

# MALDI/TOF/MS analysis of isocyanates and other hazardous workplace chemicals

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**John White**  
Health and Safety Laboratory  
Harpur Hill  
Buxton  
Derbyshire  
SK17 9JN

Mass spectrometry has long been used to analyse samples taken in the workplace, and can be combined with other techniques to increase sensitivity, selectivity and accuracy. The work summarised in this report examines the application of the recently developed Matrix Assisted Laser Desorption Ionization/ time-of-flight/ mass spectrometry (MALDI/TOF/MS) technique to the analysis of isocyanates and biocides. Isocyanates were measured on filters (to represent air monitoring) and as isocyanate/protein conjugates (representing biological monitoring). Selected biocides bound to soil samples were analysed by MALDI/TOF/MS as examples of chemicals linked to more complex matrices: this use of MALDI/TOF/MS as an extraction process is a novel application of the technique.

MALDI/TOF/MS was found to be suitable for the analysis of isocyanate-derived protein conjugates and inflammation pathway metabolites in biological samples. Further work is required to develop quantitative biological methods based on this study. MALDI/TOF/MS was also found to complement existing analytical methods, such as conventional gas and liquid chromatography, for monitoring isocyanates in workplace air and biocides in a complex environmental matrix such as soil. The lessons learned during this study can be transferred to other occupational hygiene sampling applications.

This study was carried out in collaboration with Sheffield Hallam University.

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# EXECUTIVE SUMMARY

## Introduction

A collaboration over several years of the Health and Safety Laboratory (HSL) with Sheffield Hallam University (SHU), has allowed HSL to evaluate new techniques in mass spectrometry, namely the coupling of liquid chromatography with mass spectrometry (LC/MS) and a highly sophisticated hybrid quadrupole time-of-flight mass spectrometer (Q-TOF/MS). The work summarised in this report extends these studies to incorporate the Matrix Assisted Laser Desorption Ionization (MALDI) technique. This technique is widely used (e.g. pharmaceutical industry) for the analysis of large bio-molecules.

## Objective

The objective of this project was to apply a state of the art mass spectrometric technique, Matrix Assisted Laser Desorption Ionisation/Time-of-Flight/Mass Spectrometry (MALDI/TOF/MS) to samples of occupational hygiene relevance to the HSE for the following purposes:

- Helping to define good practice in control (Mandatory/ Registration, Evaluation, Authorisation and Restriction of Chemical Substances (REACH) programme)
- Evaluating substances associated with health effects of concern in the Disease Reduction Programme (DRP)
- Evaluating "secondary human exposure" via the environment

## Main Findings

### Introduction

Isocyanates are highly reactive species widely used in the motor vehicle repair and other industries, and are a major cause of occupational asthma in the UK. Therefore they were chosen for analysis by this new technique, both bound to filters (to represent air monitoring) and as isocyanate/protein conjugates (representing biological monitoring).

Selected biocides bound to soil samples were analysed by MALDI/TOF/MS as examples of chemicals linked to more complex matrices. This use of MALDI/TOF/MS as an extraction process is a novel application of the technique. Full experimental information and data will be presented in the PhD thesis being submitted by SHU researcher Tasneem Muharib.

### Model Protein/Isocyanate Conjugates

- The results obtained show that the concept of a biological monitoring method for NCO based on MALDI/ TOF/MS is feasible.
- MALDI/TOF/MS and associated processing can determine differences in the protein fragments produced when the model proteins (GRADSPK and human serum albumin - HSA) are exposed and unexposed (control) to the diisocyanates (MDI and HDI).
- MALDI/TOF/MS gave information about the likely products of such reactions e.g. identity of protein fragments ("peptide fingerprint") etc.
- The isocyanates were found to preferentially react with the primary amine ( $-NH_2$ ) residue of the lysine group. There was also evidence of reaction but to a lesser extent with the  $-NH_2$  residue of the arginine group.

### **Protein/Isocyanate Conjugates in Urine**

- A MALDI/TOF/MS method was developed and used to measure isocyanate-derived diamines in the urine of isocyanate-exposed workers. The attempt to develop a quantitative diamine method using MALDI/TOF/MS, equivalent to the HSL method was unsuccessful probably because of interfering metabolites in the urine or because of the hydrolysis method used to produce the diamine.
- Qualitative analysis of these samples by MALDI/TOF/MS gave “target-rich” spectra showing that MALDI/TOF/MS methods can be used for biological samples.
- MALDI/TOF/MS was used to measure various metabolite marker compounds and these results were compared against the HSL diamine method results but no obvious correlation was observed.
- A slightly different design of MS, incorporating an additional ion mobility separation stage was evaluated. It was seen that this can provide useful additional information and filtering for samples in a complex biological matrix i.e. urine.

### **Protein/Isocyanate Conjugates and Proteins in Skin Cell Models**

- MALDI/TOF/MS can determine differences in the protein profiles when the metabolically active Epi-Derm FT skin model is exposed to the known irritants (toluene and 2,6-TDI) and when it is not exposed (control).
- Differences in the proteins produced by the two irritants (toluene and 2,6-TDI) could also be distinguished.
- MALDI/TOF/MS determined differences in the amounts of certain target proteins expressed when the model was tested with 2,6-TDI, toluene or nothing (control).
- MALDI/TOF/MS analysis could depth profile the differences in the nature of the proteins expressed when the skin model was exposed to 2,6-TDI.

### **Determination of Isocyanates on Coated GF/A filter Papers**

- MALDI/TOF/MS methods for the analysis of derivatized isocyanates on filters were developed and successfully applied.
- The good mass accuracy of TOF/MS can be used to provide unambiguous identification of compounds or to assist in the elucidation of an unknown compound’s structure.
- MALDI/TOF/MS analysis may be significantly faster than conventional techniques (LC analysis and “wet” chemistry extraction e.g. MDHS 25/3) for these types of sampling devices.
- The use of MALDI/TOF/MS for quantification of isocyanate MP derivatives on glass fibre filters has not been successfully demonstrated. Further work would be required to extend the method to be applicable to levels of isocyanate of occupational hygiene relevance.
- The experience gained from the qualitative and quantitative work on derivatized isocyanates is transferable to the application of MALDI/TOF/MS for the analysis of other analytes on different occupational hygiene sampling devices e.g. biocides on occupational hygiene samplers such as gloves, swabs, suits, socks etc.

### **Determination of Biocides in Soil**

- MALDI/TOF/MS methods for selected biocides (levamisol and enrofloxacin) in soil were developed.

- For levamisol, MALDI/TOF/MS was found to have significant advantages of sensitivity, applicability, specificity and speed over the conventional “wet chemistry” extraction techniques for samples in “difficult” matrices i.e. soil.
- Further work would be required to overcome soil matrix effects and develop the method for enrofloxacin.

## **Recommendations**

This study shows that the technique MALDI/TOF/MS is suitable for the analysis of isocyanate-derived protein conjugates and inflammation pathway metabolites in biological samples. The large size of some of the molecules involved means they will not be amenable to analysis by conventional analytical techniques e.g. gas and liquid chromatography. This work should form the basis for new biological monitoring methods for the determination of workplace exposure to isocyanates.

The airborne isocyanate and biocides in soil work highlight the ability of MALDI/TOF/MS to compliment conventional analytical techniques. This work should be developed further.

The long-standing collaboration of HSL with Sheffield Hallam University has been extremely productive and has enabled HSE to cost-effectively evaluate techniques that are not available at HSL. This collaboration should be continued.



# 1 INTRODUCTION

A collaboration over several years of the Health and Safety Laboratory (HSL) with Sheffield Hallam University (SHU), has allowed the Health and Safety Laboratory (HSL) to evaluate new techniques in mass spectrometry, namely the coupling of liquid chromatography with mass spectrometry (LC/MS) and a highly sophisticated hybrid quadrupole time-of-flight mass spectrometer (Q-TOF/MS) (Ford, 2002a; Ford, et al 2002b; Prideaux et al, 2007a; Prideaux, 2007b, Warburton et al, 2005; Warburton, 2006; HSL, 2002 and HSL, 2006). The information gathered in these collaborations has been used to inform purchase decisions and decisions as to the utility of the techniques under study.

