can be clearly

observed when the MP doping process is being carried out. The smaller volume used to spike on the MDI-MP does not saturate the filter and the MDI-MP is deposited at the edge of the solvent front leading to a "doughnut" shaped ring. A similar effect has previously been noticed, although not documented, during preparation of NCO-MP spiked filters for the HSL WASP (Workplace Analysis Scheme for Proficiency) quality assurance scheme.

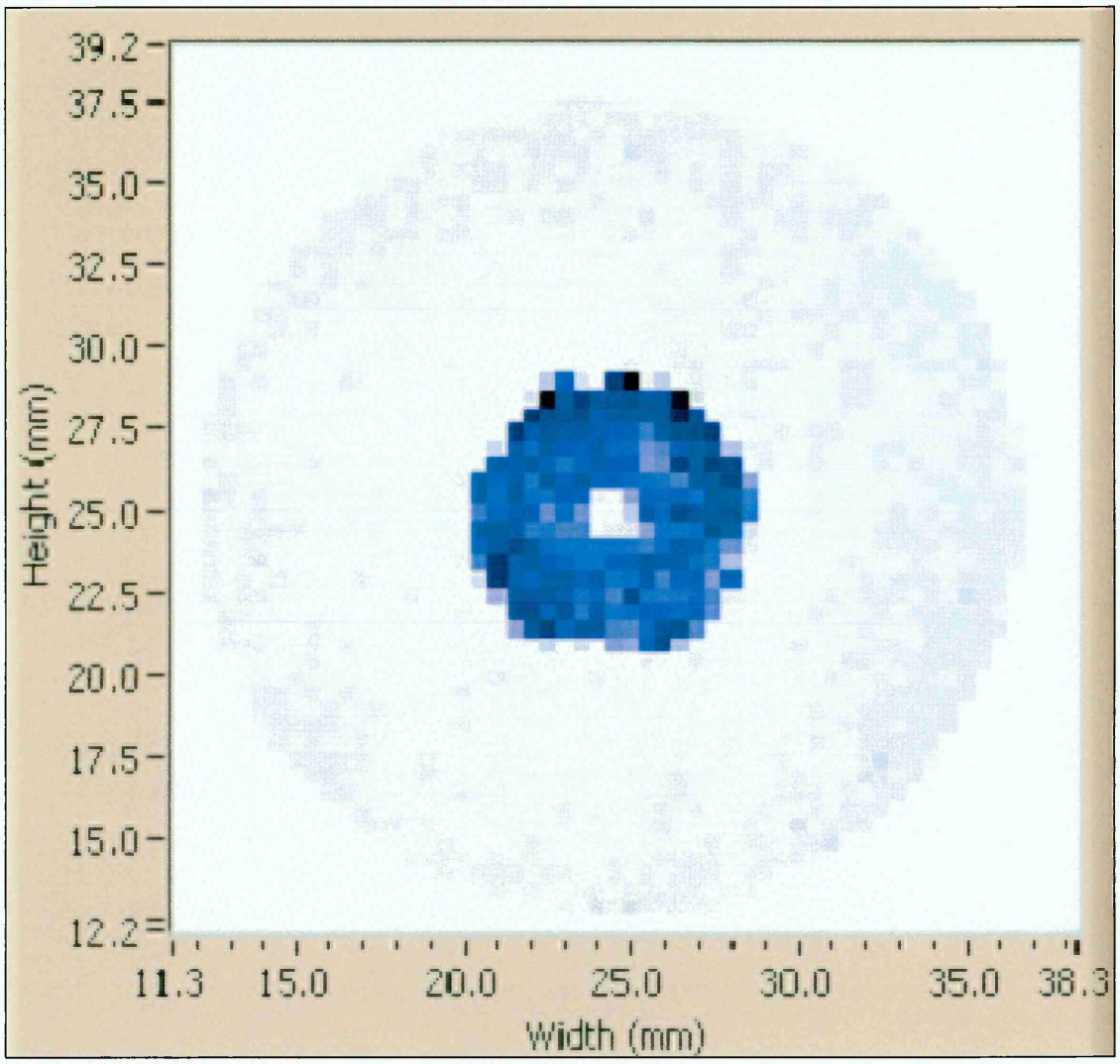


Figure 3.4 Pixel orientation of the protonated MDI-MP analyte ion at 635 Th on the filter surface

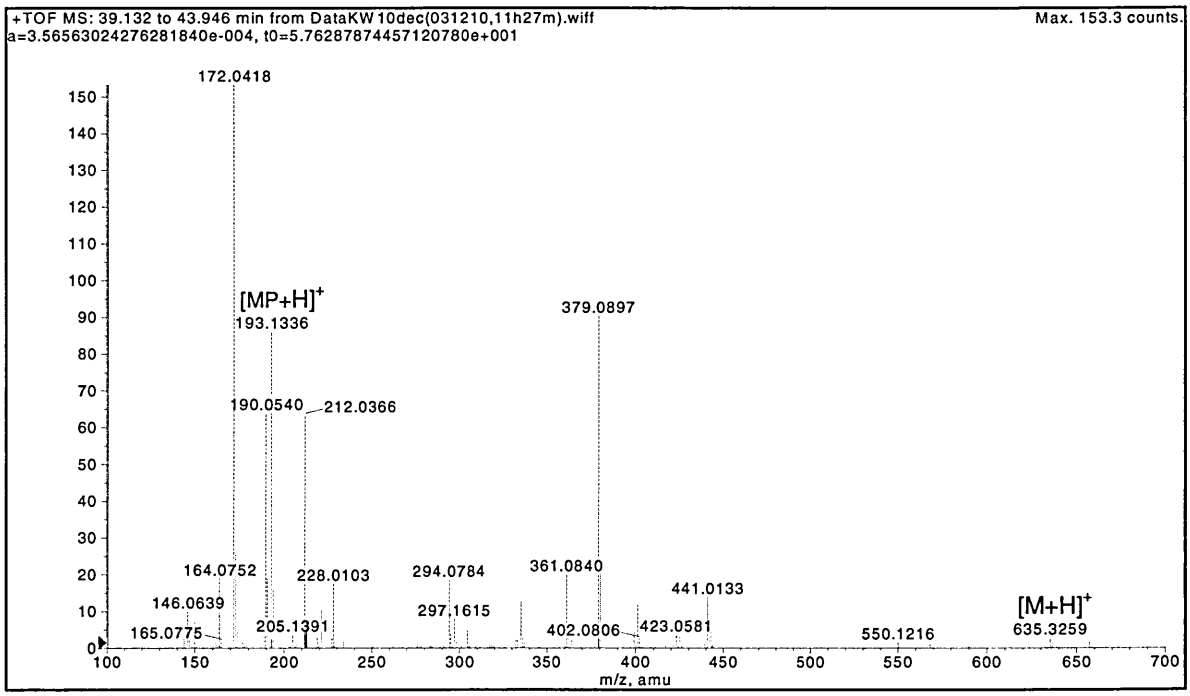


Figure 3.5 Representative spectrum from the MDI-MP spiked filter, showing protonated peaks corresponding to MP (193 Th), α-CHCA (190 Th) and MDI (635 Th)

3.3.3 *Concentration Range Study*

The five areas of MDI-MP compound could clearly be seen on the filter; however, the pixel image intensities do not directly relate to the concentration of NCO applied (Figure 3.6). This is almost certainly due to the lack of an internal standard and variability of ionisation of the MDI-MP from the matrix coated filter by the laser.

In the UK the HSE has set a STEL for NCO of 70 μg/m^3 for a 15 l sample [6]; this corresponds to 1.05 μg NCO "on filter". Therefore, the direct filter analysis methodology is capable of detecting NCO in air at levels relevant to the workplace.

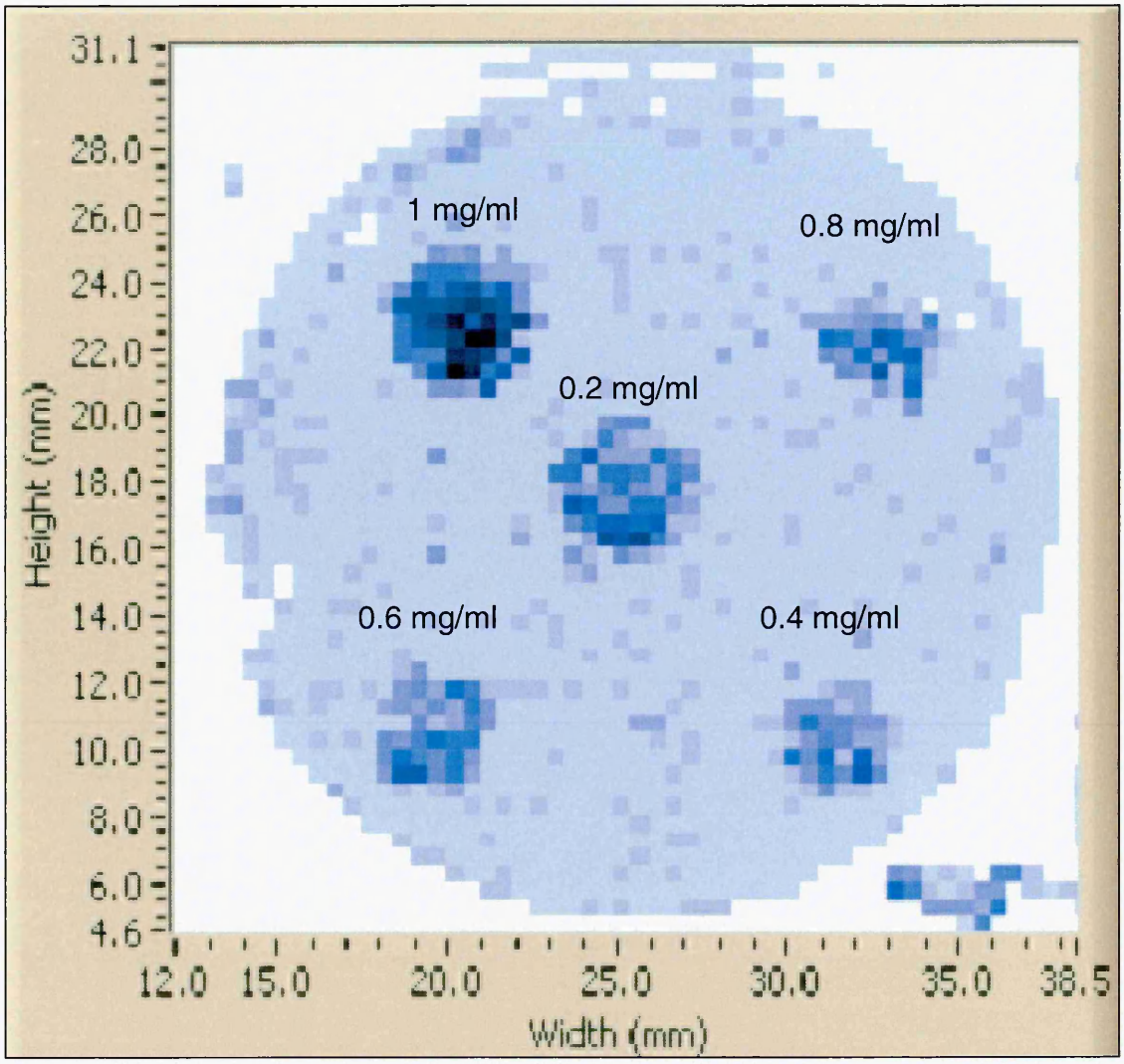


Figure 3.6 Image showing five areas of MDI-MP on the matrix coated filter

3.3.3.1 Quantitation Using α-CHCA Matrix as the Internal Standard

The calibration curve obtained (Figure 3.7) showed a certain degree of inter-sample reproducibility but the linearity was deemed to be unacceptable for quantitation.

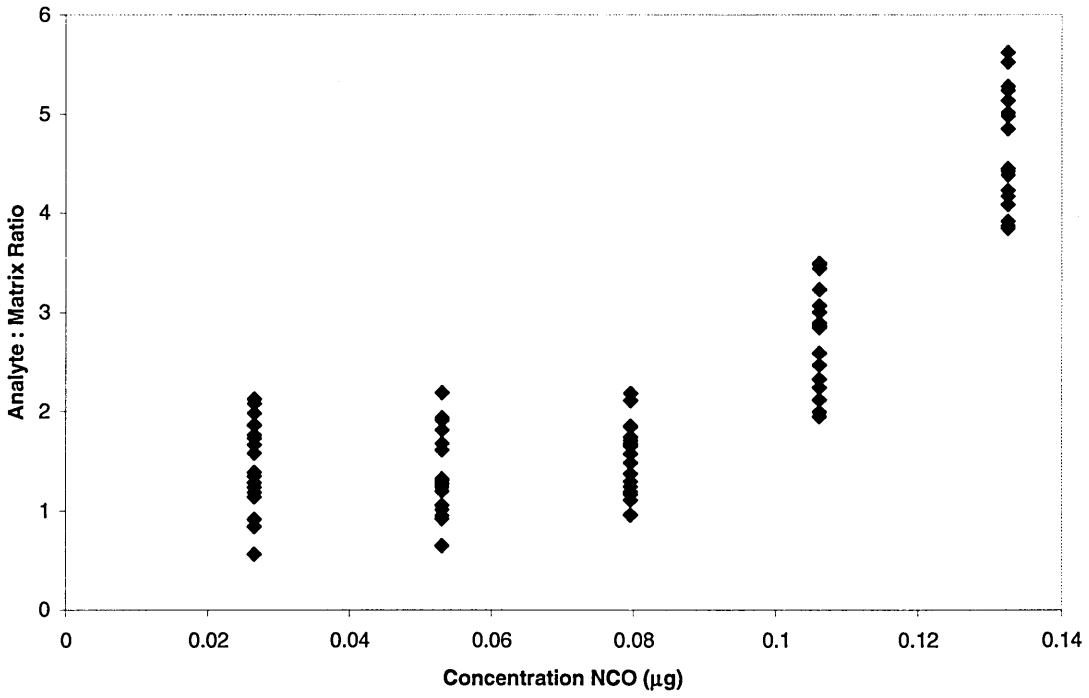


Figure 3.7 MDI-MP calibration curve using the protonated α-CHCA ion (190 Th) as an internal standard for the NCO compound

3.3.3.2 Quantitation Using Deuterated Analogues

The average sodium adduct peak area ratios depicted on the chart show good linearity up to 0.8 mg/ml (~42 ng NCO, Figure 3.8). After this point the calibration seems to arrive at a saturation point and the chart levels off. The 1 mg/ml (53 ng NCO) amount has therefore been excluded. The degree of linearity (R^2) and precision (Average Standard Deviation, n=20) were calculated from the graph. The results were $R^2 = 0.9664$ and ASD = 0.0907. A spectrum showing the protonated molecular peak $[M+H]^+$, and peaks corresponding to sodium $[M+Na]^+$ and potassium adducts $[M+K]^+$ for both MDI and its deuterated analogue is shown (Figure 3.9). The assignments for these peaks are given in Table 3.1.

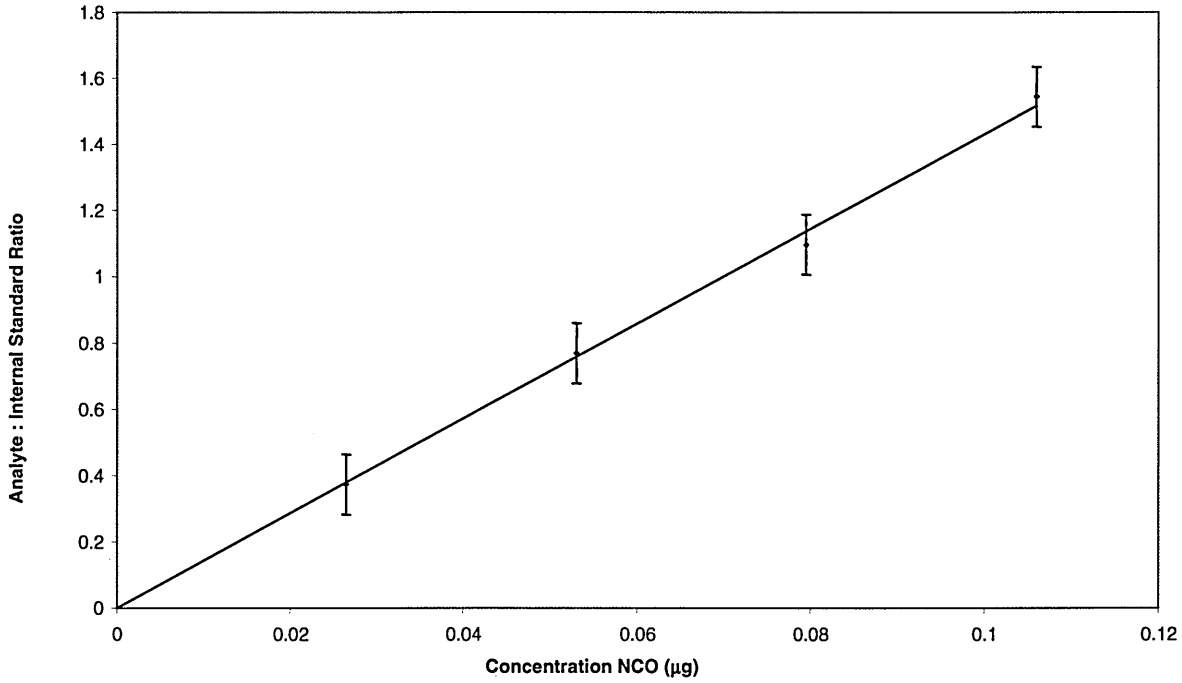


Figure 3.8 Average MDI-MP calibration curve using the sodium adduct (663 Th) from the deuterated analogue (D_6 MDI-MP) as the internal standard for the sodium adduct of MDI-MP (657 Th)

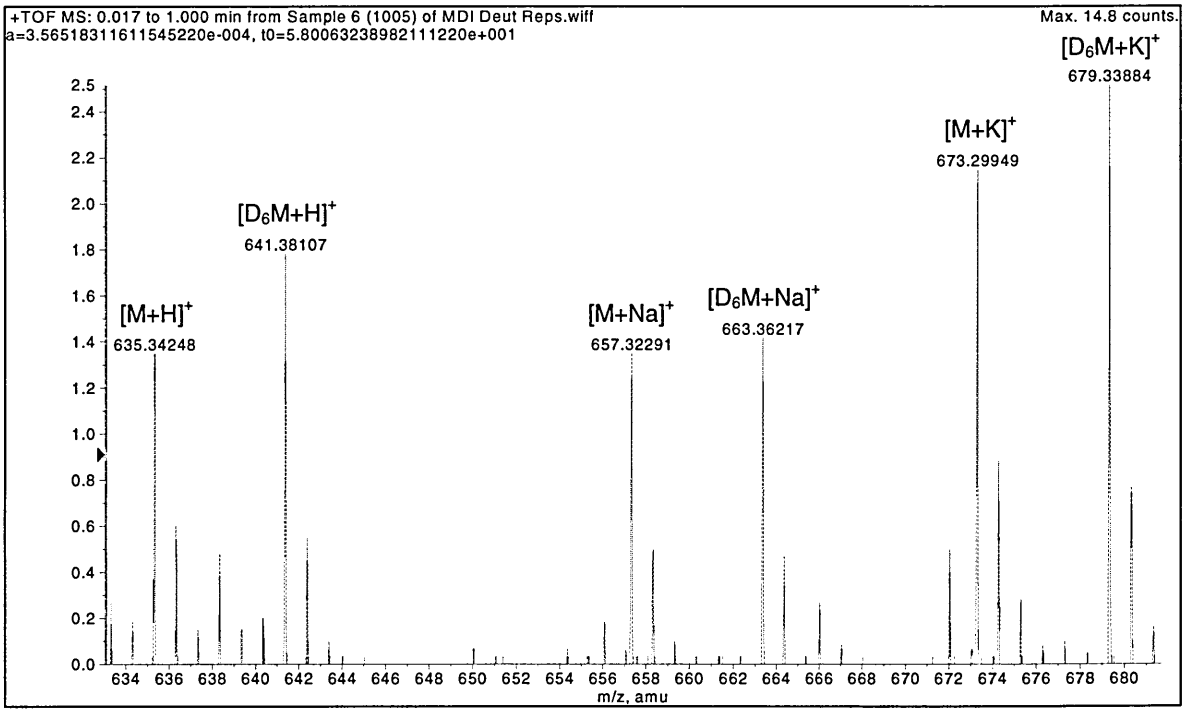


Figure 3.9 A representative spectrum from the MDI-MP calibration with D_6 MDI-MP as the internal standard

	m/z
Protonated MDI-2MP	635
Deuterated MDI-2MP	641
Sodium Adduct	657
Deuterated Sodium Adduct	663
Potassium Adduct	673
Deuterated Potassium Adduct	679

Table 3.1 Assignments for the protonated ion, sodium and potassium adduct peaks for MDI-MP and its deuterated analogue

3.3.3.3 Quantitation Using MDI-EP as an Internal Standard

The average peak area ratios depicted on the curve show good linearity up to 1mg/ml (Figure 3.10). The degree of linearity (R^2) and precision (Average Standard Deviation, n=20) were calculated from the graph. The results were R^2 = 0.9119 and ASD = 0.0662. A spectrum showing the protonated molecular peak $[M+H]^+$, and peaks corresponding to sodium $[M+Na]^+$ and potassium

adducts $[M+K]^+$ for both MDI and MDI–EP is shown (Figure 3.11). The assignments for these peaks are given in Table 3.2.

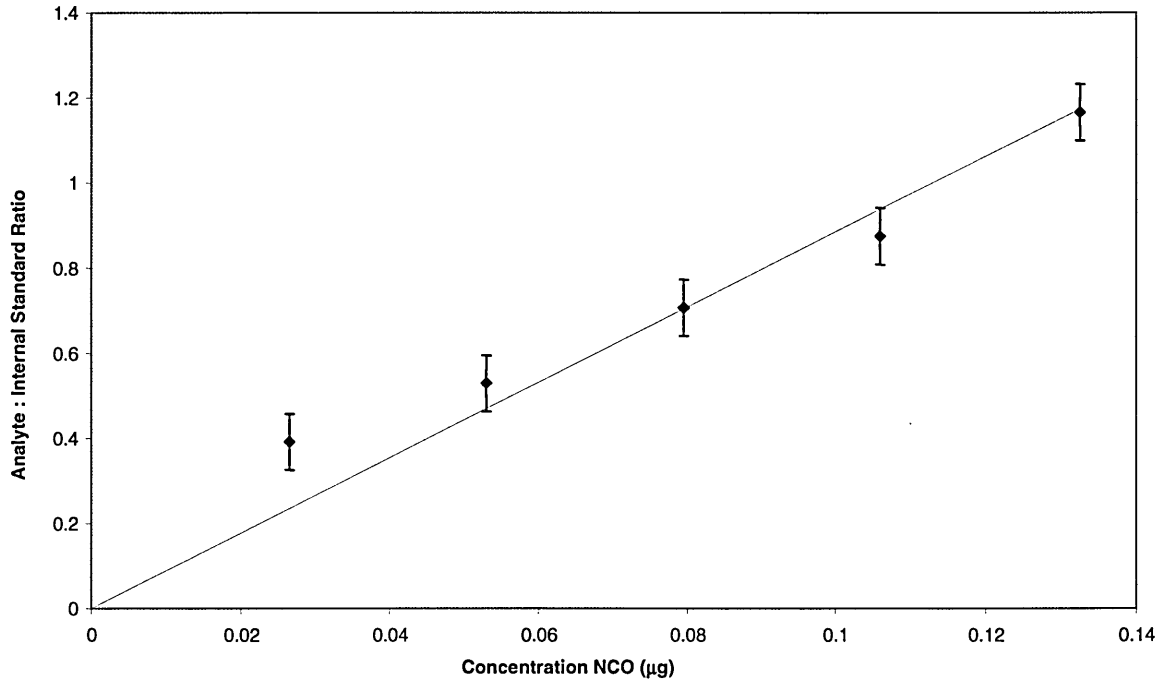


Figure 3.10 Average MDI-MP calibration curve using the sodium adduct (685 Th) from MDI–EP as the internal standard for the sodium adduct of MDI-MP (657 Th)

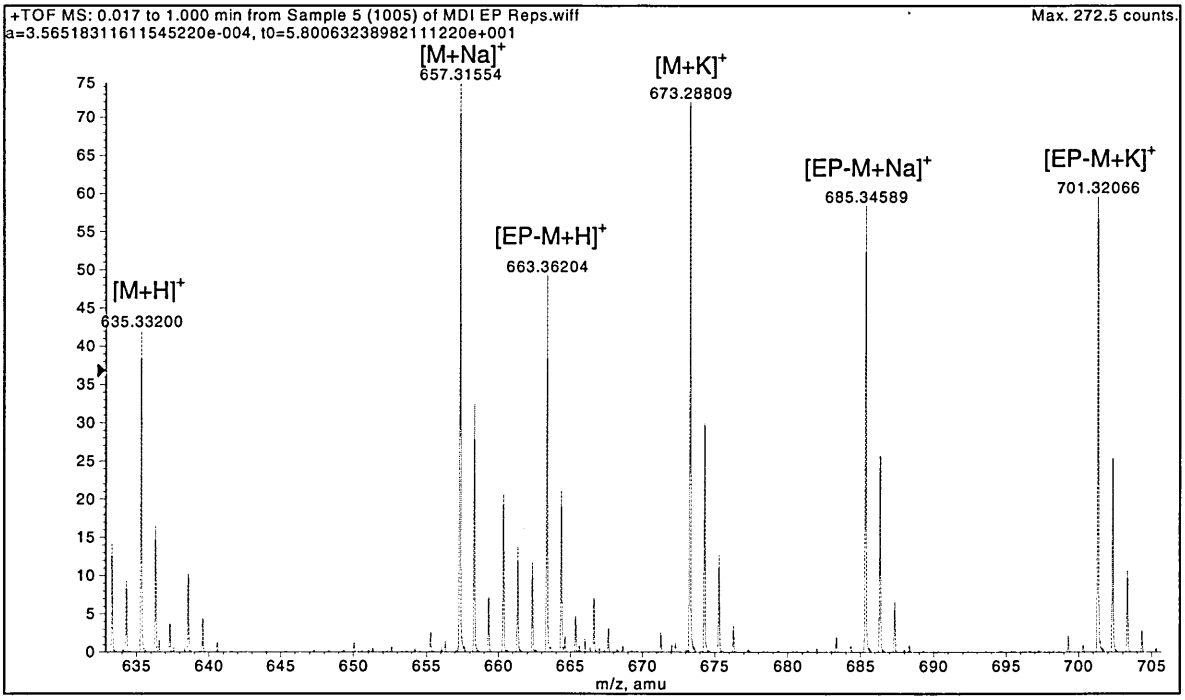


Figure 3.11 A representative spectrum from the MDI calibration with MDI-EP as the internal standard

	m/z
Protonated MDI-2MP	635
MDI-2EP	663
Sodium Adduct	657
MDI-2EP Sodium Adduct	685
Potassium Adduct	673
MDI-2EP Potassium Adduct	701

Table 3.2 Assignments for the protonated ion, sodium and potassium adduct peaks for MDI-MP and MDI-EP internal standard

3.3.5 *Acquisition Time Investigation*

The filter runs were performed to enable the assessment of increment size on calculated filter ratio. Table 3.3 depicts the gathered results for the four filters, showing average analyte:internal standard peak area ratios (sodium adducts). As the table shows, the change in increment size does not alter the calculated isocyanate results greatly. For the 0.5 mm increment, analysis time was

approximately three hours. The duration of analysis at 4 mm increments was less than a minute. It was decided that all further imaging work of the filters would be carried out with 2 mm increments, giving a sample run-time of roughly eight minutes.