The work summarised in this report extends these studies and was carried out as an HSE funded, HSL supervised, Ph.D. at SHU. For full details of the theory of MS, the TOF instrument and experimental procedures and full details of the results please see the final Ph.D. thesis (Muharib, 2009) and the reports on the earlier work (HSL, 2002 and HSL, 2006).

Mass spectrometry (MS) is the most widely used analytical detector in forensic science, industry and research (Willoughby et al, 2002). There are several mass spectrometer designs using various ion sources (e.g. electrospray, chemical ionisation, matrix assisted laser desorption ionization (MALDI) and electron impact) and ion analyzers (e.g. single quadrupole, triple quadrupole, time-of-flight, magnetic sector, Fourier transform (ion cyclotron resonance) and hybrids). All of these different designs have the following steps in common; formation of ions from the analyte (source), separation of the produced ions (analyzer) and ion collection (detector). Each of these different designs has its strengths and weaknesses that determine its field of application. TOF/MS offers high mass accuracy and excellent sensitivity.

Matrix assisted laser desorption ionization (MALDI) TOF/MS uses a different approach to conventional liquid chromatography/mass spectrometry (LC/MS) in that it dispenses with the chromatography step relying on the excellent mass accuracy of hybrid time-of-flight (Q-TOF) instruments and a knowledge of the sample chemistry to separate out the components after analysis. MALDI is a soft ionization technique that uses a pulsed laser fired at a suitably prepared analyte-containing target to generate a cloud of ionised analyte molecules that can then be pulled into the MS for analysis. To assist in this ionization step the sample is mixed with a matrix that will absorb the laser pulse and then pass this energy into the sample so producing the ion cloud. The ion cloud then passes into the MS where it is separated into its component ions in the time-of-flight tube. These ions are then detected by the multiplier array. This technique was introduced for the analysis of large bio-molecules by Tanaka et al. (Tanaka et al., 2002) and it was for this work that he received a part share in the Nobel prize for chemistry in 2002.

This project looked at the potential for MALDI/TOF/MS to be used on samples of occupational hygiene relevance. These samples were;

- model isocyanate/protein conjugates
- isocyanate/protein conjugates in the urine of workers known to have been exposed to isocyanates
- isocyanate/protein conjugates and proteins in skin cell models
- imaging of biological samples
- coated GF/A filters (air monitoring for isocyanates)
- difficult compounds on complex matrices (selected biocides in soil).

The project broke the work down into two blocks, biological monitoring and airborne monitoring. Each block was further divided into three phases;

- development of methods
- application of methods
- reporting.

It was anticipated that there would be a need for flexibility as there is considerable overlap between the blocks and phases.

The chemicals studied were isocyanates and biocides.

Isocyanates (NCO) are highly reactive species widely used in the motor vehicle repair and other industries. They are known respiratory tract and skin sensitizers and are a major cause of occupational asthma in the UK (HSE, 2009). The Health and Safety Executive (HSE) has set workplace exposure limits (WEL) for total isocyanate exposure (i.e. all NCO species), of  $70\mu\text{g}/\text{m}^3$  (short term, 15 minute) and  $20\mu\text{g}/\text{m}^3$  (8 hour TWA)(HSE, 2007). The use of isocyanates in Great Britain is regulated by the HSE under the Health and Safety at Work etc Act (1974) and supporting regulations e.g. Control of Substances Hazardous to Health (2002). The HSL method for the analysis of airborne NCO is MDHS 25/3 (HSE, 1999).

Biocidal products are used in a variety of industries to control unwanted organisms, such as animals, insects, bacteria, viruses and fungi. In the UK some biocides are regulated by the HSE under various directive and regulations. Biocides are intended to kill or otherwise exert a controlling effect by chemical or biological means. The Biocidal Products Directive gives a formal definition of a biocidal product as:

"Active substances and preparations containing one or more active substances, put up in the form in which they are supplied to the user, intended to destroy, deter, render harmless, prevent the action of, or otherwise exert a controlling effect on any harmful organism by chemical or biological means".

A biocide can be:

- a pesticide, which includes fungicides, herbicides, insecticides, algicides, molluscicides, miticides and rodenticides, or
- an anti-microbial, which includes germicides, anti-biotics, anti-bacterials, anti-virals, antifungals, anti-protozoans and anti-parasitics.

## 2 SUMMARY OF RESULTS – BIOLOGICAL MONITORING

### 2.1 MODEL PROTEIN/ISOCYANATE CONJUGATES

MALDI/TOF/MS methods for the analysis of isocyanate/protein conjugates were developed. The instrument used throughout this work, unless specified otherwise, was an Applied Biosystems Q-STAR™ MALDI/Q-TOF/MS system. To simplify interpretation of the mass spectra the initial work was carried out with a synthetic protein containing the sequence, G-R-A-D-S-P-K (H-glycine-arginine-alanine-aspartic acid-serine-proline-lysine-OH). Work was then carried out on a more complex system, the model protein, Human Serum Albumin (HSA).

The 2,6- isomer of toluene diisocyanate (2,6-TDI) was reacted at physiological temperature and pH (i.e. 37 °C and pH 7.4) with the GRADSPK protein overnight. The reaction between TDI and GRADSPK took place in the vapour phase i.e the TDI was allowed to vaporize onto the GRADSPK protein. The resulting mix was then separated on a 10% acrylamide gel. The protein containing bands were then digested using trypsin, desalted, MALDI matrix added and analysed by MALDI/TOF/MS. The MALDI matrix used was  $\alpha$ -CHCA ( $\alpha$ -cyano-4-hydroxycinnamic acid) (25mg/ml in water with 0.1% trifluoroacetic acid). 2,6-TDI was also reacted with lysine (K residue) in a similar fashion. The results of these experiments were then compared with control samples i.e. similarly processed GRADSPK protein samples that were not exposed to TDI. Trypsin is known to cleave proteins at the K residue (lysine). Similar experiments were carried out with TDI and single amino acids (lysine, K; arginine, R; glutamic acid, E; serine, S and phenylalanine, F) to assist in the interpretation of the mass spectra. Alternative proteinases (Asp-N and Glu-C), that give different isocyanate/protein fragments were used to assist in interpretation of the mass spectra. Asp-N cleaves at the nitrogen of the D residue (aspartic acid), Glu-C cleaves at the carbon (C-terminally) of the peptide bond in the E residue (glutamic acid).