Increment Size (mm)	Analyte Peak Area	Internal Std Peak Area	Analyte : I Std Peak Area Ratio	NCO on Filter μg
0.5	0.9779	1.8097	0.5404	7.1293
1.0	0.9099	1.7260	0.5272	6.9551
2.0	0.8575	1.6957	0.5057	6.6715
4.0	0.8680	1.4118	0.6148	8.1108

Table 3.3 Calculated average analyte:internal standard peak area ratios for four filters from an acquisition time investigation, each filter run in triplicate

3.3.4 *Quantitation Using a Filter Calibration and Deuterated Analogues*

A filter calibration was performed to enable quantitation of real filter samples. It was thought that using calibration data generated from stainless steel spot targets was not likely to be suitable for analysis of sample filters, due to the higher ion yields obtained from the metal target surface. The calibration graph (Figure 3.12) depicts the average analyte:internal standard peak area ratios (sodium adducts) versus amount of NCO on filter. The degree of linearity and precision (average standard deviation, n=5) were considered acceptable with values for $R^2 = 0.8474$ and ASD = 0.1036, respectively.

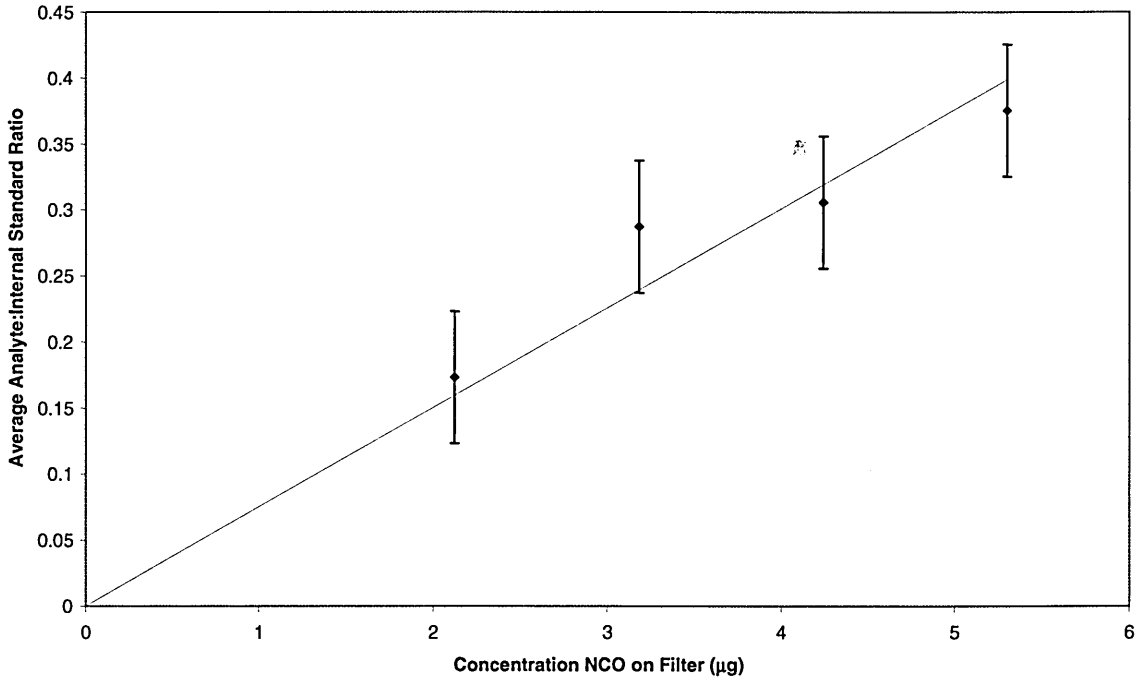


Figure 3.12 Average results for a filter calibration curve using the sodium adduct (641 Th) from D_6 MDI-MP as the internal standard for the sodium adduct of MDI-MP (657 Th)

3.3.6 *Data Extraction and Processing Methods*

Pixel Selection

The first method utilised the enlarged pixels from each of the five concentration spots from the original filter (Section 3.3.3) to collate analyte and matrix peak areas from the relevant spectra. The values were collected from 36 pixels within each spot, then the averaged analyte:matrix ratio was plotted against original isocyanate concentration. The calibration constructed using this method was not suitable for quantitation purposes. Figure 3.13 shows the pixel selection process utilised to gather peak area data.

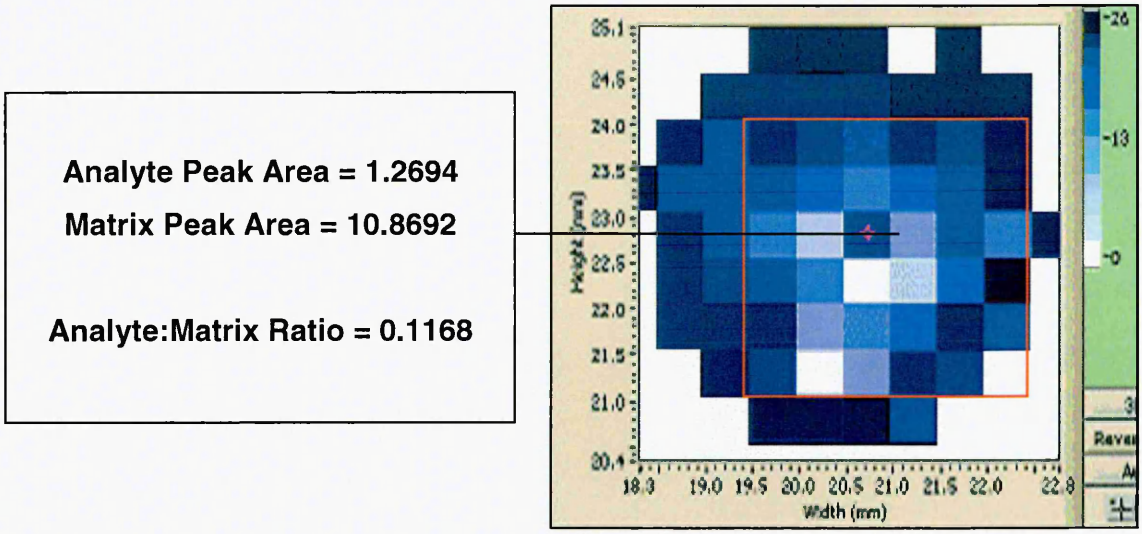


Figure 3.13 Selection process for concentration range spots on spiked sample filter

Summed Peak Intensities

This method used the averaged spectrum from an entire filter image of a prepared HSL standard filter range. The summed intensities from the filter spectrum (analyte and internal standard peaks) were used to form a calibration. The peaks employed were those of the protonated, sodiated and potassiated ions from the intact molecules for both analyte and the deuterated internal standard (635, 657 and 673 Th MDI-MP and 641, 663 and 679 D_6 MDI-MP). Calibration curves were constructed (Figures 3.14, 3.15 and 3.16) for quantitation purposes. The HSL filter sets contained 4 prepared filters each with a known concentration of NCO. Each set contains a high, low and two medium concentration filters. The initial idea was to construct a calibration curve using three of the filters and if successful, use the calibration to quantify the second intermediate concentration filter. Three filter rounds were investigated (57, 58 and 62), the concentration values of which are shown in Table 3.4.

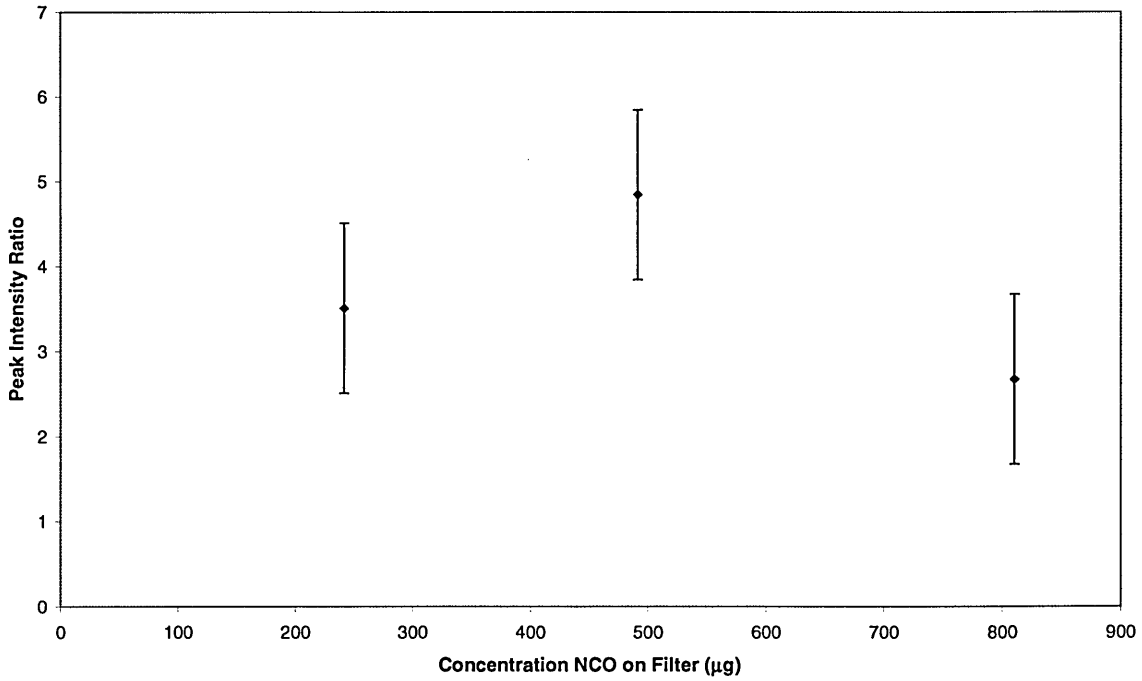


Figure 3.14 Average summed peak intensity calibration chart for round 57 HSL standard filter set

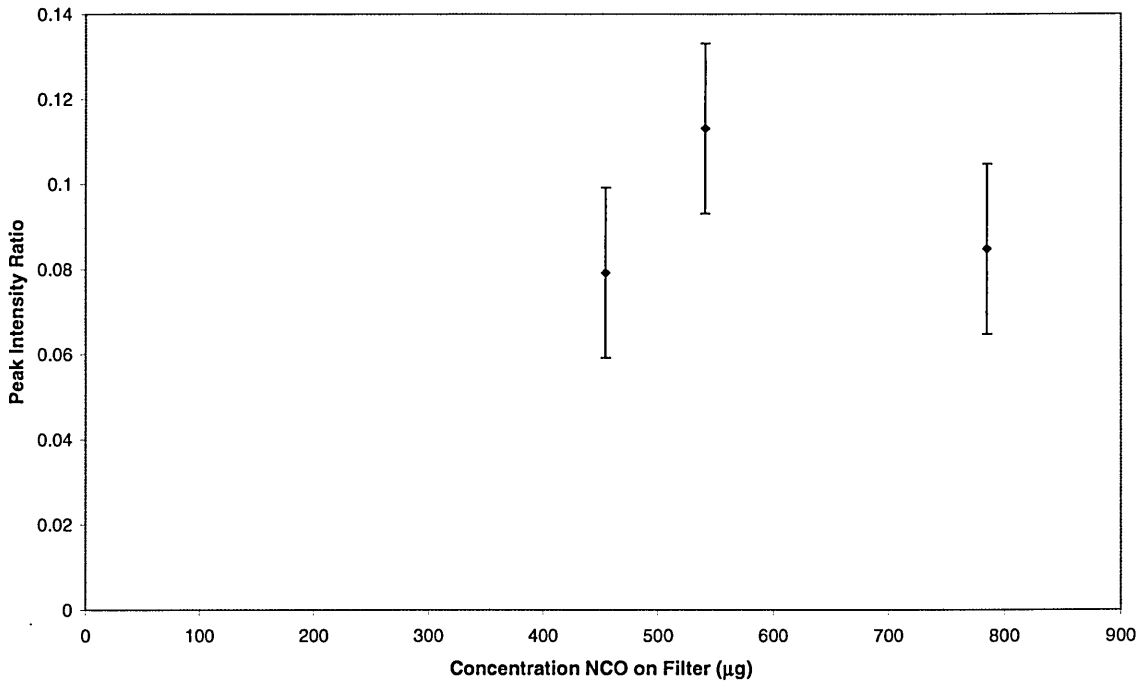


Figure 3.15 Average summed peak intensity calibration chart for round 58 HSL standard filter set

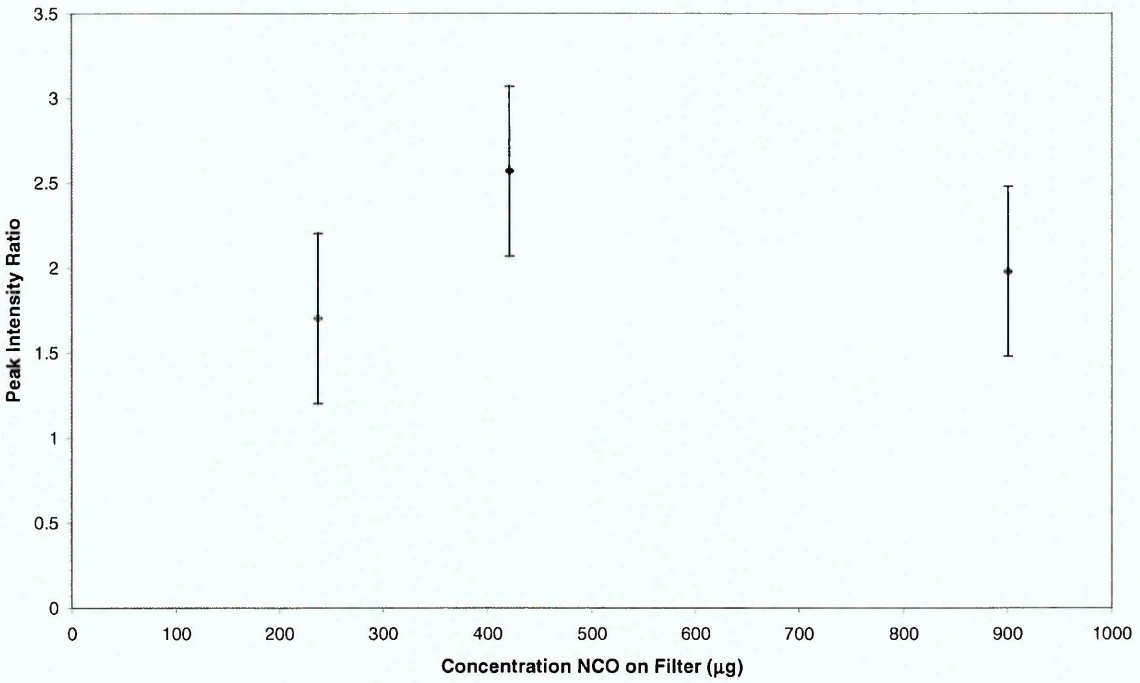


Figure 3.16 Average summed peak intensity calibration chart for round 62 HSL standard filter set

	Round 57	Round 58	Round 62
Filter # 1 (µg NCO)	241.30	300.49	236.42
Filter # 2 (µg NCO)	490.83	453.88	328.87
Filter # 3 (µg NCO)	561.69	540.23	420.30
Filter # 4 (µg NCO)	810.41	785.00	900.29

Table 3.4 A table showing the concentration values for each of the three HSL filter rounds investigated by peak intensity calculations

Regional Analysis

The computer software enables regional analysis to be performed for selected images. Unlike the previous method (summed peak intensity study) where an average spectrum is used to determine the peak intensities, this method calculates the average from the actual sample filter pixels. Figure 3.17 shows how this analysis was implemented for round 58 of the HSL standard WASP filters.

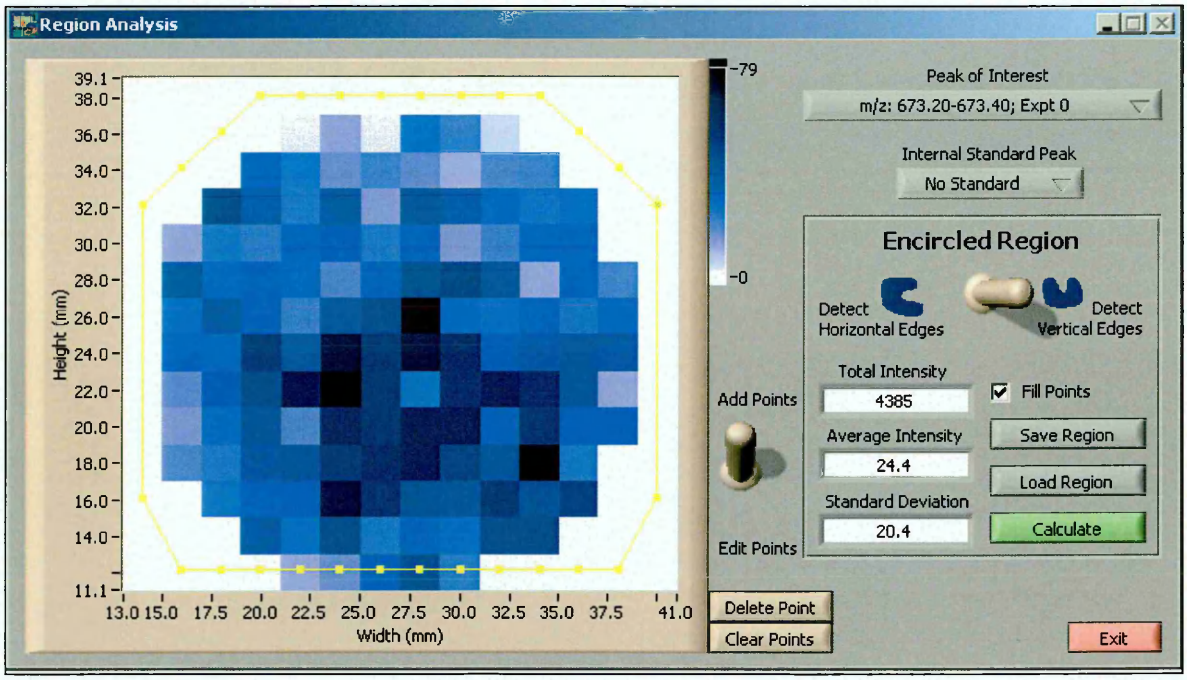


Figure 3.17 Regional analysis of a sample filter, the potassiated adduct of MDI-MP is depicted

The peak intensity ratios were calculated as previously described and a calibration was constructed. Figure 3.18 shows the average summed results for round 58 filters.

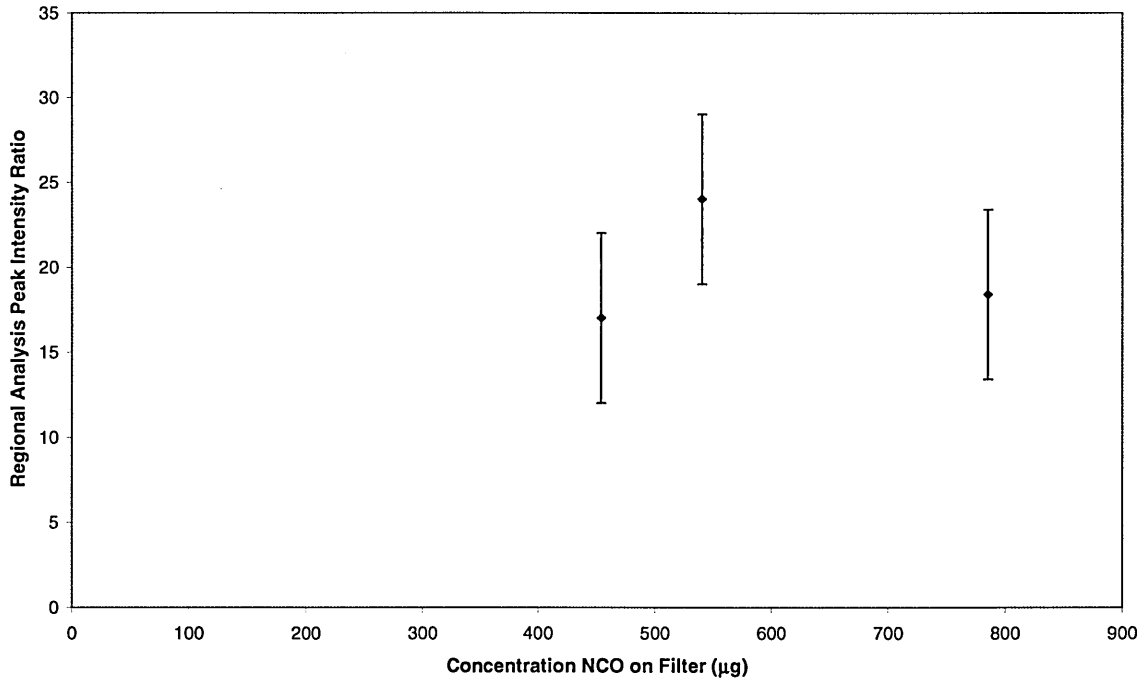


Figure 3.18 Average summed peak intensity calibration chart for round 58 HSL standard filter set performed from regional analysis calculations

3.3.7 *WASP Filter Quantitation*

Various internal standard application methods were investigated but the technique of mixing the internal standard and matrix solutions prior to application proved to yield the most even coverage of internal standard and matrix upon the filter. Both D_6 MDI-MP and MDI-EP internal standards were used on WASP sample filters for quantitation purposes. An example image from a D_6 MDI-MP filter is shown depicting the sparsely present sodium adduct of MDI-MP, 657 Th (Figure 3.19). The protonated molecules for the analyte and internal standard were absent in all images and spectral data, therefore they were not considered when quantifying the WASP filters. A representative spectrum is shown for deuterated MDI and MDI-EP internal standards from sample filters (Figures 3.20 and 3.21, respectively). The internal standards gave results that were fairly comparable.

The WASP filters used in this study were separately analysed by the HSL using MDHS 25/3 to give 'true' values of 0.4697 +/- 12% µg NCO and 0.2299 +/- 12% µg NCO, respectively. The results obtained for the same filters by direct filter

analysis by MALDI were 0.9509 +/- 27% µg NCO and 0.8566 +/- 27% µg NCO. A comparison of these data is shown in Table 3.5.