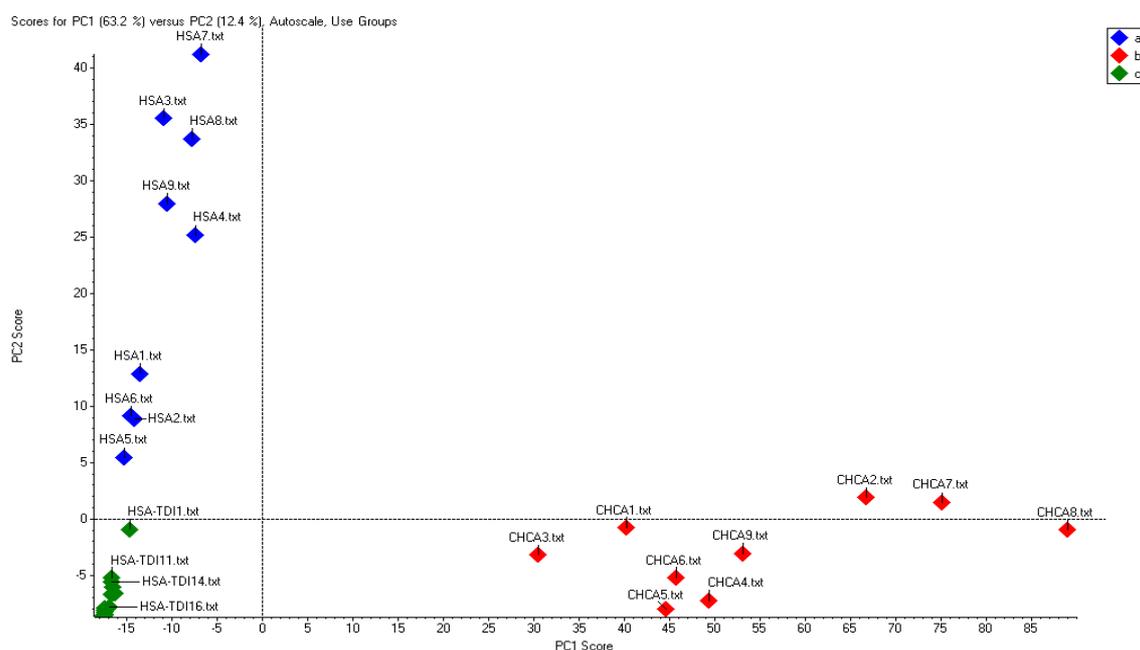
These experiments showed that the TDI was preferentially reacting with the primary amine ( $-\text{NH}_2$ ) residue of the lysine group. There was also evidence of reaction but to a lesser extent with the  $-\text{NH}_2$  residue of the arginine group. This is as expected considering the relative reactivity of isocyanates with amines and other compounds i.e. rate of reaction of isocyanate with primary amine > secondary amine > hydroxyl ~ acid ~ anhydride >> epoxide. This is in agreement with work reported elsewhere (Wisnewski et al, 2004)

MALDI/TOF/MS of the protein containing bands gave numerous peaks, the mass spectra of some of which were consistent with TDI reacted protein fragments. Several of the peaks were identified as TDI stacking adducts where the several TDI molecules had linked together to form an oligomeric TDI chain. Initially, it was considered that this was an artifact caused by the laser ionization process used in MALDI, however, further work showed that “TDI stacking” to produce oligomeric TDI was occurring on reaction and was not a laser induced artifact.

2,6-TDI was reacted, in the vapour phase, at physiological temperature and pH (37 °C and pH 7.4) with HSA overnight. The resulting mix was then separated on a SDS-acrylamide gel. The protein containing bands were then digested using trypsin, desalted, MALDI matrix added and analysed by MALDI/TOF/MS as described for the GRADSPK work. Alternative proteinases (Asp-N and Glu-C) were used to assist in interpretation of the mass spectra. The SDS-acrylamide gel clearly showed differences between the unreacted HSA (control) and the TDI exposed HSA. Upon staining (Coomassie Blue) the control showed one main protein band compared to three main bands observed for the TDI exposed HSA. MALDI/TOF/MS analysis of these protein bands gave a number of peaks that were specific to the HSA-TDI experiments i.e. were not present in the controls. The mass spectra of these peaks were consistent with TDI

reacted protein fragments. Several of the peaks were identified as TDI stacking adducts as described above for the GRADSPK work.

Principal component analysis (PCA) is a statistical technique used to simplify large and/or complex data sets. It is also used to identify differences between data sets and patterns within datasets. PCA was applied to the datasets produced during the TDI/HSA work described above. Figure 1 shows the results of a statistical analysis (principal component plot) for one of these sets of TDI/HSA experiments. This figure shows that a PCA of the MALDI/TOF/MS data described above can clearly distinguish between HSA that has been exposed to isocyanates and HSA that has not.



**Figure 1. Principal Component Analysis for HSA (control), matrix ('negative' control) and HSA-TDI (test) samples**

#### Key

Blue – peaks present in HSA only

Red – peaks present in matrix ( $\alpha$ - CHCA,  $\alpha$ -cyano-4- hydroxycinnamic acid) only

Green – peaks present in TDI-HSA only i.e. potential biomarkers of isocyanate exposure

Peaks (the majority) that were common to all three tests have been omitted for clarity

1,6-diisocyanatohexane (HDI) was reacted in the vapour phase, at physiological temperature and pH (37 °C and pH 7.4) with HSA. The resulting mix was then separated on a SDS-acrylamide gel. The protein containing bands were then digested using trypsin, desalted, MALDI matrix added and analysed by MALDI/TOF/MS. Alternative proteinases (Asp-N and Glu-C) were used to assist in interpretation of the mass spectra. The SDS-acrylamide gel clearly showed differences between the unreacted HSA (control) and the HDI exposed HSA. Upon staining (Coomassie Blue) the control showed one main protein band compared to three main bands observed for the HDI exposed HSA. MALDI/TOF/MS analysis of these protein bands gave a number of peaks that were specific to the HSA-HDI experiments i.e. were not present in the controls. The mass spectra of these peaks were consistent with HDI reacted protein

fragments. The results of this HDI/HSA work were consistent with those obtained for the TDI/HSA described above.

These experiments have showed that MALDI/TOF/MS and associated processing (i.e. proteinase digestion, gel separation and PCA) can determine differences in the protein fragments produced when the model proteins (GRADSPK and HSA) are exposed and unexposed (control) to the diisocyanates (MDI and HDI). MALDI/TOF/MS also provides information about the likely products of such reactions e.g. isocyanate binding sites, identity of protein fragments (“peptide fingerprint”) etc.

## 2.2 PROTEIN/ISOCYANATE CONJUGATES IN URINE

Biological monitoring for workplace exposure to isocyanates is currently carried out at HSL by a GC/MS method after hydrolysis by sulphuric acid of the isocyanate to the corresponding diamine (Williams et al, 1999). An LC/MS version of this method is under development (HSL, unpublished data). HSE has published a biological monitoring guidance value (BMGV - 1  $\mu\text{mol}$  isocyanate-derived diamine/mol creatinine) for the monitoring of workplace exposure to the most commonly used diisocyanates (Cocker et al, 2007; HSL, 2009).

MALDI/TOF/MS was examined to see if this technique offered any advantages for the analysis of these samples. The instruments used were the Applied Biosystems MALDI/Q-TOF/MS available at Sheffield Hallam University (as used in the rest of this work) and a MALDI/SYNAPT/HDMS<sup>TM</sup> system (Waters, UK) used at the Waters laboratory in Cheshire. The MALDI/SYNAPT/HDMS<sup>TM</sup> design of MS has a field free drift region prior to the TOF analyser. This allows a separation of the sample by ion mobility (i.e. molecular size and shape) to be carried out, in addition to the separation carried out by the TOF/MS analyser i.e. MALDI/ion mobility/Q-TOF/MS. This additional separation is useful when the matrix, sample and background have isobaric peaks (i.e. peaks with equal m/z values).

Urine samples from workers known to have been exposed to isocyanate because of prior analysis (by the HSL hydrolysis to diamine method) were analysed. MALDI/TOF/MS methods for the determination of hexamethylene diamine (HDA) and toluene diamine (TDA) were developed. These methods were found to have poor sensitivity, linearity and reproducibility in comparison to the standard HSL method possibly because of ion suppression effects caused by interferences in the urine or because of the hydrolysis method used to produce the diamine.

Qualitative MALDI/TOF/MS analysis of the unhydrolysed urine samples gave “target-rich” chromatograms containing a large number of metabolite peaks. This data was matched against commercial and in-house (Sheffield Hallam University) metabolite databases in an attempt to identify the metabolites and metabolic pathways represented in the isocyanate-exposed urine samples. Particular attention was focussed on those pathways previously established in the scientific literature as being involved with inflammation. Target metabolites associated with these pathways were then measured by MALDI/TOF/MS. The metabolites chosen were;

- Leukotriene L-D4, m/z+ 497.269 – Leukotrienes are produced in the body from arachidonic acid and are implicated in the inflammatory response usually involving the production of histamine - see section 2.3 for more information on the role of arachidonic acid in the inflammation response.
- Leukotriene L-F4, m/z+ 569.289 – another leukotriene

- Enzymes (dipeptidase and  $\alpha$ -glutamyl transferase) are involved in the conversion of LD4  $\Rightarrow$  LE4  $\Rightarrow$  LF4 as part of the arachidonic acid pathway. The ratio LF4/LD4 is often used as a proxy for the activity levels of these enzymes
- 3',5'-cyclic AMP (adenosine monophosphate) - a chemical second messenger important in many biological processes
- PAF - platelet-activating factor is a family of structurally related phospholipids (1-O-alkyl/acyl/alkenyl-2-acetyl-sn-glycero-3-phosphocholine) which possesses a wide spectrum of potent pro-inflammatory actions. One particular compound with an m/z+ of 323.112 was monitored
- Cytochrome P450 (CYP)- human CYPs are primarily membrane-associated proteins located either in the inner membrane of mitochondria or in the endoplasmic reticulum of cells. CYPs metabolize thousands of endogenous and exogenous compounds, many can metabolize multiple substrates and can catalyze multiple reactions. This accounts for their central importance in metabolizing the extremely large number of endogenous and exogenous molecules.