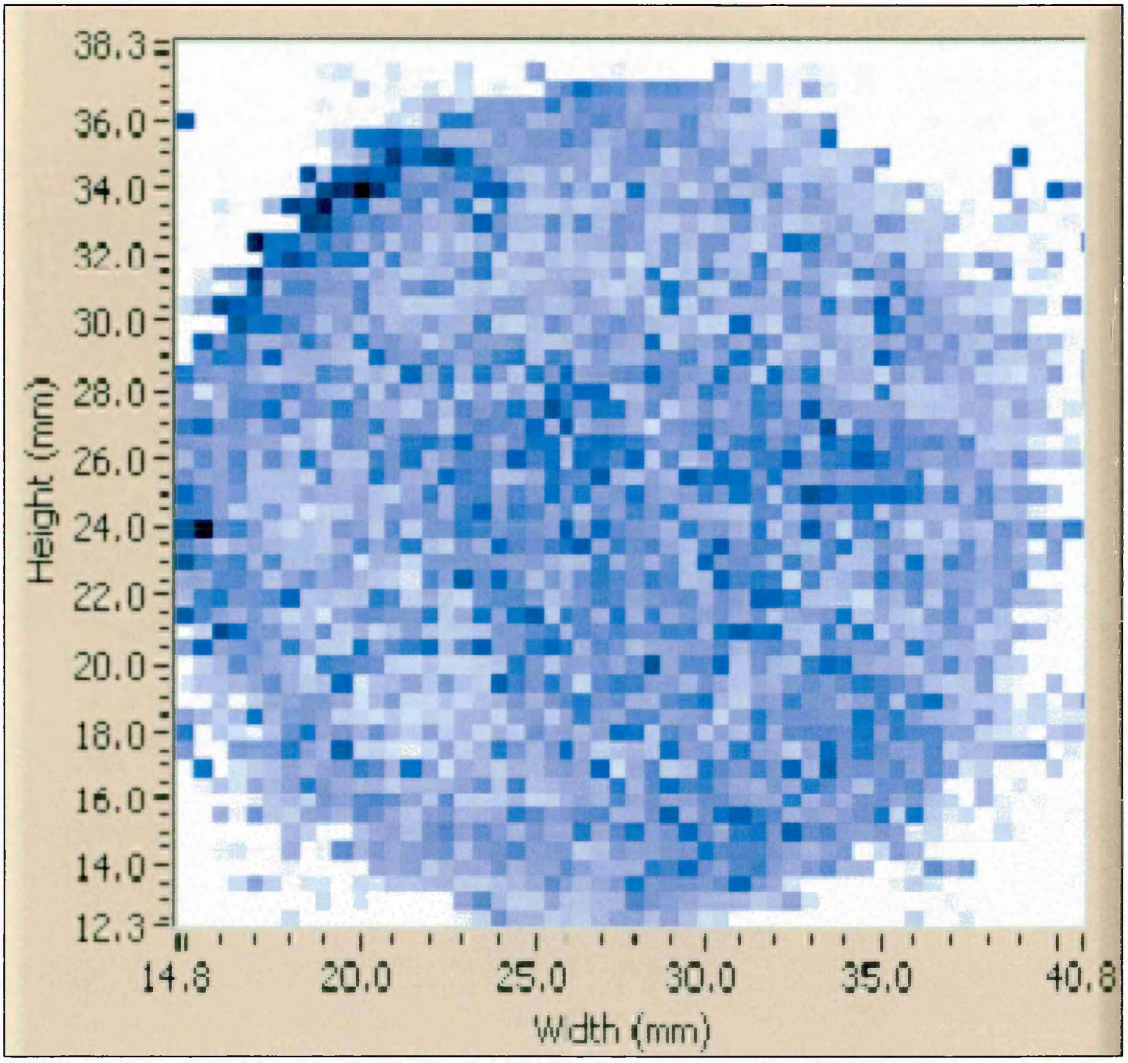


Figure 3.19 Image of the MDI-MP sodium adduct ion (657 Th) on a WASP sample filter

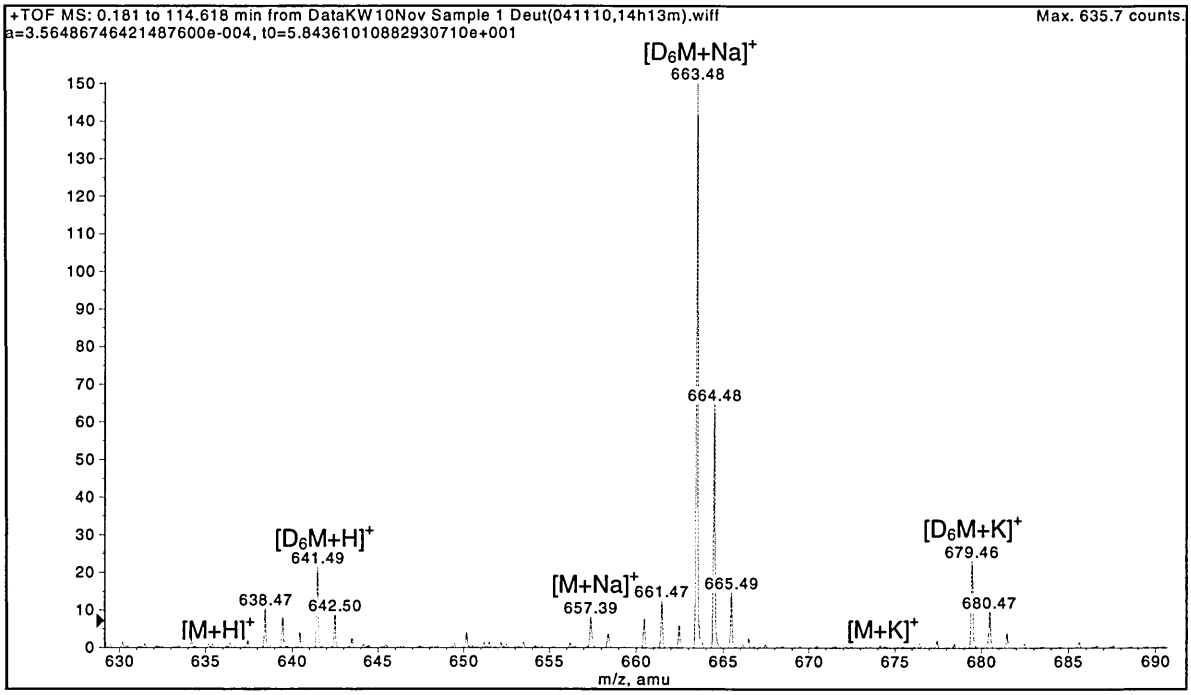


Figure 3.20 A representative spectrum from a WASP filter with D_6 MDI-MP as the internal standard

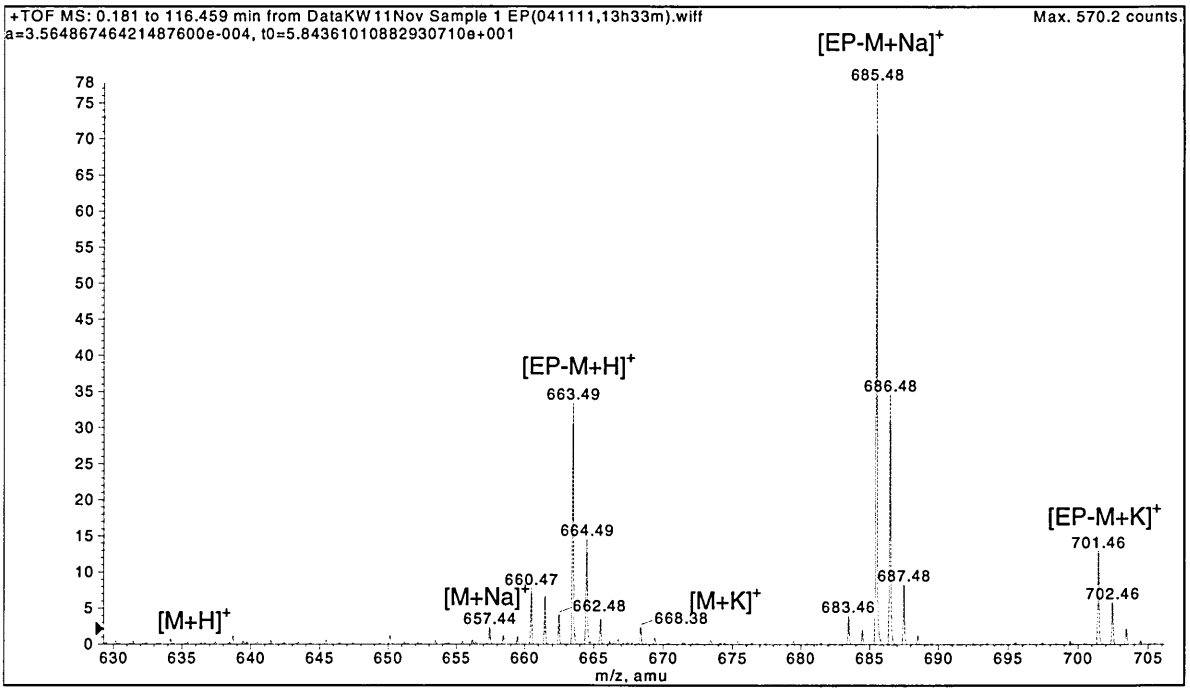


Figure 3.21 A representative spectrum from a WASP filter with MDI-EP as the internal standard

	HSL Findings	Filter Calibration
WASP Filter Round 57 #1	0.4697 µg NCO	0.9509 µg NCO
WASP Filter Round 57 #2	0.2299 µg NCO	0.8566 µg NCO
RSD	~ 12 %	~ 27 %

Table 3.5 Comparison of results for HSL analysis of WASP filters and quantitation using a direct filter calibration. For RSD values, calculations were based on filter numbers (HSL method n=60, filter method n=5)

Clearly, both the accuracy and precision of the direct filter quantitation experiments are disappointing. Incorporating the deuterated internal standard into the matrix solution and co-application would appear to be the approach that offers the best chances of success, yet the data obtained are still at best semi-quantitative. The poor quality of the data are in fact in stark contrast to our earlier work on quantitative TLC-MALDI-MS [7] where we were able to demonstrate a precision of 1-9% RSD and an accuracy of +/- 2% from known amounts of piroxicam spotted onto TLC plates. In these experiments the internal standard was incorporated into the experiment prior to matrix application by developing the TLC plate in a solvent containing it. This ensured a homogeneous coating of the plate with internal standard. It would appear from the data that we are reporting here that the incorporation of the internal standard into the matrix solution for co-application by air-spray is not as successful as this previous approach. It is, however, difficult to envisage an approach that mimics the methodology that we applied for TLC-MALDI-MS in an imaging experiment and hence there may be a limitation in the quality of quantitative data that can be obtained from imaging MALDI-MS experiments.

3.4 Conclusions

MALDI-MS has been successfully applied to the analysis of MDI-MP on MP coated filters. MDI-MP has been shown to readily form protonated molecules and adducts when subjected to MALDI-MS.

An air-spray method was developed that provides homogeneous coverage on the filter surface to allow efficient ionisation at every point. It was also determined that the MP coating method described in MDHS 25/3 was complete and produced an even coverage, therefore giving efficient derivatisation of NCO compounds when they contact the filter in the personal dust sampler.

Examination of the images produced little relation between analyte pixel intensity and concentration applied, but it was established clearly that MDI-MP could be directly analysed from sample filters. A simple calibration/normalization procedure using a matrix peak as an internal standard did not yield good quality calibration data.

The incorporation of deuterated analogue internal standards yielded a degree of success in quantitation from both spot targets and 'in-house' prepared filters. The use of the novel derivatising reagent, MDI-EP also produced an excellent linear calibration, including concentrations of up to 1 mg/ml. The MDI-EP calibration showed less spread over the concentration repetitions, with an average standard deviation calculated to be slightly lower than that using deuterated MDI-MP. EP is more easily available than deuterated MDI-MP, is fairly cheap and MDI-EP is simple to prepare. The quantitation work has shown that MALDI-MS can analyse NCO on MP coated filters at levels relevant to occupational hygiene studies (< 1.0 μg NCO).

Data manipulation investigations were unsuccessful when trying to utilise peak intensity ratios as opposed to peak areas. Summed intensities from sample filter spectra showed limited promise but were ultimately ineffective. Regional analysis work also proved inadequate for quantitation purposes by calibration.

Quantitation of WASP QA scheme filters is possible by utilising calibrations based on sodium adduct ions for both D_6 MDI-MP and MDI-EP internal standards by the use of spiked filters. Whilst reasonable quality data were obtained from quantitative studies of 'in-house' prepared filters, an attempt to apply the methodology developed to samples from a WASP round study demonstrated its limitations. Filters with 'true' values of 0.4697 +/- 12% μg NCO and 0.2299 +/- 12% μg NCO, respectively gave values of 0.9509 +/- 27% μg

NCO and 0.8566 +/- 27% μg NCO when analysed directly by MALDI-MS. These observations have negative implications for the use of imaging MALDI-MS for quantitative determinations.

[1] Health and Safety Laboratory, **2004**; International Standards Organisation – Intercomparison of methods for Organic NCO in Air – Results for MDHS 25/3, Internal Report # OMS/2004/07.

[2] Karlsson, D., Spanne, M., Dalene M. and Skarping G. *Analyst.* **1998**, 123, 117-123.

[3] Karlsson, D., Spanne, M., Dalene M. and Skarping G. *Journal of Environmental Monitoring.* **2000**, 2, 462-469.

[4] Karlsson, D., Spanne, M., Dalene M. and Skarping G. *Analyst.* **1998**, 123, 1507-1512.

[5] Health and Safety Executive, MDHS 25/3: Organic NCO in air, **1999**.

[6] Health and Safety Executive, **2002**, Occupational Exposure Limits, EH40.

[7] Crecelius, A., Clench, M. R., Richards, D. S. and Parr, V. J. *Pharmaceutical Biomedical Analysis.* **2004**, 35, 31-39.

Chapter 4

Veterinary Medicine and Pesticide Residue Analysis

4.1 Introduction

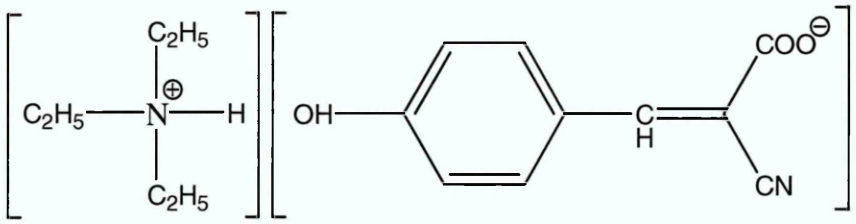
Veterinary medicines are used to treat farm animals whether as a preventative measure (prophylactic use) or on a remedy basis. Treatment includes curing disease, improving feed conversion and promoting growth. A mixture of drug and its subsequent metabolites is excreted from the animal and the manure slurry is used as a fertiliser to treat crops destined for human consumption. The leaching of these substances into water/soil systems and consequently the human food chain is therefore of great concern.

Crop growers, farmers and industry use pesticides on products to deter, destroy or mitigate pests, helping to increase quality of preservation, for transportation purposes and to increase shelf-life. Pesticides not only control insects but also the damage from other crops (such as weeds) and various fungi [1].

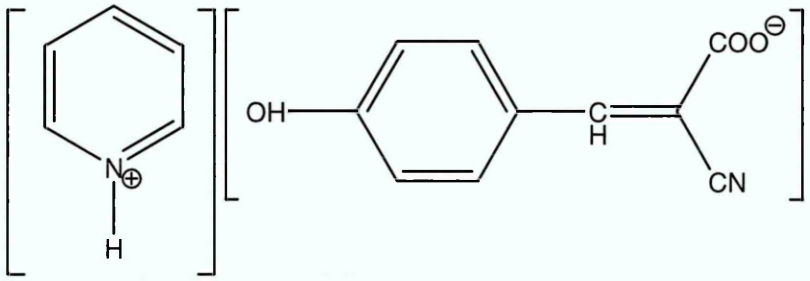
Ionic liquid matrices (ILM, also called RTIL or room-temperature ionic liquids) provide mass spectra with greatly reduced background, yielding a single peak related to the matrix [2]. These ILM have been reported to be especially useful for the analysis of low molecular weight compounds [3], but have been widely used for purposes such as organic synthesis, catalysis, electrochemical studies and gas chromatography [4-9]. These substances usually consist of a nitrogen/phosphorus-containing organic cation and a large anion [10]. They are liquid at, or just above room temperature although the precise melting point is often difficult to determine as super-cooling effects are apparent and the ultimate phase change depends on whether the temperature is being increased or decreased [11]. ILMs have many properties that make them suitable candidates for MALDI analysis. ILMs are fairly easy and inexpensive to prepare with low vapour pressures and varying viscosities. ILM are thermally very stable and have a wide liquid range [12]. ILM are good solvents for a wide range of substances and therefore applications, including polymeric, inorganic and organic analyses [13].

The ILMs investigated were prepared salts of the common solid organic acid matrix, α-CHCA. Both the tri-ethyl amine (TEA) and pyridine salts of α-CHCA

were utilised in analysis of the veterinary medicine and pesticide compounds. The structures of both these ILM are shown in Figure 4.1.



α-CHCA TEA ILM : TEA Spectral Contribution 102 Th



α-CHCA Pyridine ILM : Pyridine Spectral Contribution 80 Th

Figure 4.1 Structures of the TEA and pyridine salts of α-CHCA ionic liquid matrices

The aim of the work reported in this chapter was to analyse a range of veterinary medicines and pesticides to ultimately enable the quantitation of these substances. The structures of the veterinary medicine and pesticide compounds analysed in this chapter are shown and discussed in detail (Chapter 1, Section 1.4.6) and summarized in Table 4.1.

Compound Name	RMM
Trimethoprim	290.32
Sulphadiazine	250.28
Oxytetracycline	496.90
Florfenicol	358.21
Levamisole	240.80
Enrofloxacin	359.40
Tylosin	1027.17
Cypermethrin	416.30

Table 4.1 A summary of the veterinary medicine and pesticide compounds studied with the corresponding relative molecular masses of each

A range of ionic liquid matrices was prepared to limit the amount of background noise in the sample spectra, when compared with regular organic acid matrices.

Extracts of carrot and lettuce were run to determine if these substances could be analysed with vegetable samples. Imaging mass spectrometry was used to detect a selection of the veterinary medicines directly from the surface of lettuce leaves and carrot slices.

4.2 Experimental

4.2.1 *Chemicals and Reagents*

HPLC grade methanol was purchased from Fischer Chemicals (Loughborough, Leicester, UK). Trifluoroacetic acid (TFA), triethylamine (TEA), pyridine and α-cyano hydroxy cinnamic acid (α-CHCA, organic acid MALDI matrix) were obtained from Sigma Aldrich (Poole, Dorset, UK). Veterinary medicine and pesticide compounds were supplied by the Health and Safety laboratories (Buxton, Derbyshire, UK).

4.2.1.1 Preparation of Standards and Matrix Solutions

Stock solutions of the veterinary medicine and pesticide compounds were prepared at 1 mg/ml in methanol (0.1% TFA). α-CHCA matrix was dissolved in methanol (0.1% TFA), 25 mg/ml. Problems arose when preparing these solutions, especially with the medicine compounds. It was therefore necessary to sonicate several samples for periods of up to 5 minutes to ensure the samples dissolved. Care was taken to maintain the sonic bath temperature at 37°C to minimise evaporation and the possibility of degradation.

4.2.1.2 Preparation of Ionic Liquid Matrices

The ILM solutions were prepared by Miss Stephanie Peigne, placement student at Sheffield Hallam University. The ILM were prepared by sonication of a mixed equimolar solution of α-CHCA and organic base (TEA or Pyridine). The 0.05 mmol α-CHCA solution was prepared in 1 ml of 2:1 acetonitrile/water with 0.1% TFA before combination with the organic base.

4.2.2 *Instrumentation*

All analyses were performed using an Applied Biosystems (Foster City, USA)/MDS Sciex (Ontario, Canada) "Q-Star" hybrid quadrupole time of flight instrument, fitted with an orthogonal MALDI ion source and ion imaging software. This instrument utilises a nitrogen laser with a 337nm wavelength. The instrument resolution is 10,000 fwhm and the mass calibration is adjusted as required. The normal calibration is performed by product ion scan of renin substrate (peptide species). Data was collected and manipulated using the 'Analyst–QS' software supplied with the instrument.

The air gun employed was a Badger 100-G-F Gravity Feed, with a medium head and 200 µl solvent reservoir cup (The Airbrush Company, Lancing, West Sussex, UK).

4.2.3 *Analytical Procedure*

4.2.3.1 Preliminary Compound Analysis using α-CHCA Matrix

Initially, the compounds were analysed using α-CHCA matrix to determine the outcome of sample analysis. Veterinary medicine/pesticide stock solutions (200 µl, 1 mg/ml) were mixed with 40 µl α-CHCA matrix (25 mg/ml) and 1 µl of this resultant mixture was spiked onto the stainless steel target. Four spots for each compound were analysed on a spot target using an automated search pattern with a 1 minute acquisition time. The resultant spectra were studied to establish individual peak patterns and the suitability of α-CHCA as a matrix for analysis of these compounds.

4.2.3.2 Analysis using ILM

Both TEA and pyridine salts of α-CHCA matrix were used for analysis. This was to ascertain whether either of the ILM was superior for analysis and to determine the amount of background noise and interfering peaks from the sample spectra. 100 µl of each veterinary medicine/pesticide stock solution was mixed with 100 µl α-CHCA ILM, both TEA and pyridine. 1 µl of the resultant mixtures were spiked onto the stainless steel target for analysis. Again, four spots for each compound were analysed on a spot target using an automated search pattern with a 1 minute acquisition time.

4.2.3.3 Compound Calibration Studies

Analyte and α-CHCA (TEA) were mixed in a 1:1 ratio and 1 µl of the solutions spotted onto the stainless steel target. Samples were analysed using the automated search pattern and a 1 minute acquisition time. Duplicate analyses of four concentrations of each analyte compound were investigated; 1, 0.75, 0.5 and 0.25 mg/ml.

It was expected that the ratios calculated from the analyte:matrix peak areas could be plotted onto a chart to form a calibration for possible quantitation purposes.

4.2.3.4 Analysis Incorporating Organic Crop Samples

The ultimate aim for veterinary medicine/pesticide analysis is to be able to quantify residue limits in crop samples. The chosen crops for this investigation were carrot and lettuce samples, which had to be incorporated into the analysis method. 5 g of both carrot and lettuce were weighed and ground to a pulp in a pestle and mortar. The pulp was transferred into 50 ml falcon tubes to which 40 ml of acetone was added. The samples were centrifuged for 20 minutes and the supernatant poured off into a sealed container. During sample preparation, 15 ml of the supernatant was extracted and placed into a beaker. The acetone was evaporated on a hotplate before the residue was reconstituted with 10 ml of methanol.

The sample mixtures were composed of 20 µl of vegetable extract, 20 µl of veterinary medicine/pesticide (1 mg/ml) and 40 µl of α-CHCA TEA. 1 µl of the resultant mixtures were spotted onto a stainless steel target and analysed using an automated search pattern with a 1 minute duration.

4.2.3.5 Imaging Analysis of Veterinary Medicines

Once it was determined that the carrot and lettuce samples did not interfere with the matrix and analytes of interest, imaging analysis was performed in order to determine whether these compounds could be detected directly from the crop sample surfaces. Three veterinary medicine compounds were chosen for use from the spot target experiments as they were deemed suitable for further study. These compounds were trimethoprim, enrofloxacin and levamisole.

Three pieces of lettuce and a thin slice of carrot were blotted dry with paper towel and left under a heat lamp for an hour. 2 µl of each of the compounds (1 mg/ml) were spotted onto the individual pieces of lettuce and carrot slice and the samples left to dry for a further half-hour. The crop samples were covered with 5 coats of α-CHCA (250 µl per layer = 1250 µl) by air-spray deposition and left under a heat lamp to dry between coats.
The samples were then mounted onto a recessed stainless steel MALDI target with double-sided sticky tape and imaged by moving the sample stage at 0.5

mm increments to build up a pixel representation. The laser power was set to 25%/14.3 μJ with a repetition rate of 1000 Hz.

4.3 Results and Discussion

4.3.1 *Preliminary Compound Analysis using α-CHCA Matrix*

Data for the analytes investigated was processed to provide spectra for each compound. Spectra obtained for all veterinary medicine and pesticide compounds exhibited the standard α-CHCA peak pattern of $[M+H]^+$, $[M+Na]^+$ and $[M+K]^+$ (190, 212 and 228 Th, respectively).

Trimethoprim produced an intense peak for the $[M+H]^+$ ion at 291 Th, Figure 4.2.

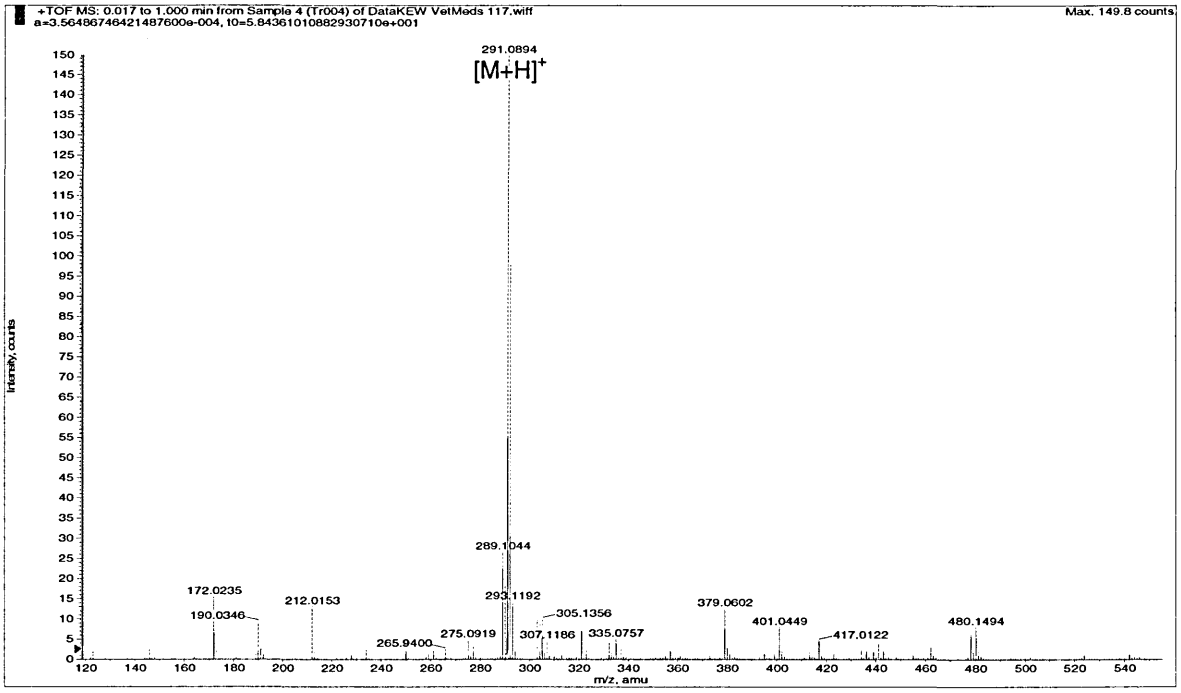


Figure 4.2 Expanded spectrum of trimethoprim showing the protonated molecular ion at 291 Th. 200 µl of the veterinary medicine stock solution was mixed with 40 µl α-CHCA matrix, 1 µl of this resultant mixture was spiked onto the target for analysis

The spectrum for sulphadiazine shows a peak for $[M+H]^+$, $[M+Na]^+$ and $[M+K]^+$ at 251, 273 and 289 Th, respectively. Also visible on the spectrum is the standard matrix peak pattern. See Figure 4.3.