The unhydrolysed urine was placed in a desalting column (Sigma-Aldrich Chemical Co, UK), these columns were then placed in phosphate buffered saline and stirred at 4 °C overnight. The columns were centrifuged at 5,000 rpm and eluent collected. The desalted urine (10 $\mu$ l) was mixed the MALDI matrix (10 $\mu$ l of  $\alpha$ -CHCA - 25mg/ml in water with 0.1% trifluoroacetic acid) and 1  $\mu$ l of the resulting mixture spotted onto the MALDI target for analysis.

Figure 2 shows a plot of some of the data obtained from the urine experiments obtained using the Sheffield Hallam University MALDI/TOF/MS system. All the data sets were normalized by setting the highest result for each analyte to 100 and referencing the other results in the data set to this.

No obvious correlations between the metabolite marker compounds and the HSL diamine results are apparent in Figure 2. Further analysis (e.g. PCA) on these results may identify correlations, this work is not reported here because of time constraints. This work has shown that MALDI/TOF/MS can be used to measure metabolites in the urine of isocyanate exposed workers. This could form the basis of a biological monitoring method if a suitable bio-marker can be identified.

The work carried out on the Waters MALDI/SYNAPT/HDMS<sup>TM</sup> system looked at the ability of ion mobility (IM) to provide extra information for these complex (urine) samples. Differences in the MALDI/IM/MS were observed for the blank urine (control) and for blank urine spiked with 2,6-TDI and HDI. These differences were noticed throughout the m/z+ and drift ranges but were particularly strong in the 600-1,000 m/z+ (mass) and 100 – 200  $\mu$ s (drift) regions. These results suggest the presence of metabolites with similar mass (m/z+) but different molecular size and shape e.g. many products of the arachidonic acid pathway are of similar mass and show that the extra separation step provided by IM can be a useful adjunct to TOF/MS.



damaged (“leaky”) cells, this induces the production of more IL-1 $\alpha$ , other pro-inflammatory cytokines (signalling molecules and polypeptide regulating molecules) e.g. IL-6, IL-8 and transcription factors (molecules that act as regulators of cellular proliferation, differentiation and invasion processes) e.g. NF $\kappa$ B and AP-1.

IL-6 was discovered in fibroblasts (a type of cell that synthesizes the extracellular matrix, collagen (and fibrin) and structural framework in animal tissues and plays a vital role in wound healing) and has a molecular weight of 8,822.45 Daltons.

IL-8 is produced in keratinocytes and is a chemokine. Chemokines control the movement of other cells e.g. directing the lymphocyte cells of the immune system towards harmful antigens so these cells may destroy them.

IL-10 is produced by Th2 cells (Helper T cells type 2 direct and activate B lymphocyte cells and, in so doing, are essential for antibody-mediated immunity) may be an important factor in the recovery of the skin after irritation.

Metabolite bio-markers are metabolites of arachidonic acid. Arachidonic acid is an essential fatty acid and has a role in cellular signalling, as a lipid second messenger involved in the regulation of signalling enzymes and as a key intermediate in the inflammatory response. Metabolites of this fatty acid include such classes of chemicals as prostaglandins, thromboxanes, leukotrienes and hydroxyecosatetraenoic acids. All of these compounds are important in the skin’s response to injury.

MALDI/TOF/MS methods for the qualitative analysis of proteins produced when isocyanates are added to a model skin system were developed. This work was a continuation of the earlier HSE funded project – (Prideaux et al, 2007a; Prideaux, 2007b). The skin model used was the commercially available Epi-Derm FT system (Mat-Tek Corporation, USA). This is an in-vitro, 3-dimensional (3D), metabolically active system that has been shown to behave very similarly to the stratum corneum (dead layer), epidermis and dermis layers of human skin. The remaining layer of human skin, the hypodermis, or sub-cutaneous tissue, is not modelled.

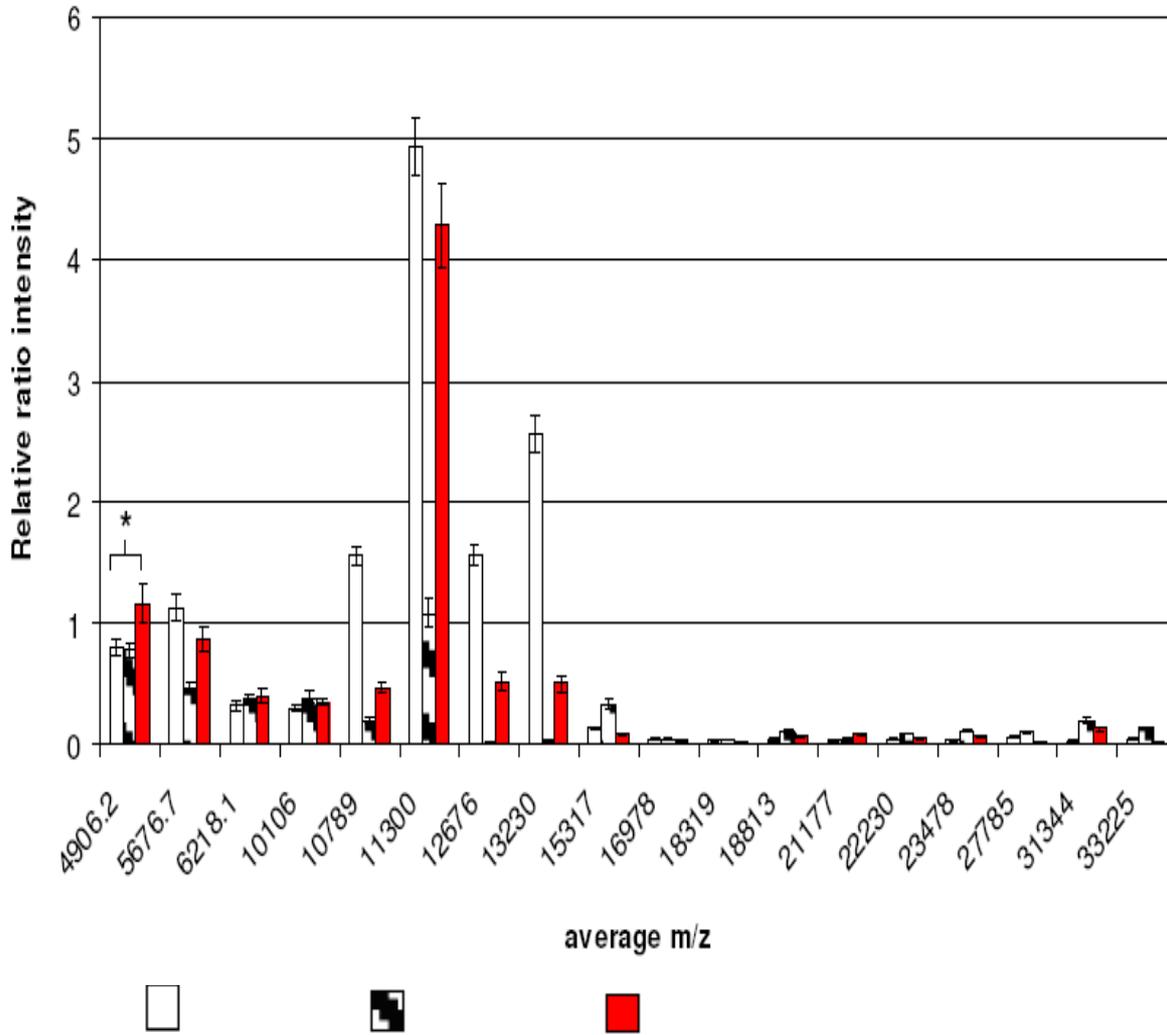
The Epi-Derm FT systems were conditioned for 24 hours as described in the manufacturer’s guidance. They were then incubated for 48 hours at 37 °C with isocyanate (2,6-TDI), with toluene or with nothing added (control). Toluene was included in these tests as it is a known irritant. The samples are then prepared for MALDI/TOF/MS as follows;

- Snap-frozen at -80 °C (isopropanol in liquid nitrogen)
- Sectioned into 20 $\mu$ m slices using a cryostat at -15 °C or -28 °C (Leica, UK)
- Washed in ethanol (several steps)
- Fixed onto a MALDI target.