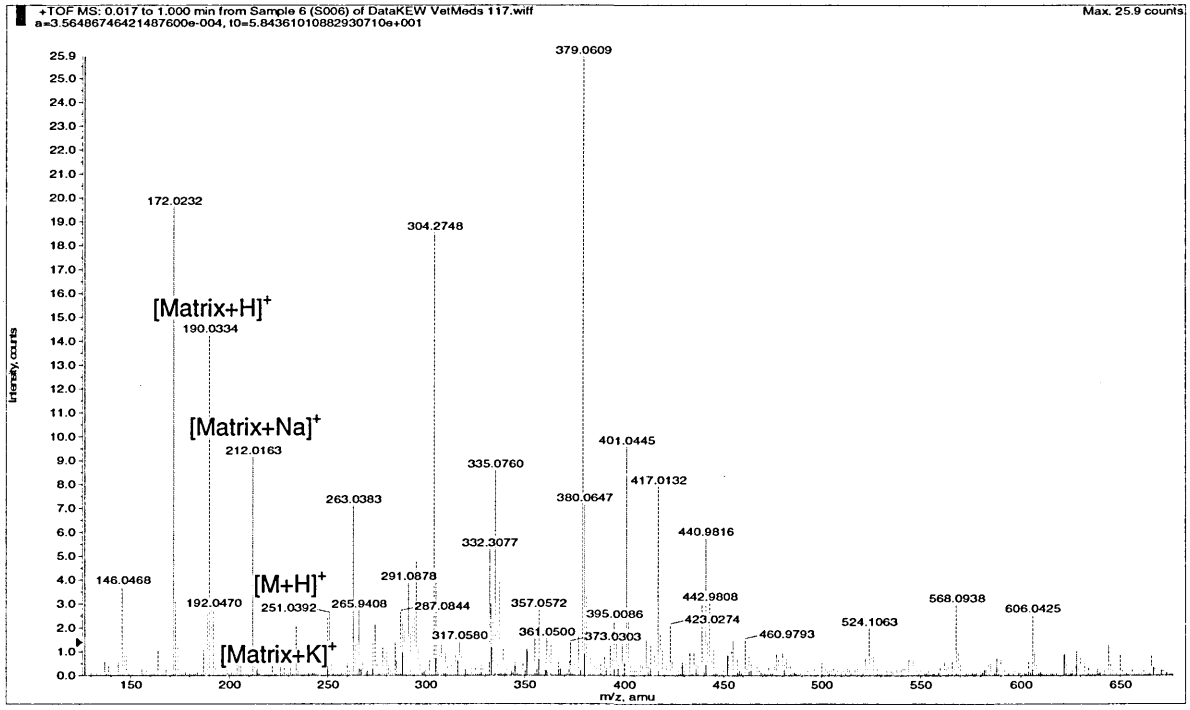


Figure 4.3 Spectrum of sulphadiazine showing the protonated molecular ion at 251 Th. The sodiated and potassiated molecular ions at 273 and 289 Th are present in the spectrum but are unlabelled due to low intensities. 200 µl of the veterinary medicine stock solution was mixed with 40 µl α-CHCA matrix, 1 µl of this resultant mixture was spiked onto the target for analysis

Oxytetracycline produced peaks corresponding to $[M+H]^+$, $[M+Na]^+$ and $[M+K]^+$ at 461, 483 and 499 Th. The matrix peak pattern can also be seen. See Figure 4.4.

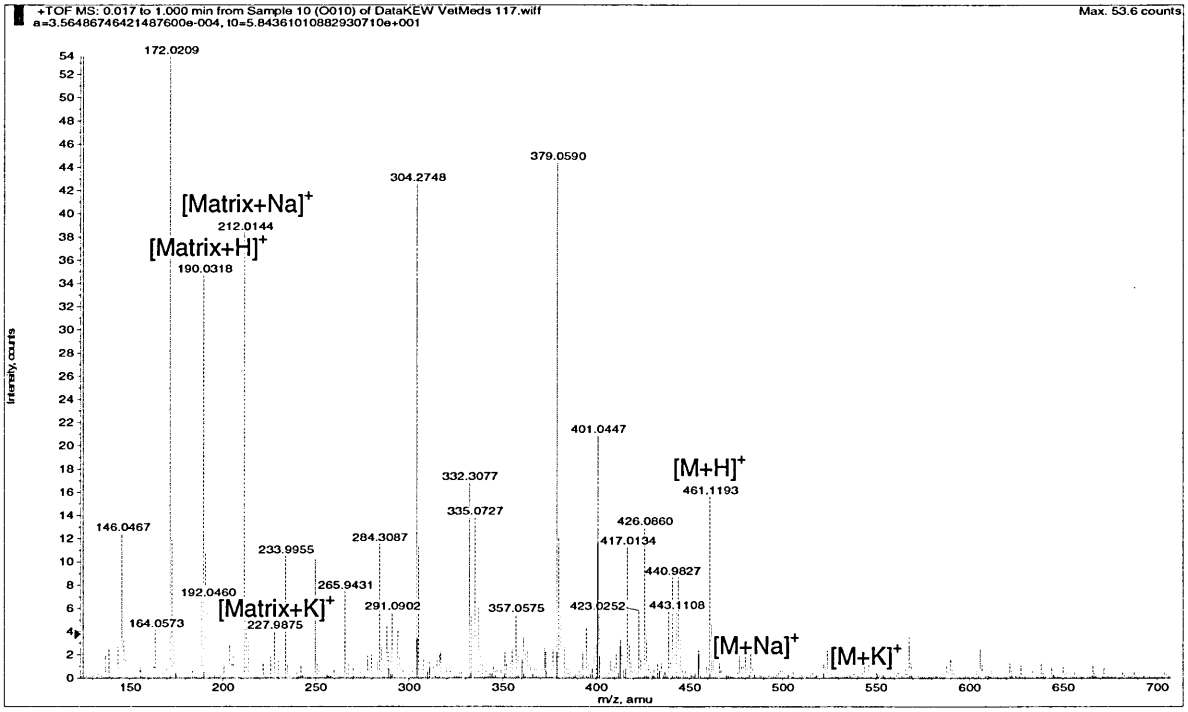


Figure 4.4 Oxytetracycline spectrum showing the protonated, sodiated and potassiated molecular ions of α-CHCA matrix and analyte at 190, 212 and 228 (α-CHCA) 461, 483 and 499 Th (oxytetracycline). 200 µl of the veterinary medicine stock solution was mixed with 40 µl α-CHCA matrix, 1 µl of this resultant mixture was spiked onto the target for analysis

The spectrum of cypermethrin with α-CHCA organic acid matrix revealed the standard matrix peak pattern and the protonated, sodiated and potassiated peaks for the analyte at 417, 439 and 455 Th, respectively. See Figure 4.5.

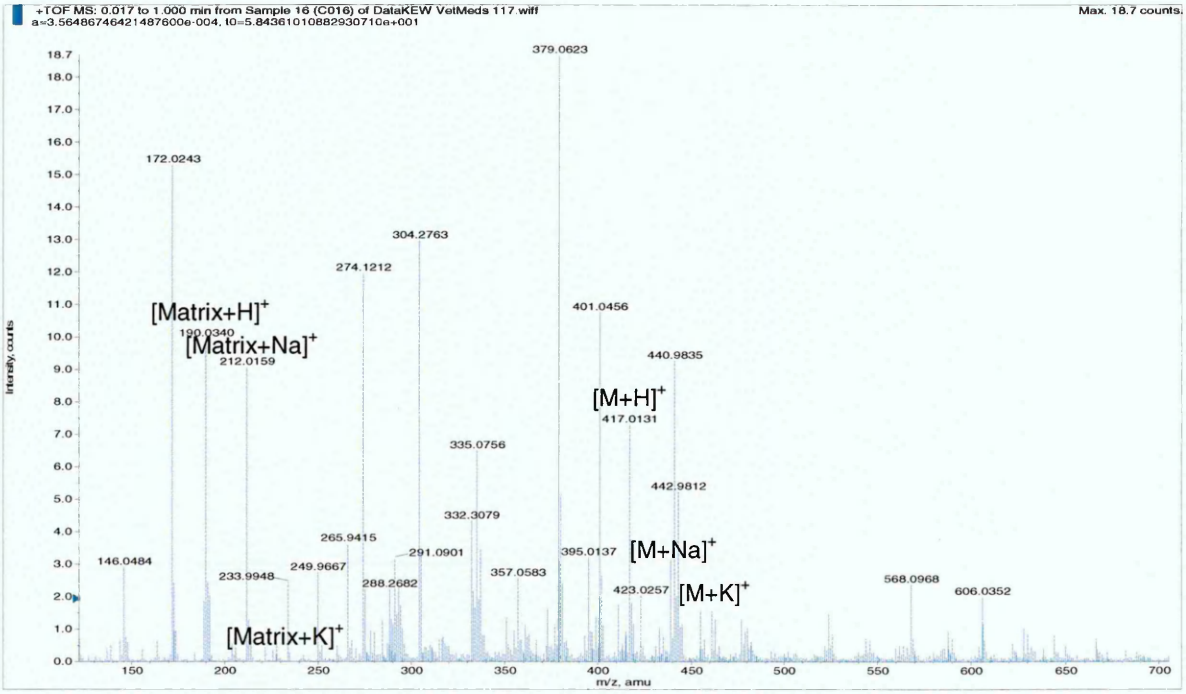


Figure 4.5 Cypermethrin spectrum showing the protonated, sodiated and potassiated α-CHCA matrix and analyte at 190, 212 and 228 (α-CHCA) 417, 439 and 455 Th (cypermethrin). 200 µl of the pesticide stock solution was mixed with 40 µl α-CHCA matrix, 1 µl of this resultant mixture was spiked onto the target for analysis

Analysis of florfenicol produced matrix peaks, but no peaks of value corresponding to the analyte of interest. The peaks for florfenicol should appear at 359 Th $[M+H]^+$, 381 Th $[M+Na]^+$ and 397 Th $[M+K]^+$. They were present only as part of the spectral background. See Figure 4.6.

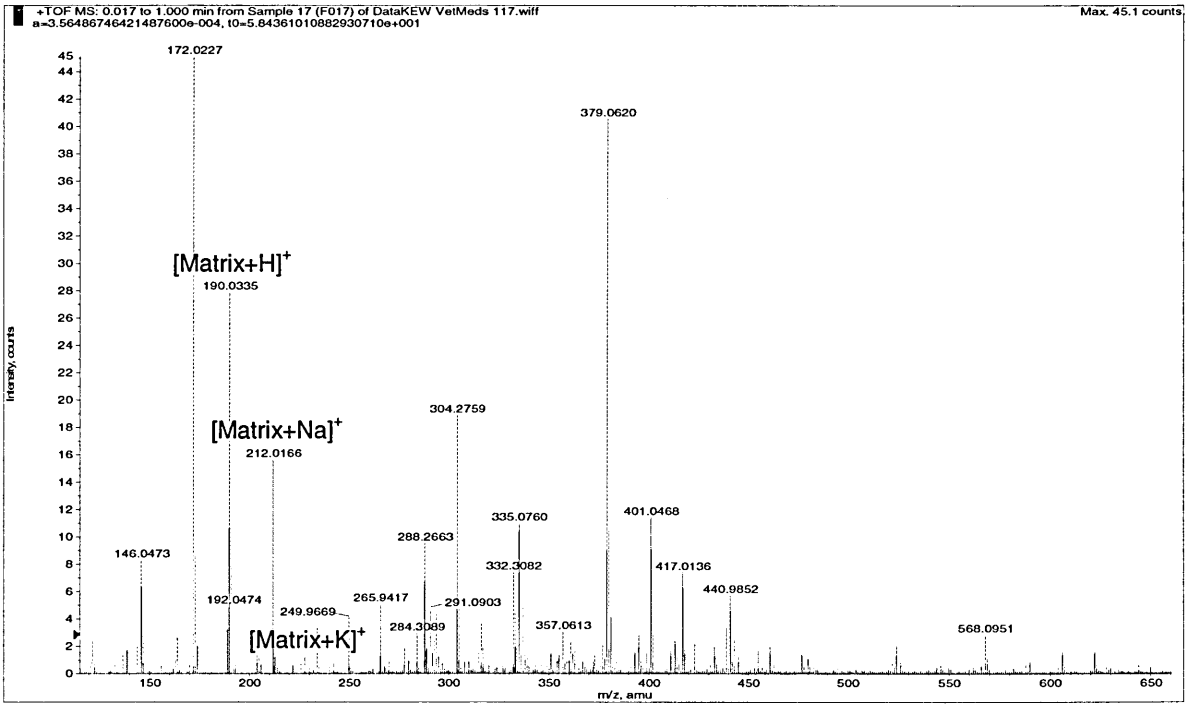


Figure 4.6 A spectrum from the analysis of florfenicol showing a standard matrix peak pattern. 200 µl of the veterinary medicine stock solution was mixed with 40 µl α-CHCA matrix, 1 µl of this mixture was spiked onto the target for analysis

Levamisol produced a spectrum with an intense peak relating to the protonated analyte ion at 205 Th. See Figure 4.7.

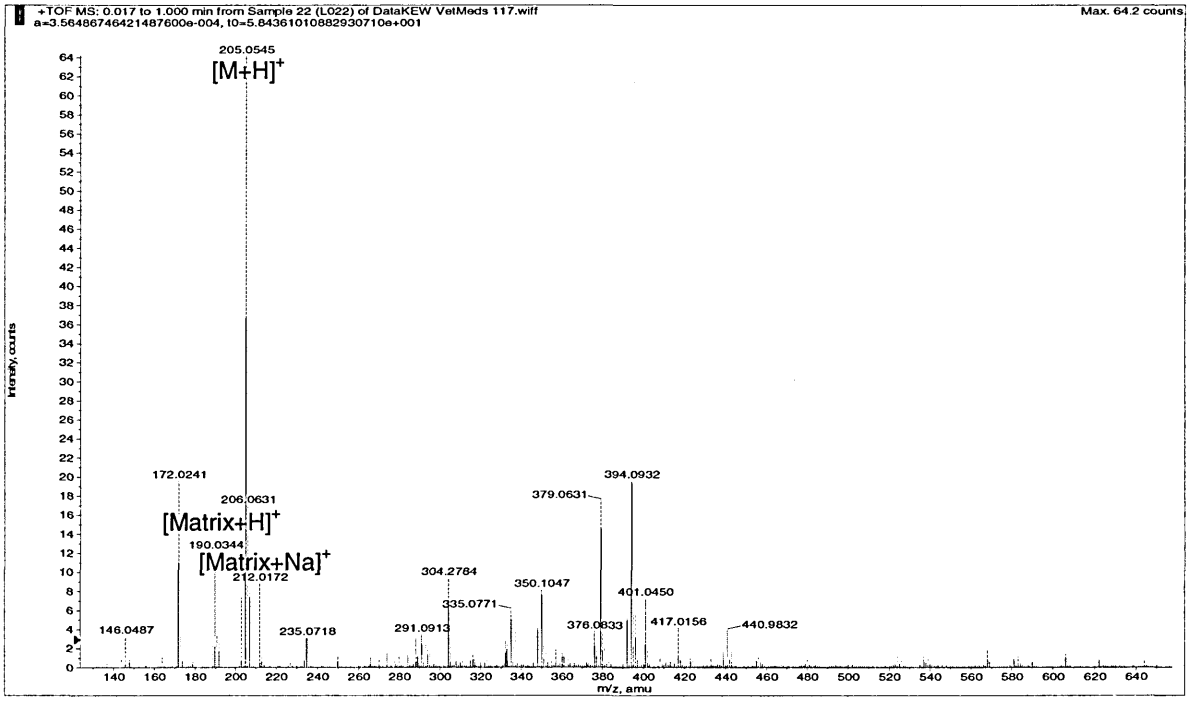


Figure 4.7 A spectrum from the analysis of levamisol showing an intense peak at 205 Th relating to the protonated molecule of the analyte. 200 µl of the veterinary medicine stock solution was mixed with 40 µl α-CHCA matrix, 1 µl of this resultant mixture was spiked onto the target for analysis

The analysis of enrofloxacin produced a spectrum showing intense peak patterns for the protonated, sodiated and potassiated adducts of both α-CHCA (190, 212 and 228 Th) and the analyte of interest (360, 382 and 398 Th). See Figure 4.8.

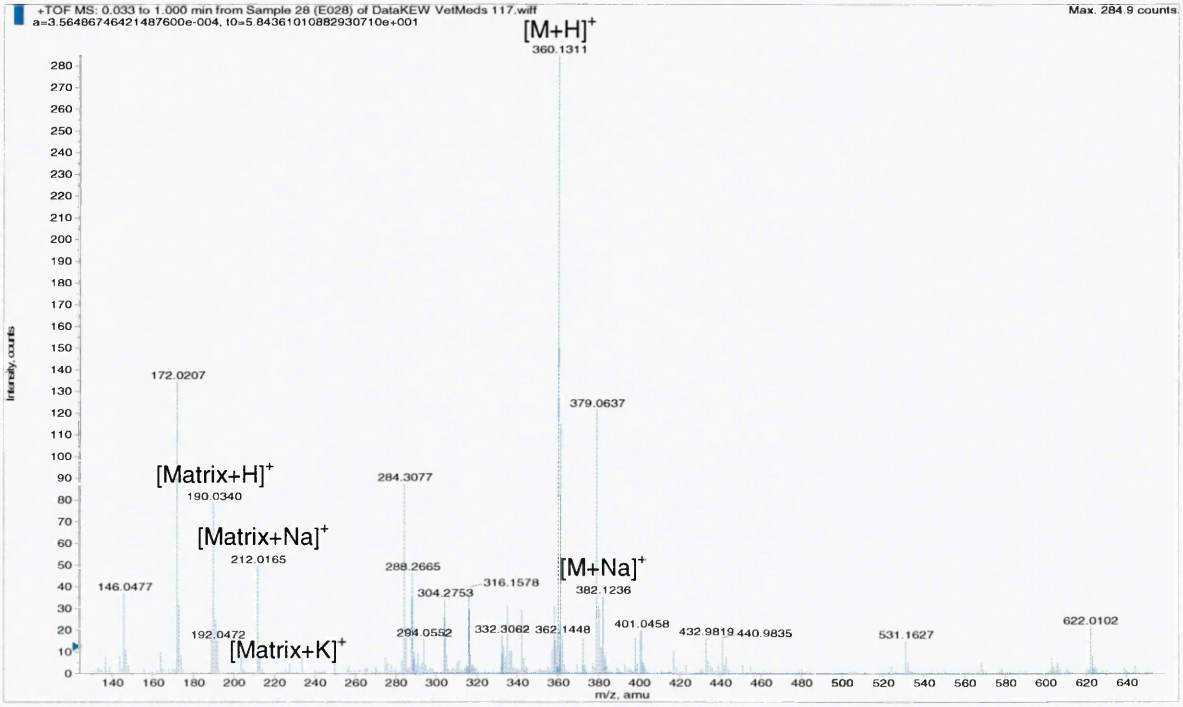


Figure 4.8 A spectrum from the analysis of enrofloxacin showing intense peaks relating to the protonated molecule (360 Th), the sodium adduct (382 Th) and the potassium adduct for the analyte (398 Th). Matrix peaks are also visible. 200 µl of the veterinary medicine stock solution was mixed with 40 µl α-CHCA matrix, 1 µl of this resultant mixture was spiked onto the target for analysis

The resultant spectrum from the analysis of tylosin showed limited peaks relating to α-CHCA matrix and none corresponding to the analyte. See Figure 4.9.

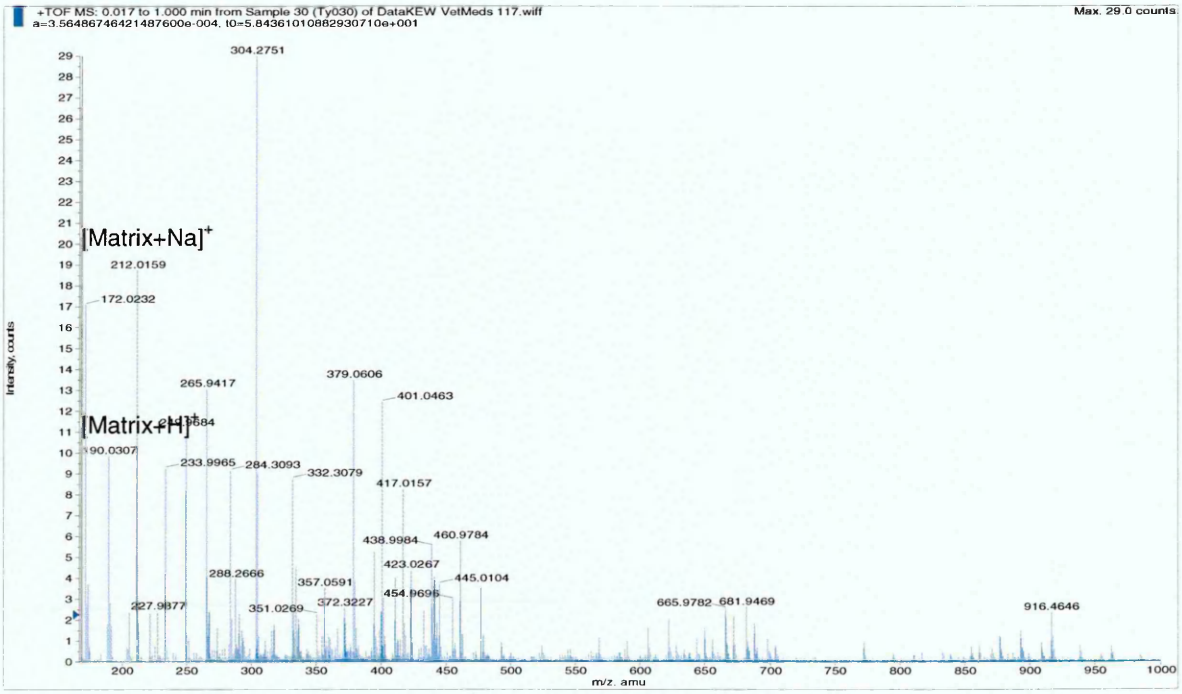


Figure 4.9 Spectrum from the analysis of tylosin showing matrix related peaks at 190 and 212 Th, respectively, for the protonated and sodiated adducts. 200 µl of the veterinary medicine stock solution was mixed with 40 µl α-CHCA matrix, 1 µl of this resultant mixture was spiked onto the target for analysis

The analysis of the chosen compounds produced spectral data that could be processed to identify more information about the target compounds and matrix choice. The relative intensity ratios for the analyte compounds and α-CHCA matrix were calculated by using the peak values related to the protonated molecules. The peak intensity chosen for α-CHCA was 190 Th $[M+H]^+$ and the veterinary medicine/pesticide values were also selected from the protonated form of the compound. The results are summarised for comparison with data from α-CHCA ILM analysis, Table 4.2.

4.3.2 *Analysis using ILM*

Analysis of the veterinary medicine and pesticide compounds with α-CHCA matrix provided the basis for further method development. Although most of the compounds produced acceptable spectra, there was still much background noise present that sometimes suppressed the analyte peaks of interest. The use of ILM was an attempt to minimise background noise to accentuate the analyte peaks for data processing. The ILM used were the TEA and pyridine salts of α-CHCA organic acid, both giving a single protonated molecule in the resultant spectra, at 102 and 80 Th, respectively. The pyridine version of the α-CHCA ILM was utilised to see if the results were better than for TEA. In the majority of cases, however, the pyridine spectra were more cluttered and the analyte peaks were almost completely suppressed. For these reasons, this ILM will not be discussed further. All α-CHCA TEA ILM spectra had virtually no background and this produced results which were much cleaner and easier to interpret.

Trimethoprim produced an intense peak for the $[M+H]^+$ ion at 291 Th. Using an ILM reduced spectral noise so that the peak relating to the sodiated ion was now visible at 313 Th and a very small peak had appeared for the potassiated ion at 329 Th. See Figure 4.10.

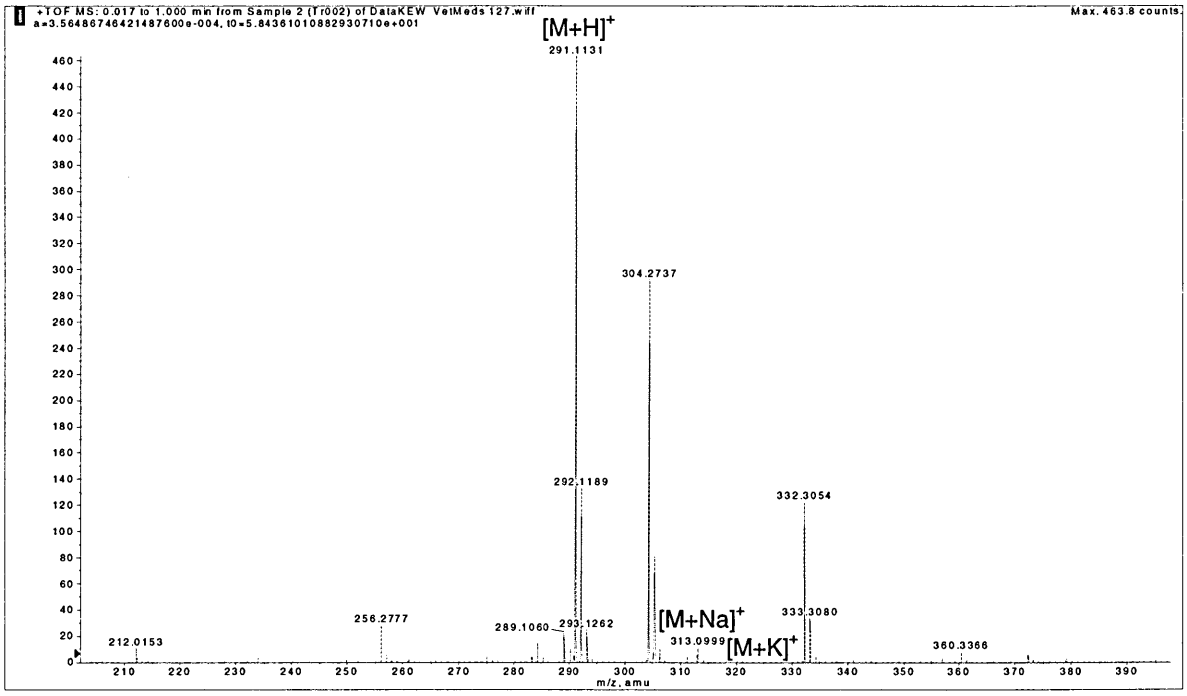


Figure 4.10 Expanded ILM spectrum from the analysis of trimethoprim showing peaks relating to the protonated, sodiated and potassiated ions of the intact molecule. 100 µl of the veterinary medicine stock solution was mixed with 100 µl α-CHCA ILM, 1 µl of the resultant mixture was spiked onto the target for analysis

The spectrum for sulphadiazine from ILM analysis shows a peak for $[M+H]^+$, $[M+Na]^+$ and $[M+K]^+$ at 251, 273 and 289 Th, respectively at much higher intensities than with the standard organic acid matrix. See Figure 4.11.

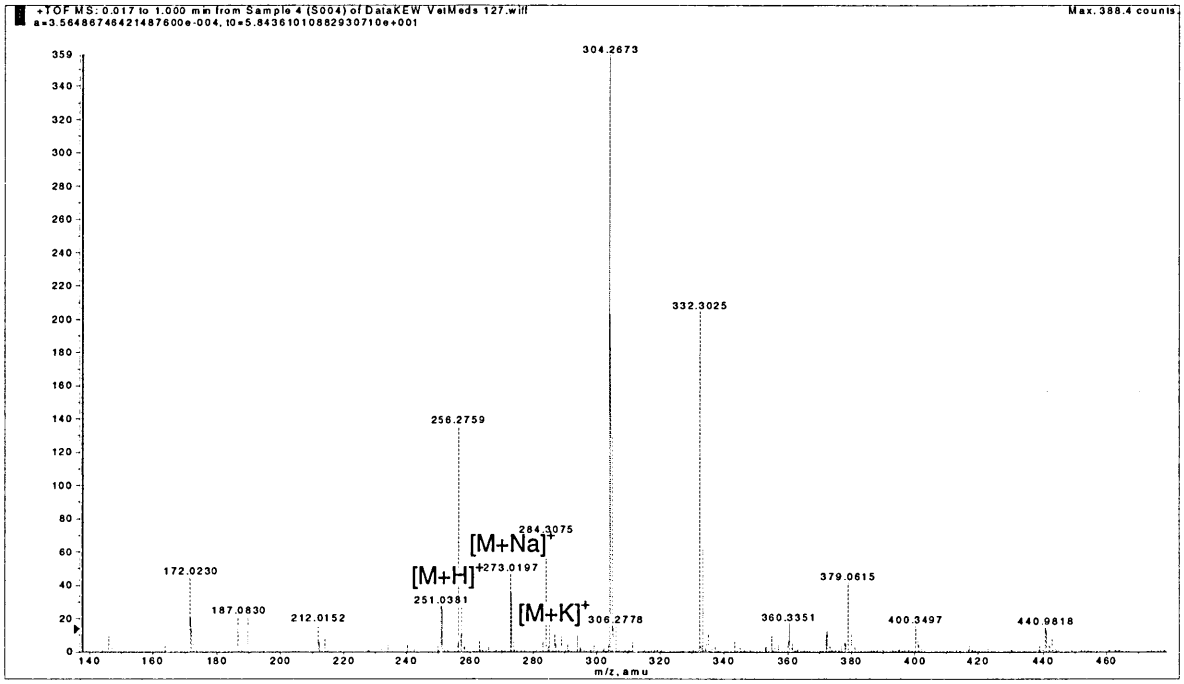


Figure 4.11 ILM spectrum of sulphadiazine showing the protonated, sodiated and potassiated molecular ions at 251, 273 and 289 Th. 100 µl of the veterinary medicine stock solution was mixed with 100 µl α-CHCA ILM, 1 µl of the resultant mixture was spiked onto the target for analysis

Oxytetracycline produced more intense peaks corresponding to $[M+H]^+$, $[M+Na]^+$ and $[M+K]^+$ at 461, 483 and 499 Th than when using standard α-CHCA matrix. See Figure 4.12.

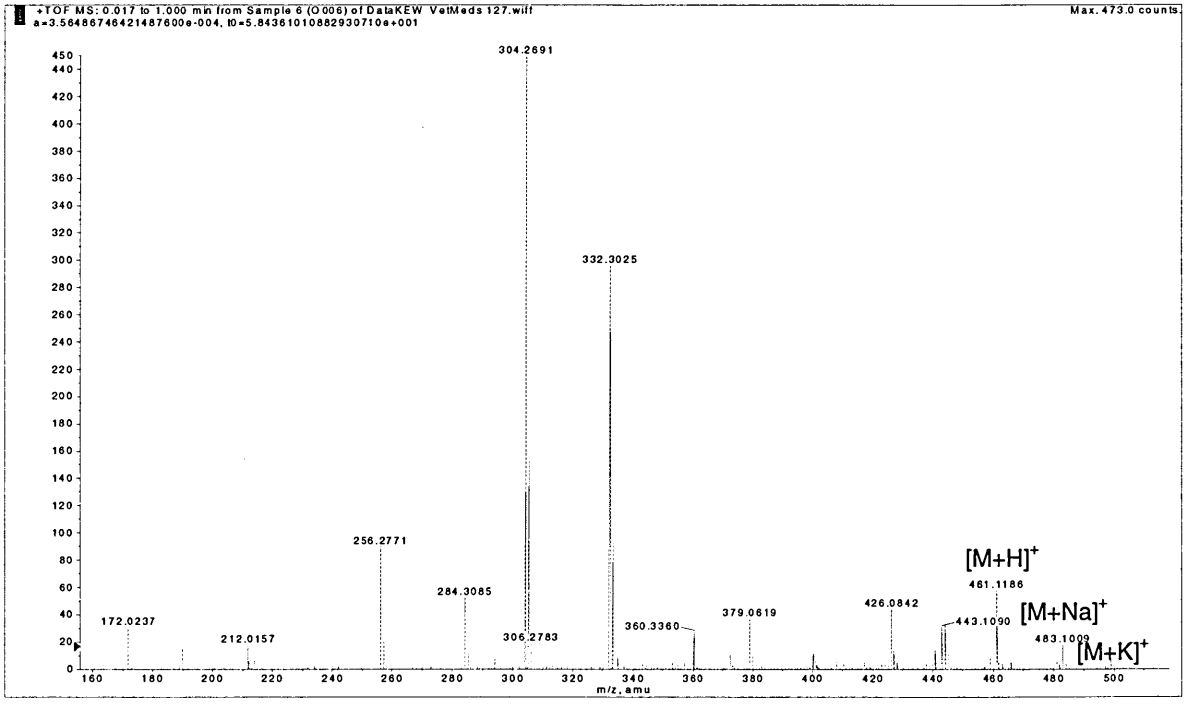


Figure 4.12 Oxytetracycline spectrum from ILM analysis showing the protonated, sodiated and potassiated molecules of the analyte at 461, 483 and 499 Th. 100 µl of the veterinary medicine stock solution was mixed with 100 µl α-CHCA ILM, 1 µl of the resultant mixture was spiked onto the target for analysis

The spectrum of cypermethrin with α-CHCA TEA ILM revealed the protonated, sodiated and potassiated peaks for the analyte at low ion intensities. The peaks appear at 417, 439 and 455 Th, respectively. See Figure 4.13.

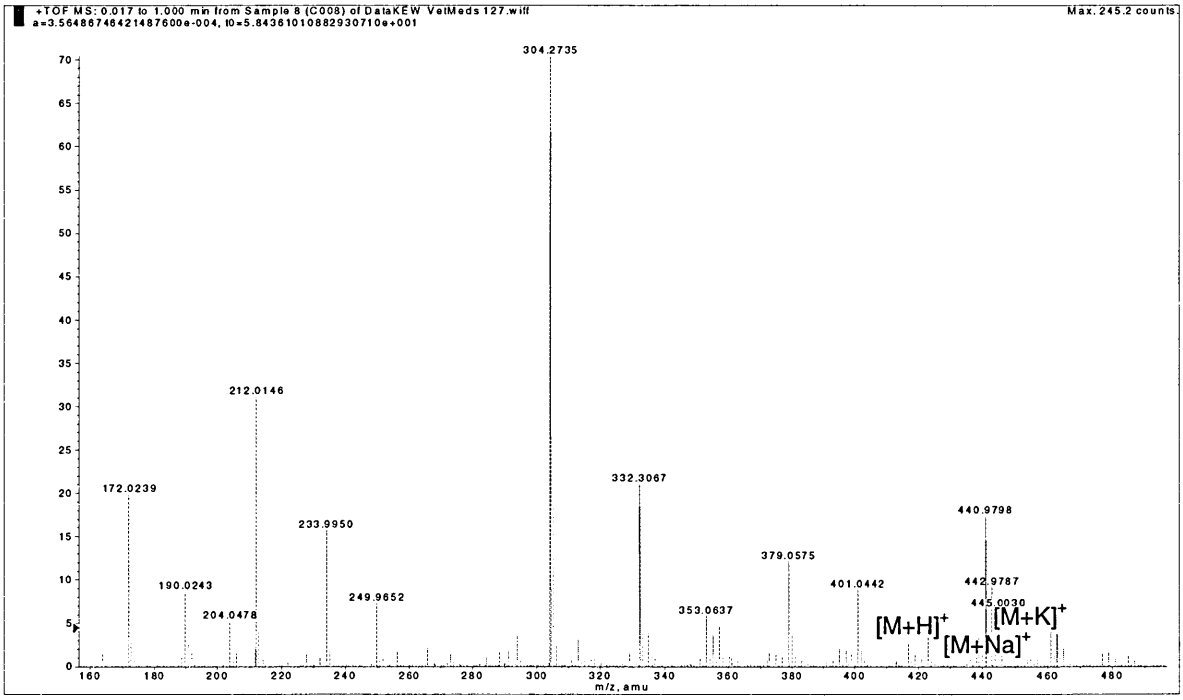


Figure 4.13 ILM spectrum from the analysis of cypermethrin showing the protonated, sodiated and potassiated molecules of the analyte at 417, 439 and 455 Th. 100 µl of the veterinary medicine stock solution was mixed with 100 µl α-CHCA ILM, 1 µl of the resultant mixture was spiked onto the target for analysis

Analysis of florfenicol utilising an ILM produced peaks corresponding to the analyte of interest. The peaks for florfenicol appeared at 359 Th $[M+H]^+$, 381 Th $[M+Na]^+$ and 397 Th $[M+K]^+$. They were present only at very low ion intensities but the use of α-CHCA TEA ILM did not completely suppress the analyte peaks as happened with the standard organic acid matrix. See Figure 4.14.

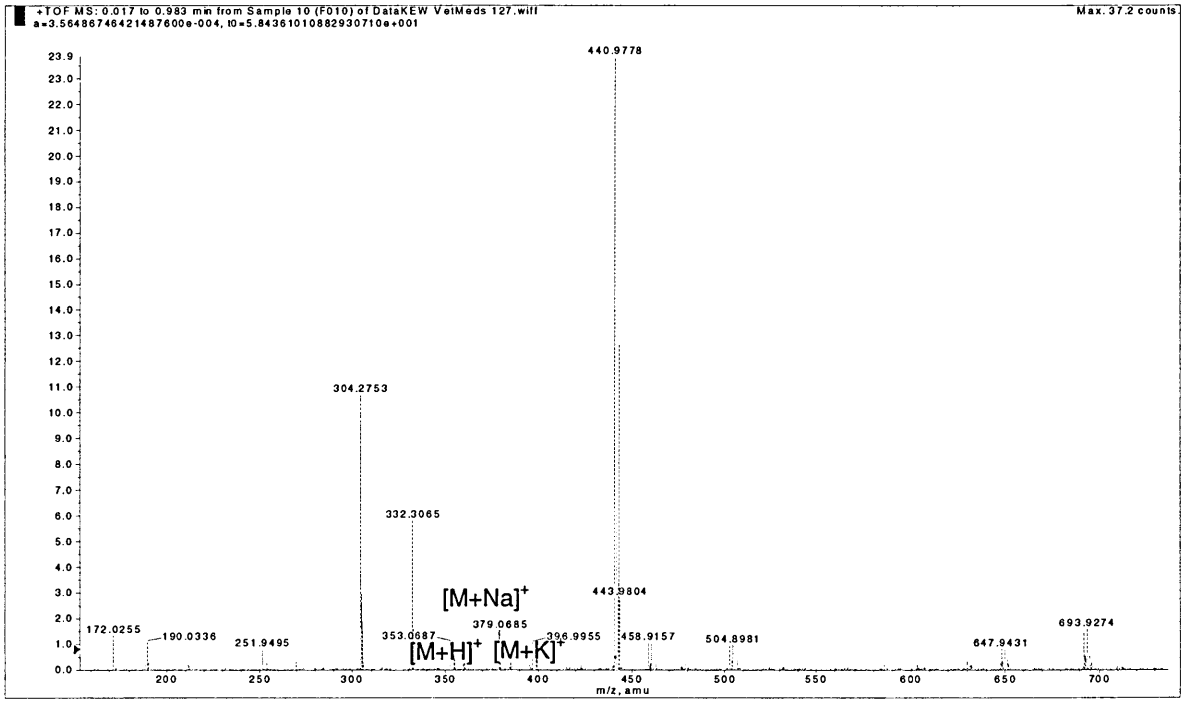


Figure 4.14 A spectrum from the ILM analysis of florfenicol showing analyte peaks at low ion intensities. 100 µl of the veterinary medicine stock solution was mixed with 100 µl α-CHCA ILM, 1 µl of the resultant mixture was spiked onto the target for analysis

Levamisol produced a spectrum with an intense peak relating to the protonated analyte ion at 205 Th and smaller peaks for the sodiated and potassiated ions at 227 and 243 Th, respectively. The resultant spectrum from the analysis using an ILM showed peaks for the sodiated and potassiated ions, whereas with the standard matrix these peaks were suppressed and only appeared amongst the background noise. See Figure 4.15.

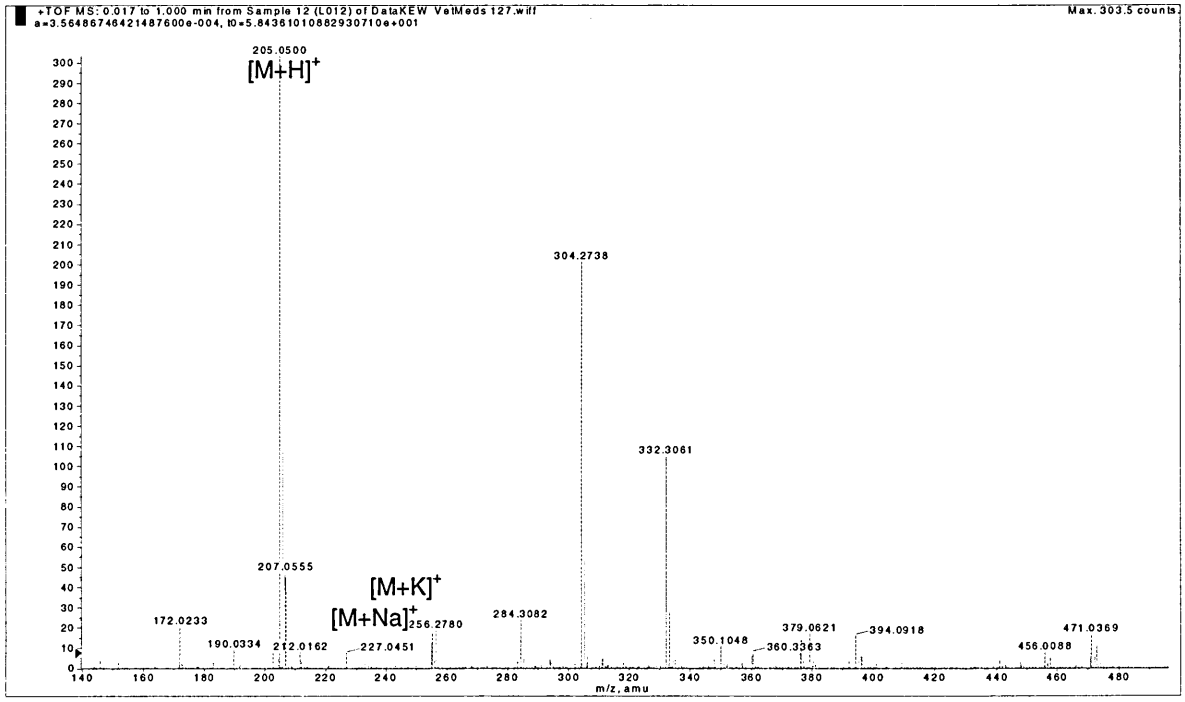


Figure 4.15 An ILM spectrum from the analysis of levamisol showing an intense peak at 205 Th relating to the protonated molecule of the analyte and lower intensity peaks for the sodium and potassium adducts. 100 µl of the veterinary medicine stock solution was mixed with 100 µl α-CHCA ILM, 1 µl of the resultant mixture was spiked onto the target for analysis

The ILM analysis of enrofloxacin produced a spectrum showing intense peak patterns for the protonated, sodiated and potassiated adducts of the analyte at 360, 382 and 398 Th. See Figure 4.16.

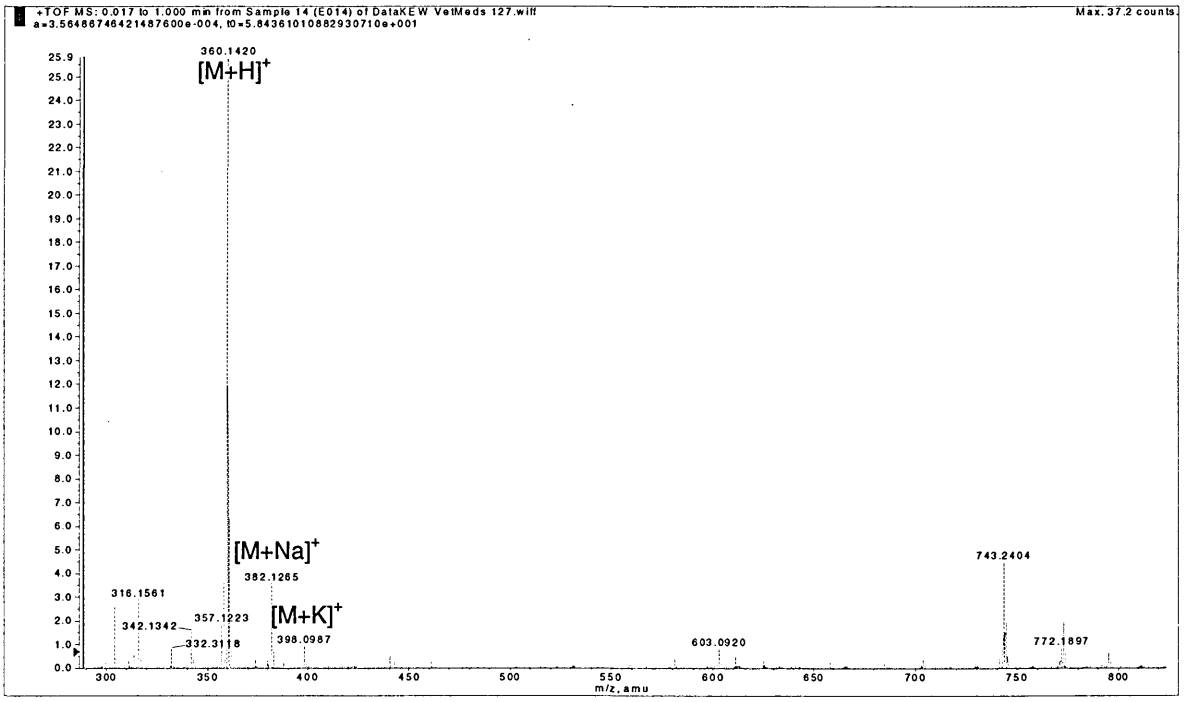


Figure 4.16 A spectrum from the analysis of enrofloxacin showing intense peaks relating to the protonated molecule (360 Th), the sodium adduct (382 Th) and the potassium adduct for the analyte (398 Th). 100 µl of the veterinary medicine stock solution was mixed with 100 µl α-CHCA ILM, 1 µl of the resultant mixture was spiked onto the target for analysis

The resultant spectrum from the analysis of tylosin again showed a matrix related peak, but none relating to the analyte. The peaks for tylosin should have appeared at 992 $[M+H]^+$, 1014 $[M+K]^+$ and 1030 $[M+K]^+$. See Figure 4.17.

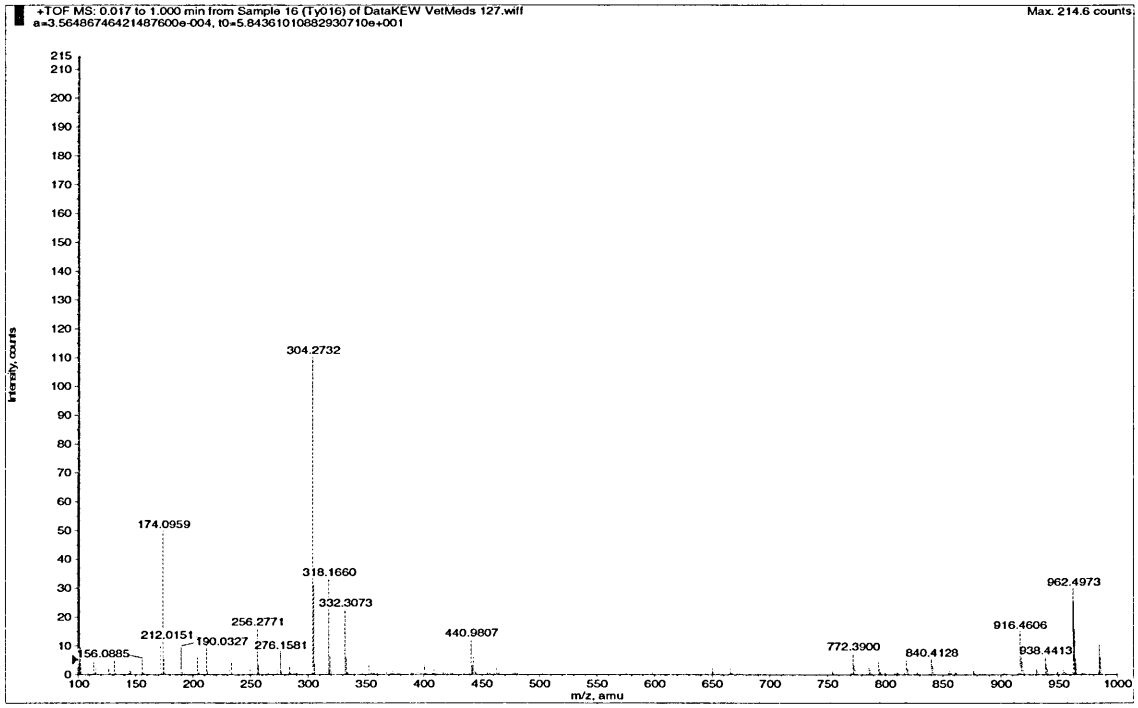


Figure 4.17 Spectrum from the ILM analysis of tylosin. 100 µl of the veterinary medicine stock solution was mixed with 100 µl α-CHCA ILM, 1 µl of the resultant mixture was spiked onto the target for analysis

The relative intensity ratios for the analyte compounds and α-CHCA TEA ILM were calculated by using the peaks relating to the protonated species from the matrix contribution and the veterinary medicine/pesticide analysed. The peak intensity chosen for α-CHCA TEA ILM was 102 Th $[TEA+H]^+$ and the respective veterinary medicine/pesticide value. The results are summarised in Table 4.2.

Compound Name	Relative Analyte:Matrix Intensity Ratio (α-CHCA)	Relative Analyte:Matrix Intensity Ratio (ILM)
Trimethoprim	15.8834	1.8263
Sulphadiazine	0.1820	0.0723
Oxytetracycline	0.4508	0.1228
Florfenicol	-	0.0364
Levamisole	5.2280	1.0338
Enrofloxacin	3.6107	0.6946
Tylosin	-	-
Cypermethrin	0.7337	0.0116

Table 4.2 Summarised values for relative intensity ratios calculated from peak values relating to the protonated molecules for α-CHCA matrix, α-CHCA TEA ILM and the veterinary medicine/pesticide compounds of interest.

There is a trend in calculated ratio values as you scan down the table for α-CHCA and the TEA ILM salt (Table 4.2). Typically, the values for trimethoprim, levamisol and enrofloxacin are much higher than the results for other compounds. When comparing the ILM with the standard α-CHCA matrix there is a definite decrease in all calculated values although the compound trend remains the same. This lowering of values can be explained by the relative peak intensity of the matrix contribution.

For the values to be smaller overall, the relative intensity of the matrix peak must be higher in comparison to the analysis compound. When using standard α-CHCA organic acid matrix, the peaks relating to the protonated, sodiated and potassiated adducts are mostly lost in noisy background of the resultant spectra and have fairly low intensities. When using the TEA ILM, the spectra do not show much background noise and the matrix related peak is prominent above any on the baseline. This means that the spectral data obtained for the TEA ILM analyses are suitable for processing due to the similar intensities of the matrix and analyte peaks and the greater influence of the single matrix related peak.

4.3.3 *Compound Calibration Studies*

The results for each veterinary medicine and pesticide compound were processed and the data collated. Results were entered into a spreadsheet to attempt the formation of calibration charts for future quantitation purposes. The analyte:ILM matrix peak area ratios were plotted against the original concentration of veterinary medicine/pesticide compound. See Figures 4.18 – 4.22.

The resultant calibration curves possessed very limited linearity and would not be successful for quantitation without further method development and the inclusion of an internal standard. In addition to the non-successful depiction of the calibration charts, neither florfenicol nor cypermethrin produced adequate analyte related peaks in this study and were therefore excluded from result processing.