Initial experiments optimised the matrix application procedure (airspraying or dropping pipette) and matrix types. Sinapinic acid in 50/50 acetonitrile/water plus 0.2% trifluoroacetic acid was used as the MALDI matrix for the high mass protein bio-markers under study (3.5 – 35 kDa). Having optimised the system, numerous MALDI/MS profiles of the control, toluene and 2,6-TDI experiments were collated and compared. The slices were analysed horizontally by rastering the laser across the slice.

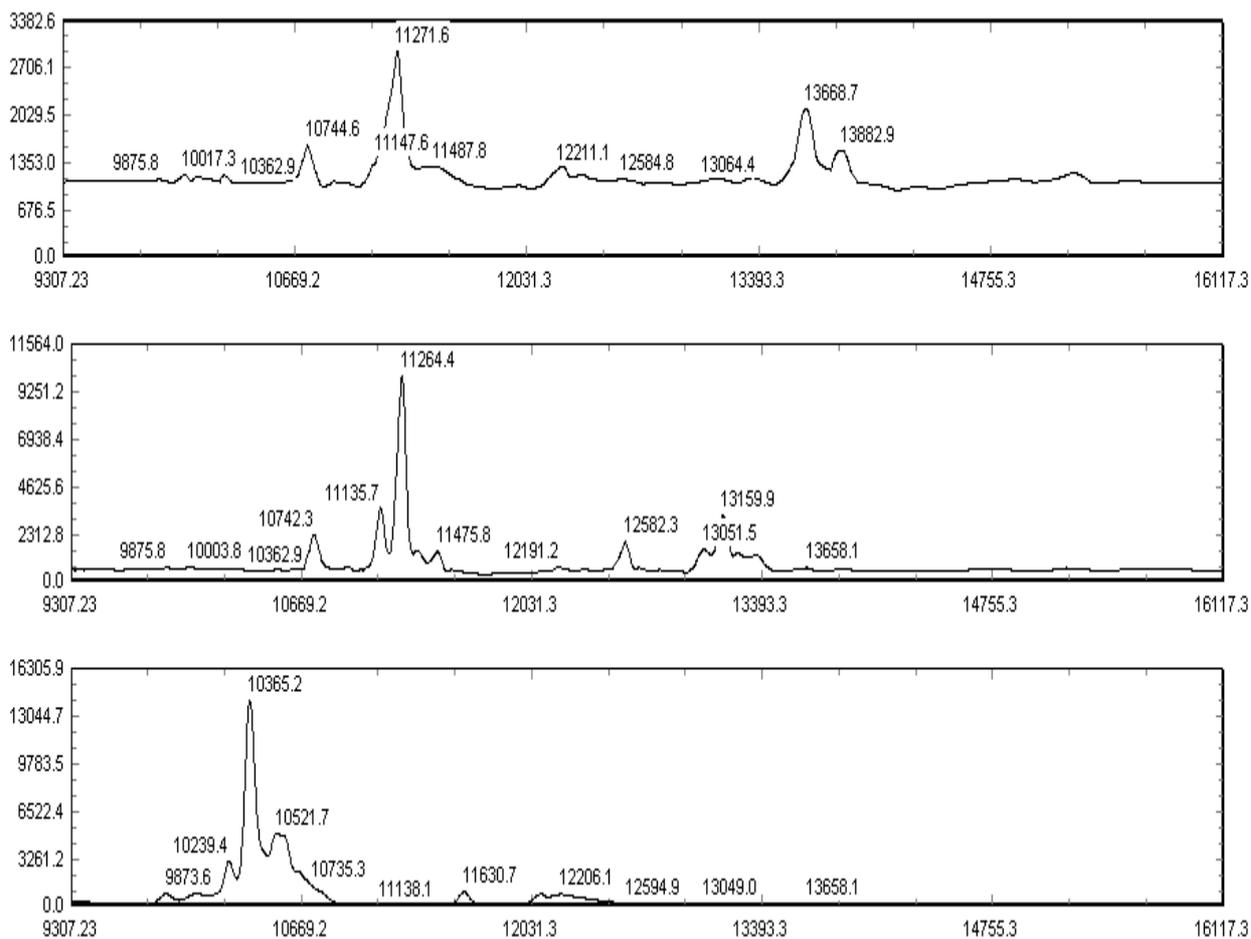
Differences were observed between the control, toluene and 2,6-TDI exposed skin cell models in both the identity of the proteins expressed (i.e. peaks of different mass observed by MALDI/TOF/MS) and the amount of a particular target protein expressed (i.e. different

intensities of a given protein peak observed). The results for one of these sets of experiments are shown in Figure 3. It can be observed that there are significant differences (either increased – up-regulated or decreased - down regulated) in the amount of the selected proteins expressed for the control, toluene and 2,6-TDI experiments. Figure 4 shows a set of example chromatograms, focussing on the 9 to 16 kDa region where most of the differences in the actual proteins expressed were observed.



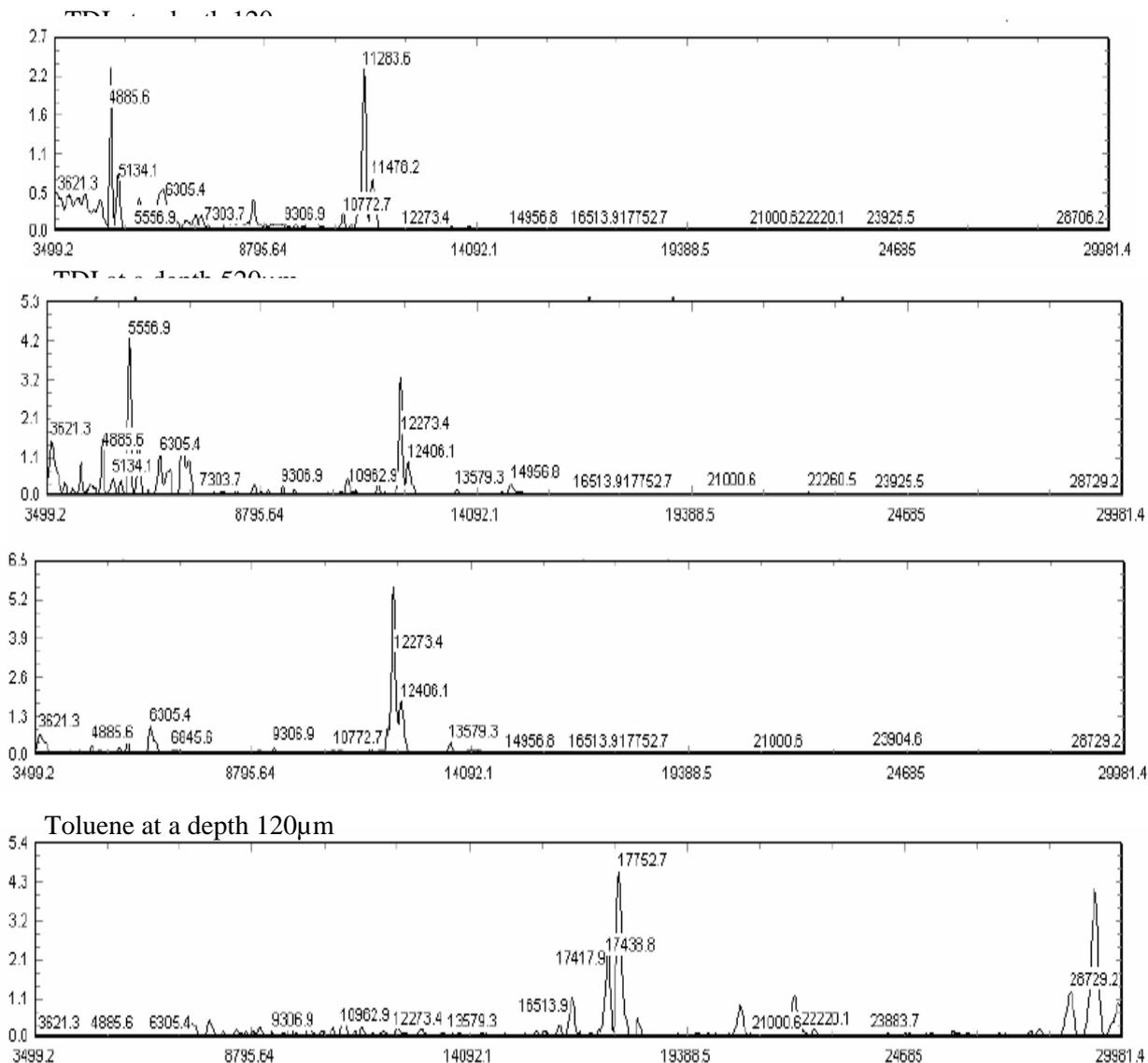
\* ANOVA analysis on the data for this protein (4.906.2 Da) found the result for 2,6-TDI to be significantly different ( $P = < 0.010$ ) to both the Control and Toluene samples. This calculation was not performed for the other data.

**Figure 3. MALDI/MS Protein Profiles of Control, Toluene and 2,6-TDI exposed Epi-Derm FT Skin Models. Mass (3.3 to 35 kDa) plotted versus Relative Expression of Protein**



**Figure 4. Example MALDI/MS Chromatograms of Control (upper), Toluene (middle) and 2,6-TDI (lower) exposed Epi-Derm FT Skin Models.**

Depth profiling was obtained by comparing the different 20 $\mu$ m slices taken through the skin model. Figure 5 shows a comparison of the chromatograms obtained for the TDI exposed skin cell system at three different depths (120 $\mu$ m, 520 $\mu$ m and 540 $\mu$ m) and the toluene exposed skin cell system (120 $\mu$ m). Clear differences in the proteins observed are seen for the comparison of the toluene and 2,6-TDI exposed 120 $\mu$ m depth slice. Differences in proteins expressed in the 2,6-TDI exposed depth slices are also observed. PCA was used to clarify these complex datasets.



**Figure 5. Example MALDI/MS Chromatograms of Toluene (@ 120µm depth slice - lower) and 2,6-TDI (@ 540µm depth slice - lower middle, @ 520µm depth slice - upper middle and @ 120µm depth slice – upper) exposed Epi-Derm FT Skin Models.**

These experiments have shown that MALDI/TOF/MS can determine differences in the protein profiles when the Epi-Derm FT skin model is exposed to the known irritants (toluene and 2,6-TDI) and when it is not exposed (control). Interestingly, differences in the proteins produced by the two irritants (toluene and 2,6-TDI) can also be distinguished. MALDI/TOF/MS could also determine differences in the amounts of certain target proteins expressed when the model was tested with 2,6-TDI, toluene or nothing (control). MALDI/TOF/MS analysis also showed that the nature of the proteins expressed when the skin model was exposed to 2,6-TDI varied with the depth through the skin model system.

## 3 SUMMARY OF RESULTS – AIRBORNE MONITORING

### 3.1 DETERMINATION OF ISOCYANATES ON COATED GF/A FILTER PAPERS

MALDI/TOF/MS methods for the quantification and qualitative analysis of 1-(2-methoxyphenyl) piperazine (MP) derivatized isocyanates on MP coated glass fibre (GF/A) filter papers were developed. This work was a continuation of the earlier HSE funded project – JR51240 (Warburton et al, 2005; Warburton, 2006; HSL, 2006).

Alternative matrix application methods (airbrushing, dropping pipette and painting) were evaluated for the filter samples and application by airbrush was found to be the preferred method. The MALDI matrix used was  $\alpha$ -CHCA ( $\alpha$ -cyano-4-hydroxycinnamic acid) (25mg/ml in water with 0.1% trifluoroacetic acid). Various internal standards were evaluated i.e. deuterated isocyanate-MP derivatives, other isocyanate-MP derivatives and use of the MP signal from the MP coated filter. The use of the background MP signal from the MP coated filter was found to give the best results. Incidentally, this finding suggests that the concentration of MP reagent is fairly constant over the glass fibre filter. Instrumental factors e.g. laser power and rastering (scanning of the laser over the target surface) speed were also explored. A new continuous rastering technique, in which the laser is continuously applied to the sample as it tracks, was found to give similar results and be much quicker than the original pulsed rastering technique, in which a laser pulse is applied, the laser is switched off, the laser moves by a set increment, the laser pulse is applied etc. until the end of the rastering track. This continuous rastering technique reduced the analysis time from ~ 3 hours to 10 minutes. The solvent desorption LC method (HSE, 1999a) for these samples routinely in use at HSL has a fifty minute run time with around twenty minutes required for sample preparation. In addition the MALDI/TOF/MS method is capable of a high degree of automation i.e. the use of multi-well plate readers, robotic sampler loaders etc. in conjunction with MALDI/TOF/MS is common in the pharmaceutical industry.

Tandem MS (MS/MS) in which the original target ion is further fragmented to daughter ions was investigated to see if this improved the sensitivity of the method. No improvement was observed in using MS/MS instead of MS.

Qualitative analysis using MALDI/TOF/MS was largely carried out on MP derivatized HDI (1,6-diisocyanatohexane) bulk products. This work was a continuation of the earlier HSE funded project – JR51184 (Ford, 2002a; Ford, 2002b; HSL, 2002). The mass spectra of these bulks showed the same peaks that have been characterised by the conventional LC/MS techniques available at HSL but with the improved mass accuracy to be expected from a TOF/MS instrument in comparison to a quadrupole/MS. This improved mass accuracy is of use for unambiguous identification of a compound or for elucidation of an unknown compound's structure.

To evaluate the performance of the quantification method a calibration curve was generated over the range 5–20 ng NCO/on filter. This curve was found to have acceptable linearity ( $R^2 = 0.9457$ ). The estimated limit of detection (based on a signal/noise ratio of 3) was calculated as 2 ng NCO/on filter, this would correspond to ~0.13  $\mu$ g NCO for a 15 l air sample i.e. ~1/500<sup>th</sup> of the current HSL STEL. It can be seen from this data that the MALDI/TOF/MS technique has the required sensitivity to measure levels of airborne isocyanate at levels of relevance to the workplace.

To test the accuracy of the method, MDI-MP (4,4'-methylene bis(phenyl isocyanate)) coated MP filters, prepared for the HSL WASP (Workplace Analysis Scheme for Proficiency) quality assurance scheme were analysed by the MALDI/TOF/MS method. These results were then compared with those obtained by the WASP scheme participants using variants of the HSL method MDHS 25/3 (HSE, 1999a). This method uses solvent desorption of the MP coated GF/A with quantification by liquid chromatography with ultra-violet/visible, electro-chemical or mass spectroscopic detection. The results of this method intercomparison are given in Table 1.