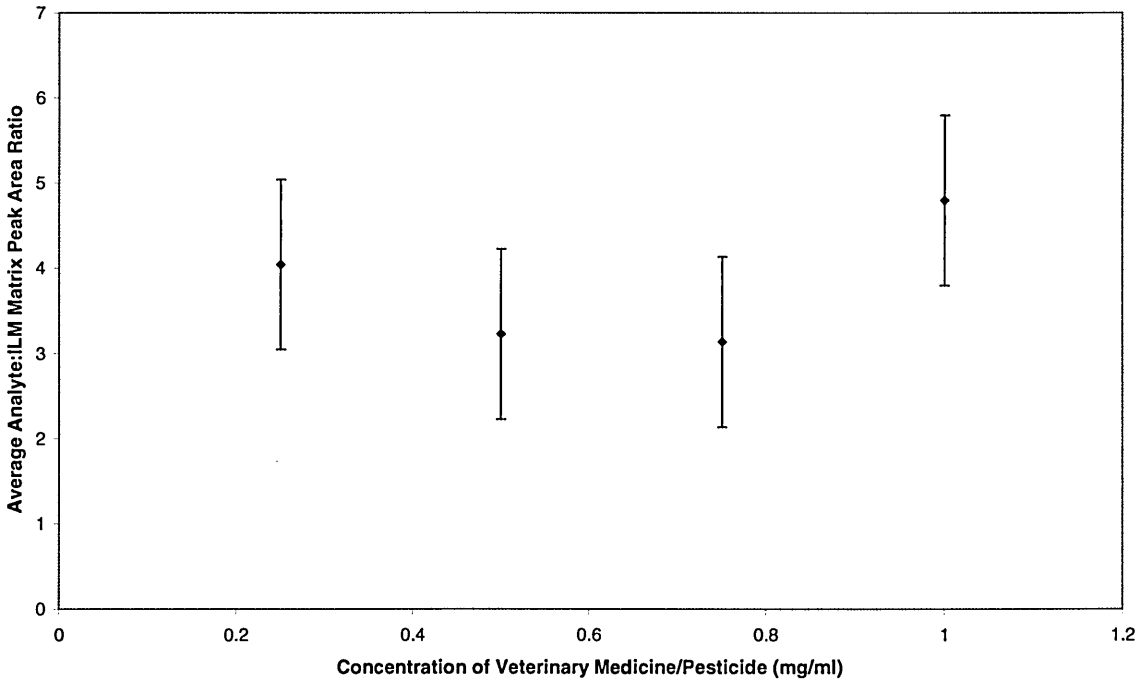


Figure 4.18 Calibration chart for the analysis of a concentration range of trimethoprim. The average analyte:ILM matrix peak area ratios were calculated from the ions for the protonated ILM and veterinary medicine (102 Th and 291, respectively) and plotted against the original concentration used

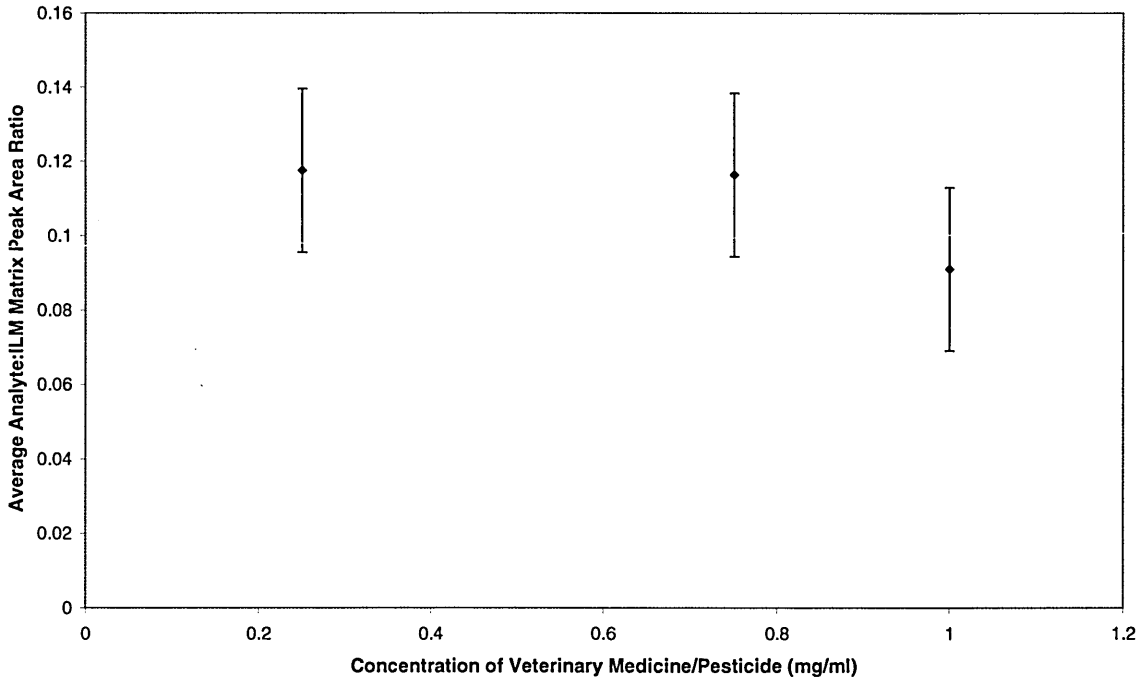


Figure 4.19 Calibration chart for the analysis of a concentration range of sulphadiazine. The average analyte:ILM matrix peak area ratios were calculated from the protonated ILM and veterinary medicine (102 Th and 251, respectively) and plotted against the original concentration used

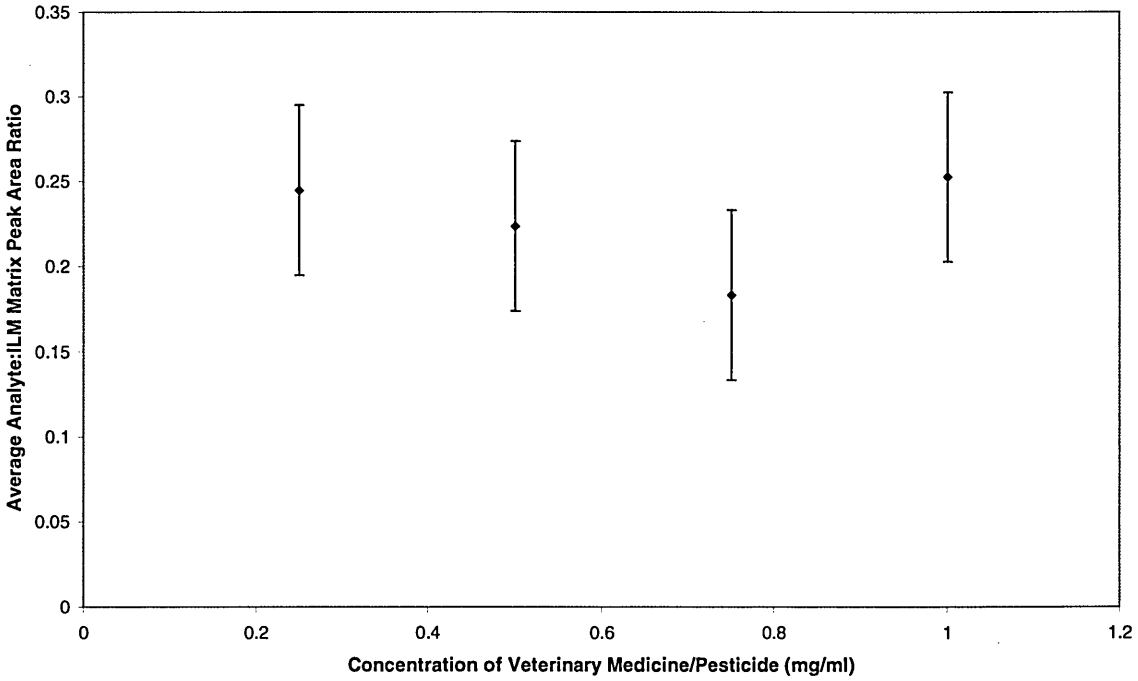


Figure 4.20 Calibration chart for the analysis of a concentration range of oxytetracycline. The average analyte:ILM matrix peak area ratios were calculated from the protonated ILM and veterinary medicine (102 Th and 461, respectively) and plotted against the original concentration used

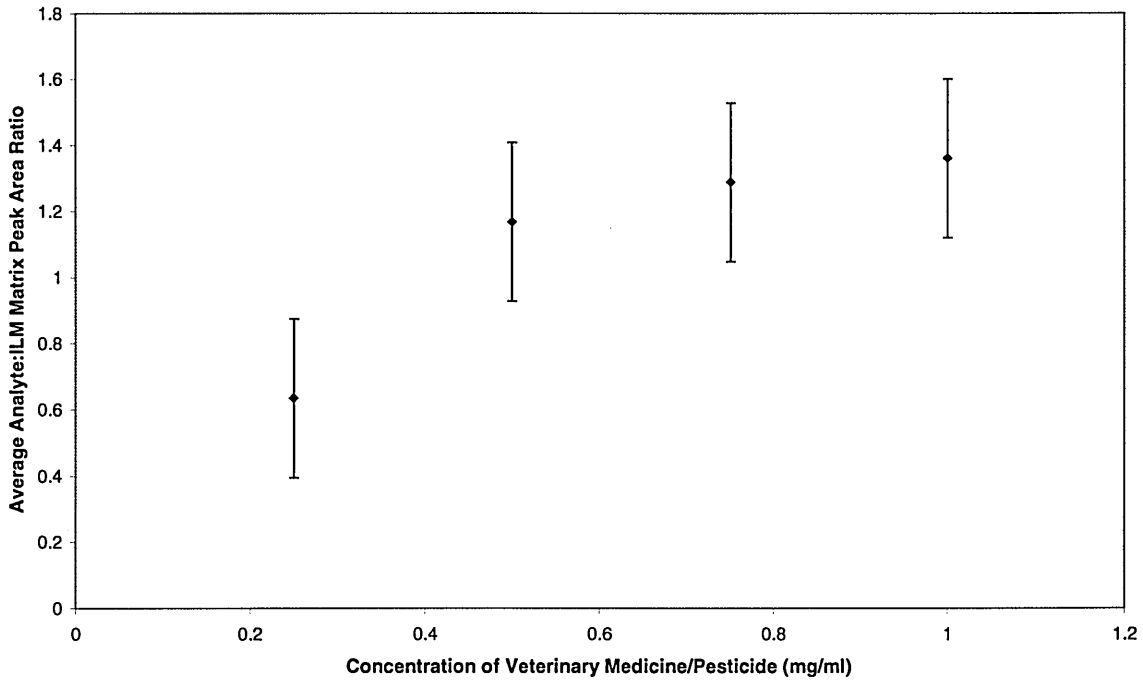


Figure 4.21 Calibration chart for the analysis of a concentration range of Levamisol. The average analyte:ILM matrix peak area ratios were calculated from the protonated ILM and veterinary medicine (102 Th and 205, respectively) and plotted against the original concentration used

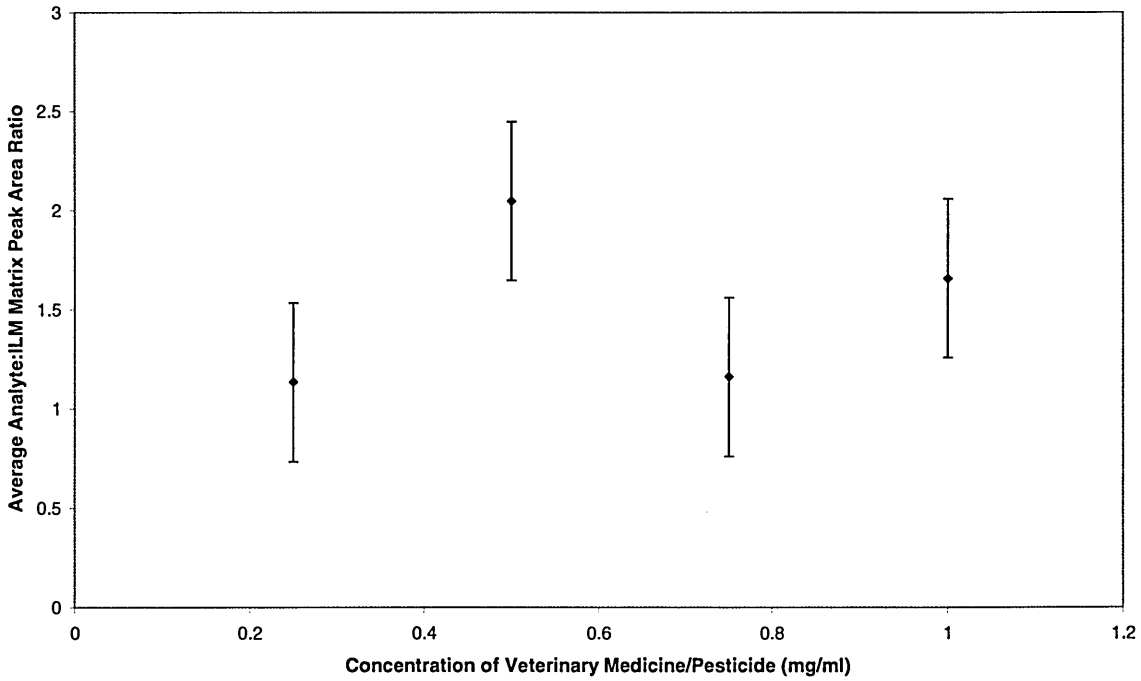


Figure 4.22 Calibration chart for the analysis of a concentration range of Enrofloxacin. The average analyte:ILM matrix peak area ratios were calculated from the protonated ILM and veterinary medicine (102 Th and 360, respectively) and plotted against the original concentration used

4.3.4 *Analysis Incorporating Organic Crop Samples*

All compounds were analysed as a mixture with carrot and lettuce extract in different samples. Tylosin was not analysed in this study due to a lack of previous results in combination with both standard organic matrix α-CHCA and the TEA ILM equivalent. The ILM α-CHCA TEA was used to minimise background peaks and produce spectral results that were easier to interpret. There were numerous peaks contributed by the vegetable extracts but neither analyte nor matrix suppression occurred.

To look for interfering peaks that originated from the vegetable extracts, a blank spectrum was produced for each and compared with a blank α-CHCA TEA ILM spectrum. Figures 4.23, 4.24 and 4.25 show the α-CHCA TEA blank and the results from the analysis of carrot and lettuce extract, respectively.

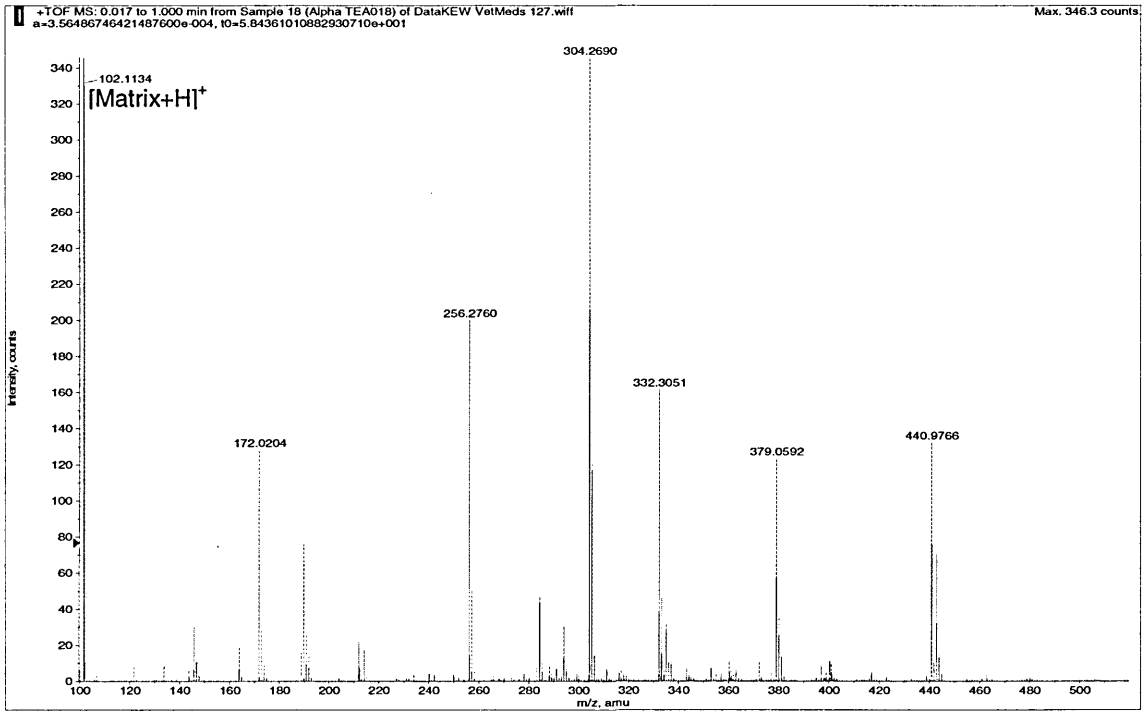


Figure 4.23 Blank α-CHCA TEA ILM spectrum

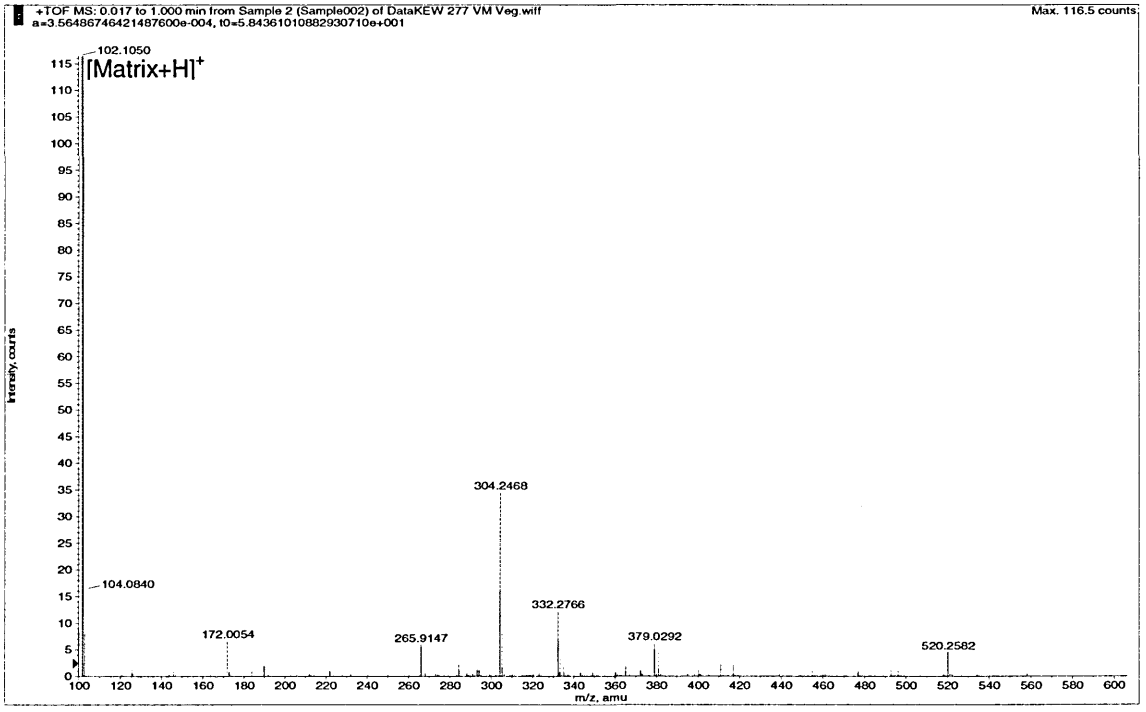


Figure 4.24 Spectrum from the ILM analysis of carrot extract

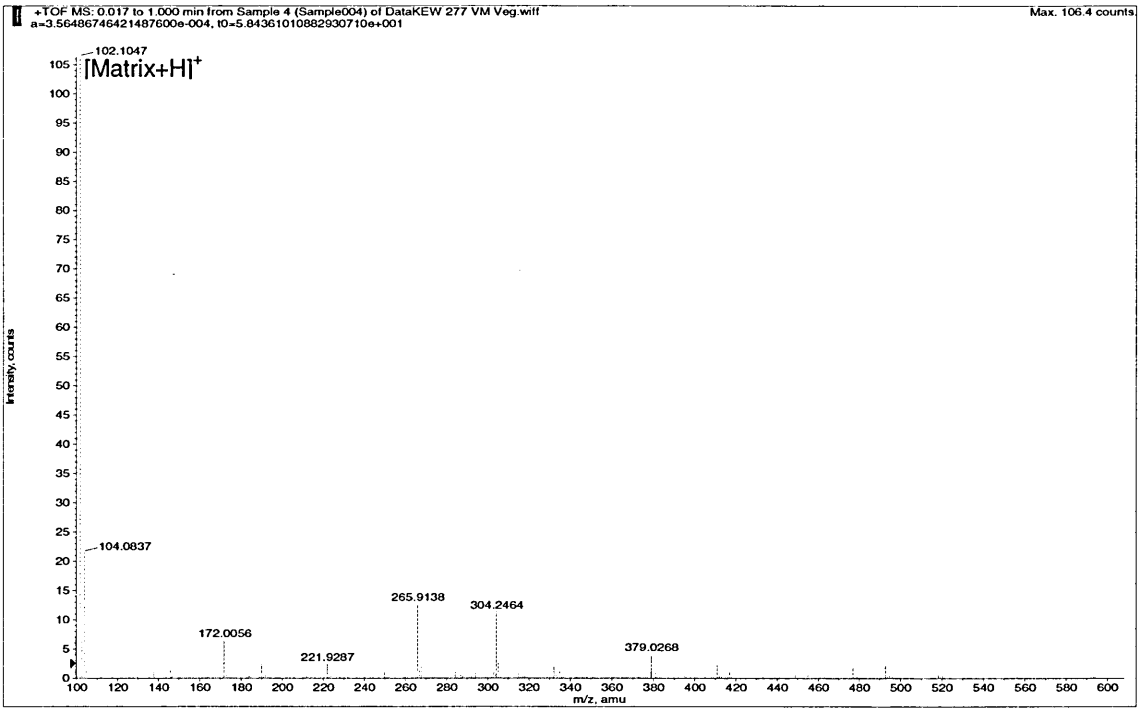


Figure 4.25 Spectrum from the ILM analysis of lettuce extract.

4.3.5 *Imaging Analysis of Veterinary Medicines*

Imaging analysis was performed to determine whether trimethoprim, levamisole and enrofloxacin could be detected directly from the carrot and lettuce surfaces. These veterinary medicine compounds were chosen due to the consistency of results and ion intensities through all analyses using standard and ILM α-CHCA matrix.

When the images were processed, these compounds could be directly detected from the crop surfaces. Suitable pixel images were obtained from the carrot and lettuce surfaces for all three compounds. The images show the protonated molecules of standard α-CHCA matrix and each compound at 190, 291, 205 and 360 Th, respectively. See Figures 4.26 and 4.27.

Standard α-CHCA matrix was utilised in these imaging experiments, the coating applied by air-spray deposition. The practical limitations of the ILM meant it was impossible to spray this onto the crop surface due to its more viscous nature and no other suitable application method has yet been developed for this purpose.

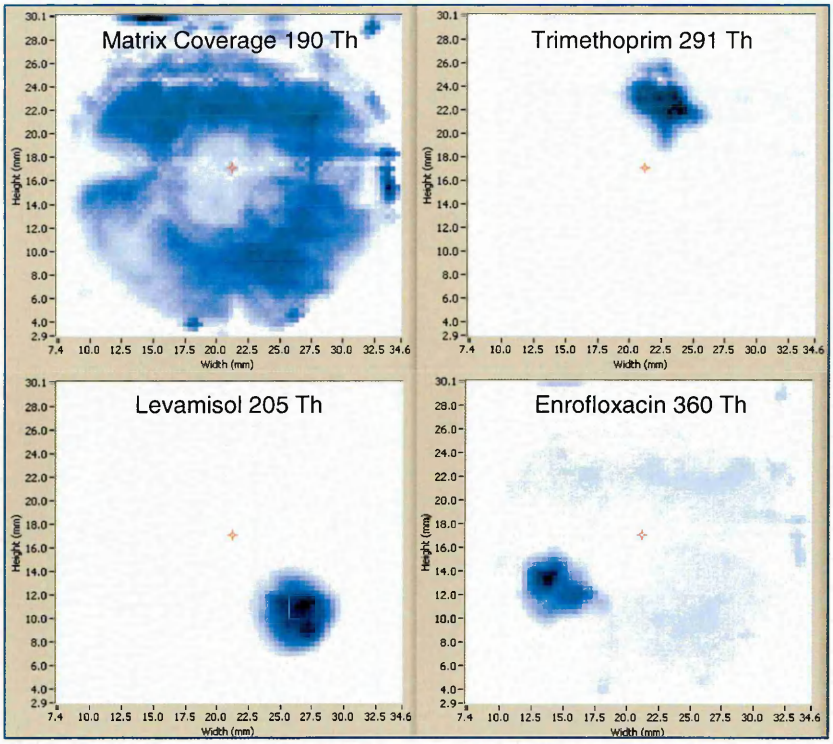


Figure 4.26 Pixel image representation from the analysis of a carrot slice previously spotted with 2 µl each of trimethoprim, enrofloxacin and levamisole, concentration 1 mg/ml.

The matrix coverage is reasonably homogenous on the lettuce surfaces, but patterns of sparse crystal cover appear on the carrot slice. The cross section of carrot shows less matrix around the edge of the slice where the skin was and also through the central core of the carrot.

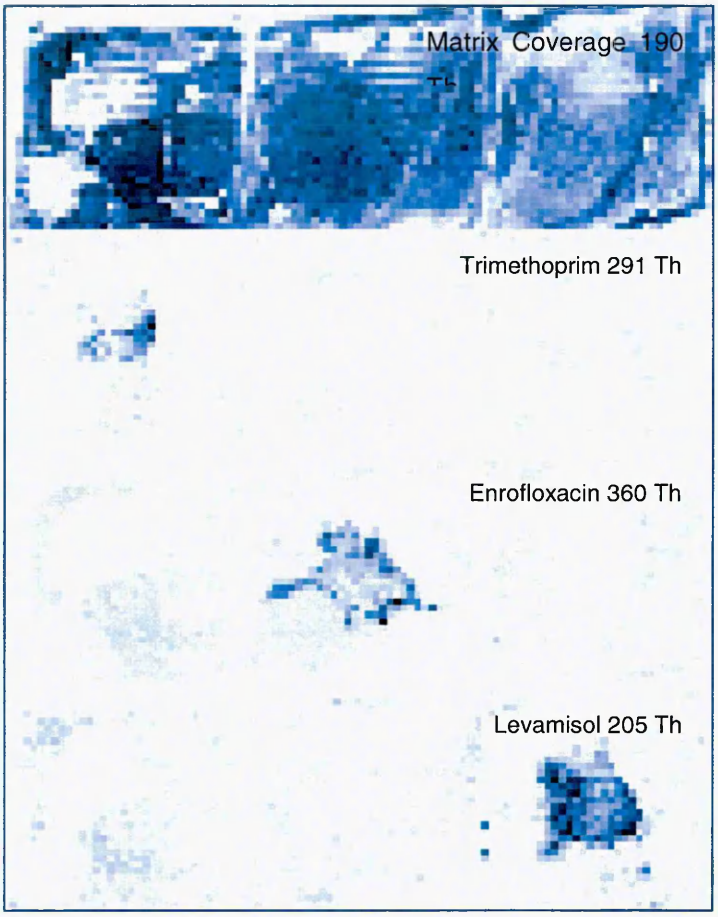


Figure 4.27 Pixel image representations from the analysis of a set of lettuce leaf sections previously spotted with 2 µl of trimethoprim, enrofloxacin and levamisole, concentration 1 mg/ml.

4.4 Conclusions

The analysis of veterinary medicine and pesticide compounds with α-CHCA organic acid matrix showed promise as a possible area for future analysis. With the exceptions of florfenicol and tylosin, which did not produce analyte peaks, all compounds yielded acceptable spectra. The spectra for most compounds depicted peaks relating to the protonated, sodiated and potassiated adducts of the intact molecule, but the strongest intensity peak relating to the protonated molecule was the only visible indication in some spectra. The lack of analyte peaks is thought to be partly due to analyte suppression from the matrix. The amount of background noise produced from using α-CHCA organic acid was extensive and therefore inhibited the appearance of analyte related peaks.

Ionic liquid matrices were utilised to attempt a reduction in background noise, enabling ionisation of the low molecular weight compounds. With ILM analysis, a single matrix related peak appeared on sample spectra allowing a clearer representation of the analyte peaks. Tylosin analysis was not successful even with reduced background noise. All other compounds produced acceptable spectra with extra analyte related peaks when compared with standard α-CHCA matrix. The spectra from florfenicol analysis demonstrated the presence of peaks relating to the protonated, sodiated and potassiated adducts at extremely low ion intensities. However, the incidence of these analyte related peaks demonstrates that reduced spectral background is effective in allowing sufficient analyte ions to be detected.

Calibration studies were relatively successful but further method development is required. Florfenicol and tylosin analysis were not successful with standard α-CHCA matrix, but the use of a different organic acid matrix could resolve this problem. The veterinary medicine/pesticide compounds produced calibration charts with some degree of linearity and could therefore be used for partial quantitation studies, taking into account the variation of results and errors. The incorporation of an internal standard into the analysis mixture would enable more accurate quantitation of unknown analyte concentrations.

Introducing vegetable extracts into the analysis mixture produced spectra with increased background noise originating from the vegetables. Combination with the ILM (single matrix-related peak) ensured that analyte peaks were still visible. No interfering peaks were encountered in sample spectra for either the ILM or the analytes in question rendering these crop choices suitable for further analytical development.

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Chapter 5

Conclusions and Future Work

5.1 Introduction

Occupational exposure to damaging or potentially harmful substances can be mostly eliminated if the correct precautions are followed. The detrimental effects produced from isocyanate and veterinary medicine compounds have been recognised and are now monitored by organisations such as the HSE to avoid unnecessary illness or ailment. Occupational exposure limits (OEL) have been set out for isocyanate compounds, and exposure to specific substances, whereas, maximum residue limits (MRL) govern veterinary medicine and pesticide restrictions. Responsibility still lies with individuals as procedures could be in place, but if they are not fully implemented their presence in the workplace is futile.

The need for rapid and accurate instrumentation and procedures is essential for the monitoring process. Science is ever changing, developing and improving which in turn enables greater protection of workforce staff.

Mass spectrometry has already proved invaluable in many areas of research and compound examination. The development of matrix application methods, improved matrix formats and higher quality instrumentation, all enable the use of MALDI as a rapid screening technique with the possibility for analyte quantitation.

5.2 Isocyanate Characterisation

MALDI-MS has successfully been applied to the analysis of NCO monomer and prepolymer samples. The results presented in Chapter 2 of this thesis represent a small example of overall data produced from the analyses of derivatised commercial formulations, based on the four most commonly encountered NCO monomers (HDI, MDI, 2,4- and 2,6-TDI). Using MALDI-MS, tandem MS and LC-MS the structures of certain experimental isocyanate compounds were confirmed. By utilising a combination of these techniques it was possible to elucidate structures of intact molecules and some fragment species from unknown polymeric isocyanate compounds. The application of tandem MS by-

passed the need for a separate on-line separation technique, proving simple and fairly rapid to execute. Tandem MS enabled fragments from possible structures to be identified, giving rise to proposed prepolymer constituents.

Analysis was based around the properties of the analyte compounds, namely the readiness to form protonated molecules when analysed by mass spectrometric methods. Problems were encountered when processing sample data due to excess amounts of derivatising reagent in the compounds and the formation of sodium and potassium adducts. The preference of the isocyanate compounds to form adducts over the protonated molecule complicated resultant spectra. The study of peaks relating to sample adducts were often more intense and separate from other interfering peaks. Looking for adduct patterns did enable easier elucidation of isocyanate structures in some cases.

The analysis of paint hardener from swab samples to obtain qualitative information was successful for the determination of isocyanate content. Derivatised monomeric and polymeric HDI compounds are both present, but further analysis is needed to determine higher mass polymeric structures.

The quantitation of isocyanate content in swab samples and other samples of interest to the HSL could be attempted using an internal standard and calibration procedures.

5.3 Direct Filter Quantitation

MALDI-MS has been successfully applied to the analysis of MDI-MP on MP coated filters. MDI-MP has been shown to readily form protonated molecules and adducts when subjected to MALDI-MS.

The developed air-spray method provided an almost completely homogeneous coverage on the filter surface to allow efficient ionisation at every point. This method also proved that the MP coating method described in MDHS 25/3 was successful, producing an even coverage (without the expected 'doughnut' ring

pattern), therefore giving efficient derivatisation of NCO compounds over the entire surface when they contact the filter.

Processing of the images showed little correlation between analyte pixel intensity and concentration of isocyanate applied, but it was proved that MDI-MP could be directly analysed from sample filter surfaces. A simple attempt was made to calibrate the procedure by using a matrix peak as an internal standard, but the outcome was limited and deemed unsuitable for quantitation purposes.

Deuterated analogues were used and have proved successful. The novel derivatising reagent, MDI-EP has also produced an excellent linear calibration, including concentrations of up to 1 mg/ml. The MDI-EP calibration showed less spread for same concentration repetitions, with an average standard deviation calculated to be slightly lower than that using deuterated MDI-MP. The quantitation work on both metal target and 'in-house' prepared filter samples has shown that MALDI-MS can analyse NCO on MP coated filters at occupational hygiene monitoring levels (< 1.0 μg NCO).

Data manipulation investigations into the use of peak intensities were unsuccessful. Peak area ratios for all analyses produced linear calibrations whereas those from peak intensity studies were of little value. Summed intensities from sample filter spectra showed limited promise but were ultimately ineffective. Utilising the data manipulation software on the instrument enabled regional analysis work to be performed by automatic calculation of isocyanate presence on the filter, but unfortunately this also proved inadequate for quantitation purposes.

Quantitation of WASP QA scheme filters is possible by employing calibrations based on sodium adduct ions for both D_6 MDI-MP and MDI-EP internal standards by using spiked NCO standard filters. The data obtained from quantitative studies of 'in-house' prepared filters were reasonable; however, method transferal to WASP round filters produced unacceptable results with the information gained having major limitations. Filters with HSL derived 'true' values of 0.4697 +/- 12% μg NCO and 0.2299 +/- 12% μg NCO, respectively

gave values of 0.9509 +/- 27% μg NCO and 0.8566 +/- 27% μg NCO when analysed directly by MALDI-MS.

The calibration method should be refined in future as an attempt to minimise the RSD and spread of results. The methods and procedures that have been implemented and developed in Chapter 3 of this thesis could now be expanded to cover other monomeric and polymeric NCO and the analysis of genuine workplace air samples if the application of developed methodology could be mastered.

5.4 Veterinary Medicine and Pesticide Residue Analysis

The initial analysis of veterinary medicine and pesticide compounds has been performed previously and provided the foundation for further study. Analysis using α-CHCA organic acid matrix enabled determination of these analyte compounds and the potential to move onto quantitation.

Tylosin and florfenicol could not be successfully analysed using α-CHCA matrix. No analyte related peaks were identified in sample spectra. The spectra for most compounds contained peaks relating to the protonated, sodiated and potassiated adducts of the intact molecule, but the strongest intensity peak relating to the protonated ion was the only visible indication in some spectra. There were many interfering peaks that appeared as background noise on spectra which made data manipulation extremely difficult in some situations. The lack of analyte peaks is thought to be partly due to analyte suppression from the matrix. The amount of background noise produced from using α-CHCA organic acid was extensive and therefore inhibited the appearance of analyte related peaks.

Ionic liquid matrices (ILM) were synthesised and utilised in an attempt to reduce background noise on sample spectra, enabling ionisation of the low molecular weight compounds. With ILM analysis, a single matrix related peak appeared on sample spectra with very few other matrix related peaks appearing, at extremely low intensities when they did occur. This method allowed a clearer

representation of the analyte peaks which aided the interpretation of sample results. Tylosin analysis was not successful even with reduced background noise although florfenicol results were obtained with the use of ILM. The spectra from florfenicol analysis demonstrated the presence of peaks relating to the protonated, sodiated and potassiated adducts at extremely low ion intensities. However, the incidence of these analyte related peaks demonstrates that reduced spectral background is effective in allowing sufficient analyte ions to be detected. All other compounds produced acceptable spectra with extra analyte related peaks compared with standard α-CHCA matrix at greater ion intensities.

Calibration studies were relatively successful but further method development is required. As a standard α-CHCA organic acid matrix was being utilised, florfenicol and tylosin compounds were excluded, although the use of a different organic acid matrix could resolve this problem. The veterinary medicine/pesticide compounds produced calibration charts with some degree of linearity and could therefore be used for partial quantitation studies, taking into account the variation of results and errors. Studies were not developed enough, but it is thought that incorporating an internal standard into the analysis mixture would enable more accurate calibration charts and therefore quantitation of unknown analyte concentrations.

Introducing vegetable extracts into the analysis mixture produced spectra with increased background noise originating from the vegetables. Carrot and lettuce extracts were employed as these were crops that the HSL are particularly interested in monitoring for real sample analysis. Combination with the ILM (single matrix-related peak) ensured that analyte peaks were still visible. No interfering peaks were encountered in sample spectra for either the ILM or the analytes in question rendering these crop choices suitable for further analytical development.

Imaging analysis of crop samples produced promising results, depicting areas corresponding to the applied veterinary medicine compounds. Small amounts of the chosen compounds (trimethoprim, levamisol and enrofloxacin) were applied to the crop surfaces and allowed to dry. The samples were covered with α-CHCA matrix and analysed by imaging MALDI-MS. There are definite areas of

the compounds on resultant images with some spatial resolution being maintained. If spatial resolution was important to the investigation, other methods for matrix application could be considered such as rapid evaporation air-spray deposition utilising acetone, for drier sample coverage.

Direct quantitation of veterinary medicine and pesticide residues could be accomplished with a suitable calibration chart and the introduction of an appropriate internal standard.

Appendices

Appendix 1

Refereed Publications

Characterisation of Derivatised Monomeric and Prepolymeric Isocyanates by MALDI-TOF-MS and Structural Elucidation by Tandem MS

Karen E. Warburton[1], Malcolm R. Clench*[1], Michael J. Ford[1,3], John White[2], Duncan A. Rimmer[2] and Vikki A Carolan[1]

[1] Biomedical Research Centre, Sheffield Hallam University, Howard Street, Sheffield, S1 1WB, UK

[2] Health and Safety Laboratory, Broad Lane, Sheffield, S3 7HQ, UK

[3] Oak Ridge National Laboratory, Oak Ridge, TN, USA

*European Journal of Mass Spectrometry.***2005**, 11, 6, 565-574.

Imaging Matrix Assisted Laser Desorption Ionisation Mass Spectrometry; A New Technique for Drug Distribution Studies

Sally J. Atkinson[1], Brendan Prideaux[1], Josephine Bunch[1,2], Karen E. Warburton[1] and Malcolm R. Clench*[1]

[1] Biomedical Research Centre, Sheffield Hallam University, Howard Street, Sheffield, S1 1WB, UK

[2] Centre for Analytical Science, University of Sheffield, Western Bank, Sheffield, S10 2TN, UK

Chemistry Today, **2005**, 23, 6, 5-8.

Quantitative Determination of NCO Compounds on Air Sampling Filters by Matrix Assisted Laser Desorption Ionisation - Mass Spectrometry

Karen E. Warburton [1], Malcolm R. Clench [*1], John White [2], Duncan A. Rimmer [2] and Vikki A. Carolan [1]

[1] Biomedical Research Centre, Sheffield Hallam University, Pond Street Sheffield, S1 1WB, UK

[2] Health and Safety Laboratory, Harpur Hill, Buxton, SK17 9JN, UK

Rapid Communications in Mass Spectrometry.

Appendix 2

Conference Attendance and Presentations

The Chromatographic Society

One day event in Cheshire, UK, October 2002

Colloquia

One day MALDI event at UMIST, UK, March 2003

51st American Society of Mass Spectrometry Conference

Montreal, Canada, June 2003

Presented poster entitled "The application of MALDI-TOF-MS to the qualitative determination of isocyanate derivatives"

Analytical Research Forum

Sunderland, UK, July 2003

Presented poster entitled "The applications of MALDI-TOF-MS and MALDI-MS-MS to the qualitative and quantitative determination of isocyanate derivatives"

16th International Mass Spectrometry Conference

Edinburgh, Scotland, September 2003

Presented poster entitled "The application of MALDI-TOF-MS-MS to the qualitative determination of isocyanate derivatives"

ASN Meeting on Mass Spectrometry

One day event at Sheffield Hallam University, UK, March 2004

Oral presentation entitled "Direct determination of isocyanates on industrial air sampling filters by MALDI mass spectrometry"

2nd National Meeting on Environmental Mass Spectrometry

One day event in Chester, UK, April 2004

Oral presentation entitled "The characterisation of isocyanates by MALDI mass spectrometry"

52nd American Society of Mass Spectrometry Conference

Nashville, America, May 2004

Invited speaker at an imaging mass spectrometry meeting

Oral presentation entitled "Direct determination and quantification of isocyanates on industrial air sampling filters by MALDI-MS"

Analytical Research Forum

Preston, UK, July 2004

Presented poster entitled "Direct determination and quantification of isocyanates on industrial air sampling filters by MALDI mass spectrometry"

British Mass Spectrometry Society Conference

Derby, UK, September 2004

Poster presentation entitled "Direct determination and quantification of isocyanates on industrial air sampling filters by MALDI mass spectrometry"

53rd American Society of Mass Spectrometry Conference

San Antonio, America, June 2005

Presented poster entitled "Direct analysis and quantification of isocyanate compounds from industrial air-sampling filters by MALDI mass spectrometry"

Analytical Research Forum

Plymouth, UK, July 2005

Oral presentation entitled "Direct analysis and quantification of isocyanates from industrial air-sampling filters by MALDI mass spectrometry"

British Mass Spectrometry Society Conference

York, UK, September 2005

Oral presentation entitled "Direct analysis and quantification of isocyanates from industrial air-sampling filters by MALDI mass spectrometry"

Light – Tandem Mass Spectrometry (MALDI-TOF-MS-MS) to the Qualitative Determination of Isocyanate Derivatives

Karen E. Warburton[1], Michael J. Ford[1], Malcolm R. Clench[1], John White[2] and Duncan Rimmer[2]

[1]Biomedical Research Centre, School of Science and Mathematics, Sheffield Hallam University, Pond Street, Sheffield, S1 1WB, UK
[2]The Health and Safety Laboratory, Broad Lane, Sheffield, S3 7HQ, UK

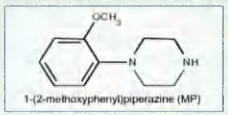
Introduction

Isocyanates [NCO] are a group of highly reactive compounds which can produce an adverse effect in humans following prolonged contact [1]. They are known respiratory sensitisers and are the most common cause of occupationally induced asthma, bronchitis and emphysema [2-6]. Sensitisation is an irreversible allergic reaction which results in the loss of the ability to cope with even one ppb of isocyanate compound in the atmosphere for a short period of time, before an asthmatic response is triggered [7,8]. There are numerous methods for NCO determination, but the Health and Safety Executive monitors and implements safe working practice in the U.K. The HSE documentation regarding exposure patterns is set out in the Methods for the Determination of Hazardous Substances number 25/3 (MDHS 25/3) [9]. Mono functional NCOs are commonly used as intermediate compounds in the production of herbicides, crop-protection agents and anti-diabetic drugs. However, the most important industrial form of NCO is the di-functional group including hexamethylene diisocyanate (HDI) and 4,4-methylenebiphenyl Diisocyanate (MDI). In this poster, the analyses of derivatised monomeric NCO standards and oligomeric pre-polymer NCO samples are reported. The samples were analysed as 1-(2-methoxyphenyl)piperazine (MP, $C_{11}H_{17}N_2O$:193.1341) derivatives by MALDI/MS/MS.

Method

All analyses were performed using an Applied Biosystems/MDS Sciex "Q-Star" hybrid quadrupole time of flight instrument, fitted w orthogonal MALDI ion source. The two diisocyanate compounds investigated were MP derivatised Trimeric HDI and Polymeric MDI diisocyanate concentrations were 1 mgmL^{-1} made up in methanol with 0.1% Trifluoro Acetic Acid (TFA). The organic acid matrix use α-Cyano-4-Hydroxy Cinnamic Acid (α-CHCA), at a concentration of 25 mgmL $^{-1}$ in methanol / 0.1% TFA. 200 µL of the sample and of the matrix were mixed and 1µL of the resulting solution was placed on the stainless steel MALDI target for analysis.

The two samples were analysed in normal MS mode and their mass spectra obtained. Main peaks in the spectra were then f analysed by tandem mass spectrometry using accurate mass calibration of the instrument. The purpose of the MS/MS analysis was t



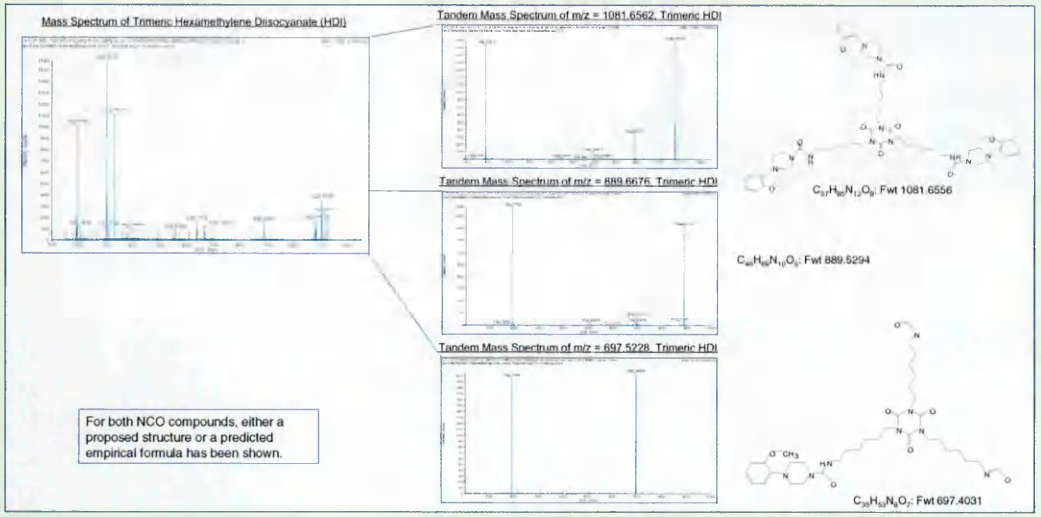
1-(2-methoxyphenyl)piperazine (MP)

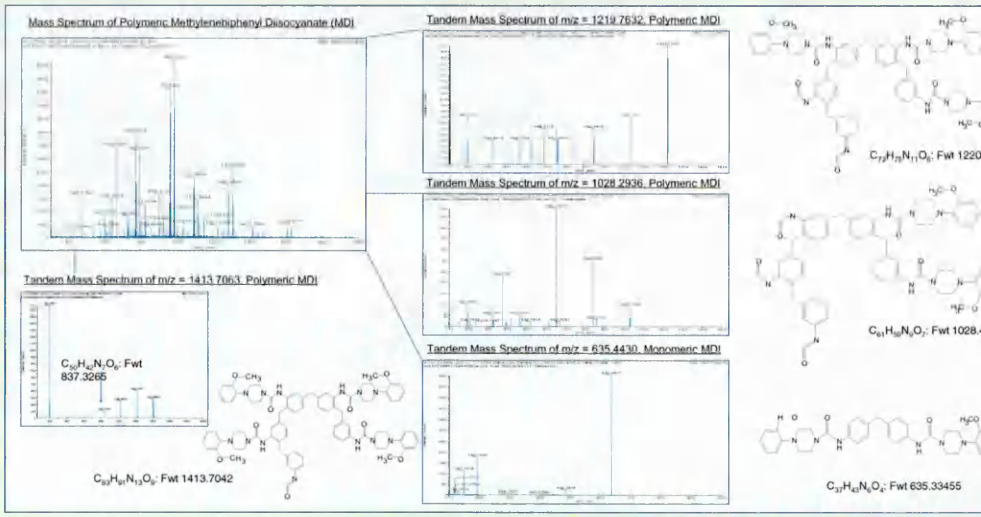
study the fragmentation patterns of the two compounds and to determine the structures of unknown fragments that occur when the sample is ionised. The determination of the structural properties of thes species is an important area of investigation, the main concern is whether or not the high mass ions a NCO species. If the known structural formula for a protonated MP derivatised trimeric HDI species, equivalent to the ion at m/z 1082, can be confirmed by analysis then the same experimental methods and findings can be applied to unknown species.

Results and Discussion

It was found that MP derivatised NCO yield excellent quality MALDI mass spectra characterised by intense protonated molecules with sodium and potassium adduct ions. Using MS/MS and accurate mass measurement it has been possible to tentatively assign structures to compounds of RMM more than 1000 Da, observed in the pre-polymer samples. A possible method of identifying the degree of NCO functionality is proposed, based on the observation of ions corresponding to loss of the derivatising reagent. The results presented here show mass spectra and product ion scans of the different fragment ions for each compound.

All spectra show the presence of the rearrangement / α-cleavage product m/z 193, analogous to the protonated MP derivatising ag The main peaks on both mass spectrum showed the losses of subsequent MP groups. HDI showed losses of two MP groups at m/z a product ion relating to the loss of one derivatising group (192 Da) and 697 showing the loss of two MP. MDI showed apparent losse three MP groups at m/z values of 1220, 1028 and 837. The proposed structures for each of the fragments are superimposed beside relevant spectra.





Conclusion

A derivatised HDI trimer precursor ion, m/z 1082, containing three NCO functional groups, dissociates to produce ions corresponding to the loss of two, but not three derivatising reagents. The species obtained from HDI analyses, shown above, were expected and their structures were known prior to investigation. It is believed that a species that retains one derivatising reagent may be electronically and structurally stable enough to avoid further dissociation. It may be possible to postulate that the total number of NCO functional groups is equal to the number of observed losses of derivatising reagent plus one.

The MDI spectrum produced by product ion experiments on m/z 1413, shows the characteristic loss of three MP groups, which would suggest a four NCO group functionality species. The structure for the ion corresponding to m/z 1413 is shown above, as are the proposed structures of relatively low masses evident from product ion scanning. MALDI/MS/MS was applied to four MDI related ions, m/z 1413, 1220, 1028 and 635.

Further work will include the proposal of structures other than those mentioned here, including other isocyanate fragments and unknown species that do not contain NCO functionality.

Special thanks to Dr. Detlev Schleuder of Applied Biosystems, Germany, for his assistance in acquiring the MDI mass spectra

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[1] Butcher, B. T., Mapp, C. E. & Fabbri L. M. "Polyisocyanates and Prepolymers" in *Asthma in the Workplace*. Bernstein, I. L. **1993**, 415. Marcel-Dec
[2] Wegman, D. H., Peters, L., Pagnotto, L. & Fine, L. J. *British Journal of Ind Medicine*. **1997**, 34, 196-200.
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[9] The Health and Safety Executive, MDHS 25/3. *Isocyanate Measurement Met*

(MALDI-TOF-MS-MS) to the Qualitative Determination of Isocyanate Derivatives

Karen E. Warburton[1], Michael J. Ford[1], Malcolm R. Clench[1], John White[2] and Duncan Rimmer[2]

[1]Biomedical Research Centre, School of Science and Mathematics, Sheffield Hallam University, Pond Street, Sheffield, S1 1WB, UK
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ntroduction

yanates [NCO] are a group of highly reactive compounds which can produce an adverse t in humans following prolonged contact [1]. They are known respiratory sensitisers and are most common cause of occupationally induced asthma, bronchitis and emphysema [2-6]. nsitisation is an irreversible allergic reaction which results in the loss of the ability to cope with one ppb of isocyanate compound in the atmosphere for a short period of time, before an matic response is triggered [7,8]. There are numerous methods for NCO determination, but the lth and Safety Executive monitors and implements safe working practice in the U.K. The HSE umentation regarding exposure patterns is set out in the Methods for the Determination of ardous Substances number 25/3 (MDHS 25/3) [9]. Mono functional NCOs are commonly used intermediate compounds in the production of herbicides, crop-protection agents and anti-betic drugs. However, the most important industrial form of NCO is the di-functional group luding hexamethylene diisocyanate (HDI) and 4,4-methylenebiphenyl Diisocyanate (MDI). In poster, the analyses of derivatised monomeric NCO standards and oligomeric pre-polymer O samples are reported. The samples were analysed as 1-(2-methoxyphenyl)piperazine (MP, $H_{17}N_2O$:193.1341) derivatives by MALDI/MS/MS. MS Imaging has also been used to ermine Isocyanate content upon industrially used glass fibre filters.