Table 1. Determination of MDI-MP on WASP QC scheme samples

WASP Round 57	MALDI/TOF/MS	WASP Participant Mean	Comparison (MALDI/WASP)
	ng NCO/filter	ng NCO/filter	
Filter 1	950	491	1.9
Filter 2	860	241	3.6
Filter 3	850	562	1.5
Filter 4	1,210	810	1.5

Notes

*The participant mean results are the “true” results calculated from the results given by the 18 laboratories that submitted results for this round. The participating laboratories use a variety of LC based methods. The relative standard deviation of the laboratories, for the LC methods, of all the data available (34 rounds) was calculated as ~12%.*

*The relative standard deviation for the MALDI/TOF/MS method, from the calibration curve, was calculated as ~27%.*

The results given in Table 1 show that, although the MALDI/TOF/MS has been demonstrated (by the calibration curve work) to have the required sensitivity for the determination of levels of isocyanate of occupational hygiene relevance, the accuracy of the method is not satisfactory. Further work may be carried out at SHU to attempt to solve this problem.

## 4 SUMMARY OF RESULTS – COMPLEX MATRIX

### 4.1 DETERMINATION OF BIOCIDES IN SOIL

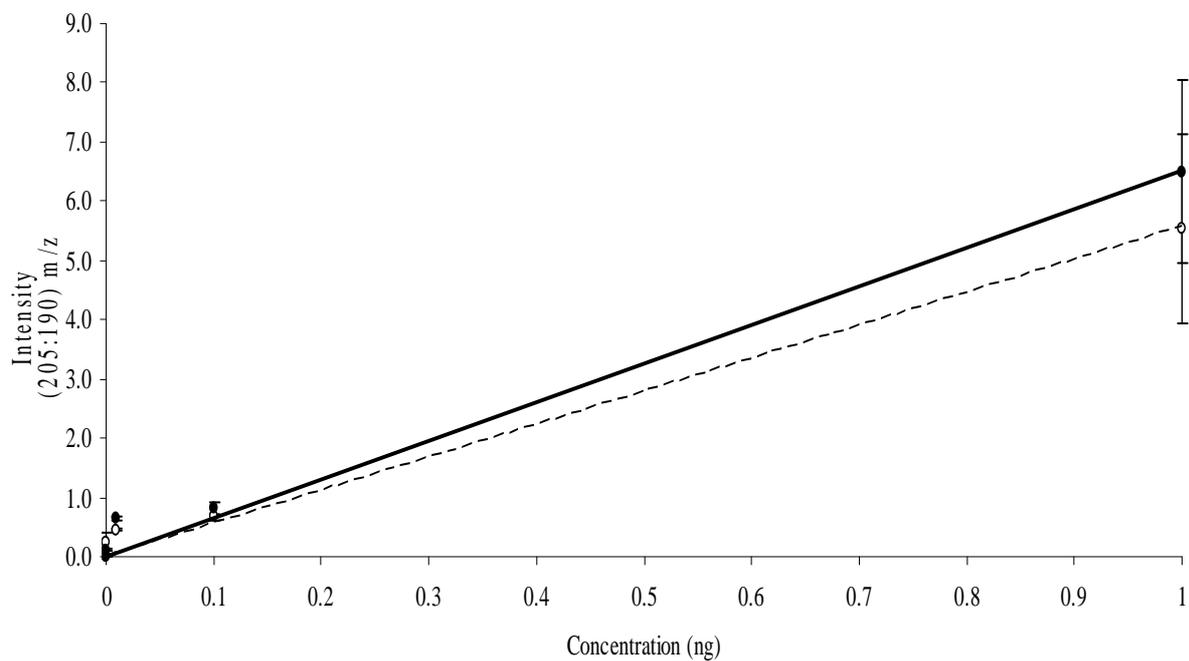
MALDI/TOF/MS methods for the quantification and qualitative analysis of biocides in soil were developed. This work was a continuation of the earlier HSE funded project – JR51240 (Warburton, 2006; HSL, 2006). The conventional method (liquid/liquid extraction followed by LC/MS analysis) has been found to give low recoveries (<5%, HSL, unpublished data) for the biocides studied (enrofloxacin and levamisole). This is a novel application of MALDI i.e. using the MALDI technique as an extraction technique.

Stock solutions of levamisole and enrofloxacin were prepared in ethanol. Calibration curves were prepared by serial dilution into ethanol and by spiking known amounts of these solutions onto soil. These samples were incubated at room temperature for 1 hour, an aliquot of 10  $\mu$ l taken, the MALDI matrix was added (10 $\mu$ l of 25mg/ml of  $\alpha$ -CHCA in water with 0.1% trifluoroacetic acid), 1  $\mu$ l of the resultant mixture was spiked onto a stainless steel MALDI target plate and allowed to dry at room temperature. These samples were then analysed by MALDI/TOF/MS. Initial work optimised the system for these compounds and backgrounds.

Figure 6 shows example calibration curves (ethanol and spiked soil extracts) for levamisole. It can be seen that these curves have a similar slope, also noteworthy are the low levels (ng) of levamisole being detected. The two slopes (Kruskal-Wallis analysis performed using the StatsDirect software) were not significantly different (calculated  $P = 0.8484$  i.e.  $P \gg \gg 0.010$ ). These results show that the MALDI/TOF/MS analysis is relatively unaffected by the matrix, this finding is markedly different to the situation observed for the conventional method in use at HSL (liquid/liquid extraction and LC/MS/MS analysis of levamisole spiked soil - HSL, unpublished data). It should also be noted that the conventional method is time-consuming (a sample work-up of ~ a day per set of ~ 10 samples) and in comparison the MALDI/TOF/MS method is much quicker (~ 30 minutes per sample). As stated in section 3.1 MALDI/TOF/MS methods are also capable of a high degree of automation i.e. automated matrix addition robots and multi-well plate readers and stackers capable of loading hundreds of samples in a sample set.

Figure 7 shows a comparison of the mass spectra obtained for blank soil, enrofloxacin in soil and enrofloxacin in ethanol. The enrofloxacin peak at  $m/z^+ \sim 360.16$  can clearly be seen in the enrofloxacin in soil and ethanol samples but is not in the blank soil. The excellent mass accuracy of the TOF/MS technique enables the potentially interfering peak from the soil ( $m/z^+ \sim 360.35$ ) seen in the blank soil and enrofloxacin spiked soil samples to be discounted. This improved mass accuracy enables improved filtering of interfering peaks and so gives improved specificity over conventional (quadrupole) LC/MS. Calibration curve work, similar to that described for levamisole, was carried out. Unfortunately, a marked difference was seen for the gradient of the enrofloxacin in soil and enrofloxacin in ethanol calibrations. This strongly suggests the presence of an extracted compound from the soil that is interfering with the laser ionisation process (i.e. ion suppression/competition)

This work shows that the MALDI/TOF/MS technique can have significant advantages of sensitivity, applicability, specificity and speed over the conventional techniques for samples in “difficult” matrices i.e. soil, although interferences from the environmental matrix may still occur for some compounds.



Key

- (solid black line) levamisole in soil
- o (dotted line) levamisole in ethanol

**Figure 6. Example Calibration Curves for Levamisole (from ethanolic solution and from spiked soil extracts)**



## **5 CONCLUSIONS**

### **5.1 MODEL PROTEIN/ISOCYANATE CONJUGATES**

The results obtained show that the concept of a biological monitoring method for NCO based on MALDI/ TOF/MS is feasible.

MALDI/TOF/MS and associated processing (i.e. proteinase digestion, gel separation and PCA) identified differences in the protein fragments produced when the model proteins (GRADSPK and HSA) were exposed to the diisocyanates (MDI and HDI) when compared to unexposed (control) samples. MALDI/TOF/MS also provided information about the likely products of such reactions e.g. isocyanate binding sites and identity of protein fragments (“peptide fingerprint”).

A MALDI/TOF/MS method would have some advantages over the “hydrolysis to amine” method (Williams et al, 1999) currently in use e.g. samples for the existing method have to be taken within a few hours of exposure because of the short NCO/amine half life, the hydrolysis method cannot determine low molecular weight isocyanate (e.g. methyl isocyanate and isocyanic acid), the MALDI/TOF/MS method would give information on the isocyanate species present and the proteins to which they were conjugated.