Method

All analyses were performed using an Applied Biosystems/MDS Sciex "Q-Star" hybrid quadrupole time of flight instrument, fitted with an orthogonal MALDI ion source. The diisocyanate compound investigated was MP derivatised Trimeric HDI. The diisocyanate concentration was 1 mgmL^{-1} made up in methanol with 0.1% Trifluoro Acetic Acid (TFA). The organic acid matrix used was α-Cyano-4-Hydroxy Cinnamic Acid (α-CHCA), at a concentration of 25 mgmL $^{-1}$ in methanol / 0.1% TFA. 200 µL of the sample and 40 µL of the matrix were mixed and 1µL of the resulting solution was placed on the stainless steel MALDI target for analysis.

The sample was analysed in normal MS mode and a mass spectrum obtained. Main peaks in the spectrum were then further analysed by tandem mass spectrometry using accurate mass calibration of the instrument. The purpose of the MS/MS analysis was to study the fragmentation patterns of the HDI compound and to determine the structures of unknown fragments that occur when the sample is ionised.

1-(2-methoxyphenyl)piperazine (MP)

The determination of the structural properties of these species is an important area of investigation, the main concern is whether or not the high mass ions are NCO species. If the known structural formula for a protonated MP derivatised trimeric HDI species, equivalent to the ion at m/z 1082, can be confirmed by analysis then the same experimental methods and findings can be applied to unknown species.

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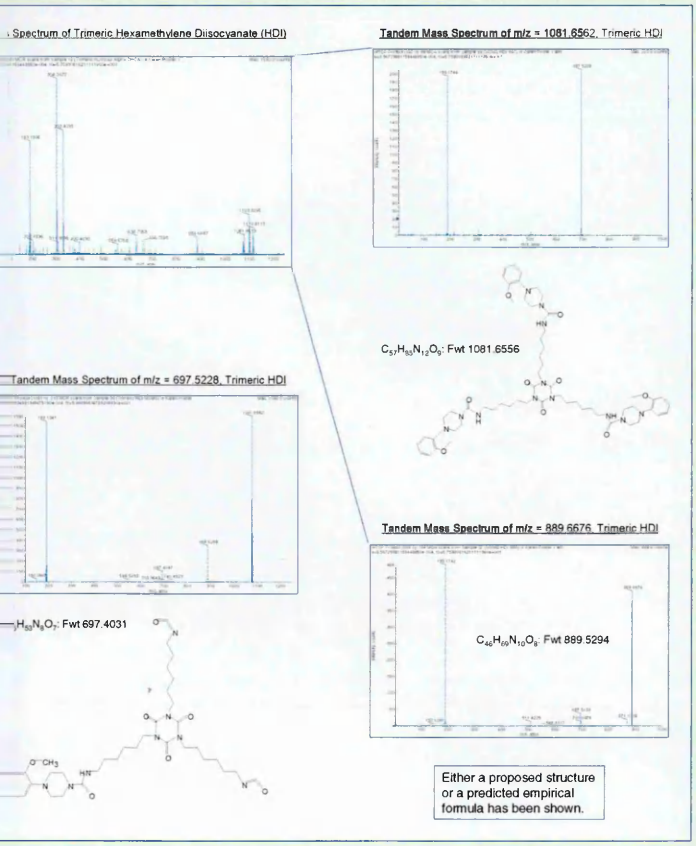
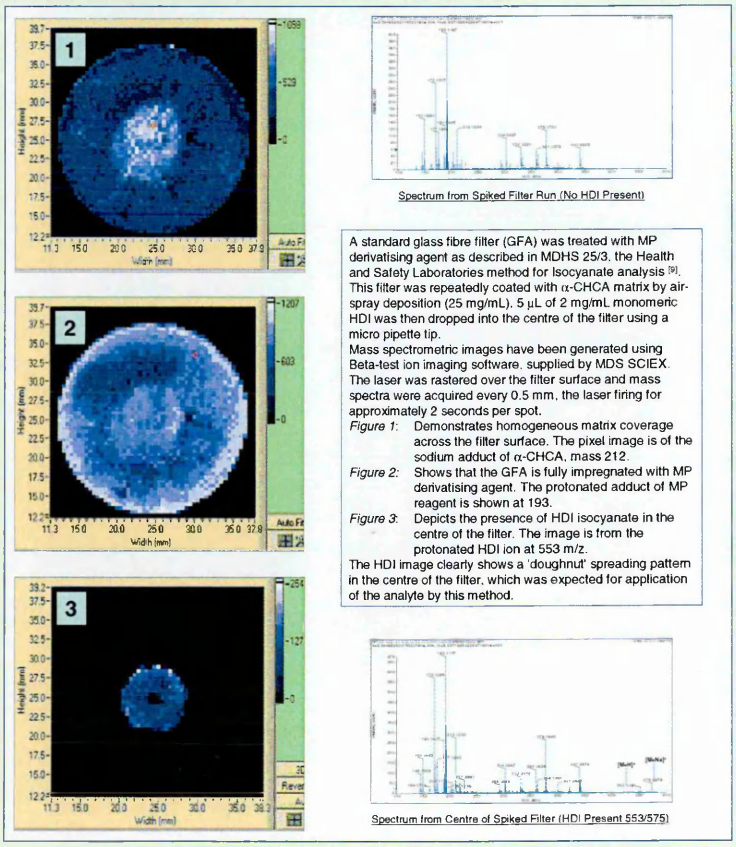
as found that MP derivatised NCO yield excellent quality MALDI mass spectra characterised intense protonated molecules with sodium and potassium adduct ions. Using MS/MS and urate mass measurement it has been possible to tentatively assign structures to compounds FRMM more than 1000 Da, observed in the pre-polymer samples. A possible method of tifying the degree of NCO functionality is proposed, based on the observation of ions responding to loss of the derivatising reagent. The results presented here show mass spectra product ion scans of the different fragment ions for each compound.

All spectra show the presence of the rearrangement / α-cleavage product m/z 193, analogous to the protonated MP derivatising agent. The main peaks on the HDI mass spectrum showed the losses of subsequent MP groups. HDI showed losses of two MP groups at m/z 889, a product ion relating to the loss of one derivatising group (192 Da) and 697 showing the loss of two MP. The application of this method to other pre-polymer samples such as MDI, should indicate the number of MP groups present in the compound and therefore its NCO functionality. Structures for each of the fragments are superimposed beside the relevant spectra.





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r work will include the analysis of other pre-polymer isocyanate es and the proposal of structures other than those mentioned here. predicted structures will include other isocyanate fragments and wn species that do not contain NCO functionality.

Imaging of the GFA's showed excellent potential for direct filter analysis. The matrix application method has been optimised to ensure a homogeneous layer is present, enabling successful ionisation of the analyte over the entire filter surface.

Future work using GFA filters will be carried out on actual sample filters collected from industrial processes to determine the presence and possible quantitation of differing isocyanate compounds using MALDI/MS. Quantitation will however require a calibration to be performed with the inclusion of an internal standard (deuterated isocyanate compounds are proposed for this purpose).

Karen Warburton would like to acknowledge the financial support received from BMSS to enable attendance at the IMSC conference 2003

References

[1] Butcher, B. T., Mapp, C. E. & Fabbri L. M. "Polyisocyanates and their Prepolymers" in *Asthma in the Workplace*. Bernstein, I. L. **1993**, 415. Marcel-Decker.
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Ionisation - Time of Flight – Mass Spectrometry (MALDI-TOF- MS) to the Qualitative & Quantitative Determination of Isocyanate Derivatives

Karen E. Warburton[1], Malcolm R. Clench[1], John White[2] and Duncan Rimmer[2]

[1]Biomedical Research Centre, School of Science and Mathematics, Sheffield Hallam University, Pond Street, Sheffield, S1 1WB, UK
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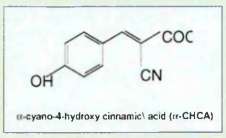
Method

All analyses were performed using an Applied Biosystems/MDS Sciex "Q-Star" hybrid quadrupole time of flight instrument, fitted with an orthogonal MALDI ion source. The isocyanate compound investigated was MP derivatised MDI. The original working concentration was 1 mgmL^{-1} made up in methanol with 0.1% Trifluoro Acetic Acid (TFA). The organic acid matrix used was α-Cyano-4-Hydroxy Cinnamic Acid (α-CHCA), at a concentration of 25 mgmL $^{-1}$ in methanol / 0.1% TFA.

Various methods of matrix application were investigated to coat the filter surface. The most homogeneous and successful method for matrix application was airspray deposition. The filter was sprayed with 5 coats of α-CHCA matrix, being left to dry under a heat lamp between layers.

1-(2-methoxyphenyl)piperazine (MP)

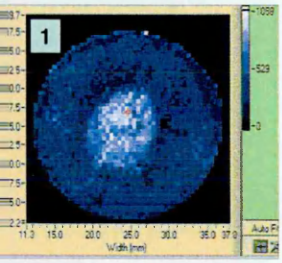


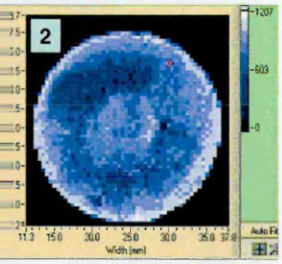
α-cyano-4-hydroxy cinnamic acid (α-CHCA)

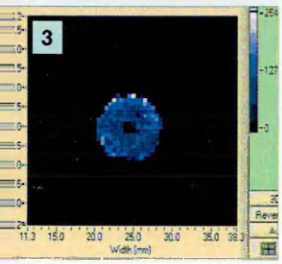
esults and Discussion

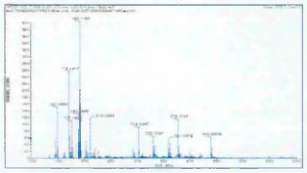
as found that MP derivatised NCO yield excellent quality MALDI mass spectra characterised intense protonated molecules $[M+H]^+$, sodium $[M+Na]^+$ and potassium $[M+K]^+$ adducts. Before anate analysis could be carried out, it had to be determined if the matrix application method derivatisation method carried out by the HSE (using MP) produced even coverage and were efore successful. As an MP solution is dropped into the centre of the filter, it was necessary to mine the MP spread on the filter surface. It was thought that the distribution of MP would be a ghnut' shape, sparse in the centre and around the edge but with an intense ring pattern.

Once it had been decided that the matrix application and derivatising methods were adequate, 5 µL of 1 mgmL^{-1} MDI was dropped onto the centre of the filter to determine if isocyanates could be detected directly from the GFA, which they could. A quantitation process was necessary if an industrial monitoring program was to be established. A sample filter was spotted with a concentration range of MDI and again imaged. The isocyanate was detected for each concentration, but the pixel intensities did not directly relate to the concentration of isocyanate applied. A deuterated MDI analogue was investigated for use as an internal standard, with promising results.








Spectrum from Spiked Filter Run (No MDI Present)

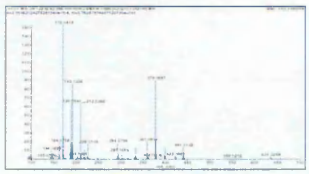
A standard glass fibre filter (GFA) was treated with MP derivatising agent as described in MDHS 25/3, the Health and Safety Laboratories method for isocyanate analysis [9]. This filter was repeatedly coated with α-CHCA matrix by air-spray deposition (25 mg/mL). 5 µL of 2 mg/mL monomeric MDI was then dropped into the centre of the filter using a micro pipette tip.
Mass spectrometric images have been generated using Beta-test ion imaging software, supplied by MDS SCIEX. The laser was rastered over the filter surface and mass spectra were acquired every 0.5 mm, the laser firing for approximately 2 seconds per spot with 25% power.

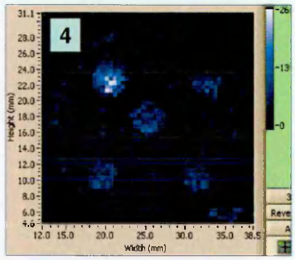
Figure 1: Demonstrates homogeneous matrix coverage across the filter surface. The pixel image is of the sodium adduct of α-CHCA, mass 212.

Figure 2: Shows that the GFA is fully impregnated with MP derivatising agent. The protonated adduct of MP reagent is shown at 193.

Figure 3: Depicts the presence of MDI isocyanate in the centre of the filter. The image is from the protonated MDI ion at 635 m/z.

The MDI image clearly shows a 'doughnut' spreading pattern in the centre of the filter, which was expected for application of the analyte by this method.


Spectrum from Centre of Spiked Filter (MDI Present 635)

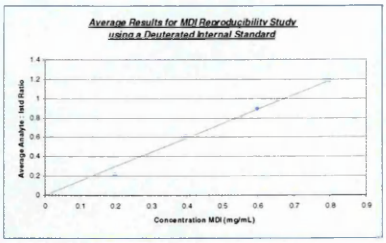


The MP impregnated filter was coated with 25 mg/mL α-CHCA matrix by air-spray deposition (5 layers, left to dry under a heat lamp between coats). 1 µL aliquots of an MDI concentration range were spotted onto the filter. The concentrations of MDI were 1, 0.8, 0.6, 0.4 and 0.2 mg/mL. The filter was then imaged by rastering the laser, again with an increment size of 0.5 mm and a laser power of 25%.

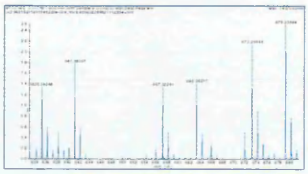
Figure 4: Depicts the five isocyanate spots on the filter surface at mass 635.

A deuterated analogue of the isocyanate was employed as the internal standard for a separate quantitation calibration. The run was carried out using an automatic search pattern. 20 spots for each concentration level were run on a spot target (1 – 0.2 mg/mL. 200 µL analyte mixed with 80 µL matrix) using an automated spiral pattern with a 1 minute acquisition time.

The average analyte : internal standard peak area ratios were plotted to give a calibration chart.



A representative spectrum showing the protonated molecular peak $[M+H]^+$, and peaks corresponding to sodium $[M+Na]^+$ and potassium adducts $[M+K]^+$ for both MDI and its deuterated analogue is shown.


Spectrum from MDI Quantitation using a Deuterated Analogue

	MDI
Protonated MDI Peak	635
Deuterated MDI Peak	641
Sodium Adduct	657
Deuterated Sodium Adduct	663
Potassium Adduct	673
Deuterated Potassium Adduct	679

nclusion

g of the GFA's showed excellent potential for direct filter is. The matrix application method has been optimised to ensure a eneous layer is present, enabling successful ionisation of the over the entire filter surface. The MP derivatisation method also to be successful for on filter derivatising of isocyanates in the lace.

alyte pixel intensities from the concentration range filter did not to the applied concentration. For the purposes of quantification a number of isocyanate compounds and suitable analogues were for use as an internal standard. The most successful internal rd for MDI was discovered to be the deuterated equivalent of the nate compound (10&11), a linear calibration was obtained.

It is necessary to develop a procedure for internal standard application when working with sample filters. The most probable method for internal standard application will be combination with the matrix followed by airspray deposition onto the filter.

Future work will be carried out on actual sample filters collected from industrial processes to determine the presence of differing isocyanate compounds. Quantitation will hopefully be performed using an internal standard calibration, that of the deuterated analogue if another suitable internal standard is not found to be more successful.

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Direct Analysis and Quantification of Isocyanate Compounds from Industrial Air-Sampling Filters by MALDI Mass Spectrometry

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OVERVIEW

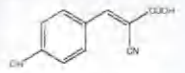
Isocyanates [NCO] are a group of highly reactive compounds which can produce an adverse effect in humans following prolonged contact [1]. NCO are known respiratory sensitisers and are the most common cause of occupationally induced asthma, bronchitis and emphysema [2-6]. Repeated exposure to low concentrations of NCO leads to progressive impairment of pulmonary function, shortness of breath and increased stress on the heart. In a sensitised individual, exposure to NCO even for a short period of time, will trigger an asthmatic response [7]. Sensitisation may be the result of a single case of high exposure (e.g. leak or spill) or prolonged exposure to low levels (e.g. poor quality atmosphere in the workplace). The degree of injury as a result of NCO exposure is proportional to the type of compound and exposure level [8]. Occupational asthma is a disease characterised by variable airflow limitation and/or airway hyper-responsiveness attributable to a particular occupational environment [9].

There are numerous methods for NCO determination, but the Health and Safety Executive (HSE) monitors and implements safe working practice in the U.K. The HSE documentation regarding exposure patterns is set out in the Methods for the Determination of Hazardous Substances number 25/3 (MDHS 25/3) [10].

Mono functional NCOs are commonly used as intermediate compounds in the production of herbicides, crop-protection agents and anti-diabetic drugs. However, the most important industrial form of NCO is the di-functional group including hexamethylene diisocyanate (HDI) and 4,4-methylenebiphenyl Diisocyanate (MDI). In this poster, the analyses of derivatised monomeric NCO standards and samples are reported. The samples were analysed as 1-(2-methoxyphenyl)piperazine (MP $C_{11}H_{16}N_2O$;193.1341) derivatives by MALDI/MS, MS imaging and utilisation of a deuterated internal standard have also been used to determine Isocyanate content upon industrially used glass fibre filters (GFA).

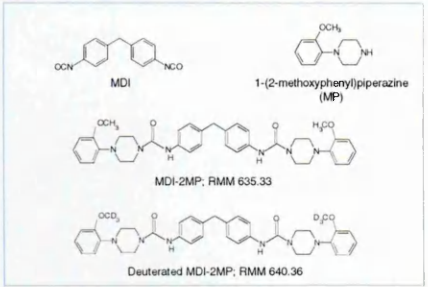
INTRODUCTION

All analyses were performed using an Applied Biosystems/MDS Sciex "Q-Star" hybrid quadrupole time of flight instrument, fitted with an orthogonal MALDI ion source and ion imaging software. This instrument utilises a nitrogen laser with a 337 nm wavelength. Data were collected and manipulated using the 'Analyst – QS' software supplied with the instrument. The air gun employed was a Badger 100-G-F gravity feed with a medium head and 200µl solvent reservoir cup. The isocyanate compound investigated was MP derivatised MDI. The original working concentration was 1 mg/ml made up in methanol with 0.1% Trifluoroacetic Acid (TFA). The organic acid matrix used was α-Cyano-4 Hydroxy Cinnamic Acid (α-CHCA), at a concentration of 25 mg/ml in methanol/0.1% TFA.



α-cyano-4-hydroxy cinnamic acid (α-CHCA)

Various methods of matrix application were investigated to coat the filter surface. The most homogeneous and successful method for matrix application was air-spray deposition. The filter was sprayed with 5 coats of α-CHCA matrix, being left to dry under a heat lamp between layers. The internal standard was also applied by air-spray deposition as a mixture with the matrix. A deuterated analogue of MDI-2MP was chosen for use as an internal standard (0.5 mg/ml) as there were no interfering peaks in resulting spectra and the reference range was close to the analyte peaks of interest.



METHODOLOGY & PRELIMINARY RESULTS

A standard glass fibre filter (GFA) was treated with MP derivatising reagent as described in MDHS 25/3, the Health and Safety Laboratories method for Isocyanate analysis [10]. This filter was repeatedly coated with α-CHCA matrix by air-spray deposition (5 layers of concentration 25 mg/ml, 250 µl per layer). 5 µl of 1 mg/ml monomeric MDI was then dropped into the centre of the filter using a micro pipette tip. A second filter was prepared in the same way with 1 µl aliquots of an MDI concentration range spotted onto it. The concentrations of MDI were 1, 0.8, 0.6, 0.4 and 0.2 mg/ml.

Mass spectrometric images have been generated using ion imaging software, supplied by MDS SCIEX. The laser was fired at the filter surface as the sample stage moved beneath and mass spectra were acquired every 0.5 mm. The laser was set to fire for approximately 2 seconds per spot with 25% power (14.3 µJ).

Figure 1: Demonstrates homogeneous matrix coverage across the filter surface. The pixel image is of the sodium adduct of α-CHCA, mass 212.

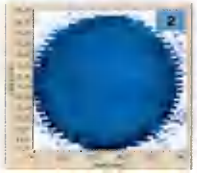


Figure 2: Shows that the GFA is fully impregnated with MP derivatising agent. The protonated adduct of MP reagent is shown at 193.

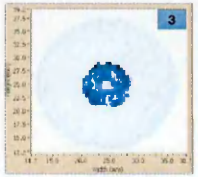


Figure 3: Depicts the presence of MDI isocyanate in the centre of the filter. The image is from the protonated MDI ion at 635 m/z. The MDI image clearly shows a 'doughnut' spreading pattern in the centre of the filter, which was expected for application of the analyte by this method.

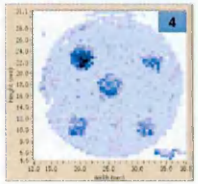
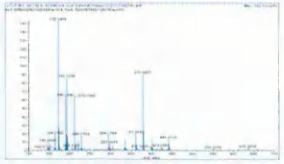
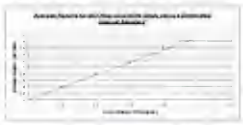


Figure 4: Depicts the five isocyanate spots present on the filter surface at mass 635.


Spectrum from Centre of Spiked Filter (MDI Present 635)

QUANTITATION

A deuterated analogue of MDI was employed as the internal standard for a quantitation calibration. The run was carried out using an automatic search pattern. 20 spots for each concentration level were run on a spot target (1 – 0.2 mg/ml, 200 µl analyte mixed with 200 µl internal standard and 80 µl matrix) using an automated spiral pattern with a 1 minute acquisition time. The average analyte : internal standard peak area ratios were plotted to give a calibration chart.



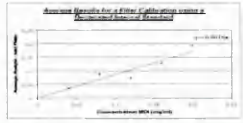
A representative spectrum depicting the protonated molecular peak $[M+H]^+$, and peaks corresponding to sodium $[M+Na]^+$ and potassium adducts $[M+K]^+$ for both MDI and its deuterated analogue is shown below. A table of the corresponding values for the two compounds is also given.


Spectrum from MDI Quantitation using a Deuterated Analogue

	MDI
Protonated MDI Peak	635
Deuterated MDI Peak	641
Sodium Adduct	657
Deuterated Sodium Adduct	663
Potassium Adduct	673
Deuterated Potassium Adduct	679

A small investigation was completed to determine what effect the change of increment size when imaging, has on the calculated filter concentration. The increment sizes tested were 0.5, 1, 2 & 4 mm. The four filters were prepared in exactly the same way and concentrations calculated using the analyte / internal standard peak area ratios.

A filter calibration was attempted to enable, if successful, the direct quantification of isocyanates from air-sampling filters. The filters were prepared over a concentration range (0.2—0.04 mg/ml) by spotting 200 µl of the solution onto the filter and allowing it to dry. The surface of the filter was then coated with matrix / internal standard mixture by air-spray deposition (5 layers, as previously described, containing 200 µl of internal standard overall). The average analyte : internal standard peak area ratios were plotted to give a calibration chart from the resulting images. The sum of $[M+H]^+$,$[M+Na]^+$ & $[M+K]^+$ peak areas were used when calculating the ratios.



DISCUSSION

It was found that MP derivatised NCO yield excellent quality MA spectra characterised by intense protonated molecules $[M+H]^+$ $[M+Na]^+$ and potassium $[M+K]^+$ adducts. Before isocyanate analysis carried out, it had to be determined if the matrix application me derivatisation method carried out by the HSE (using MP) produ coverage and were therefore successful. As an MP solution is droppe centre of the filter, it was necessary to examine the MP spread or surface. It was thought that the distribution of MP would be a 'doughn sparse in the centre and around the edge but with an intense ring patt it had been decided that the matrix application and derivatising meth adequate, 5 µl of 1 mg/ml MDI was dropped onto the centre of th determine if isocyanates could be detected directly from the GFA, w could.

A quantitation process was necessary if an industrial monitoring progr be established. A sample filter was spotted with a concentration ran and again imaged. The isocyanate was detected for each concentrati pixel intensities did not directly relate to the concentration of isocyana A deuterated MDI analogue was investigated for use as an interna and proved to be successful. Spot target calibrations and direct filter c were performed to enable the quantitation of either extracted filter liq sampling filters.

CONCLUSION

Imaging of the GFA's showed excellent potential for direct filter ana matrix application method has been optimised to ensure a homogen is present, enabling successful ionisation of the analyte over the e surface. The MP derivatisation method also proved to be successful derivatising of isocyanates in the workplace.

The analyte pixel intensities from the concentration range filter did no the applied concentration. For the purposes of quantification work, a isocyanate compounds and suitable analogues were tested for u internal standard. The most successful internal standard for discovered to be the deuterated equivalent of the isocyanate compou linear calibration was obtained for spot target samples.

It was necessary to develop a procedure for internal standard applic sample filters. The most suitable method for application was to co internal standard with the matrix and use air-spray deposition to coa with the mixture. 2 mm increments were used when imaging the filte change in increment size did not appear to affect the calculated cor from the sample filters (but did cut analysis time from 2 hours to app 10 minutes). A calibration chart was constructed by using summed p from the protonated molecular and adduct peaks. This enabled cal concentrations from known sample filters, the results being compa only a small amount of error. It is hoped that this method can be u future for test filters, of which the concentration is not known.

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