### **5.2 PROTEIN/ISOCYANATE CONJUGATES IN URINE**

Urine samples from isocyanate-exposed workers were analysed by MALDI/TOF/MS. An attempt to develop a diamine method equivalent to the HSL method was unsuccessful. Qualitative analysis of these samples by MALDI/TOF/MS gave “target-rich” spectra showing that the methods developed in phase 1 can be used for biological samples. Various metabolite marker compounds were measured and compared against the HSL results but no obvious correlation was observed.

The opportunity to use a slightly different design of MS, the Waters MALDI/SYNAPT/HDMS™ system was taken and the use of ion mobility as an additional separation step evaluated. It was seen that this can provide useful additional information and filtering for samples in a complex biological matrix i.e. urine.

### **5.3 PROTEIN/ISOCYANATE INTERACTIONS IN SKIN CELL MODELS**

This work has shown that MALDI/TOF/MS can determine differences in the protein profiles when the Epi-Derm FT skin model is exposed to the known irritants (toluene and 2,6-TDI) and when it is not exposed (control). Differences in the proteins produced by the two irritants (toluene and 2,6-TDI) could also be distinguished. MALDI/TOF/MS determined differences in the amounts of certain target proteins expressed when the model was tested with 2,6-TDI, toluene or nothing (control). MALDI/TOF/MS analysis could depth profile the differences in the nature of the proteins expressed when the skin model was exposed to 2,6-TDI.

### **5.4 DETERMINATION OF ISOCYANATES ON COATED GF/A FILTER PAPERS**

MALDI/TOF/MS methods for the analysis of derivatized isocyanates on filters were developed. Qualitative analysis takes advantage of the good mass accuracy of TOF/MS to provide unambiguous identification of compounds or to assist in the elucidation of an unknown

compound's structure. Quantification of isocyanate MP derivatives on glass fibre filters has not been successfully demonstrated at levels of relevance to occupational hygiene monitoring. Further work may be carried out to remedy this situation. The experience gained from this work can be transferred to the application of MALDI/TOF/MS for the analysis of other analytes on different matrices e.g. biocides on occupational hygiene samplers (gloves, swabs, suits, socks etc.).

## **5.5 DETERMINATION OF BIOCIDES IN SOIL**

MALDI/TOF/MS methods for selected biocides (levamisole and enrofloxacin) in soil were developed. For levamisole, the MALDI/TOF/MS technique was found to have significant advantages of sensitivity, applicability, specificity and speed over the conventional "wet chemistry" extraction techniques for samples in "difficult" matrices i.e. soil. Further work would be required to develop the method for enrofloxacin.

## 6 APPENDICES

### 6.1 ABBREVIATIONS

$\alpha$ -CHCA	$\alpha$ -cyano-4-hydroxycinnamic acid
DRP	Disease Reduction Programme
GC	Gas Chromatography
GF/A	filter paper - Glass Fibre class A filter paper
GRADSPK	synthetic protein (H-glycine-arginine-alanine-aspartic acid-serine-proline-lysine-OH)
HDI	1,6-diisocyanatohexane a.k.a. HexaMethylene Diisocyanate
HDMS	High Definition Mass Spectrometry
HSA	Human Serum Albumin
HSE	Health and Safety Executive
HSL	Health and Safety Laboratory
IM	Ion Mobility
LC	Liquid Chromatography
MALDI	Matrix Assisted Laser Desorption Ionisation
MDHS	Methods for the Determination of Hazardous Substances
MS	Mass Spectrometry
NCO	Isocyanate (nitrogen-carbon-oxygen)
PCA	Principal Component Analysis
Q-STAR	registered trademark of Applied-Biosystems MDS Sciex – refers to their Quadrupole-Time Of Flight design of mass spectrometer
Q-TOF	Quadrupole-Time Of Flight
REACH	Registration, Evaluation, Authorisation and Restriction of Chemical Substances
SHU	Sheffield Hallam University
SYNAPT	registered trademark of Waters – refers to their Quadrupole-Time Of Flight design of mass spectrometer
TDI	Toluene DiIsocyanate
TOF	Time Of Flight

TWA	Time Weighted Average
WEL	Workplace Exposure Limit

## 6.2 PUBLICATIONS AND PRESENTATIONS

One important output of this project is to publicise the work of HSL/HSE in this area. This was achieved by the student (Tasneem Muharib) attending relevant conferences and meetings, giving talks, presenting posters and publishing in refereed journals.

### Conferences

2008

BMSS (British Society for Mass Spectrometry), York, UK, September  
HuPO (Human Proteomics Organisation) Amsterdam, The Netherlands, August  
RSC (Royal Society of Chemistry), Hull, UK, July  
EMS (Environmental Mass Spectrometry), Chester, UK, April  
ASMS (American Society for Mass Spectrometry) Conference, Denver USA, June

2007

BMSS (British Society for Mass Spectrometry), Edinburgh, UK, September  
ASMS, Indiana, USA, June  
RSC, Glasgow, UK, July

2006

ASMS, Seattle, USA, June  
RSC, Cork, Ireland, July  
IMSC, International Mass Spectrometry Conference, Czech Republic, September

### Publications

(In preparation)

Muharib T, White J, Clench M. R "Protein Biomarkers of skin disease using *in vitro* skin models and MALDI-MS-Imaging".

Muharib T *et al.*, "Quantification of Derivatized airborne Isocyanates using MALDI-MS-I and MALDI-MS/MS-I".

Muharib T *et al.*, "Metabolomic profiling of urine of workers exposed to Isocyanates using MALDI-MS".

### Oral Presentations

2008

RSC, "Detection of low and high Molecular Weight skin disease biomarkers in 3D metabolically *in vitro* skin models by MALDI-MSI".

EMS, "Quantification of MDI-2MP on filters using MALDI-MSI"

### Poster Presentations

2008

BMSS, "Low MW Biomarkers of skin disease: Lipids and Metabolites by MALDI-MS-I"

HuPO, "Protein and *in situ* digest peptide Biomarkers of skin disease by MALDI-MS-I"

EMS, "Quantification of MDI-2MP on filters using MALDI-MS-I"

ASMS, RSC, BMSS, "Isocyanate adducts at specific amino acid on HSA adducts by MALDI-MS"

2006

ASMS, RSC, IMSC, “Nature of Isocyanate HSA, peptide and amino acid adducts by MALDI-MS”

**AWARD:** First prize for poster presentation at EMS (Chester, UK, April, 2008).

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# MALDI/TOF/MS analysis of isocyanates and other hazardous workplace chemicals

Mass spectrometry has long been used to analyse samples taken in the workplace, and can be combined with other techniques to increase sensitivity, selectivity and accuracy. The work summarised in this report examines the application of the recently developed Matrix Assisted Laser Desorption Ionization/ time-of-flight/mass spectrometry (MALDI/TOF/MS) technique to the analysis of isocyanates and biocides. Isocyanates were measured on filters (to represent air monitoring) and as isocyanate/protein conjugates (representing biological monitoring). Selected biocides bound to soil samples were analysed by MALDI/TOF/MS as examples of chemicals linked to more complex matrices: this use of MALDI/TOF/MS as an extraction process is a novel application of the technique.

MALDI/TOF/MS was found to be suitable for the analysis of isocyanate-derived protein conjugates and inflammation pathway metabolites in biological samples. Further work is required to develop quantitative biological methods based on this study. MALDI/TOF/MS was also found to complement existing analytical methods, such as conventional gas and liquid chromatography, for monitoring isocyanates in workplace air and biocides in a complex environmental matrix such as soil. The lessons learned during this study can be transferred to other occupational hygiene sampling applications.

This study was carried out in collaboration with Sheffield Hallam University.

This report and the work it describes were funded by the Health and Safety Executive (HSE). Its contents, including any opinions and/or conclusions expressed, are those of the author alone and do not necessarily reflect HSE policy.